Isolation and characterization of bacteriophage from environmental water samples specific for *Klebsiella pneumoniae*

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Arka Roy

Student ID: 13336006

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Biotechnology Program

Department of Mathematics and Natural Sciences

BRAC University

Dedicated To

My beloved Mother and Father who have always been there for me

Declaration

I hereby declare that this thesis entitled "**Isolation and characterization of bacteriophage from environmental water sample specific for *Klebsiella pneumoniae***" is submitted by me, Arka Roy, to the Department of Mathematics and Natural Sciences under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Department of Mathematics and Natural Sciences, BRAC University. I also declare that the thesis work presented here is original, and has not been submitted elsewhere for any degree or diploma.

Candidate

_____

Arka Roy

Certified

_____

Dr. M, Mahboob Hossain

Supervisor

Professor

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

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Abstract

Klebsiella pneumoniae is a clinically important pathogen that is frequently associated with multidrug resistance. Recently, some of its strains have acquired superbug status. Over time it is getting difficult to treat *Klebsiella pneumoniae* induced infection using antibiotics and it is necessary to identify new approach. Bacteriophages are bacteria-infecting viruses that were previously used to treat various bacterial infection in human. Hence, in this study, *Klebsiella pneumoniae* specific bacteriophage designated as Klpp1 was isolated and characterized for the first time in Bangladesh in order to develop a potential therapeutic agent. Upon host range examination, Klpp1 phage indicated to have a broad host range within genus *Klebsiella* since it was able to lyse 8 out of 13 different *Klebsiella* culture but could not infect bacteria from other genera. The isolated phage was able to survive at a temperature up to 60-degree Celsius and was infective in pH range of pH 4.0 to pH 11. The phage was perfectly stable in chloroform but totally lost its infectivity in ethanol. Klpp1 was found to have a latent period of 20 minutes with a burst size of approximately 140 plaque forming unit per infected cell. Therefore, this study provides an addition to the growing number of bacteriophages isolated, particularly *Klebsiella pneumoniae* specific bacteriophage. The studies on its biological characteristics may provide useful information and knowledge in establishing potential therapeutic agent against *Klebsiella pneumoniae* infection.

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List of Abbreviations

AMR	Antimicrobial Resistance
BAM	Bacteriophage Adherence to Mucus
CNS	Central Nervous System
CPS	Capsular Polysaccharide
DLA	Double Layer Agar
DNA	Deoxyribonucleic acid
ds	double stranded
EPA	Environmental Protection Agency
ESBL	Extended Spectrum Beta Lactamase
ETT	endotracheal tubes
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
i.n	intranasal
i.p	intraperitoneal
ICDDRB	International Centre for Diarrhoeal Disease Research, Bangladesh
ICTV	International Committee on Taxonomy of Viruses
INSDC	International Nucleotide Sequence Database Consortium
KPC	*Klebsiella pneumoniae* carbapenemase
KPC-KP	KPC-type carbapenemases producing *Klebsiella pneumoniae*
LPS	Lipopolysaccharide
MDR	Multidrug Resistance
ml	milliliter
MOI	Multiplicity of Infection
NDM	New Delhi metallo-beta-lactamase

NIDCH	National Institute of Diseases of the Chest and Hospital
PFGE	Pulse Field Gel Electrophoresis
pfu	plaque forming unit
PLA	Pyogenic Liver Abscess
RNA	Ribonucleic acid
rpm	rotations per minute
RTE	Ready-To-Eat
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ssDNA	single stranded DNA
TEM	Transmission Electron Microscopy
USA	United States of America
UTI	Urinary Tract Infections
UV	Ultraviolet
VAP	ventilator associated pneumonia
XDR	Extremely Drug Resistance
μl	microliter
μm	micrometer

Chapter 1: Introduction

Bacteriophages or phages for short are a group of viruses that infect specific bacteria (Abedon, 2012). They are the most abundant as well as the most genetically diverse biological entities on Earth, with global population number estimated at 10^{30} to 10^{32} (Hemminga et al., 2010). It is estimated that there are 5 to 10 viruses for each bacteria (Weinbauer, 2004). Phages are ubiquitous in nature and they are found in all environments that support bacterial proliferation (Kęsik-Szeloch et al., 2013). It is now acknowledged that phages play a crucial role in the cycling of organic matter in the biosphere and play an important role in bacterial diversity in addition with maintaining bacterial balance in the ecosystem (Chibani-Chennoufi et al., 2004; Guttman et al., 2004). Phages are thought to lyse 10-20 % of the marine bacterial community every day (Suttle, 1994). Like other viruses, phages are an obligate intracellular parasite and their life cycle is completely dependent on their host bacterial cell as they lack cell structure and enzyme system required for proliferation (Carlton, 1999). By 2017, more than 25000 phage nucleotide sequence has been submitted in International Nucleotide Sequence Database Consortium (INSDC) (Adriaenssens and Brister, 2017) and researchers think that many more phages are waiting to be discovered.

Klebsiella pneumoniae, a Gram-negative bacillus, is a pathogenic microorganism that causes hospital-acquired Urinary Tract Infections (UTIs), pneumonia and septicemia (Podschun and Ullmann, 1998). In recent times, community-acquired Pyogenic Liver Abscess (PLA) caused by *K. pneumoniae* complicated with metastatic meningitis and endophthalmitis has emerged worldwide, particularly in Asia (Hsu et al., 2013). As the global antibiotic use increasing rapidly the more and more bacterial pathogen are showing antibiotic resistance and maybe *Klebsiella pneumoniae* is the most worrisome one. Previously several strains of *Klebsiella pneumoniae* were identified that showed resistance to a variety of antibiotics. However, in recent times KPC-type Carbapenemases producing *Klebsiella pneumoniae* (KPC-KP), which very regularly show multidrug-resistant (MDR) or even extremely drug resistant (XDR) phenotype, including antibiotics of last resort like colistin, are spreading globally (D'Andrea et al., 2017). As a result, this pathogen has gained the "superbug" status (Wu and Li, 2015). These KPC-KP have become

endemic in few countries (e.g. USA, China, Taiwan, Israel, Greece, Italy, and Colombia) and are now a vital cause of healthcare-associated infections associated with high morbidity and mortality (D'Andrea et al., 2017). Therefore, it is getting increasingly difficult to treat *Klebsiella pneumoniae* induced infection and the search for alternative therapeutic agents is now a major challenge.

Bacteriophages have been used to treat bacterial infection in human since its discovery in the early twentieth century (Sulakvelidze and Kutter, 2004). So far phages were used successfully in different agricultural settings such as treating plant bacterial disease and showed potentiality in livestock and aquaculture (Sulakvelidze and Barrow, 2004). Recently, researchers are trying to use phage as molecular tool in vaccine delivery, gene therapy (Clark and March, 2006) and as a diagnostic tool to detect bacterial species in the clinical and environmental sample (Funatsu et al., 2002). However, out of all these usages of phage, the ability to lyse specific bacterial cell especially those that are antibiotic resistant and prevent or cure bacterial infections makes phages an interesting alternative antimicrobial agent where chemically synthesized antibiotics may fail.

Till now, bacteriophages specific to *Klebsiella pneumoniae* have been isolated but the number is not very high considering the amount of diversity available in phage population. Currently, there is no record of *Klebsiella pneumoniae* specific phages being isolated in Bangladesh. Therefore, an attempt was made in this study to isolate *Klebsiella pneumoniae* specific phages from different water sample across Dhaka city, Bangladesh.

All the previously isolated phages specific to *Klebsiella pneumoniae* demonstrated variation as well as similarities in their phenotypic and genotypic characteristics which corroborate the phage diversity. Thus, *Klebsiella pneumoniae* specific phage isolated from the water sample in Dhaka city could possibly show variation and similarities to previously isolated *Klebsiella pneumoniae* specific phages and might have the potential to be added in the International Committee on Taxonomy of Viruses (ICTV) database. The basic understanding of phage biology of the isolated *Klebsiella pneumoniae* phage could be useful in the development of therapeutic agent against *Klebsiella pneumoniae*.

Objectives:

The main aim of this project was to isolate bacteriophage against *Klebsiella pneumoniae* and to identify its various characteristic to determine the therapeutic potential as an antimicrobial agent.

Specific aims:

1. To isolate Strong lytic bacteriophage against *Klebsiella pneumoniae* from the different water sample.

2. To characterize the isolated page based on

a) host range
b) thermal stability
c) pH stability
d) chloroform and ethanol stability
e) one step growth curve

Chapter 2: Literature Review

2.1 Bacteriophages:

Bacteriophages or 'phages' for short are naturally occurring bacterial viruses which infect bacterial cells (Abedon, 2012). They are highly host specific and have the ability to proliferate inside bacterial cell (Clark and March, 2006; Hagens and Loessner, 2007; Hanlon, 2007; Nishikawa et al., 2008; Viazis et al., 2011). Phages are considered as natural killer of bacteria that can infect and lyse the host organism (Abuladze et al., 2008; Nishikawa et al., 2008). In recent times, it has been widely acknowledged that bacteriophages are abundant in the environment and they influence the biosphere extensively. Bacteriophages are estimated to kill between 20-40 % of oceanic bacteria every day, play a key role in nutrient and energy cycle of an ecosystem and forms the pool of most genetically diverse 'life form' on earth (Suttle, 2005).

2.1.1 Early history of bacteriophages:

Bacteriophages were first observed in 1896 by a British bacteriologist, Ernest Hankin (Ackermann, 2012). In the water of Ganga and Jumna River in India, he observed the presence of an antibacterial activity against bacteria *Vibrio Cholerae*. He suggested that this unknown agent, which was heat sensitive and could pass through porcelain filter, causing the bactericidal activity is responsible for preventing the spread of cholera disease. Two years later, while working with Bacillus subtilis, a Russian bacteriologist named Gamalaya witnessed similar phenomenon (Sulakvelidze et al., 2001). In 1901 Emmerich and Löw reported that sample from a culture which demonstrated autolysis was able to lyse different culture, was capable of curing experimentally induced infection (Summers, 2004). Almost two decades after Hankin's observation, Frederick William Twort, a British pathologist observed a "glassy transformation" of Micrococcus colonies grown on solid agar media. He hypothesized that the unknown substance causing the watery transformation of the bacterial colonies could be a virus. Two years later of Twort's documentation, Felix d'Herelle a French Canadian microbiologist observed similar kind of incidence. He proposed that it was

"ultravirus" that was causing lysis of bacterial cell in liquid media and created clear patches on the bacterial lawn which he primarily called it *taches*, then *taches vierges*, and later *plaques.* Felix d'Herelle also named the virus responsible for these phenomena as "bacteriophage" which derived from "bacteria" and Greek word "phagein" which means to "eat" or "devour". While Twort, for numerous reasons including financial difficulties could not pursue his findings, d'Herelle continued and devoted his research career to bacteriophage study (Calendar, 2005; Sulakvelidze et al., 2001; Summers, 2004) and he concluded bacteriophage as "exogenous agents of immunity" following the observation of an increase in phage titer in the stool sample of recovering patients suffering from dysentery and typhoid (Deresinski, 2009).

The first documented therapeutic use of bacteriophage was done by Bruynoghe and Maisin in 1921 from Louvain where they noted reduction in pain, swelling, and fever upon injection of staphylococcal phage preparation in the local region of cutaneous boils (Summers, 2004). However, the first therapeutic use of phage conducted by Felix d'Herelle at the Hospital Des Enfants-Malades in Paris in 1919 under the supervision of Professor Victor-Henri Hutinel, the hospital's Chief of Pediatrics. They administrated anti-dysentery phage preparation to a 12-year boy with severe dysentery and observed consecutive cease of symptoms and full recovery within a few days. He ran several other trails after that where patients recovered within 24 hours using only one dose of phage preparation. As the results from these trials were not published immediately, therefore, Bruynoghe and Maisin were credited for the first stated application of phage in treating infectious disease (Sulakvelidze et al., 2001; Summers, 1999).

While several early phage therapeutic trials were considered as successful and many prominent pharmaceutical companies and research laboratories such as D'Herelle's commercial laboratory in Paris and Eli Lilly Company in the United States sold phage preparation to treat various infectious disease, they ultimately became a failure (García et al., 2008; Sulakvelidze et al., 2001; Thiel, 2004). The discovery of broad range antibiotics played the major role in declining the interest of producing phage commercially. The lack of understanding of phage biology and inadequacies in the diagnostic bacteriology techniques available at the time aided the shift in interest from phage in the western world. However, phage therapy was continued to be offered in the Eliava Institute and later by others also such as the Hirszfeld Institute of Immunology and Experimental Therapy

in Wroclaw, Poland (Deresinski, 2009). One the other hand phage research continued at a fundamental level in the west where the study of phage played a major role in some momentous discoveries in biological science. It led to the identification of DNA as genetic material (Van Valen et al., 2012), understanding of genetic code and phenomenon of restriction-modification and to the development of molecular recombinant technology. Phage derived proteins are now being used as diagnostics agents (Smith et al., 2001), therapeutic tools (Loeffler et al., 2001; Schuch et al., 2002) and for discovering new drug (Liu et al., 2004).

2.1.2 Bacteriophage classification:

Phages are enormously diverse and vary from one another based on structural, physicochemical, and biological properties. When in 1917 d'Herelle discovered bacteriophage, he presumed that there were only one species of phage containing many races. However, in 1933, Burnet showed heterogenicity among enterobacterial phages and in 1943, Ruska observed three morphological types of bacteriophage which evoked the necessity of proper classification of phages. Holmes proposed a classification system of phages based on plaque and particle size, host range, and resistance to urea and heat which was not accepted by scientific community. Lwoff, Horne, and Tournier published a classification scheme in 1962 based on nucleic acid type and morphology. Later the International Committee on Taxonomy of Viruses (ICTV) classified phages based on nucleic acid and gross morphology and grouped them into six genera (Ackermann, 2004).

Table 2.1: Overview of phage families (Adapted from Ackermann, 2007).

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	φX174
	2, C, S	*Corticoviridae*	complex capsids, lipids	PM2

Table 2.1: Overview of phage families (continued).

Shape	Nucleic acid	Virus group	Particulars	Example
	2, L	*Tectiviridae*	inner lipid vesicle, pseudotail	PRD1
	2, L	SHI, group*	inner lipid vesicle	SH1
	2, C	STV1 group*	turret-shaped protrusion	STIV
	RNA, 1, L	*Leviviridae*	poliovirus-like	MS2
	2, L, seg	*Cystoviridae*	envelope, lipids	Φ6
Filamentous	DNA, 1, C	*Inoviridae*	a. long filaments	fd
			b. short rods	MVL1
	2, L	*Lipothrixviridae*	envelope, lipids	TTV1
	2, L	*Rudiviridae*	TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*	same, lemon-shaped	SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttaviridae*	droplet-shaped	SNDV
	2, L	*Ampullaviridae**	bottle-shaped	ABV
	2, C	*Bicaudaviridae**	two-tailed, growth cycle	ATV
	2, L	*Globuloviridae**	paramyxovirus-like	PSV

C Circular; *L* linear; *S* superhelical; *seg* segmented; *1* single-stranded; *2* double-stranded

*Awaiting classification

Over the years the edifice of phage classification grew slowly by addition of new families and genera. the current classification of bacteriophage by the International Committee on Taxonomy of Viruses (ICTV) consists of 1 order, 14 families, 37 genera while 5 other potential families waiting for classification (Ackermann, 2009). Over 96 % of all phages defined in the literature are tailed double-stranded (ds) DNA phage and belong to the order *Caudovirales*. The phages under the order *Caudovirales* are further classified into three main large families, *Siphoviridae*, *Myoviridae,* and *Podoviridae*, differentiated by their tail length and contractile ability (Ackermann, 2004). Having long flexible tail, 61 % of the phage under *Caudovirales* falls in

Siphoviridae; 25 % are *Myoviridae* having double-layered contractile tails; and with short noncontractile tails, 15 % are *Podoviridae*. Other types of phages, Polyhedral, filamentous, and pleomorphic phages comprising less than 4 % of observed phages (Ackermann, 2007).

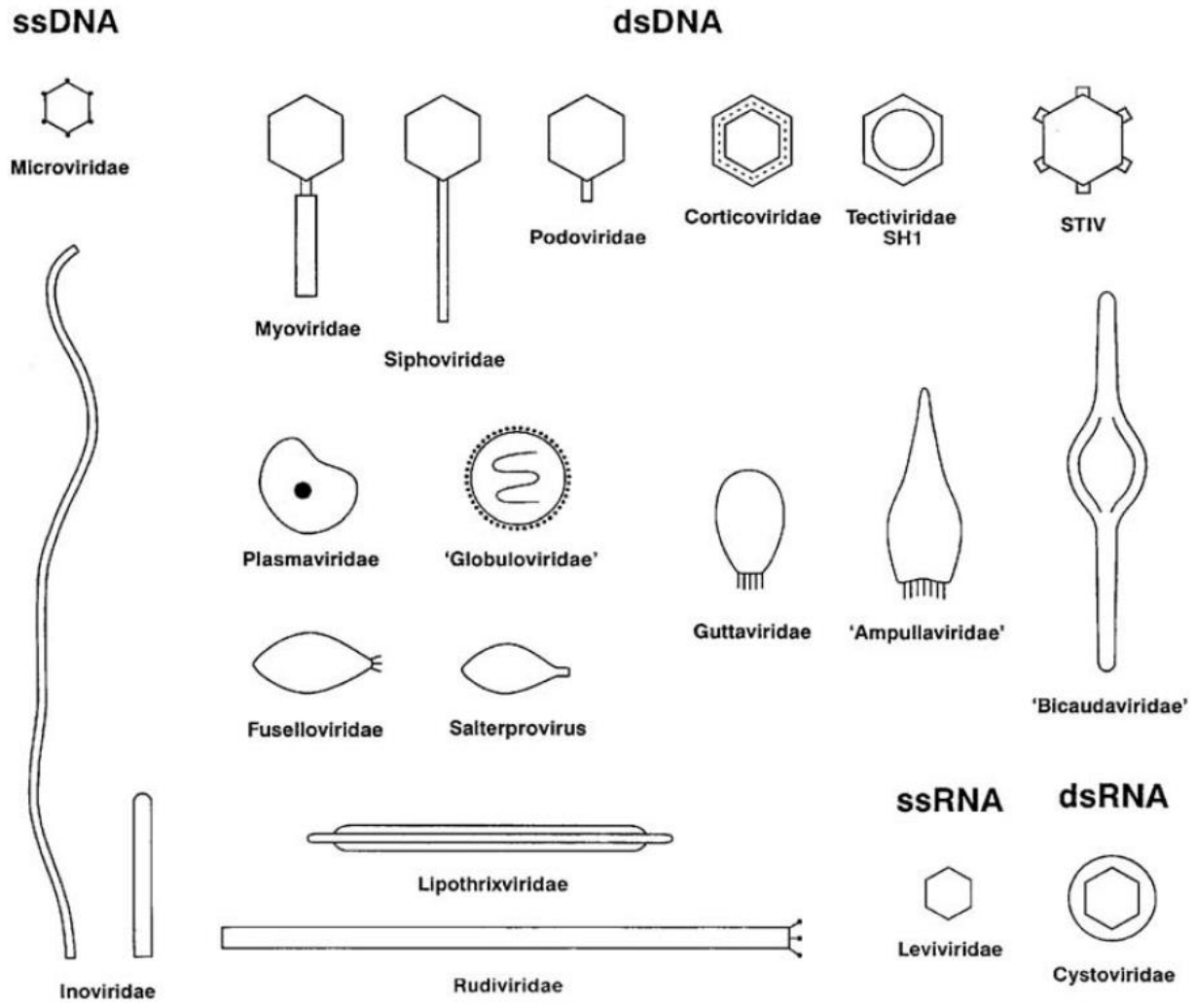


Figure 2.1: Moprphotypes of different bacteriophage family. (Adapted from Ackermann, 2009).

The ICTV taxonomic system requires visualization of the phage structure using electron microscopy. As ICTV ignores genomic information to establish a detailed and specific classification system, its methods are currently reconsidered since it often gives rise to conflicting ideas. For instance, current classification system, on the basis of common characteristics of short tails, places both the *Salmonella* P22 and T3 phage in the *Podoviridae* family (Ackermann et al., 2012). However, it has been demonstrated that p22 phage is gnomically much more related to λ phage (long tailed and belong to family *Siphoviridae*) that functional hybrid of λ-P22 genome in vivo was formed (Byl and Kropinski, 2000). These kinds of contradictory results encouraged scientists to reevaluate the current taxonomic method and to push forward a more molecular basis classification system. Forest Rohwer and Rob Edwards proposed a phage classification system in 2002 based on metagenomics data which is actually identification of phage using genomic data in a cultured independent process. This new method of classification was proposed to rectify the divergence between the current taxonomic system and available genomic data but this has been found to be problematic due to the fact that there is no universal gene specific for different existing phage family (Paul et al., 2002). As of now, there is no proof that shows metagenomics can substitute morphological classification done by electron microscopy as both of them answers a different question.

2.1.3 Bacteriophage abundance in the environment:

Bacteriophages are considered to be the most prominent biological entities on the planet with an estimated population size of 10^{30} or more (Chibani-Chennoufi et al., 2004). Phages have been isolated from different environmental setting such as acidic hot springs (higher than 80°C with pH=3.0), solar salterns (10 times saltier than the ocean), alkaline lakes (pH=10), in the terrestrial subsurface (greater than 2000 m deep), below 30 m of ice in polar lakes (Breitbart and Rohwer, 2005), from soil (Ashelford et al., 2003), sewage sludge (Carey-Smith et al., 2006) and mammalian faeces (O'Flynn et al., 2004).

Marine water is one of the major resources of bacteriophage and several studies indicate a greater variance in overall phage prevalence in these ecosystems. sea water is one of the major reservoirs

of dense phage population (about 9×10^8 virion ml^{-1}) and roughly 70 % of aquatic bacteria are infected by those phages (Ackermann et al., 2012). Phage abundance across aquatic system varies between less than 10^4 $m1^{-1}$ to more than 10^8 ml^{-1} (Wommack and Colwell, 2000). This variation in number is generally correlated with the variation in associated host organism which ultimately depends on the productivity of the system. For instance, in marine system phage abundance is highest in the coastal environments (10^6–10^7 ml-1), intermediate in offshore surface water (10^5–10^6 ml-1) and lowest in the deep sea (104–105 ml-1). It has also been demonstrated that the phage abundance decreases along the transect from coastal to offshore (Weinbauer, 2004).

Bacteriophage prevalence in freshwater is higher than in marine water. In sea ice, phage abundance was reported 10 to 100 times higher than in surrounding water (Maranger et al., 1994). The phage abundance variation also exists in the fresh water system. A study done by Tapper and Hicks (1998) documented that phage prevalence in first 20 μm or the surface microlayer of Lake Superior was 2 to 15 times higher than in 20 m depth. Though benthic viruses were found to be 10 to 1000 times higher than in the overlaying water column, the phage abundance decreased with sediment depth (Weinbauer, 2004).

2.1.4 Life cycle of bacteriophages:

Bacteriophages have multiple possible life cycles which determine their role in bacterial or archaeal biology. Two major phage life cycles are the lytic and lysogenic cycle. Both the life cycle includes 2 common steps: i) absorption of phage i) penetration of genetic material (Salmond and Fineran, 2015). Phage at first will interact with the receptors expressed on the surface of the bacterial cell. Some phages require a cluster of one specific receptor for proper absorption. Whereas, for some phage absorption stage requires different stage involving the different set of receptors such as T4-like phages (Guttman et al., 2004). After irreversible attachment of the phage to the bacterial surface, it injects its genome into the bacterial cell involving mechanism specific for each phage.

The replication strategies following the successful penetration of phage genetic material dictate the virulent (lytic) or temperate nature of bacteriophage. In lytic cycle, injected phage genome take command of the bacterial replication machinery and produce necessary components for new progeny phage. When all the essential components are manufactured, the phage particles are assembled into infective virions. This followed by lysis of the host cell, where the new progeny phages are liberated from the bacterial cell via disruption of the cell wall and cell membrane. In tailed phages, this process is accomplished by two protein lysin and holing. Holin is a natural timer protein which assembles pores in the membrane at an appropriate time to allow lysin to reach peptidoglycan layer and initiate cell lysis. The lytic life cycle results in the destruction of the host cell (Guttman et al., 2004).

Lysogenic cycle is another phage proliferation technique. Here, new phage particles are not immediately produced and the host cell is not destroyed. In this process, phage genome is either integrated into the host genome or prevail as a plasmid within the cell. In lysogenic relationship lytic genes are inhibited by the phage gene product termed a repressor. Due to lytic inhibition, the phage gene will remain integrated into the host chromosome which is called a prophage and will replicate along with the host replication process (Little, 2005).

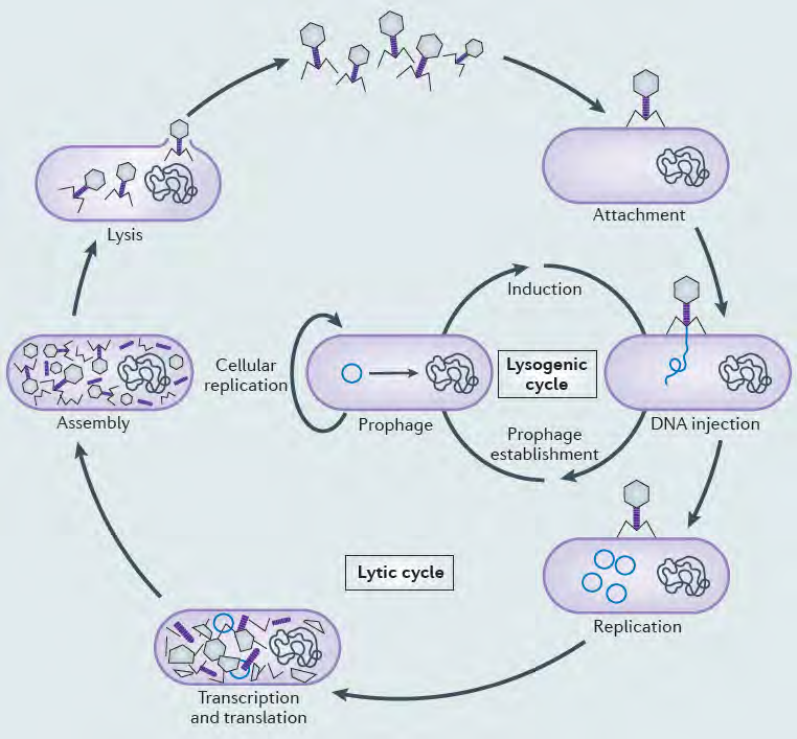


Figure 2.2: Lytic and Lysogenic replication cycle of bacteriophages (Adapted from Salmond and Fineran, 2015).

Though lysogenic phase is really stable it can be switched to initiate the lytic cycle. Temperate phages such as λ phages are able to proliferate via both lytic and lysogenic cycle (Little, 2005). This introduction of lytic cycle in prophage can be induced by various physical and chemical agents such as poly-aromatic hydrocarbons, mitomycin C, hydrogen peroxide, temperature, pressure and UV radiation (Williamson et al., 2001). In order for virulent phages to replicate and survive in the environment, the rate of phage-host encounter required to exceed the virus decay and inactivation rate. However, the temperate phages are not dependent on the host cell density, rather requires a small number of lysogenic carrier cell and occasional induction of lytic cycle and release of free phages (Wommack and Colwell, 2000).

2.1.5 Phage impact on bacterial population:

In recent times, studies on marine biodiversity have shown that bacteriophages influence their host bacterial organism in a density-dependent manner, basically targeting and infecting a few bacterial

species at any one time (Ventura et al., 2011). This is in harmony with "kill-the-winner" model where the predation is directed towards "winner" (abundant) bacterial population in that environment (Rodriguez-Valera et al., 2009). As a result, the nutritional resources are more accessible to other bacterial species and provides the opportunity for a new bacterial species to become abundant. Studies involving genome size distribution have demonstrated that over a period of time specific virus become abundant, then reduce to an undetectable level and then again become abundant (Wommack et al., 1999). A recent study conducted by Middelboe et al. (2009) have demonstrated how phages can drive *Flavobacterium* strain diversification in sea water. Bacteriophages also play a key role in bacterial population inside animal intestinal tract. A Study involving horse feces have shown that diversity and abundance of *E.coli* strains in horse gut are directly correlated to the relative abundance of specific coliphages (Golomidova et al., 2007). Barr et al. (2013) documented that phages adhered to mucus (BAM) of the human gut can provide an innate protection to the underlying epithelial tissue from outside pathogens. Moreover, the presence of prophage in bacteria residing in gut flora provides a competitive advantage to the host which makes the pathogenic organism hard to outcompete commensal organism hence, maintain the stability of human gut microbiome (Ventura et al., 2011).

2.1.6 The impact of bacteriophages on host fitness:

Bacteriophage plays a key role in bacterial evolution and augmenting survivability by altering genetic combination. Temperate phages can carry bacterial genes from one cell to another via transduction method. Many temperate phages integrate at a specific site in bacterial genome and during the transition to lytic growth, they often mistakenly cut out a portion of bacterial DNA along with prophage which may later get incorporated into another bacterial cell. Hence, changing the cells genomic composition by specialized transduction process. Other phages, such as Mu, are able to alter host genome by generalized transduction method where prophages are integrated randomly in the host cell genome and then always carry some host DNA with them (Guttman et al., 2004). This phage-mediated gene mobility increases the possibility of gene maintenance in bacterial population (Miller, 2001) and increases the chance of genes spread that are costly but advantageous such as gene rendering antibiotic resistance and gene for xenobiotic compound degradation (Abedon and LeJeune, 2007).

Table 2.2: Examples of bacteriophage-encoded virulence factors involved in various stages of bacterial pathogenesis (Adapted from Fidelma Boyd, 2004).

Bacterial Host	Bacteriophage	Virulence Factor (gene)	Function of gene product	Reference
S. mitis	SM1	Coat protein (pblA, pblB)	Required for host attachment	(Bensing et al., 2001)
S. flexneri	Sf6	O-antigen (oac)	Alter antigenic recognition	(Clark et al., 1991)
S. pyogenes	H4489A	Hyaluronidase (hylP)	Involved in cellular invasion	(Hynes and Ferretti, 1989)
E. coli	λ	OMP (bor)	Required for intracellular survival	(Barondess and Beckwith, 1990)
C. botulinum	C1	Neurotoxin (c1)	Extracellular toxins	(Barksdale and Arden, 1974)
C. diphtheriae	ß-phage	Diphtheria toxin (tox)	Extracellular toxins	(Freeman, 1951)
V. cholerae	CTXΦ	Cholera toxin (ctxAB)	Extracellular toxins	(Waldor and Mekalanos, 1996)
E. coli	H-19B	Shiga toxin (stx-1)	Extracellular toxins	(Neely and Friedman, 1998)
S. enterica	Fels-1	Neuraminidase (nanH)	Putative virulence factors	(Figueroa-Bossi et al., 2001)

It has been documented several times that various bacterial virulence factors agents contributing to the emergence of infectious disease in eukaryotes are encoded by integrated prophage (Brüssow et al., 2004). In this phage conversion process, bacteriophage encoded genes convert nonpathogenic host bacteria to pathogenic one or amplify their existing virulence by providing a mechanism for invasion of host tissue and the avoidance of host immunity system (Fidelma Boyd, 2004). One of the most known examples of this phage conversion occurs in *Vibrio cholerae*, a generally harmless bacteria that become highly virulent by incorporating phage cholera toxin

(ctxAB) genes in the genome (Waldor and Mekalanos, 1996). There are other human bacterial infectious diseases such as botulism, diphtheria, and disease associated with Shiga Toxigenic *Escherichia coli* (STEC) caused by endotoxin respectively botulism toxin, diphtheria toxin, and Shiga toxin which are expressed from phage-encoded genes (Abedon and LeJeune, 2007). Many other examples of virulence inducing genes are known including genes that help in host attachment, altering antigenic recognition, cellular invasion, and intracellular survival (Table 2.2).

2.1.7 Bacteriophage application:

Once the potential of bacteriophage as antibacterial agents was realized following the initial discovery and characterization in the early twentieth century, there was a flurry of interest in phages using them as therapeutic tools. However, poor understanding of the biological mechanism of phage activity and subsequent discovery and general application of broad-spectrum antibiotics in the late 1930s and 1940s, interest in the therapeutic use of bacteriophage declined and for many years was only considered as a research tool in molecular biology (Clark and March, 2006).

Bacteriophages are now again reconsidered as an antimicrobial tool due to the current upward trend of bacterial resistance and availability of necessary molecular techniques and tools to precisely assess the safety and efficacy of using phage, thanks to the advancement of modern biotechnology. They are also being evaluated as a delivery vehicle for gene therapy, as a biocontrol agent, uses in the development of phage-derived vaccine and in phage display technique.

2.1.7.1 Phage Therapy:

Using bacteriophages as therapeutic agents over antibiotics has many advantages, but there are some concerns also. Phages have been used in the treatment of plant, animal and human beings with varying degree of success. Phages host specificity acts as an advantage since it is less likely to interfere with the natural flora of host. It has been reported that after administration, phages dissipate swiftly through the body and reach most organ (Dabrowska et al., 2005). However, having protein and/or lipid structure, phages can elicit an immune response which can result in quick removal of phages from circulation. There are studies going on to resolve this problem.

There are proposals of using phage lytic enzymes rather than using whole phage (Fischetti, 2005) or using genetically modified non-lytic phage to deliver bactericidal protein-encoding DNA to pathogenic bacteria (Westwater et al., 2003).

2.1.7.2 Phage Display:

In phage display method, a DNA encoding desired peptide or protein is ligated with phage coat protein gene which ultimately is expressed on the surface of bacteriophage (Clark and March, 2006). Using this technique a library can be generated and screened to isolate proteins or peptide with particular application in mind. It can be used to isolate protein with high affinity that can act as a diagnostic tool in detection of pathogen or agents posing a biological threat (Petrenko and Vodyanoy, 2003). Phage-display library can also help in identifying the protein with enhanced enzymatic activity by screening a library of proteins with a randomly altered active site (Fernandez-Gacio et al., 2003). Another phage-display technique has been used to treat cocaine addiction in a rodent model. Here, antibody fragments are expressed on the phage surface and when phages were administrated nasally they traveled to the central nervous system (CNS) and bound with the cocaine molecule, effectively prevented the action of cocaine on the brain (Dickerson et al., 2005).

2.1.7.3 Phages as vaccine delivery vehicle:

Bacteriophages have been used as transport for vaccine delivery in two ways: one is directly vaccinating with phages expressing vaccine antigens on their surface and another is by incorporating a DNA vaccine expression cassette into phage genome and using the phage particle to deliver that DNA cassette (Clark and March, 2004). In phage-display vaccination method, the target antigen can either be generated by transcriptional fusion to coat protein or by artificially conjugating antigen protein to the phage surface which enables broad range antigen display ability (Molenaar et al., 2002). It has been demonstrated that unmodified phages deliver DNA vaccine more efficiently compared to standard DNA vaccine procedure as phage coat protein protects the DNA vaccine more efficiently and shows greater antibody response (Clark and March, 2006).

2.1.7.4 Phage Typing:

The specificity of phages for bacterial cells enables them to be used as a diagnostics tool for detection of bacterial species and typing of the bacterial cell. For these purposes, several methods can be employed such as delivery of reporter gene (e.g. lux or green fluorescent protein) using phages that would be expressed after successful infection of target bacteria (Funatsu et al., 2002; Kodikara et al., 1991). Another method involves detecting specific absorption of phage that had fluorescent dye covalently attached to its surface (Goodridge et al., 1999; Hennes et al., 1995). Detection of the cellular components that are released after bacterial lysis caused by phages specific to those bacteria, such as adenylate kinase provides an alternative way for identifying pathogenic bacteria (Corbitt et al., 2000). Phage amplification assay is another technique that has been extensively used to detect virulent bacteria such as *Salmonella, Pseudomonas, Mycobacterium tuberculosis, E.coli, Campylobacter* and *Listeria* species (Barry et al., 1996).

2.1.8 Current state of phage therapy on human:

Although phages have been explored intensively for therapeutic purpose in the Western countries over the last few decades and even before that in Eastern European countries, currently there are no phage products for humans that have either been approved by governmental agencies or are in phase-III clinical trials in the European Union or the USA (Viertel et al., 2014). However, they are several placebo-controlled clinical human trials are going on to demonstrate the safety of phages. For a long time, phages have been used for the treatment of Dysentery, dyspepsia, salmonellosis, enterocolitis, colitis and for other bacterial infections in human in the eastern part of Europe mainly in Georgia and Poland. However, results of much of those trials were published in non-English literature hence the knowledge was confined within the regional researchers. Additionally, most of these trials were non-randomized uncontrolled trials (Wittebole et al., 2014). Even though those trials didn't report any adverse effect on human, scientist around the world demanded systemically designed controlled trials to prove the efficacy and safety of phages. In 2009, the first phase-I randomized controlled trial conducted in the United States was published. The study carried out on 42 patients with a chronic venous leg ulcer, used phage cocktail directed against *Pseudomonas*

aeruginosa, *E. coli,* and *S. aureus*, did not find any adverse effect of the treatment (Rhoads et al., 2009).

Another double blinded placebo-controlled study was done in the United Kingdom on patients suffering from chronic *Pseudomonas aeruginosa* related otitis. Patients were treated with phage solution containing a mixture of 6 bacteriophages. The group treated with phage reported lower intensity of symptoms such as discomfort, wetness, unpleasant odor and itching and the physicians in charge reported decreased clinical observation such as inflammation (Wright et al., 2009). At the Burn Wound Centre of the Queen Astrid Military Hospital, Brussels, Belgium, a small scale phase-I study of 9 patients was performed. A large burned section of the patients was treated with BFC-1 phage cocktail containing 3 lytic phage and no adverse events were reported (Wittebole et al., 2014).

In Bangladesh, a phage therapy study funded by Nestlé Research Center was conducted where 15 healthy adults were orally administrated a mixture of phages containing nine different T4 phages against E. coli diarrheal strains. No adverse effects were observed even after giving high phage titer of 3×10^9 pfu dose. The phages did not multiply inside healthy humans due to the absence of the pathogenic host bacteria (Sarker et al., 2012).

2.1.9 Current state of commercial phage based product:

The recent surge of interest in bacteriophage has led many companies to innovate novel phage-based product. Currently, several biotechnological companies all over the world are developing and testing phage derived product, some of which are already approved by the government and available to use while others are in different stage of development, trials, and licensing (Table 2.3). For example, ListShield™ (formerly LMP-102) a bacteriophage-based product invented by Intralytix Incorporated (USA) was approved by the Food and Drug Administration (FDA) on August 18, 2006. The product contains six individually purified phages infective against *Listeria monocytogenes*. ListShield™ was permitted to use on Ready-To-Eat (RTE) meat and poultry products and was reported to be effective against 170 strains of *Listeria* (Housby and Mann, 2009). It was also approved by US EPA for application on surfaces in food facilities and other

establishments. A similar phage-based product named Listex^{TM} P-100 also targeting *Listeria monocytogenes* was developed by EBI Food Safety, a food safety phage company located in Wageningen, The Netherlands. The product was accepted by US FDA in 2006 under GRAS (Generally Recognized As Safe) status.

Table 2.3: Commercial phage-based products (Adapted from Basdew, 2012).

Product	Target organism	Company	Country
AgriPhage™	*Xanthomonas campestris* pv. vesicatoria or *Pseudomonas syringae* pv. tomato	Omnilytics www.omnilytics,com	Israel
BioTector	*Salmonella* spp. in poultry	CheiIJdang Corporation http://eng.cjcheiljedang.com/	China
EcoShield™	*Escherichia coli* in foods and food processing facilities	Intralytix www.intralytix.com	USA
FASTPlaque-Response™	Detection of rifampicin resistance in *Mycobacterium tuberculosis*	Biotech Laboratories www.biotec.com	United Kingdom
FASTPlaqueTB™	Detection of *M. tuberculosis*	Biotech Laboratories www.biotec.com	United Kingdom
ListShield™	*Listeria monocytogenes* in foods and food processing facilities	Intralytix www.intralytix.com	USA
LISTEX™ P100	Targets *L. monocytogenes* strains on food products	EBI Food Safety www.micreosfoodsafety.com	Netherland
MRSA/MSSA blood culture test	Detects *Staphylococcus aureus* methicillin resistance/susceptibility	Microphage www.microphage.com	USA
MRSA screening test	MRSA	Microphage www.microphage.com	USA
MicroPhage MRSA/MSSA test	Differentiation of methicillin resistant (MRSA) and methicillin-susceptible (MSSA)	Microphage	USA
Bacteriophage DNA vaccines	Bacteriophage DNA vaccination via phage encoded DNA delivered intravenously or orally	BigDNA (http://www.bigdna.com/)	UK

Although phage therapy has not been yet permitted for treating human disease in the USA, there are companies In Georgia such as Biochimpharm, who prepare and market various phage centric liquid preparation and tablet for a wide range of bacterial infectious disease and are sold as over the counter drug. There are several other phage centered products being developed by various companies that are in different stage of clinical trials. For instance, GangaGen Inc., a clinical-stage biotechnology company that is trying to develop phage solution capable of treating antibiotic resistant infections in human (Housby and Mann, 2009).

2.1.10 Advantages of using phage therapy:

Loc-Carrillo and Abedon, (2011) denoted some bacteriophage characteristics that can give them an upper hand in therapeutic circumstance over chemical antibiotics. These properties are as follows:

1. Bacteria infected by obligate lytic phages are not capable of regaining their viability (Carlton, 1999). Whereas, some antibiotics such as chloramphenicol and tetracycline are bacteriostatic, i.e., those antibiotics do not kill the bacterial cell, which allows bacterial organism become resistant to those antibiotics (Stratton, 2003).
2. During the bactericidal (lytic) replication process phages increase in number mainly where host are abundant. Though there are some limitations associated with this such as requirement of relatively high bacterial density (Abedon and Thomas-Abedon, 2010; Kutter et al., 2010; Skurnik and Strauch, 2006), it can be noted as auto “dosing” since phages themselves contribute to determining phage dose (Abedon and Thomas-Abedon, 2010).
3. Phages are inherently non-toxic as they are generally composed of protein and nucleic acid (Abedon and Thomas-Abedon, 2010; Kutter et al., 2010; Skurnik et al., 2007). In theory, they can induce a harmful immune response. However, in practical application, there was not enough evidence to support this idea (Carlton, 1999; Kutateladze and Adamia, 2010).
4. Phages being able to infect only a few strains of a specific bacterial species or rarely infecting closely related bacterial genus (Hyman and Abedon, 2010), is an advantageous feature since they most likely will not affect the normal flora bacteria (Gupta and Prasad,

2011; Skurnik et al., 2007). On the other hand, treatment involving broad-spectrum antibiotics not only disrupt the beneficial bacteria but also often lead to superinfection e.g., antibiotic-associated *Candida albicans* yeast infections and *Clostridium difficile* colitis (Carlton, 1999).

5. Having comparatively narrower host range confines the bacterial types that can evolve phage specific resistance mechanism contrasting with the wide range of bacterial species that can be affected by most chemical antibiotics and lead to antibiotic resistance (Carlton, 1999). Moreover, often in the process of acquiring phage resistance, bacterial organism lose their virulence when bacteria cell remove their phage receptors from the surface which are important component in their pathogenicity (Capparelli et al., 2010; Skurnik and Strauch, 2006).
6. Because of the fact that phages lyse bacterial cell in a mechanism that is different from the chemical antibiotic, the evolution of antibiotic resistance doesn't correlate with the phage resistance. Therefore, phages can be used to treat infections caused by antibiotic resistant strain such as multi-drug resistant *Staphylococcus aureus* (Gupta and Prasad, 2011; Mann, 2008).
7. It was observed that Biofilms are significantly more resistant to chemical antibiotics than planktonic bacterial cells (Abedon et al., 2017). However, in some cases, phages were documented to have the ability to clear biofilms. Some phages have the ability to produce exopolymer-degrading depolymerase which helps them to degrade the outer surface of the cell and easily access the cell membrane to infect and replicate (Chaudhry et al., 2017).
8. Bacteriophages can be versatile in formulation development and application form. They can be administrated along with antibiotics, which was found to be more effective in some instance than any single treatment (Chaudhry et al., 2017). Phages can be applied in different forms, such as cream, liquid, impregnated in solid etc. Additionally, phages are suitable for the most route of administration (Carlton, 1999; Krylov, 2001; Kutateladze and Adamia, 2010; Kutter et al., 2010). Different phages with different host specificity can be mixed to widen their range of antibacterial activity for the formulation (Goodridge, 2010; Kutter et al., 2010).
9. Because phages will not infect all the bacterial species due to their specific host range, discarded therapeutic phage will have a less environmental impact, unlike road spectrum

antibiotics which will interact with all the organism it comes in contacts with (Ding and He, 2010). Moreover, phages are susceptible to various environmental factors such as high temperature, desiccation or sunlight which can quickly inactivate them.

10. The phage production involves multiplication of phage in host bacterium by culturing and subsequent purification (Gill and Hyman, 2010). Depending on the host bacterium, the cost of host growth varies but as the technology advances the cost of phage purification is expected to be going down (Kramberger et al., 2010). The general cost of phage production per unit is no that atypical compared to pharmaceutical production while isolation and characterization of potential therapeutic phages can be relatively low (Skurnik et al., 2007).

2.1.11 Drawbacks associated with phage therapy:

1. Narrow host range can be a disadvantage as the specific phage might not be able to constantly lyse all the pathogenic strains of that certain infection. There are several options to circumvent this problem: using phage with broad host range (Ross et al., 2016), using host range mutant bacteriophage (Flaherty et al., 2001) or using a mixture of different phages (Chan et al., 2013).
2. An unpurified phage preparation can cause several biological effects during phage therapy. Phage multiplication using host cell is a primary step for phage production. During cell lysis, lipopolysaccharide, a component of the cell wall of gram-negative bacteria are released. Lipopolysaccharide acts as an endotoxin and if they are present in high concentration then they can trigger a coagulation cascade, modify hemodynamics, invoke fever, endotoxic shock, and hypotension (Boratyński et al., 2004). Purifying phage preparation using chromatography and ultrafiltration can produce endotoxin-free preparation (Boratyński et al., 2004).
3. Not all phages can be used for therapeutic propose. Only obligate lytic phages that lyse the bacterial cell directly instead of integrating its genome in bacterial DNA (temperate) are usable for phage therapy. Temperate phages play a major role in the exchange of genetic material between different bacterial strain (Rice et al., 2009) and often they contribute to the pathogenicity. Some known examples are Cholera toxin from CTXΦ phage and Shiga toxin from H-19B phage acquired by *Vibrio cholerae* and *E.coli* respectively. Additionally,

The origin of some bacteriocins (Daw and Falkiner, 1996) and even the bacterial pili (Karaolis et al., 1999) points towards phages.

4. Development of phage-resistant mutation can make the phage therapy unproductive. However, using phage cocktail (a mixture of phages) that uses different cell receptors can restrain rise of phage resistance (Tanji et al., 2004).
5. Phage inactivation by human serum can pose a limitation in phage therapy. Some studies have indicated inactivation of phage by human serum (Jerne and Avegno, 1956; Kallings, 1961) while other experiments have documented little or no inactivation (Curtin and Donlan, 2006; Fu et al., 2010) or inactivation after a long period of incubation (Kucharewicz-Krukowska and Slopek, 1987).
6. The broad negative concept towards viruses may play a key role in the acceptance and popularity of phage-based product among the general public. Even though some phage centered product has been accepted by US FDA as GRAS (Generally Regarded As Safe) (Kutter et al., 2010), phages as 'viruses' can be misunderstood by common people being equivalent to pathogenic viruses that cause various human disease. Clearly differentiating bacteriophages from other pathogenic viruses is a must to avoid public resistance which until now has not yet capitalized maybe due to reason that bacteriophages are mostly known as phages instead of as a bacterial virus to the majority of the people.

2.2 *Klebsiella pneumoniae:*

Klebsiella pneumoniae is a rod-shaped, non-motile, encapsulated and facultative anaerobic Gram-negative bacterium that is regularly found in the flora of skin, mouth, intestines and also in surrounding environment. (Wu and Li, 2015). *Klebsiella pneumoniae*, is a member of the group of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) that are capable of escaping the bacteriocidal action of antibiotics and are more prone to develop antimicrobial resistance (AMR) (Pendleton et al., 2013). It is the most important clinical member of the genus *Klebsiella* which belongs to the family *Enterobacteriaceae* and is the third most frequently isolated microorganism in blood cultures from sepsis patients that can cause severe epidemic and endemic nosocomial infections (Wu and Li, 2015). *K. pneumoniae* is an opportunistic pathogen and majority of the infection occurs in immunodeficient patients, infants and the elderly. Patients who are already hospitalized for other conditions are particularly vulnerable to *K. pneumoniae* infections, and the risk of being colonized increases as the duration of hospital stay increases (Podschun and Ullmann, 1998). *Klebsiella pneumoniae* is found to be developing rapid resistance against various antimicrobial agents, including broad-spectrum cephalosporins and β-lactams (Gouby et al., 1994). Though there are several mechanisms that lead to antibiotic resistance, the emergence of *Klebsiella pneumoniae* carbapenemase (KPC) (Yigit et al., 2001) and the New Delhi Metallo-beta-lactamase (NDM) (Kumarasamy et al., 2010) poses some serious threat especially in nosocomial infections (Daikos and Markogiannakis, 2011).

2.2.1 Clinical significance:

Klebsiella induced infections, occur almost exclusively in the weak patient subgroup and include pneumonia, urinary tract infections primary and secondary bacteremia, bronchitis, meningitis, and surgical wound infections (Cryz, 1994). *Klebsiella* was found responsible for being the third most common cause of all types of lower respiratory tract infections and primary bacteriemia as shown by the Nosocomial Infection Surveillance System (Horan et al., 1986). *Klebsiella* is a prominent cause of pneumonia among, chronic care, elderly nursing home and community patients (Garb et al., 1978).

The spread of *Klebsiella pneumoniae* in the hospital environment is influenced by the fact that humans play the reservoir role for the pathogen (Jarvis et al., 1985; Montgomerie, 1979). Major studies have denoted that *Klebsiella* is excreted for a very short period of time, indicating that it is not part of the normal gut flora (Cryz, 1994). Carriage rates increase significantly among patients hospitalized, particularly those receiving antibiotics (Rose and Schreier, 1968). Prior infection *K. pneumoniae* colonizes in the intestinal tract (Selden et al., 1971; Smith et al., 1973). A study found that infection rate increased to 45 % in colonized patients from 10 % in non-colonized patients (Cryz, 1994). Nasopharyngeal carriage in the general people has been documented at 1 % to 6 % and approximately 10 % of the severe patients carry *Klebsiella pneumoniae* in their upper respiratory tract (Montgomerie, 1979). This colonization rate increases to 100 % for premature infants and neonates. The antibiotic administration also increases the rate of *Klebsiella* presence. The respiratory tract was the second most common portal of entry associated with *Klebsiella* bacteremia (García de la Torre et al., 1985).

Common-source outbreaks as a result of *Klebsiella* have been traced back to a variety of materials, including breast milk and lipid emulsions fed to infants, blood sampling probes, hand cream, and nebulizer solutions (Jarvis et al., 1985). A major find was that in three of those outbreaks, the common source of contamination was traced to the hands of medical personnel which is not unexpected given it is known that hospital staff sometimes carry *Klebsiella* on their hands which can transfer and infect patients (Garb et al., 1978). The mortality rate associated with *Klebsiella* induced pneumonia and bacteremia varies between 24 % and 48 % (Haddy et al., 1987; Kreger et al., 1980). Factors adversely influencing outcome include (a) an underlying disease state characterized as rapidly fatal; (b) insufficient antibiotic therapy; and (c) when the pulmonary tract or abdomen harbors the foci of infection (García de la Torre et al., 1985; Jay, 1983).

2.2.2 *Klebsiella pneumoniae* virulence factors:

When it comes to virulence factors, *K. pneumoniae* have quite a few of them up its sleeve. These include capsular polysaccharide (CPS), lipopolysaccharide (LPS), Adherence factors,

Siderophores, Urease, Outer-membrane proteins and Biofilms (Table 2.4). Because of the collective threat of several virulence factors, *K. pneumoniae* has attained superbug status and is one of the most common antibiotic-resistant bacteria (Wu and Li, 2015).

2.2.2.1 Capsular polysaccharide:

Like few other bacteria, *K. pneumoniae* contains a polysaccharide 'coat', either as a distinct layer of exopolysaccharide called a capsule or as a lightly attached slime layer. Capsule polysaccharide (CPS) has the ability to form biofilm using electrostatic and Van der Waals forces and specific ligand-receptor interactions (Costerton et al., 1995). Moreover, the capsule structure provides extra protection from environmental threats, such as dryness or heat (Dutton et al., 1989; Ophir and Gutnick, 1994). The capsule covering the bacterial surface can also protect the microbe from host immune machinery in vivo, such as the serum bactericidal molecules (Tomás et al., 1986). Additionally, it was observed that neutralization of the bactericidal effect of some antibodies occurred by released capsular polysaccharide from the bacterial surface (Baer and Ehrenworth, 1956). Till now, around 80 types of capsule have been identified for *K. pneumoniae* but only some of the capsules were found to be correlated with the severity of infection (Wu and Li, 2015). Clinical isolates of *Klebsiella* strains generally produce a large amount of CPS on the cell surface. It is estimated that K2 antigen is a major virulence associated antigen based on the point that K2 serotype of *K. pneumoniae* is highly clinically isolated. K1 serotype *K. pneumoniae* was found particularly to cause the pyogenic liver abscess (PLA) (Chuang et al., 2006). Serotype K1 strains may contain magA (mucoviscosity associated gene A) in the cps locus of NTUH-K2044, a hyper mucoviscosity strain, hence magA is considered a virulence factor (Wu and Li, 2015). Although the overall genomic structure is highly conservative among different pathogenic strains of *K. pneumoniae*, the genetic distinction of the polysaccharide compound and linkage facilitates a great diversity of the CPS molecules that may help to escape host immune system.

Table 2.4: Major virulence factors of *K. pneumoniae* (Adapted from Wu and Li, 2015).

Name	Activity in Virulence
Capsular polysaccharides	Evade phagocytosis
LPS	Evade serum killing factors
Adherence factors	Adhere to host surface
Siderophores	Acquire iron
Urease	Allow growth in urinary tract
Antibiotic resistance	Tolerate antibiotics
Outer-membrane proteins	Resist antibiotics
Biofilms	Survive in harsh host

2.2.2.2 Lipopolysaccharide:

Another crucial surface component of *K. pneumoniae* is lipopolysaccharide (LPS, the O antigen) (Domenico et al., 1989). Lipopolysaccharide of *K. pneumoniae* is made of three structural domains: (i) the hydrophobic lipid A, a major component of the outer leaflet; (ii) the core oligosaccharide, which is linked to lipid A and to O antigen; and (iii) the critical O antigen (O chain), a long chain species of LPS providing antigenic epitope and inducing humoral immunity (Vinogradov et al., 2002). The lipid A attaches the LPS molecule into the outer membrane and itself is also an endotoxin, which can stimulate the immune system mainly as an agonist of Toll-like receptor 4 (TLR4). TLR4 is generally expressed on the surface of dendritic cells, macrophages, epithelial cells and other cell types. Binding of TLR4 initiates cell signaling cascades which result in the production of pro-inflammatory cytokines (Alexander and Rietschel, 2001). The O antigen and the lipid A linking core region contains a small number of mono di or oligosaccharide. *K. pneumoniae* was found to have 11 different O antigen. The binding of complement(s) to the bacterial surface is affected by the structure of LPS which also influences the serum sensitivity for different strain (Ciurana and Tomás, 1987). Over 90 % of the clinical isolates from Denmark, Spain, and the US are O antigen positive, majority of which were O1 serotype (Hansen et al., 1999; Jenney et al., 2006). Generally, the structure of LPS helps *K.*

pneumoniae to escape from the complement killing effects which helps the bacterium spread throughout the body. Smooth LPS strain of *K. pneumoniae* cannot bind complement C1q, allowing them to avoid the complement activities. Despite the serum-resistant O1 serotype can bind C3b, it cannot activate the C5b-9 membrane attack complex (MAC). As a result, these pathogens cannot be killed by the alternative complement pathway (Albertí et al., 1996).

2.2.2.3 Adherence factors:

Like many other enteric pathogens, *K. pneumoniae* produces a range of different adherence factors on the surface (Podschun et al., 1987). These adherence factors, particularly type 1 and type 3 pilior fimbriae, are associated with biofilm formation (Di Martino et al., 2003; Fader et al., 1979). During the virulent attack, the type 1 and type 3 fimbriae-proteinaceous structures facilitate adhesion of the bacterium to the epithelial cells. Both clinical and environmental isolates of *K. pneumoniae* express type 3 fimbriae (Di Martino et al., 2003) which gives bacterial cell the ability to adhere different types of cell including human urinary bladder cells, endothelial cells, trypsinized buccal cells, tracheal cells, and respiratory cells (Fader et al., 1979; Favre-Bonte et al., 1999; Fortis et al., 1998). Clinical *K. pneumoniae* isolates can be found adhered to different antibiotic surfaces in the hospital environment (Podschun and Ullmann, 1998). Moreover, *K. pneumoniae* adhered to cardiac valve prostheses and to urinary and intravenous catheters was also identified (Khardori and Yassien, 1995; Stewart and Costerton, 2001). The production of adhesin that mediates adherence to intestinal cells is an important phase in *K. pneumoniae* pathogenesis. Carrying adhesin genes on large plasmids associated with antibiotic-resistance determinants indicates a selective advantage for bacteria containing such plasmids. Due to the significance of fimbriae for attachment to host cells during infection, vaccines based on these structures have displayed some protection against later pathogen invasion (Wu and Li, 2015).

2.2.2.4 Siderophores:

Klebsiella produces and secretes iron-chelating compounds to obtain iron from the host iron-binding proteins which is similar to other *Enterobacteriaceae*. Obtaining iron is crucial for *Klebsiella* pathogenesis in vivo as iron is a cofactor for bacterial metabolism and depleted iron may also hinder host immune cells. These iron-binding compounds, called 'siderophores', are usually released under iron-limiting conditions. As soon as the iron binds to the siderophores, the bacterial surface receptors recognize the complex and the bacterial cell reabsorbs it. *K. pneumoniae* produces two different types of siderophores: one is Enterochelin and the other is Aerobactin (Koczura and Kaznowski, 2003; Neilands, 1995). When iron is scarce, *Klebsiella* cannot produce certain outer-membrane proteins which are generally produced in high amount under iron-rich conditions (Lodge et al., 1986). Therefore, some of these outer-membrane proteins could take part in uptake mechanism for exogenous siderophores (Williams et al., 1989).

2.2.2.5 Urease:

Most of the *K. pneumoniae* isolates that cause urinary tract infections (UTI) can produce urease as a virulence factor (Mobley et al., 1995). Urease-producing strain of *K. pneumoniae* can sometimes cause severe catheter encrustation during infection (Wu and Li, 2015). The microorganism hydrolyzes urea into ammonia which elevates the pH of urine, procedures calcium and magnesium phosphates crystals in the urine and develops biofilm on the catheter (Broomfield et al., 2009).

2.2.2.6 Outer-membrane proteins:

Multiple-antibiotic-resistant mutants of *K. pneumoniae* may show altered levels of outer-membrane proteins. These mutants may evolve spontaneously and demonstrate resistance to a variety of antimicrobials, including trimethoprim, nalidixic acid, chloramphenicol and β-lactams (Wu and Li, 2015). Additional outer-membrane porin proteins associated with antibiotic resistance have been documented in clinical isolates of *K. pneumoniae* (Gutmann et al., 1985; Pangon et al., 1989). Mutation in OmpK35 and OmpK36, two major outer membrane porins, in *K. pneumoniae* resulted in heightened antimicrobial resistance, strong resistance to serum killing, high susceptibility to neutrophil phagocytosis, and decreased virulence (Tsai et al., 2011). Carbapenem

resistance resulted due to the loss of OmpK35 and/or OmpK36 coupled with a DHA-1 AmpC β-lactamase and was associated with poor clinical outcome (Chen et al., 2010).

2.2.2.7 Biofilms:

Bacterial biofilms are regularly observed at the site of persistent infections (Myllymaa et al., 2013) adhered to tissue surfaces (Aparna and Yadav, 2008), suggesting that biofilms are associated with virulence and chronic infection. In the proximity of foreign bodies, immune responses are impaired making the medical implants susceptible to biofilm (Eberhard et al., 2009). Biofilms were found to shorten the lifetime of many medical devices (Heuer et al., 2011) and a major cause of implant failure (Mombelli and Décaillet, 2011). Extracellular polymeric substances residing in the biofilm protect bacteria from opsonization and phagocytosis, making treatment highly difficult (Günther et al., 2009). In vitro experiment have demonstrated that *K. pneumoniae* in biofilms are less susceptible to antibiotics compared to their planktonic equivalents (Singla et al., 2013). Treatment of a biofilm established infection with current medication is often ineffective (Reid and Bailey, 1996). In some cases, the only way to solve this problem is to mechanically remove the biofilm or implant which is not only costly but also traumatic to patients (Reid and Bailey, 1996; Smeets et al., 2003). The mechanism of biofilm formation is not completely understood but biofilm formation is important for the pathogenesis of *K. pneumoniae*, especially for antibiotic-resistant plasmids caring strains (Hennequin et al., 2012). Type 3 fimbria was characterized as an accessory for enabling biofilm formation on biotic and abiotic surfaces (Alcántar-Curiel et al., 2013).

2.2.2.8 Antibiotic resistance:

When taking about virulence factors of a pathogen, the mechanism allowing the bacteria to grow in presence of high concentration of antibiotic must be considered (Bauernfeind et al., 1989; Reig et al., 1993). The evolution of extended spectrum beta lactamase (ESBL) producing *K. pneumoniae* is becoming a serious concern as it reduces the available effective therapy. ESBLs contain three major genetic groups: SHV, TEM, and CTX-M types (Chong et al., 2011). Several mobile genetic elements, such as insertion sequences, transposons, and integrins are associated with the spread of ESBL genes. The SHV-type β-lactamase is the most common enzyme

attributing to *K. pneumoniae* drug resistance (Jacoby, 1994). Often times the antibiotic resistance occurs due to increase gene expression resulting in a high-level production of β-lactamase (Wu and Li, 2015). This is suspected to occur because of mutation in the promoter region where a single base difference in the promoter region of one family of ESBL may create dual overlapping promoters, resulting in a 4 to 30 fold increased expression of the enzyme (Jacoby, 1994). The second class of β-lactamase is generally associated with plasmid from clinical isolates of *K. pneumoniae* (Gonzalez Leiza et al., 1994). The enzyme encoded by this β-lactamase gene actually has two functional isoforms. Genetic analysis directs toward altered sequence recognition by the bacterial signal peptidase as a viable reason for the existing differences between the two active enzymes.

The AmpC β-lactamases are clinically important cephalosporinases integrated on the chromosomes of *K. pneumoniae*, giving the organism resistance ability to various antibiotics: cefoxitin, cefazolin, cephalothin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. AmpC enzymes are inducible and Integration of these enzymes renders increased drug resistance which can be triggered by therapy. Transmissible plasmids can transfer the resistance by delivering genes required for AmpC enzymes to other bacteria that lack or poorly expresses a chromosomal *bla* (AmpC) gene (Wu and Li, 2015).

2.2.3 Global spread of antibiotic resistant *K. pneumoniae*:

Antimicrobial resistance in microbial population is on the rise, aided by the inappropriate usage of the chemical antibiotics in humans, livestock and poultry resulting in the rapid emergence of resistance or even multi-drug resistant strain. Not only the rate of antimicrobial resistance is increasing but also the range of antibiotic resistant infections is broadening world-wide (Hegreness et al., 2008). A study done by Sanchez et al. (2013) documented a significant increase in resistance over a decade In the United States for *K. pneumoniae* for almost all antibiotics except tetracycline (Figure 2.3).

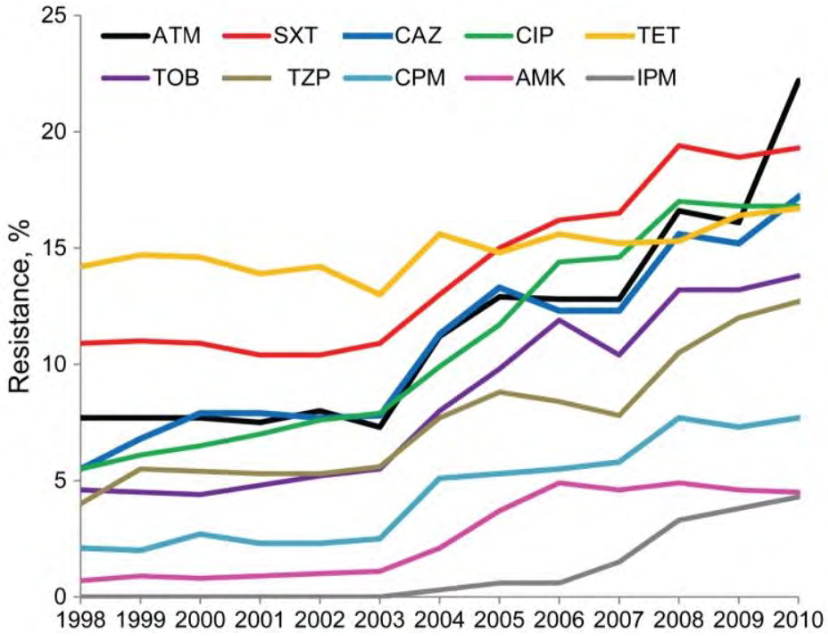


Figure 2.3: *Klebsiella pneumoniae* antimicrobial drug resistance in the United States between 1998 and 2010. ATM, aztreonam; SXT, trimethoprim/sulfamethoxazole; CAZ, ceftazidime; CIP, ciprofloxacin; TET, tetracycline; TOB, tobramycin; TZP, piperacillin/tazobactam; CPM, cefepime; AMK, amikacin; IPM, imipenem. (Adapted from Sanchez et al., 2013).

A similar trend is also observed in Europe (Figure 2.4) where a substantial increase in *K. pneumoniae* isolates resistance to different antibiotics: aminoglycosides, fluoroquinolones, third-generation cephalosporins, and carbapenems were identified.

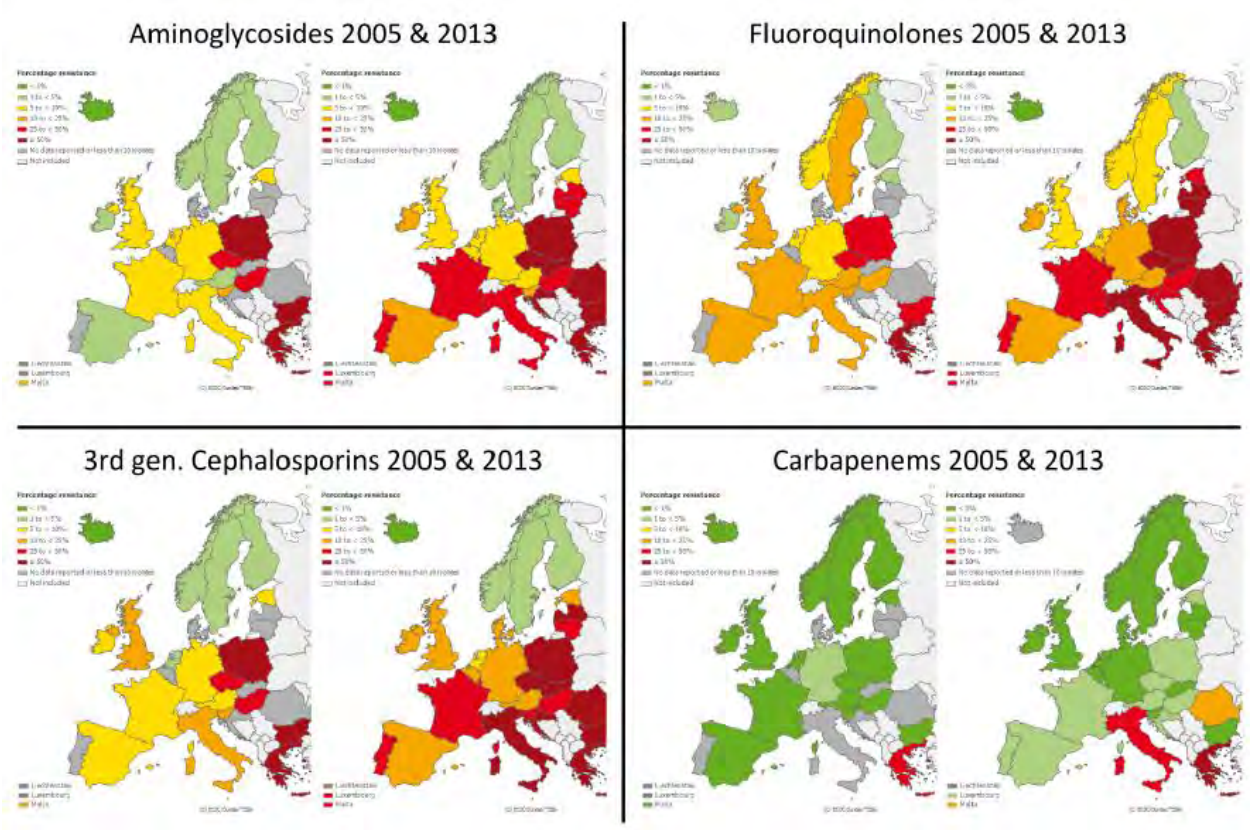


Figure 2.4: Development of AMR in *K. pneumoniae* in Europe between 2005 and 2013. Maps showing the development of aminoglycoside-, fluoroquinolone-, third-generation cephalosporin- and Carbapenem non-susceptibility of *K. pneumoniae* in Europe between 2005 and 2013. Colors indicate percentage of resistance: green < 1 %; light green 1 - 5 %; yellow 5 - 10 %; orange 10 - 25 %; red 25 - 50 %; dark red ≥ 50 %; dark gray no data; light gray not included (Adapted from Bruchmann, 2015).

The available information regarding antibiotic resistance in South Asia is scarce but the data presented by Center for Disease Dynamics Economics & policy shows the percentage of *Klebsiella pneumoniae* resistance in India is quite high. The average rate of antibiotic resistance in *K. pneumoniae* is ~60 %, and the third generation Cephalosporin resistance being most prevalent, around 80 %. The antibiotic resistance pattern in South Africa shows a similar tendency, having average antimicrobial resistance close to ~40 % and gaining highest resistance against third-generation Cephalosporin, around 67 % ("ResistanceMap - Antibiotic Resistance," n.d.).

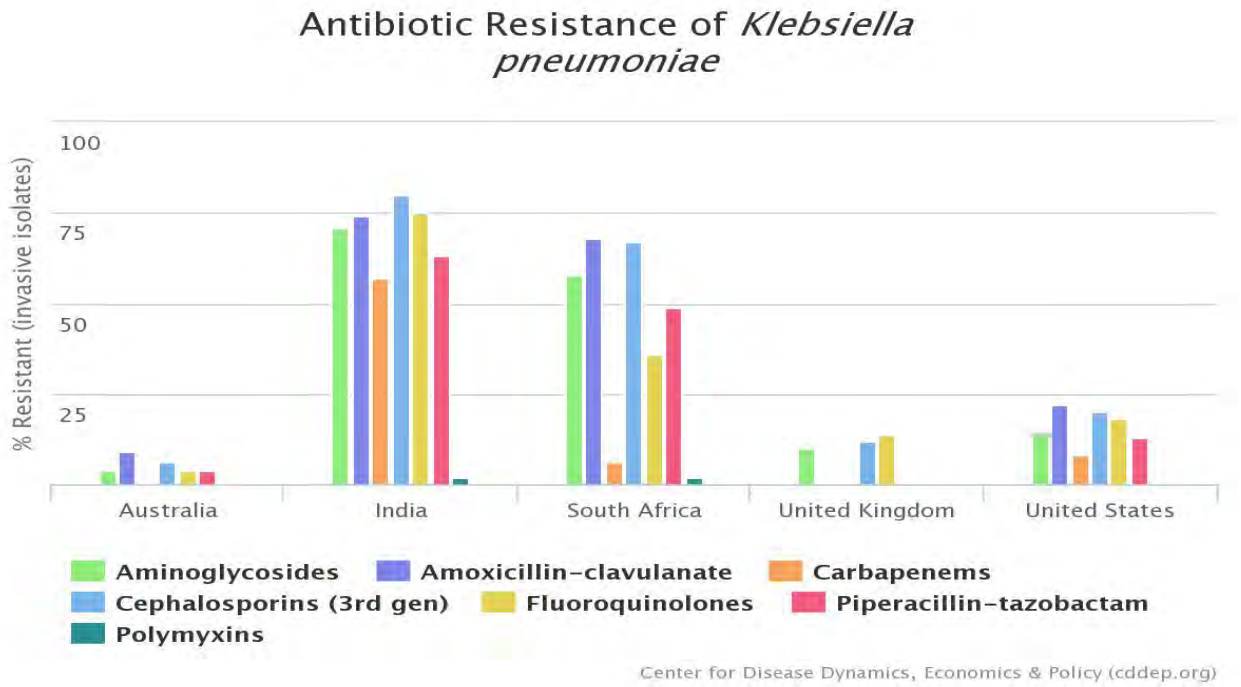


Figure 2.5: Trends in global antibiotic resistance, 2014-2015. The data used to create this figure can be accessed at the Center for Disease Dynamics, Economics & Policy (CDDEP) ResistanceMap website at https://resistancemap.cddep.org/AntibioticResistance.php.

2.2.4 Phage therapy in treating *K. pneumoniae* infection:

Bacteriophages have been successfully used to treat *K. pneumoniae* infection in human for a long time, especially in Eastern Europe. One of the most detailed phage therapy on human published in English language was done by Slopek et al. who published six consecutive paper and in seventh paper published in 1987, he summarized the results of several phage therapy case studies presented by a multiple Polish physicians working with the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland (Abedon, 2015; Sulakvelidze et al., 2001). Five hundred and fifty patients were treated with different infection condition caused by a range of bacterial pathogen among which *Klebsiella* was one of them and the results of those studies were positive. Antibiotics were found ineffective for majority these cases (Sulakvelidze et al., 2001) and the results of these phage therapies were significant as it shows the phage effectiveness against the antibiotic resistant pathogen.

Weber-Dabrowska et al. in their 2001 publication reported the effectiveness of phage therapy against twenty cancer patients, among whom 6 patients were suffering from pneumonia infected by *Klebsiella pneumoniae* (Weber-Dabrowska et al., 2001). All the patients demonstrated complete healing of the local lesions and termination of the suppurative process and in some case, additional general improvements were observed as well.

The results of those previous trials encouraged researcher around the world and recently we have seen some publication evaluating different phage and phage components effectiveness in animal models against *Klebsiella pneumoniae* strains that are antibiotic resistant and even multidrug resistant. Vinodkumar et al., (2005) showed that a single intraperitoneal (i.p.) injection of a lytic phage administrated 45 minutes after the bacterial challenge was adequate to rescue all the mice infected by a multidrug resistant (MDR) *Klebsiella pneumoniae* strain that caused fatal bacteremia. A study conducted by Chhibber et al. (2008) also reported similar result where single intraperitoneal injection of 10^{10} plaque forming unit (pfu) per ml phage administered directly after intranasal (i.n.) injection of *K. pneumoniae* B5055 was enough to rescue 100 % of animals from *K. pneumoniae*-mediated respiratory infections. However, 6-hour delay in phage administration after infection introduction rendered the treatment ineffective.

Kumari et al. (2009) assessed the efficacy of five different phages separately as well as in mixture to cure burn wound infection caused by *K. pneumoniae* in murine model. Phages were found to be non-toxic and a single dose of phages administrated intraperitoneally (i.p.) at an MOI of 1.0 was sufficient to completely cure *K. pneumoniae* infection and significantly decrease mortality in burn wound model. All the phages, as well as the phage mixture, were effective in the treatment and it was suggested that phages could be used to treat burn wound infection especially caused by the antibiotic resistant pathogen.

Another study performed by Cao et al. (2015) demonstrated that phage 1513, a lytic bacteriophage was able to protect mice from lethal pneumoniae upon Intranasal administration of a single dose of 2×10^9 pfu/mouse phage 2 h after KP 1513 (a multidrug resistant *K. pneumoniae*) inoculation. In a sublethal pneumonia model, lower level of *K. pneumoniae* burden in the lungs was observed in phage-treated mice as compared to the untreated control. The overall conditions of these mice were

improved such as low body weight loss, low levels of inflammatory cytokines in their lung and improvement of lung lesion conditions.

One important drawback of phage therapy is the rapid clearance of phage by the human reticuloendothelial system and poor pharmacokinetics profile (Merril et al., 1996; Westwater et al., 2003), which some researchers are trying to circumvent by using phage delivery system. A study conducted by Singla et al. (2015) assessed liposome as phage delivery vehicle. Here, a depolymerase-producing lytic bacteriophage (KPO1K2) was entrapped by liposome and administrated to mice suffering from lobar pneumonia induced by *K. pneumoniae* B5055 strain. For liposome encapsulated phage, the intraperitoneal injection was found more effective than intranasal administration as systemic delivery ensured phages greater access to the bacterial pathogen. As with some previous studies that reported total ineffectiveness of phage treatment due to a delay of few hours in administration (Bull et al., 2002; Chhibber et al., 2008; Smith and Huggins, 1982; Watanabe et al., 2007). Authors reported that liposome entrapped phages were able to protect mice even when the phage administration was delayed up to 3 days whereas, free phage was able to protect mice only up to 24 hours delay in administration. Additionally, non-liposomal phages provided protection against infection administrated only when 6 hours prior to intranasal bacterial challenge compared to 48 hours prior to intranasal bacterial challenge for liposomal phages. Moreover, liposomal phage treatment resulted in significant reduction in the levels of proinflammatory cytokines and an increase in the levels of anti-inflammatory cytokines, compared with non-liposomal phages which are desirable for therapeutic treatment since excess inflammatory response can be damaging (Mukhopadhyay et al., 2006; Nonas et al., 2005).

A similar study was undertaken by Chadha et al. (2017) where they used nano-lipid carriers (liposomes) as a delivery system but this time they used phage cocktail composed of five different bacteriophages to treat burn wound infection caused by *Klebsiella pneumoniae*. The phage mixture of equal proportion was entrapped by liposome and was administrated to BALB/c mice with burn wound infection caused by *K. pneumoniae* B5055 bacteria. The result demonstrated that the mice treated with liposomal encapsulated phage cocktail showed, greater reduction in bacterial load in blood and major organs compared to free phage cocktail. Also, complete removal of bacterial burden occurred in a much shorter period of time. Authors suggest that these findings were due to

the enhanced protection provided by the liposome encapsulated cocktail which increased the phage retention in blood and other organs. In this study, researchers found that liposome entrapped phages were able to protect mice from death even after a 24-hour delay in phage administration after bacterial inoculation whereas, free phage cocktail became ineffective in protecting mice from death.

Bacteriophages were also evaluated for liver abscesses caused by *K. pneumoniae*. Hung et al. (2011) reported positive result in mouse animal model infected by *K. pneumoniae*. In this study, mice were treated with φNK5 lytic phage. A single dose of lower than 2×10^8 pfu was administrated through intraperitoneal and intragastric route in a different mouse model 30 minute after *K. pneumoniae* injection. In both case, mice were protected from death. The intraperitoneal treatment was more efficient than intragastric treatment when phage was administrated as late as 24 hours after *K. pneumoniae* inoculation. In phage treated mouse, *K. pneumoniae*-induced liver injury, such as inflammatory cytokine production, liver necrosis, and blood levels of aspartate aminotransferase and alanine aminotransferase was significantly inhibited. All these recent experiments show the potentiality of phage successfully treating *K. pneumoniae* induced infection, particularly for infection caused by antibiotic resistant strain.

Chapter 3: Methods and Material

3.1 Place of study:

The study was done in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural sciences, BRAC University, Dhaka, Bangladesh.

3.2 Standard laboratory practice:

All the glassware such as test-tube, conical flask, beakers were washed once with tap water followed by second time wash with distilled water. Culture media (both agar based and broth), pipette tips, centrifuge tubes, empty test-tube for double-layer ager method were autoclaved at 121°C at 15 psi for 15 minutes before use and stored culture media at 4°C and autoclaved equipment in aseptic condition. While performing experiments, clean lab coat was worn and hand gloves were used and the experiments were performed inside a vertical laminar flow cabinets which in prior was cleaned with 70 % ethanol to avoid contamination.

3.3 Preparation of Culture Media, Reagents and Solutions:

Preparation of Nutrient Agar for bacterial culture and bottom agar of DLA:

According to the manufacturer's instruction, to produce 1000 ml of nutrient agar media, 28 grams of powder should be added. Keeping this ratio constant, necessary volume of nutrient agar medium was prepared each. An appropriate amount of media powder was measured using an electronic balance machine before adding to a flask containing the exact amount of deionized water. The flask was heated on a Bunsen burner to properly mix the solution until it became clear and produced bubble. Then the media containing flask was autoclaved at 121°C for 15 minutes covering is opening by an aluminium foil.

After sterilization, the media was poured on to sterilized petri dishes inside a vertical laminar flow cabinet and left to solidify at room temperature. Petri dishes were previously sterilized in an oven

at 160°C for 1 hour. For medium-sized plate, around 15-20 ml media was poured on each plate. After the media became solid, they were used that day and unused plates were stored at 4°C.

Preparation of 0.7 % top Nutrient Agar:

In order to perform double layer ager assay, 0.7 % nutrient agar was prepared separately. To produce this, at first, 13 gram of nutrient broth powder was measured and added to 500 ml of distilled water, to which 7 gram bacteriological agar was added. Then more deionized water was added to make the final volume 1000 ml. it was then heated on a Bunsen burner to make the solution homogenous until it was clear and formed bubble. Then the solution was poured into multiple 50 ml falcon tube, and autoclaved at 121°C for 15 minutes with the screw cap opened in half turn. After taking them out of autoclave the screw caps were tightly closed and media containing falcon tubes were stored at 4°C.

Preparation of Nutrient Broth:

The nutrient broth was prepared by weighing 13 grams of nutrient broth powder in 500 ml of distilled water and then adding more distilled water to make a final volume of 1000 ml. the mixture was then mixed well and 9 ml of this solution were put in each test-tube. Several such test-tubes were prepared and autoclaved at 121°C for 15 minutes. After sterilization, the test-tubes were stored at 4°C.

SM buffer:

This buffer was prepared by adding 5.8 gram NaCl, 2 gram $MgSO_4 \cdot 7H_2O$, 50 ml Tris-Cl (1M, pH 7.4), and 5 ml Gelatin (2 % w/v) to a container and adding deionized water to make final volume 1000 ml. the buffer was put in several 50 ml falcon tube with screw cap and autoclaved at 121°C for 15 minutes. After sterilization, the buffer was stored at room temperature.

$CaCl_2$ solution (0.001M):

To prepare this solution 0.111 gram solid calcium chloride was dissolved in 1000 ml deionized water followed by autoclaving at 121°C for 15 minutes which in turn was stored at room temperature.

$MgCl_2$ solution (0.001M):

This solution was prepared by measuring 0.095 gram of solid magnesium chloride and dissolving it in 1000 ml deionized water and followed by autoclaving at 121°C for 15 minutes which in turn was stored at room temperature.

70 % ethanol:

To prepare 70 % ethanol, deionized water was added to the 737 ml of 95 % ethanol to make a final volume of 1000 ml.

3.4 Bacterial culture:

All the bacterial cultures used in this project were collected from patients of, National Institute of Diseases of the Chest and Hospital (NIDCH), Dhaka Shishu (Children) Hospital and International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB). Bacterial samples were streaked on the fresh nutrient agar plate and incubated for 24 hours at 37°C. After checking growth, plates were wrapped with parafilm and stored at 4°C for further use. Before each experiment, bacterial samples were freshly subcultured and 24-hour cultures were used. By regular subculturing, viability and purity of the organisms were maintained.

3.5 Bacteriophage isolation:

3.5.1 Environmental water samples collection:

A total of four water samples were collected for this study in order to isolate lytic bacteriophage specific to *Klebsiella pneumoniae*. The sample K1 was collected from Dhanmondi Lake which

appeared to be clear of sediment, turbid and a little bit greenish in color and the water was placid. The sample K2 was collected from Hatirjheel Lake and the water in this site at the time of collection appeared black, turbid, placid and clear of sediment. The sample K3 was from a drain pipe near NIDCH with water in it being relatively clear with a little bit mixture of sediment and was flowing in slow motion. The sample K4 was taken from a sewerage pipe situated inside NIDCH hospital area. The water was black looking and turbid. The water collected from this site contained small beads like black particle floating inside the water. These water samples were collected in 50 ml sterile falcon tube during the month of May 2017 and kept at 4°C as recommended by Olson et al. (2004) until processed. During water sample collection the sample water at the site was mixed thoroughly before collection.

3.5.2 Bacteriophage enrichment:

A modified version of Twest and Kropinski (2009) was adopted for bacteriophage enrichment from collected water samples. The water samples were first well shaken for a couple of minutes then they were centrifuged at 10000 rpm for 10 minutes to remove sediment, large particulates, and bacteria. Ten milliliters of sterile nutrient broth containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was taken in a 250 ml conical flask and 10 ml of the clear supernatant was added. The flask was inoculated with 100 μl of an overnight culture of *Klebsiella pneumoniae*. The inoculated flask was incubated at 37°C for 24 hours inside a shaker incubator at 120 rpm. After the incubation period, the contents of the flask was centrifuged at 10000 rpm for 10 minutes. The clear supernatant was then filtered by passing through 0.22 μm low protein binding PES syringe filter and collected in another sterile falcon tube and stored at 4°C. It was consecutively tested for the presence of bacteriophage by spot test.

3.5.3 Phage detection:

After bacteriophage enrichment, the presence of *Klebsiella pneumoniae* specific phage was detected using spot test. In the spot test, 100 μl of an overnight culture of *Klebsiella pneumoniae* was placed in a sterile test-tube. To the test-tube, 3 ml of 48-50°C warm 0.7 % top nutrient agar

was added and properly mixed. Before the added nutrient agar solidifies, the culture –agar mixture was poured into a fresh nutrient agar plate inside a vertical laminar to maintain aseptic condition. The plate was left for 10-15 minutes to solidify the top agar. Then 5 μl of the supernatant was spotted on the plate surface. Then again the plate was left for 15-20 minutes to dry out the spotted supernatant. After that, the plate was incubated at 37°C for overnight in an inverted position. The next day, the plate was checked for clear lysis zone caused by bacteriophage.

3.5.4 Phage purification by double layer agar assay:

Enriched and filtered water samples that gave clear bacterial lysis zone during spot test was taken to isolate and purify bacteriophage using double layer assay method of Adams (1959). Before starting the experiment a falcon tube containing 50 ml of top agar was melt in an oven and put in a water bath maintaining 48ºC. When the melted top agar came down to the water bath temperature, the experiment started. First, phage containing filtrate was serially diluted up to 10^{-6} of initial concentration by tenfold dilution in SM buffer. Then 100 μl of an overnight culture of *Klebsiella pneumoniae* was put in different sterile test-tube each for different dilution concentration of phage. In each test tube, 100 μl of diluted phage suspension was added specific for each concentration and was mixed thoroughly. The bacterial culture and phage mixture was left for 10 minutes as an incubation time to allow phages to bind the bacterial cells. After the incubation period, the pre-melted and temperature maintained top agar was taken from the water bath and 3 ml of the top agar was added to the bacteria-phage mixture containing test-tube. The whole thing was mixed properly by capping the test tube, holding it at 45° angle and rotating quickly in a circular motion to avoid bubble formation. Then the mixture was poured on to a fresh nutrient agar plate marked by different dilution concentration and left for 10-15 minutes to solidify. The mixing and pouring of the top agar were done swiftly before agar solidifies inside test tube. When the upper agar layer became solid the plate was incubated at 37ºC for 24 hours and checked for plaque formation.

Plates with distinguishable and distant plaque formation were selected for phage isolation and purification. Phage was picked from a plaque that had the highest opacity and largest area size by slightly touching the surface of the plaque using a sterile micropipette tip. The tip was dipped and

swirled in an Eppendorf tube containing 300 μl of SM buffer to release the phage in the buffer solution.

The phage containing suspension was again subjected to double layer assay followed by plaque picking and dissolving in fresh SM buffer. This procedure was performed up to three times until homogenous plaque formation was observed to ensure pure phage preparation.

3.5.5 Phage stock preparation:

Large liquid phage stock was prepared according to Bonilla et al. (2016). In this stage, 100 μl of an overnight culture of host bacteria, *Klebsiella pneumoniae*, was added to a 250 ml conical flask containing 100 ml sterilized nutrient broth supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The flask was incubated for 1 hour at 37ºC with shaking (120 rpm) inside a shaker incubator. Then, 100 µl of purified phage solution was added to the flask and was let it sit for 10 minutes. After that, the flask was incubated at 37ºC for overnight to form a clear lysate.

The next day phage lysate was centrifuged at 10000 rpm for 10 minutes. The clear supernatant was collected into multiple 50 ml sterile falcon tube by filtering the supernatant through 0.22 µm low protein binding PES syringe filter membrane and stored at 4ºC.

3.5.6 Phage titer determination:

Bacteriophage was titered using double layer agar method of Adams (1959). The phage stock solution was subject to serial dilution in SM buffer up to 10^{-6} of initial concentration. One hundred microliters of each dilution were taken into different sterile test tubes. To each of these test tube, 100 µl of overnight grown host bacteria was added and left for 10 minutes at room temperature without any movement to allow the page to attach to the host bacteria. After initial incubation 3 ml of molten (48-50ºC) 0.7 % top agar was added. The contents were mixed carefully and pour on to fresh nutrient agar plate without bubble formation as bubbles in the agar are quite hard to remove and can be misread as plaque. The plates were left for 10-15 minutes to solidify the top agar layer. Then they were incubated at 37ºC for 18-24 hours.

After overnight incubation, the plates were checked for plaque formation and plate with an individually distinguishable plaque was selected for phage titer determination. Counted plaque number was used to determine the titer of stock solution using this formula:

$$\text{Titre (PFU/ml)} = \frac{Number\ of\ plque\ (PFU)}{dilution\ x\ volume\ of\ phage\ added\ to\ plate\ (ml)}$$

3.6 Phage characterization:

3.6.1 Host range determination:

Different bacterial cultures (Table 3.1) were used to assess the host range of isolated bacteriophage. It was done according to Verma et al. (2009). Three milliliters of 0.7 % warm (48-50ºC) top agar was mixed with 100 µl of each bacterial culture in different sterile test-tube and poured on separate fresh solid nutrient agar plate. After solidification of the top agar layer, 5 µl of the high titer phage stock was spotted along with 5 µl of sterilized nutrient broth as a control. The plates were incubated for 24 hours at 37ºC and checked for the presence of bacterial lysis.

Table 3.1: Bacterial cultures used for host range determination.

Bacterial culture	Source
Klebsiella isolate 1	NIDCH
Klebsiella isolate 2	NIDCH
Klebsiella isolate 3	NIDCH
Klebsiella isolate 4	NIDCH
Klebsiella isolate 5	NIDCH
Klebsiella isolate 6	NIDCH
Klebsiella isolate 7	NIDCH
Klebsiella isolate 8	NIDCH
Klebsiella isolate 9	NIDCH
Klebsiella isolate 10	NIDCH
Klebsiella varicella (reference strain)	ICDDR,B

Table 3.1: Bacterial cultures used for host range determination (continued).

Bacterial culture	Source
Klebsiella spp	NA
Shigella flexneri	ICDDR,B
Shigella dysenteriae	ICDDR,B
Salmonella typhi	ICDDR,B
Bacillus subtilis	ICDDR,B
Bacillus cereus	ICDDR,B
Bacillus licheniformis	ICDDR,B
Escherichia coli ATCC: 25922	ICDDR,B
Escherichia coli ATCC: 13706	ICDDR,B
E. coli 0157:H7	ICDDR,B
Enterobacter cloacae	NA
Enterotoxigenic *Escherichia coli* (ETEC)	ICDDR,B
DH5α	NA
Streptococcus pneumoniae	SHISHU
Pseudomonas aeruginosa	SHISHU
Streptococcus agalactiae	SHISHU
Staphylococcus aureus ATCC: 25923	SHISHU
Proteus vulgaris	SHISHU
Streptococcus pyogenes	SHISHU
Acinetobacter baumannii ATCC: 19606	ICDDR,B
Enterococcus faecalis ATCC: 29212	ICDDR,B
Pseudomonas aeruginosa ATCC: 27853	ICDDR,B
Hafnia alvei	ICDDR,B
Salmonella paratyphi	ICDDR,B

[a] National Institute of Diseases of the Chest and Hospital

[b] International Centre for Diarrhoeal Disease Research, Bangladesh

[c] Dhaka Shishu (Children) Hospital

[*] Not Available

3.6.2 Thermal stability test:

The stability of the isolated bacteriophage at different temperature was tested. 1ml of phage solution was taken in different sterile Eppendorf tube. These tubes were kept in the water bath for each temperature of 30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC, 90ºC, and 100ºC for 60 minutes. Then the titer of bacteriophage after treatment was determined using double layer assay method.

3.6.3 pH stability test:

pH stability test was done according to Verma et al. (2009) with a slight modification. A pH range of pH 2 to pH 12 was prepared by adjusting the pH of fresh nutrient broth. 1M HCl was added drop by drop and tested by pH meter to achieve desired acidic pH and 1M NaOH was used to achieve desired basic pH. One milliliter phage suspension was mixed with 9 ml of pH adjusted medium and incubated for 3 hours at 37ºC. Phage suspension having pH 7 was used as a control. After the incubation period, phage titer was determined by double layer agar method against host bacteria *Klebsiella pneumoniae*.

3.6.4 Organic solvent sensitivity test:

Isolated bacteriophage stability in organic solvent chloroform and ethanol was tested. One milliliter of $6x10^7$ pfu/ml phage suspension was mixed with equal volume of organic solvent separately and incubated at room temperature for 1 hour with shaking by hand from time to time. After incubation, the mixtures were centrifuged at 10000 rpm for 10 minutes and the phage titer in the aqueous phase was determined by double layer agar method. Phage suspended in SM buffer and held in 4ºC was considered as control and the results were compared to it for relative inactivation of phage by organic solvents.

3.6.5 One step growth curve:

One step growth experiment was performed according to the method of Bao et al. (2011). Host bacteria, *Klebsiella pneumoniae* was grown on nutrient broth and optical density of the culture was

adjusted (OD 0.6 at 600 nm) to reach mid-exponential phase to give a 10^8 cfu/ml. phage stock solution was diluted to 10^6 particles per ml and 1 ml of this was added to 1 ml of exponential phase bacteria to achieve a multiplicity of infection (MOI) of 0.01. This mixture was incubated for 5 minutes at 37ºC. Then it was centrifuged at 10000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended in 1 ml of fresh nutrient broth. One hundred microliters were taken immediately, was diluted and subject to phage titration by double layer agar method. Samples were taken in a 5-minute interval for 45 minutes and were titered by double layer agar method. The plaque count was converted to pfu/infected-cell by dividing each phage titer in pfu/ml by the expected initial infected bacterial cell number which is 10^6. The latent period and burst size were determined according to Hyman and Abedon, (2009). The latent period was identified as time in between after phage absorption and the initial rise in plaque number. The burst size was identified by dividing the average of pfu/infected-cell in the post-rise period of growth curve by the average of pfu/infected-cell in the pre-rise period of the growth curve.

Chapter 4: Results

4.1 Isolation and purification of bacteriophage:

4.1.1 Spot test:

The four water samples that were collected and enriched for *Klebsiella* specific bacteriophage, only one of them gave clear lytic zone. The analysis of that enriched sample water by double layer assay resulted in individual plaque. The plaque with most clarity and bigger diameter was chosen for further study.

Table 4.1: Spot test result of different water samples:

Water samples	Location	Collection date	Spot test	Remarks
K1	Dhanmondi Lake	14-5-2017	-	Water was dirty and no rain a week before collection
K2	Hatirjheel Lake	15-5-2017	-	Water was dirty and no rain a week before collection
K3	Drain pipe near NIDCH*	18-5-2017	-	Clear water with solid particles and no rain a week before collection
K4	Sewerage pipe near NIDCH*	20-5-2017	+	Black water with solid particle and rained the day before collection

* National Institute of Diseases of the Chest and Hospital

- could not produce clear lytic zone

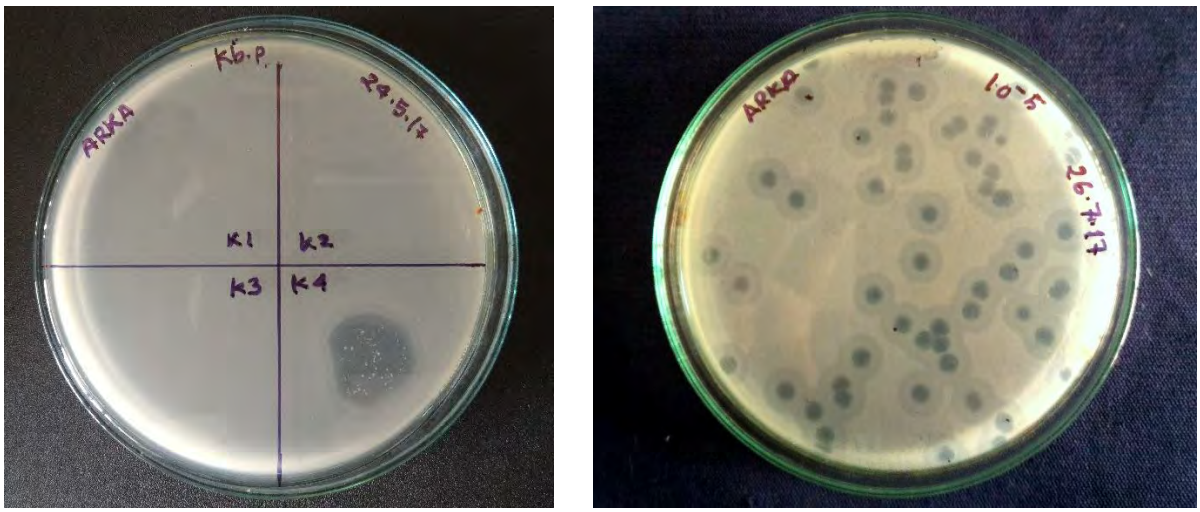
+ produced clear lytic zone

4.1.2 Plaque morphology:

The isolated phage produced circular individual plaque against host bacteria on the double layer agar plate. The plaque had two circular layers with inner 3 millimeters wide completely clear center surrounded by 8 millimeters wide opaque outer circle.

4.1.3 Determination of phage titer:

After the initial purification and following phage propagation for 24 hours in liquid culture, phage titer was determined by DLA method and that gave 10^8 pfu/ml. the propagation and subsequent enumeration were repeated and both the time the phage titer was above 10^8 pfu/ml.



(a) (b)

Figure 4.1: Spot test of enriched sample waters against host bacteria *Klebsiella pneumoniae* (a) and individual plaque formation of Klpp1 bacteriophage on double layer assay plate (b).

4.2 Bacteriophage characterization:

4.2.1 Host range specificity:

The ability to infect and produce lytic zone by the Klpp1 bacteriophage against different bacteria was tested by spot test and among the 11 *Klebsiella* isolates from NIDCH, Klpp1 was able to infect 7 of them and was unable to create any lytic zone on other 4 isolates. The Klpp1 bacteriophage was also able to lyse *Klebsiella* spp but did not infect any other bacteria the result of host range specificity test as follows:

Table 4.2: Host range spectrum of Klpp1 bacteriophage against various bacterial cultures.

Bacterial culture	Spot test	Source
Klebsiella isolate 1	-	NIDCH[a]
Klebsiella isolate 2	+	NIDCH
Klebsiella isolate 3	+	NIDCH
Klebsiella isolate 4	-	NIDCH
Klebsiella isolate 5	+	NIDCH
Klebsiella isolate 6	+	NIDCH
Klebsiella isolate 7	-	NIDCH
Klebsiella isolate 8	+	NIDCH
Klebsiella isolate 9	+	NIDCH
Klebsiella isolate 10	+	NIDCH
Klebsiella isolate 11	-	NIDCH
Klebsiella varicella (reference strain)	-	ICDDR,B[b]
Klebsiella spp	+	NA[*]
Shigella flexneri	-	ICDDR,B
Shigella dysenteriae	-	ICDDR,B
Salmonella typhi	-	ICDDR,B
Bacillus subtilis	-	ICDDR,B

Table 4.2: Host range spectrum of Klpp1 bacteriophage against various bacterial cultures (continued).

Bacterial culture	Spot test	Source
Bacillus cereus	-	ICDDR,B
Bacillus licheniformis	-	ICDDR,B
Escherichia coli ATCC: 25922	-	ICDDR,B
Escherichia coli ATCC: 13706	-	ICDDR,B
E. coli 0157:H7	-	ICDDR,B
Enterobacter cloacae	-	NA*
Enterotoxigenic *Escherichia coli* (ETEC)	-	ICDDR,B
DH5α	-	NA*
Streptococcus pneumoniae	-	SHISHU[c]
Pseudomonas aeruginosa	-	SHISHU
Streptococcus agalactiae	-	SHISHU
Staphylococcus aureus ATCC: 25923	-	SHISHU
Proteus vulgaris	-	SHISHU
Streptococcus pyogenes	-	SHISHU
Acinetobacter baumannii ATCC: 19606	-	ICDDR,B
Enterococcus faecalis ATCC: 29212	-	ICDDR,B
Pseudomonas aeruginosa ATCC: 27853	-	ICDDR,B
Hafnia alvei	-	ICDDR,B
Salmonella paratyphi	-	ICDDR,B

[a] National Institute of Diseases of the Chest and Hospital

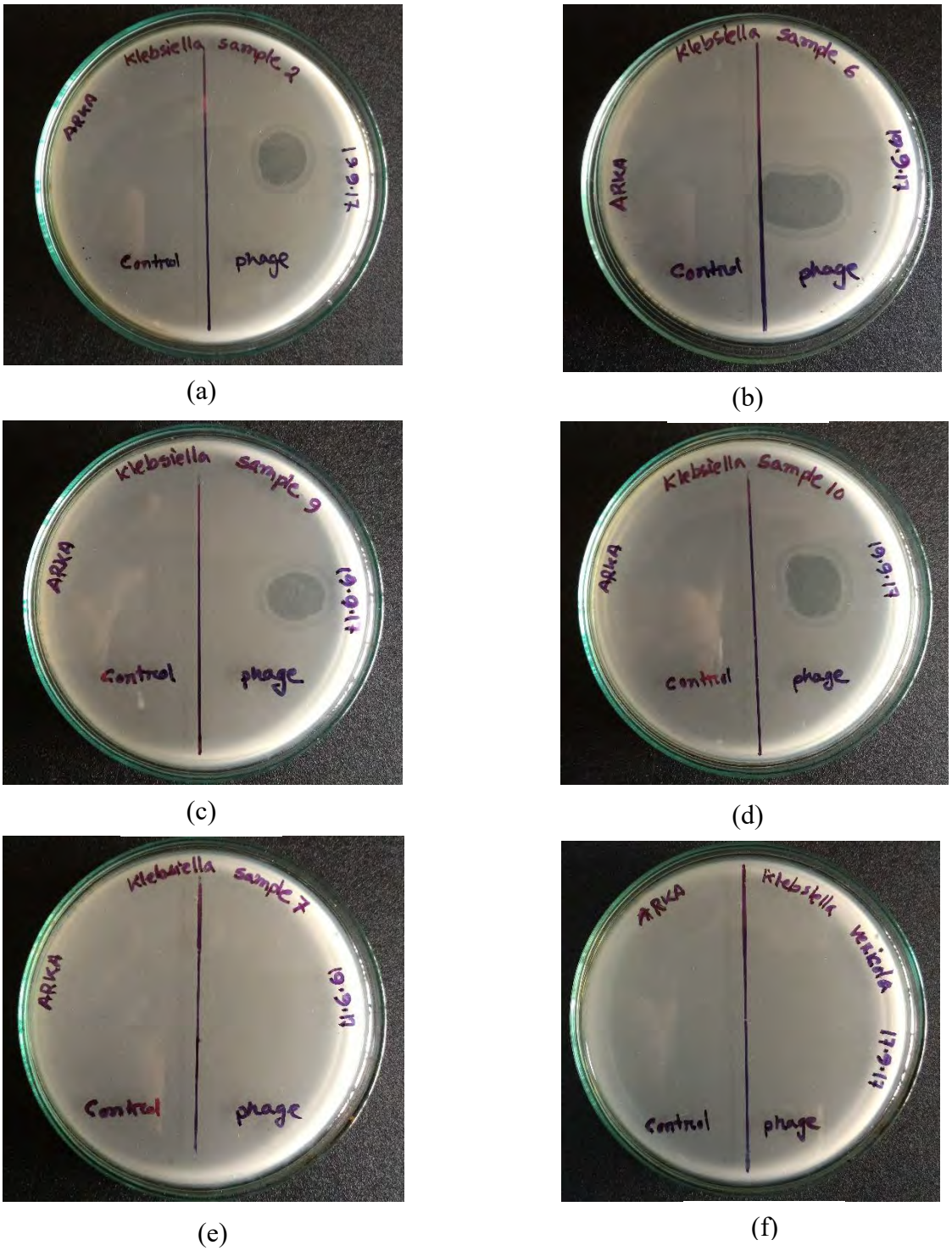
[b] International Centre for Diarrhoeal Disease Research, Bangladesh

[c] Dhaka Shishu (Children) Hospital

* Not Available

- was unable to produce lytic zone

+ produced clear lytic zone



(a) (b)

(c) (d)

(e) (f)

Figure 4.2: Host range specificity of Klpp1 bacteriophage against various *Klebsiella* isolates: *Klebsiella* sample 2 (a), *Klebsiella* sample 6 (b), *Klebsiella* sample 9 (c), *Klebsiella* sample 10 (d), *Klebsiella* sample 7 (e), and *Klebsiella variicola* (f).

4.2.2 Thermal stability:

The thermal stability test was carried out to measure the heat resistance of Klpp1 phage. After 60 minute incubation, the phage was stable at 30ºC, 40ºC, and 50ºC but reduced its infectivity by two logs at 60ºC and completely lost its infectivity from 70ºC and upward temperature (Figure 4.3).

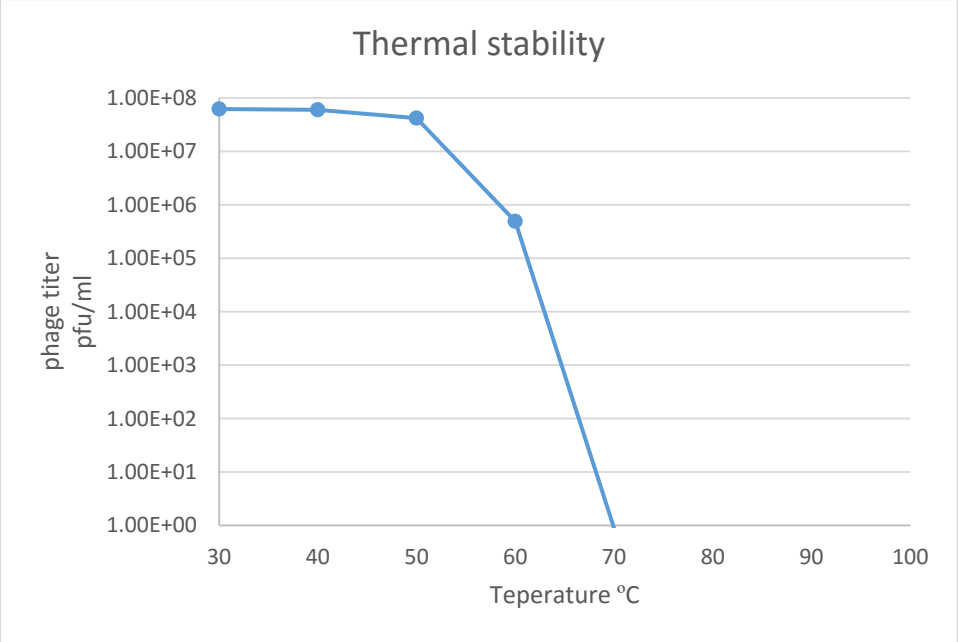


Figure 4.3: Thermal stability of Klpp1 bacteriophage at various temperatures.

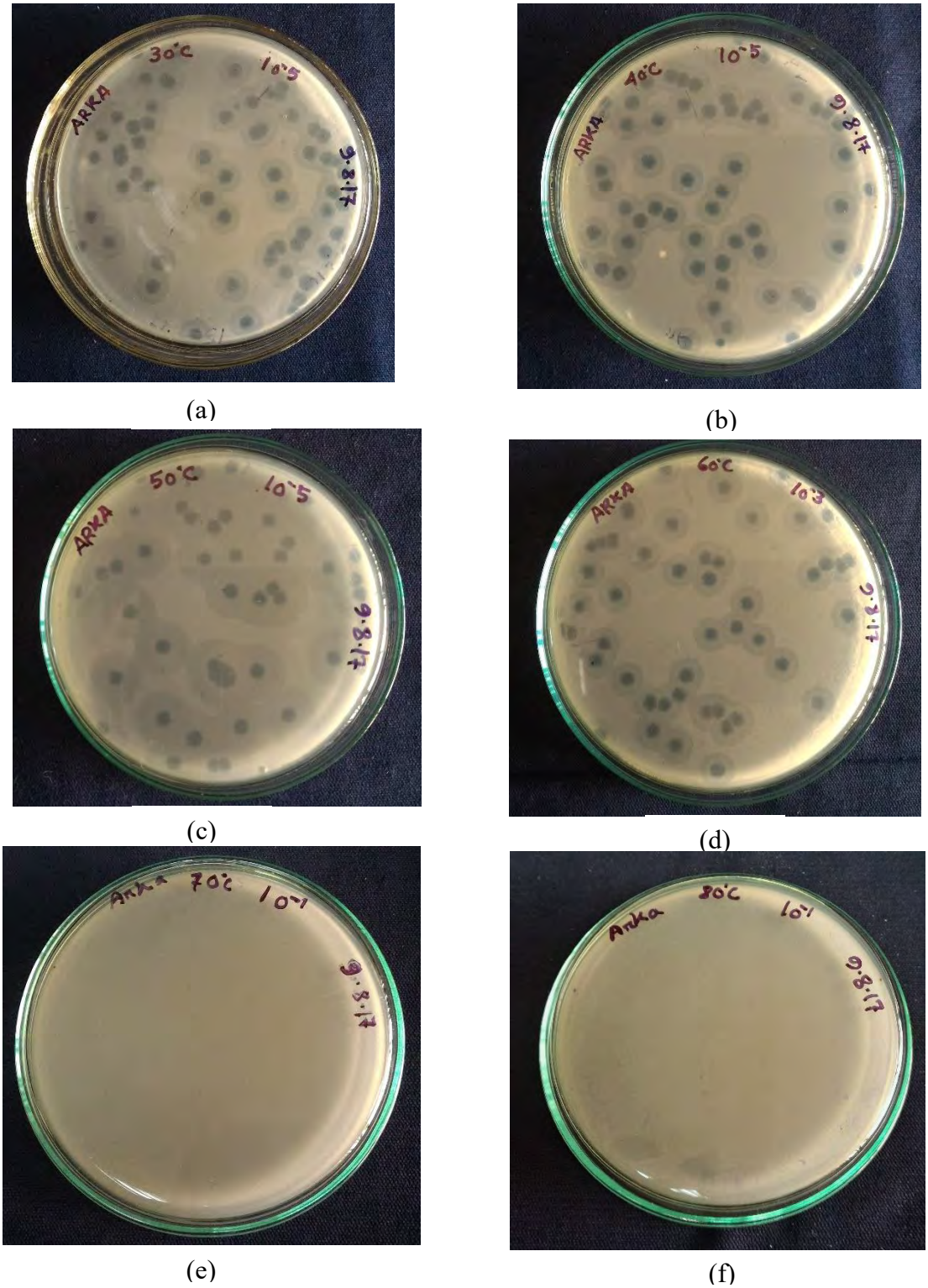


Figure 4.4: Plate with countable plaque number after heat treatment at different temperature in thermal stability test: 30°C (a), 40°C (b), 50°C (c), 60°C (d), 70°C (e), and 80°C (f).

4.2.3 pH stability test:

pH stability test was carried out in order to determine the optimal pH of the Klpp1 phage at different pH for 3 hours. Klpp1 was stable in the pH range 6.0-8.0. The titer of the phage drops by one order of magnitude at pH 5.0 until it was reduced to 10^2 pfu/ml at pH 4.0 and lost its infectivity at pH 3.0 and 2.0. Phage titer continued to drop dramatically after pH 8.0 and reached 10^4 pfu/ml at pH 11.0 but it became inactive at pH 12.0 (Figure 4.5).

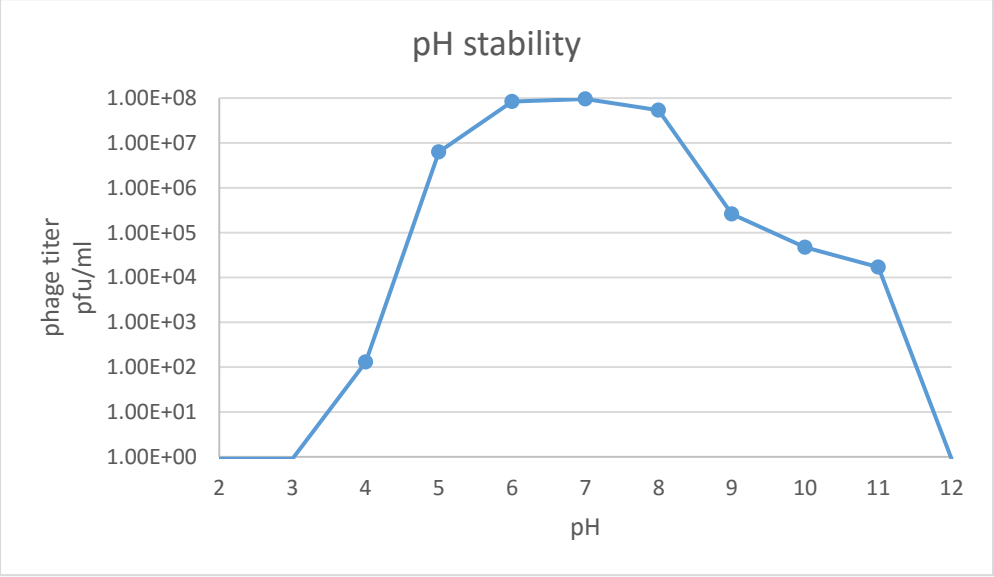
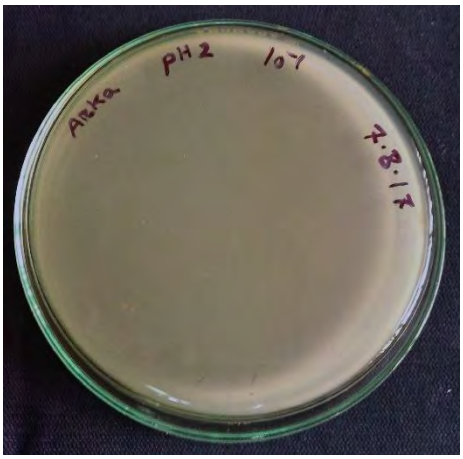
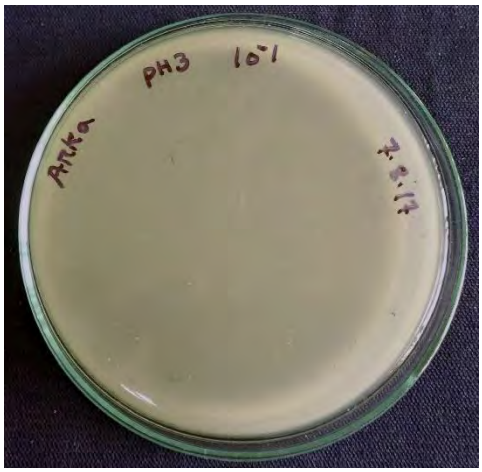


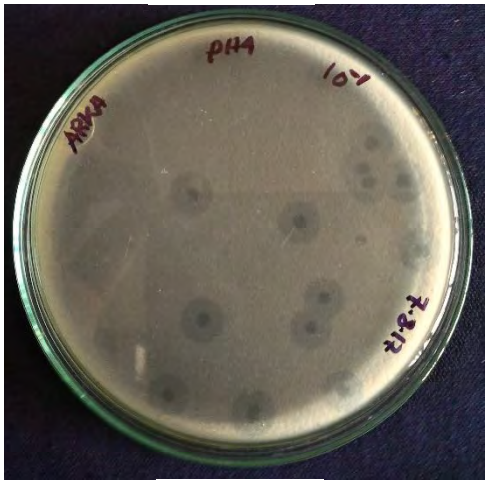
Figure 4.5: Effect of pH on stability of Klpp1 bacteriophage.


Arka
PH 2
10-1
7.8.12

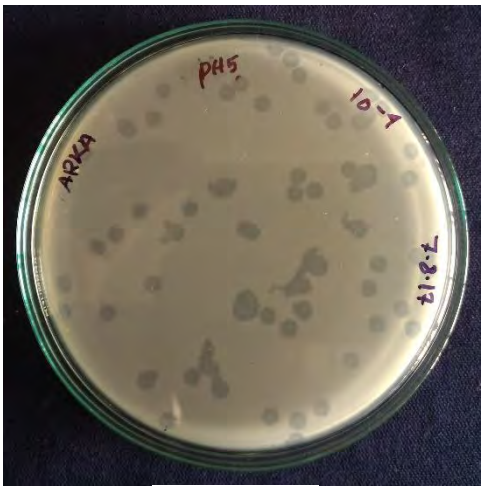
(a)


PH3
10-1
ARKA
7.8.12

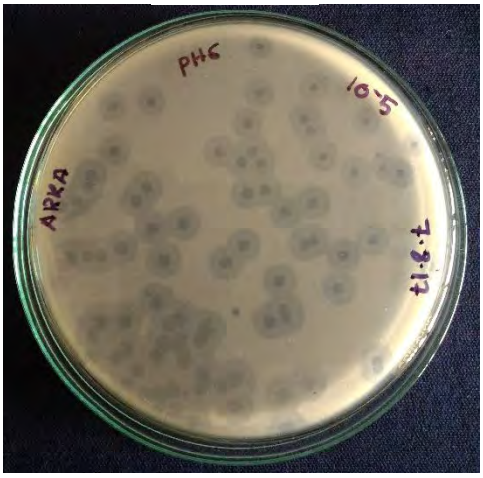
(b)


PH4
10-1
ARKA
7.8.12

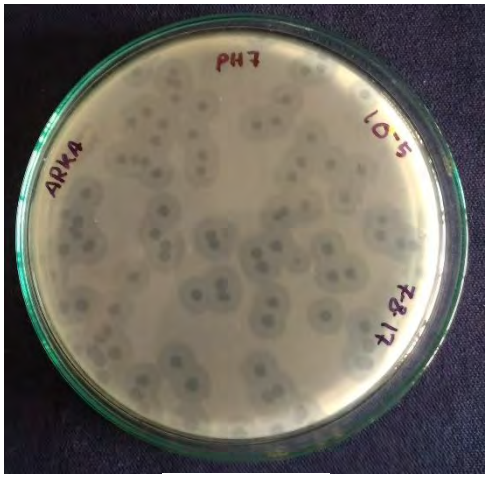
(c)


PH5
10-1
ARKA
7.8.12

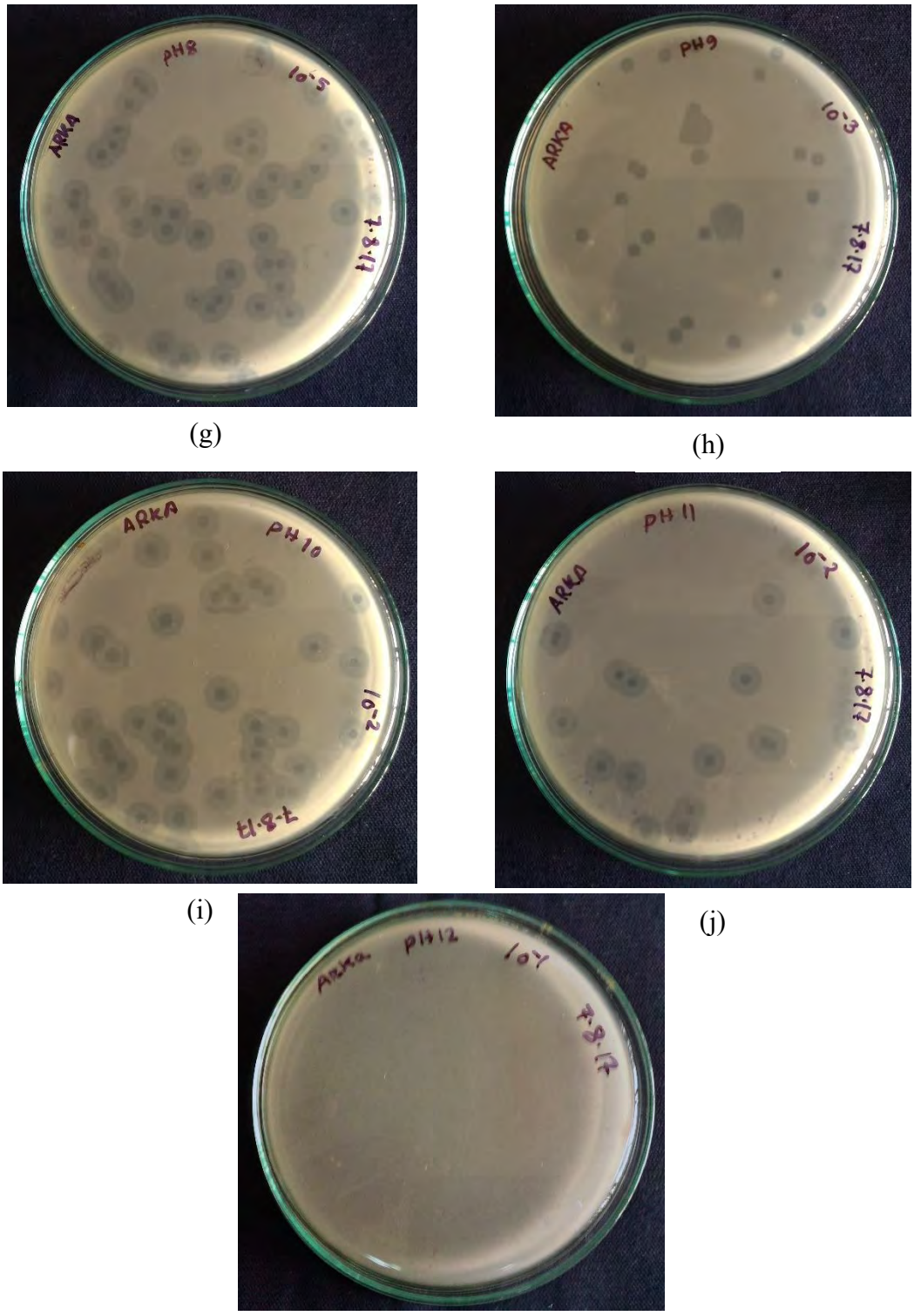
(d)


PH6
10-5
ARKA
7.8.12

(e)


PH7
10-5
ARKA
7.8.12

(f)



(g) (h)

(i) (j)

(k)

Figure 4.6: Plate with countable plaque number after treatment with different pH: pH 2 (a), pH 3 (b), pH 4 (c), pH 5 (d), pH 6 (e), pH 7 (f), pH 8 (g), pH 9 (h), pH 10 (i), pH 11 (j), and pH 12 (k).

4.2.4 Organic solvent sensitivity:

The sensitivity of Klpp1 phage against organic solvent chloroform and ethanol was tested. After 60 minutes incubation with chloroform, no significant change in titer was observed compared to control, whereas the incubation with ethanol for the exact same amount of time resulted in the complete inactivity of Klpp1 phage (Figure 4.7).

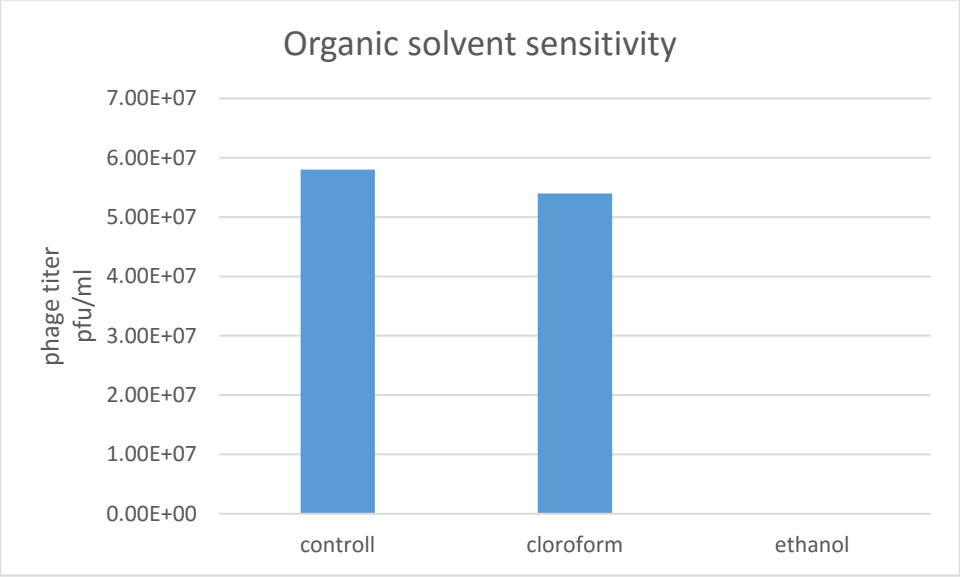
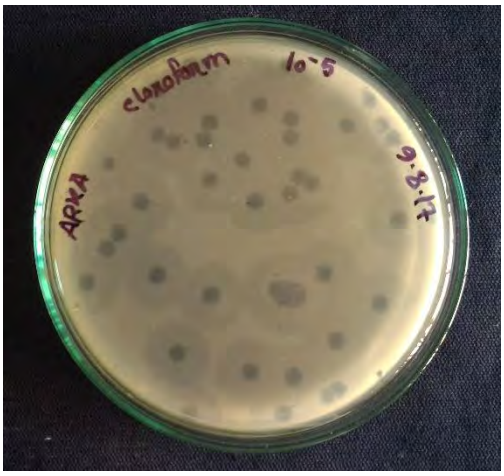
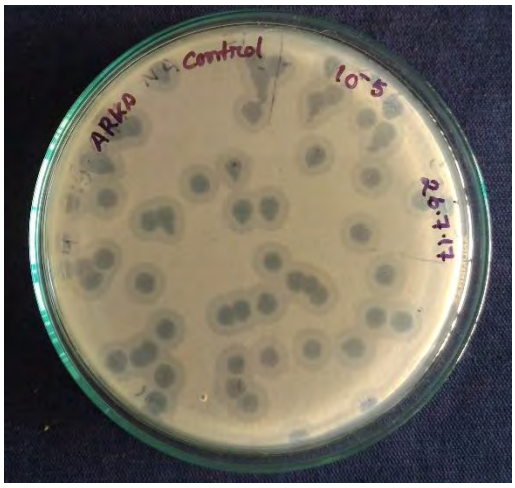


Figure 4.7: Effect of organic solvent on stability of Klpp1 bacteriophage.



(a)

(b)



(c)

Figure 4.8: Plate with countable plaque number after organic solvent sensitivity test: Chloroform (a), Ethanol (b), and Control (c).

4.2.5 One step growth curve:

Latent period and burst size, two phage growth parameter of Klpp1 were determined from the change in the number of phages during one replicative cycle. Following the initial steady period, the rise in phage titer was observed after 20 minutes. Therefore, the latent period for Klpp1 was identified as 20 minutes. The burst size was calculated by dividing the average pfu/infected-cell in the post-rise period of the growth curve by the average pfu/infected-cell in the initial steady period. This way the burst size was calculated as approximately 140 pfu/infected-cell.

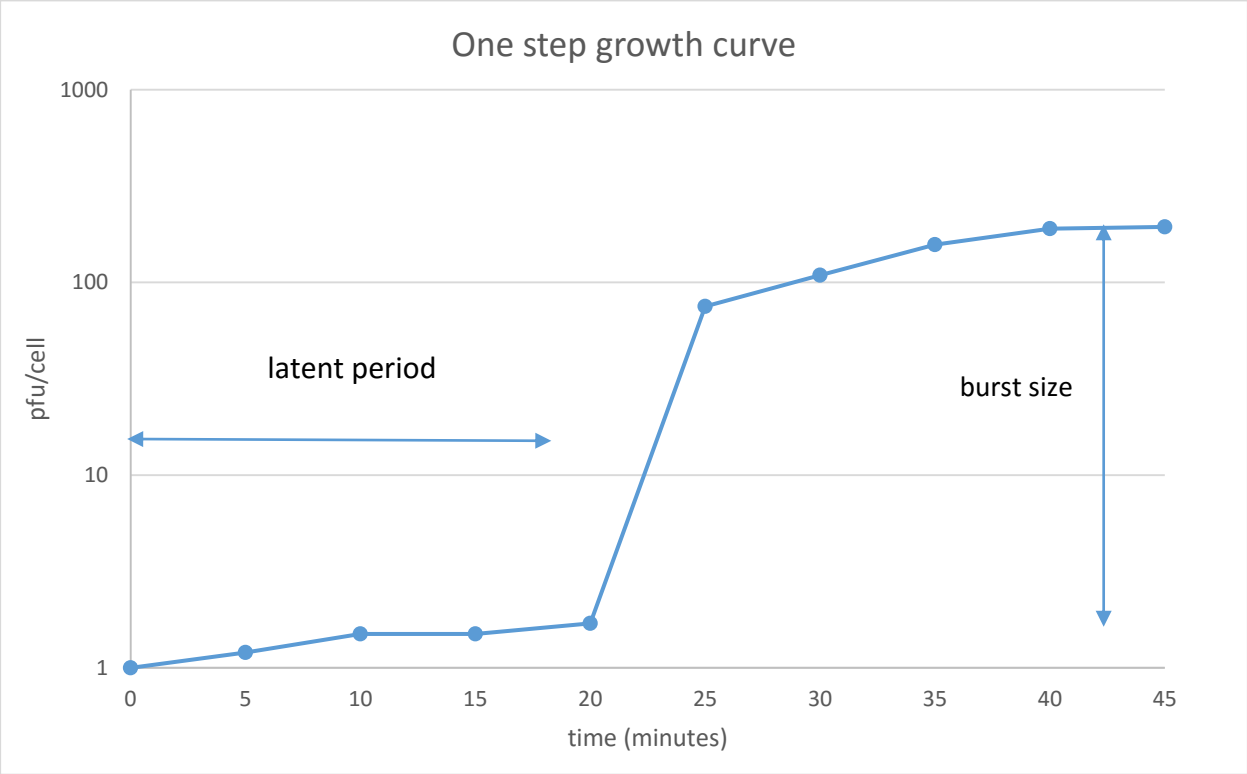
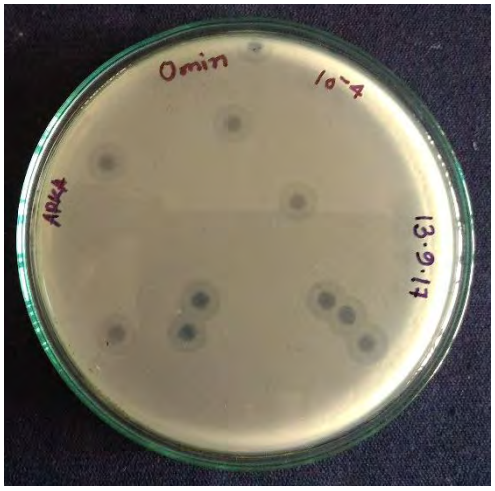
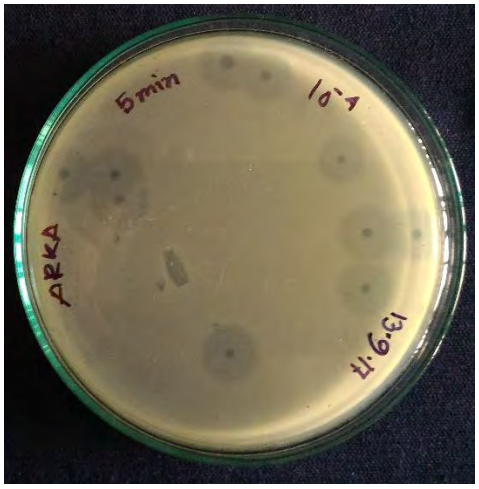


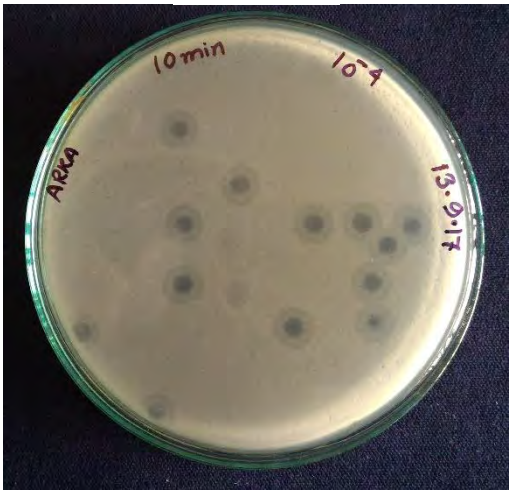
Figure 4.9: One stop growth curve of bacteriophage Klpp1.


0min
10^-4
ARKA
13.9.17

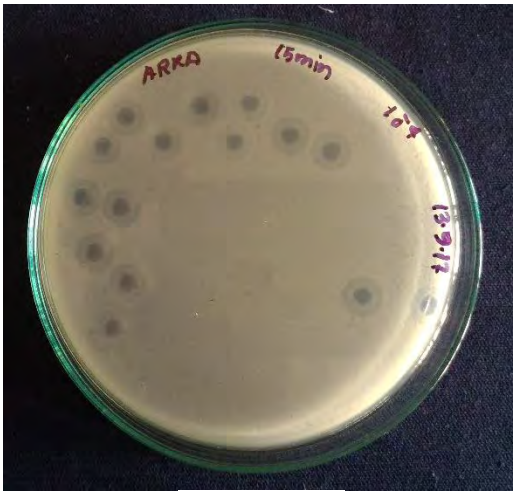
(a)


5min
10^-4
ARKA
13.9.17

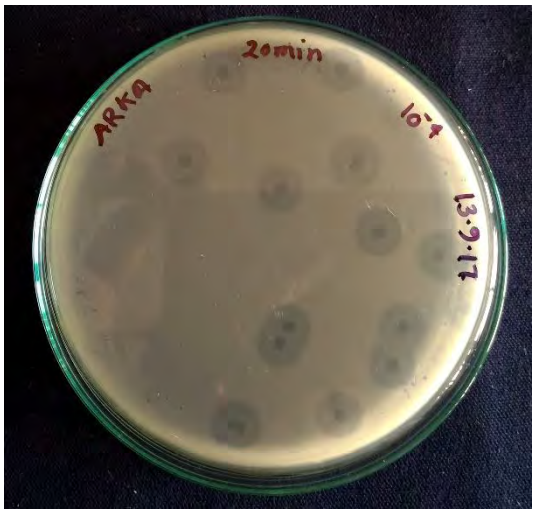
(b)


10min
10^-4
ARKA
13.9.17

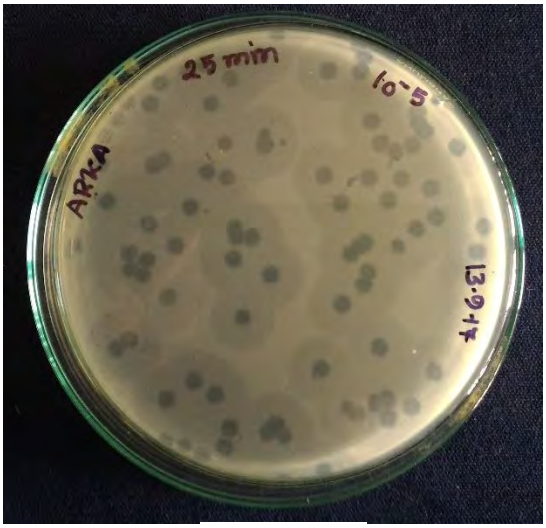
(c)


ARKA
15min
10^-4
13.9.17

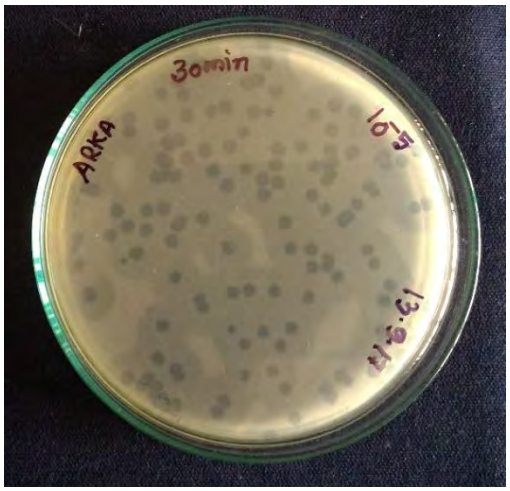
(d)


ARKA
20min
10^-4
13.9.17

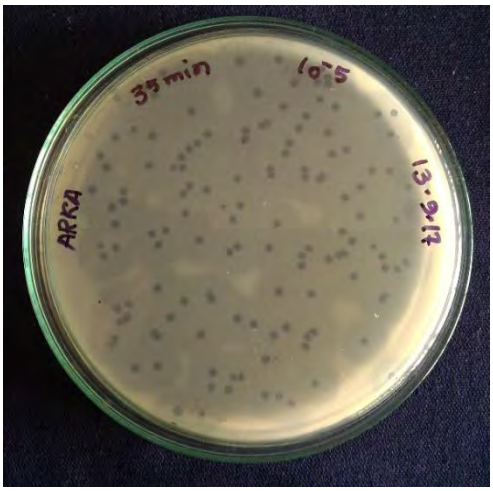
(e)


ARKA
25min
10^-5
13.9.17

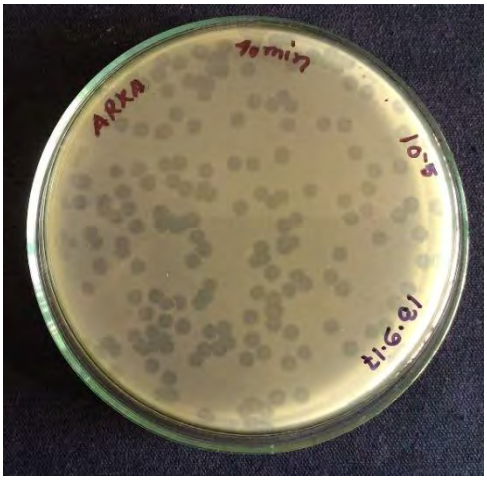
(f)



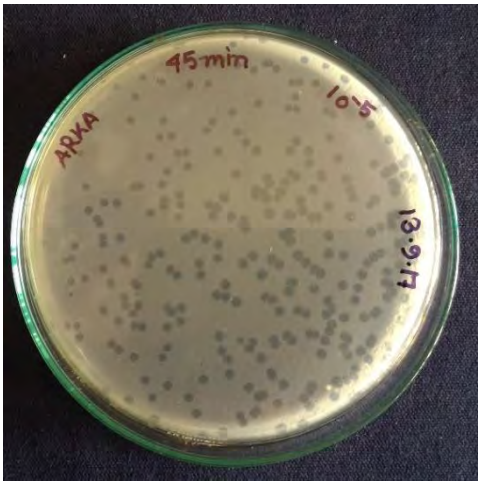
(g)



(h)



(i)



(j)

Figure 4.10: Plate with countable plaque number after one step growth experiment: 0 min (a), 5 min (b), 10 min (c), 15 min (d), 20 min (e), 25 min (f), 30 min (g), 35 min (h), 40 min (i), and 45 min (j).

Chapter 5: Discussion

5.1 Isolation of bacteriophage:

Water samples were stored at 4ºC and processed within 14 days which is way under 40 days recommendation of Olson et al. (2004) since storage of phage at -20ºC is not recommended because of the ice crystal structure destroying phage as shown by Warren and Hatch, (1969).

Out of the four water samples, only one sample gave *Klebsiella pneumoniae* infective bacteriophage in spot test while other 3 samples failed to produce any lytic zone even after phage enrichment of those water sample was repeated multiple times. Among these phage negative water sample, K1 and K2 were collected from the open freshwater body and K3 was from a drain pipe containing relatively clear water. One reason behind this scenario can be because of difference in abundance of host bacteria in sample collection sites as bacteriophages are obligatory parasite their proliferation depends on the growth of the host bacteria (Uchiyama et al., 2007). If the host bacteria are present in lower density in source water there is less chance for host specific phages to interact with each other and consecutively phage proliferation may not occur as for most phage a successful multiplication requires at least ~10^3-10^4 cfu per ml (Wiggins and Alexander, 1985). Moreover, Podschun et al. (2001) found that *Klebsiella* species is not present in all water body. Only half (53 %) of the samples contained *klebsiella* bacteria with *Klebsiella pneumoniae* being the most common species (52 % of total isolate) and also present in very low density (1-5 cfu per 250ml). If *Klebsiella pneumoniae* in the K1, K2, and K3 sampling site was present in insufficient density then the phage could not proliferate and was extant in a low amount to collect for isolation. On the other hand, the K4 water sample was from a sewerage pipe coming out form NIDCH hospital where the host bacteria is most likely abundant since patients with various chest disease are treated there and *Klebsiella pneumoniae* might have come into the sewer with sputum from infected patients.

One reason for not founding phage in those 3 water samples could be collecting water without sediment. As phage particle tends to attach to sediment and over time these phage attached sediment aggregate in the bottom of the water body and with a little bit of shaking the ground floor phages are released into the water (De Flora et al., 1975). As a result, phages are generally more

abundant in the sediment than the overlying water (Drake et al., 1998). The two lakes from which waters were collected, were placid and sediments were deposited at the bottom of the lake which reduced the overall phage attached sediment available for collection at the surface level. As the sewage water sample contained beads like black particle, the phage might have attached to those particles and was collected along with those beads. Before initial phage enrichment, the water samples were shaken for a couple of minutes which might have detached those phage form the particle and mixed them thoroughly with the water sample.

Phages as a virus can be rendered noninfective in aquatic environment via several factors. The virucidal organism, mainly some heterotrophic bacteria that can degrade viruses considering them as another nutrient source and some protist that also destroy large viruses (Weinbauer, 2004; Fujioka et al. 1980). Heavy metal can also render phages non-infective by binding with them mostly in polluted water (Bitton, 1980). High energy photon can also disrupt phages (Murray and Jackson, 1993). However, the most important factor for viral decay in an aquatic environment is sunlight, specifically ultraviolet (UV) light that damages Viral genomic material to an extent which cannot be repaired (Kirchman 2012). UV-B in the sunlight has the greatest effect at about 300 nm wavelength (Caldwell, 1971; Setlow, 1974) and accounting for 50-90 % of inactivation rate of virus caused by full sunlight (Suttle & Chen, 1992). The sampling site of K1, K2 is open water body and here UV light may have played an impotent role since water sample was collected from surface water during the summer season when the sunlight intensity is highest. However, the sampling site of K3 and K4 was in a continuous flow and is mostly covered so very little to no sunlight interacted with the waters.

By observing the plaque morphology found in plaque assay during isolation, only the clear plaques were considered for further isolation, because the clear zone indicates complete lysis of all bacteria within the area. Therefore, the phage present in this zone were lytic. After each successful infection and replication of phage, it caused the lysis of the host cell and released a large number of progeny-phage which in turn infected the surrounding bacteria and repeated the cycle to increase the zone of lysis. However, all the clear plaques did not have the same size. This difference in plaque size denotes the presence of more than one phage type in the water sample. A slowly proliferating phage, one which yields a low number of progeny phage, will more likely to produce a smaller plaque compared to quickly proliferating phage (Irving et al., 1990). The physical size of a phage

also influences the overall size of plaque it produces. As smaller phage has less physical size, it will diffuse more easily and quickly through semi-solid agar in plaque assay plate in order to produce a zone of lysis. Whereas larger phage will face difficulty to diffuse, hence, it will produce smaller plaque size (Irving et al., 1990). Therefore, phage was picked and isolated from water sample which produced biggest plaque size assuming that the isolated phage had either higher burst size or smaller physical size or both to achieve greater lysis of host organism *Klebsiella pneumoniae*.

The titer found after propagation of the isolated phage Klpp1 in liquid culture is in sync with the idea considered for selecting bigger plaque size for phage isolation. The high titer of 10^8 pfu/ml indicates high efficacy of Klpp1 in terms of host bacterial control in liquid culture. A high titer is a desirable trait since it directs higher affinity of phage towards receptors displayed by host bacteria along with greater phage replication. Having a higher affinity for host bacteria increase the probability of phages to attach to the bacterial host and may require less time for infection to occur under a low multiplicity of infection (MOI) condition, which results in a low requirement of phage titer to achieve greater bacterial pathogen control.

The halo around the plaque indicates the decapsulation of the bacterial host cell by phage produced soluble enzyme such as depolymerase (Hughes et al., 1998). The hazy ring suggests that phage produced a depolymerase enzyme that defused through the agar layer and degraded the bacterial capsular polysaccharide (CPS) into different oligosaccharide components. Early studies showed that certain *Klebsiella pneumoniae* bacteriophages produced depolymerase during phage proliferation and released the enzyme from infected bacteria that targeted other bacteria's CPS (Adams and Park, 1956). Phage induced depolymerase positively affect the phage absorption to the bacterial cell surface. These enzymes might be linked with the phage particles (Adams and Park, 1956; Eklund and Wyss, 1962; Maré and Smit, 1969) as free enzyme that is produced during phage proliferation and lytic cycle (Bartell et al., 1966; Kimura and Itoh, 2003) or as both free and bound enzyme (Eklund and Wyss, 1962; Maré and Smit, 1969).

Biofilm formation is a problem in treatment procedure and Bacteriophages producing depolymerase can be advantageous often over some antibiotic in treating the bacterial infection that tends to form a biofilm. *Klebsiella pneumoniae* was documented as being able to grow biofilm

in vitro condition since the late 1980s (LeChevallier et al., 1988) but evidence of in vivo biofilm formation emerged in 1992 (Reid et al., 1992). Later many studies showed that *Klebsiella pneumoniae* isolated from blood, sputum, wound swabs, urine of catheterized patients suffering from UTIs, endotracheal tubes (ETT) of patients affected by ventilator associated pneumonia (VAP) were capable of producing biofilm (Niveditha et al., 2012; Singhai et al., 2012; Yang and Zhang, 2008). In the formation of the structure of biofilm communities, bacterial Capsule and LPS has been proven to be involved (Balestrino et al., 2008). Moreover, various studies to date, have demonstrated that some correlation exists between the biofilm-forming ability of *Klebsiella pneumoniae* strains and antibiotic resistance (Subramanian et al., 2012; Vuotto et al., 2014; Yang and Zhang, 2008). A major drawback of antibiotics in treating these biofilms is that as the biofilm ages antimicrobial agents are required in higher concentration to inactivate or eradicate the biofilm cells (Amorena et al., 1999; Anwar et al., 1992) and often effective only on younger biofilm but ineffective against older biofilm due to the heterogeneity and thickness of the biofilm (Cernohorská and Votava, 2004). Whereas, biofilm age did not considerably decrease susceptibility to phage (Hanlon et al., 2001). Combination of phage producing-depolymerase enzymes was even effective against mixed-species biofilm (Chhibber et al., 2015). Because of these advantages of bacteriophage over antibiotics against biofilm, the viability of using phage as therapeutic agents needs to be evaluated.

5.2 Host specificity:

Klpp1 phage was examined for its lytic ability against multiple host species. It was able to lyse majority of the *Klebsiella* isolates from NIDCH (63 %) and one *Klebsiella* spp bacterial culture. However, Klpp1 did not produce any plaque in any other bacterial cultures which includes *Klebsiella variicola* and other *Enterobacteriaceae* such as *E. coli* (both toxic and non-toxic variants), *Salmonella typhi, Hafnia alvei* and *Enterobacter* cultures. From the result, it seems that Klpp1 phage has host specificity within the genus *Klebsiella*. Though Klpp1 can infect multiple isolates of *Klebsiella* it can't be said for sure that the phage has broad host range since the species of the *Klebsiella* isolates were not identified and whether they are different strains or not was also not determined. Nevertheless, the result seems to indicate a broad host range contained within genus *Klebsiella* and this correlates with the idea that some phages have strain level specificity

while others have broader host range infecting only multiple strains of a single species to closely related several species (Donlan, 2009).

5.3 Thermal stability:

Temperature is an important factor influencing the bacteriophage stability (Hurst et al., 1980; Nasser and Oman, 1999; Yates et al., 1985). It influences the whole phage replication process including attachment, penetration, and multiplication (Olson et al., 2004). At suboptimal temperature, less amount of phage can enter its genetic material into the host organism, hence fewer phages can participate in phage replication. Additionally, temperature regulates the viability, occurrence, and storage of bacteriophage (Jończyk et al., 2011).

The Klpp1 phage was found to be stable up to 50ºC without any significant change in titer. Whereas, two log reduction in phage titer at 60ºC and complete reduction in phage titer at 70ºC indicate that the phage is not resistant to extreme temperature. As Klpp1 phage is stable at both 30ºC and 40ºC it can be said that the average human body temperature of 37ºC will not thermally deactivate its lytic activity if Klpp1 was considered as a therapeutic agent.

5.4 pH stability:

Acidity or alkalinity of the environment is another crucial factor in phage survivability (Krasowska et al., 2015). Klpp1 phage was resistant from pH range of 4.0 to 11.0 after 3 hours of incubation and became completely inactive at pH 2.0 and pH 12.0. The optimal pH value for Klpp1 was pH 6.0 to pH 8.0 where it was mostly stable. As the phage becomes inactive at pH lower than 4.0 Klpp1 is not suitable for direct oral delivery of the phage for therapeutic purpose without some kind of protective layer such as alginate and chitosan (Malik et al., 2017) since the baseline pH of 1.5 in human stomach (Beasley et al., 2015) will deactivate the phage. However, as Klpp1 is stable for a wide range of pH it can be used as a therapeutic agent for *Klebsiella pneumoniae* induced Urinary Tract Infection (UTI). It can be used in impregnation of urinary catheters to inhibit bacteriological biofilm as previously suggested by Verma et al. (2009).

5.5 Organic solvent sensitivity:

The sensitivity of bacteriophage towards different organic solvents is another important factor if phages are to be used for different therapeutic or biocontrol preparations where numerous organic solvents are involved. The stability of phage in the different organic solvent is mainly dependent on the stability of its protein which is influenced by the solvents ability to strengthen or destroy certain inter and intramolecular hydrophobic and electrostatic interactions (Olofsson et al., 1998). Competition between stabilization and destabilization of different interaction exist upon exposure to organic solvents (Olofsson et al., 1998).

Klpp1 phage was found to be resistant upon exposure to chloroform in a chloroform-aqueous environment which paves the way for storage of Klpp1 in chloroform which will keep the phage solution from bacterial contamination as chloroform is a known antimicrobial agent and it has been used often in preparation of phage stock (Cotton and Lockingen, 1963). On the other hand, Klpp1 was sensitive towards ethanol which correlates with the similar result found by Verma et al. (2009). Previously Olofsson et al. (1998) demonstrated that the ethanol concentration higher than 40 % would decrease the phage viability significantly it can be said that the ethanol with 70 % concentration, which is generally used in different laboratory to maintain aseptic condition, is a good option to preserve sterile environment where the Klpp1 presence is not acceptable.

5.6 One step growth curve:

One step growth experiment allows one to identify the effect of changes in the yield of virus per infected host cell and in chemical and physical properties on the period of an infectious cycle (Adams, 1959). Through one-step growth experiment, latent period and burst size were measured for Klpp1 phage. These two parameters are influenced by host culture, the temperature of incubation, medium on which the experiment was done, and specific growth rate (Keogh, 1973). In this study, Klpp1 showed a short latent period of 20 minutes and a high burst size of around 140 pfu/infected-cell. This short latent period and high burst size are of an obvious advantage since its results in the production of more infective phages in a short period of time which in turn increases the probability of host bacteria to interact and consecutively lysis of host organism. A previous study conducted by Abedon, (1989) relating the length of phage latent period and host density

found that the high host cell density select for phages with a shorter latent period, as phages with a short latent period can utilize the high bacterial host more quickly to proliferate. Therefore, it again indicates the presence of *Klebsiella pneumoniae* in high density in the sewage water sample where rich nutrient supports the rapid growth of host culture.

5.7 Limitations and future study:

The most common problem encountered in this study was high bacterial contamination occurred in the double layer assay plates. Even though the aseptic condition was maintained as long as possible during different experiments, different bacterial colonies would sometimes appear on the surface of the assay plates which would often cover a potential plaque resulting in a false plaque count which can affect the reliability and accuracy of the result. The source of these contaminations was traced back to the micropipettes used in the experiment which were not externally cleaned using ethanol before starting the experiment and often contamination in the phage stock solution. Cleaning the micropipette via ethanol and removing bacterial cells from phage stock by centrifugation and followed by filtration through a 0.22 μm syringe filter solved the bacterial contamination problem. Moreover, chloroform can be added to phage stock solution to prevent bacterial contamination (Cotton and Lockingen, 1963).

During double layer assay experiment, sometimes multiple plaques would overlap each other and would form a cluster which would make identifying individual plaque difficult. As the average plaque size was relatively big, using greater dilution would reduce the total plaque number which would decrease the probability of plaque overlap. Properly mixing the top agar with phage-host mixture would eliminate aggregation of plaque in a small area in double layer assay plate. As bubble tends to form during phage-agar mixing step which can interfere with the result, it was found that either rolling the test-tube quickly using inside of both palms or placing the top of the test-tube at the center of palm and then rotating the test-tube at a horizontal circular motion at a tilted position would decrease the chance of bubble formation significantly without sacrificing the appropriate mixing.

A total of 36 bacterial strains were used to specify the host range of Klpp1 phage, however, only 13 of them were from *Klebsiella* genus and none of them had species or strain identified. In order

to use phage as an antibacterial agent, it needs to have broad host range. Despite the Klpp1 indicates a broad host range, it needs to be determined more precisely using a larger pool of strain-specific *Klebsiella pneumoniae* and other *Klebsiella* species along with some antibiotic resistant strain bacteria so that the therapeutic potential of Klpp1 can be precisely measured.

Detailed characterization of different bacteriophage properties is a requirement for assessing its potential as a therapeutic agent. Some important characteristic of a phage, such as sensitivity towards temperature and pH fluctuation, stability in different organic solvent and phage growth dynamics were identified. However, none of the molecular analysis was done. In future, more study needs to be done to characterize the Klpp1 phage genetically and physiologically. These include estimation of phage genome length via Pulse Field Gel Electrophoresis (PFGE), Creating restriction digestion profile, cloning and sequencing the phage genome for a thorough understanding of the genes involved in the phage life cycle. Moreover, documentation of the phage morphology using transmission electron microscopy (TEM) needs to be done since according to guidelines of International Committee on Taxonomy of Viruses, phage morphology visualization is a prerequisite for classification of individual phage. Protein profiling of the phage needs to be carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the content of structural proteins of phage.

Proper isolation and characterization of the depolymerase enzyme produced by Klpp1 phage are required to properly identify mechanism involved in the phage absorption process. Additionally, the effectiveness of the depolymerase enzyme as a separate therapeutic tool needs to be investigated since previous studies indicate positive result achieved by depolymerase treatment which increased survivability of tested animal models (Lin et al., 2014; Majkowska-Skrobek et al., 2016; Mushtaq et al., 2004; Scorpio et al., 2008) and often increased the susceptibility of the bacterial host towards antibiotics when depolymerase in combination with antibiotic was used in vivo condition (Bansal et al., 2014). Therefore, the idea of using depolymerase enzyme alone, in combination with other antimicrobial agent or as a part of treatment procedure need to be assessed.

Conclusion:

This work aimed to isolate and characterize *Klebsiella pneumoniae* specific lytic bacteriophage from different environmental water samples in an attempt to isolate a therapeutically potential phage. This study led to the isolation of bacteriophage Klpp1 from a water sample which was further characterized to identify its host range, stability under the different adverse condition, and a couple of important parameter of its growth dynamics. The result of those experiments demonstrated that Klpp1 phage is pretty stable under pH fluctuation and can endure moderate temperature and remains stable in Chloroform. The phage may produce depolymerase enzyme and indicates a broad host range confined within genus *Klebsiella*. The stability of Klpp1 under different conditions along with phage-associated depolymerase production makes it a worthy candidate for further study to find out whether Klpp1 can be used for the treatment of *Klebsiella pneumoniae* related infections.

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Appendix - I

Media composition

The following media were used during the study. All components were autoclaved at 121ºC, 15 psi for 15 minutes unless mentioned otherwise

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Appendix-II

Reagent

The following reagent was used throughout the study:

SM buffer	Per 1000 ml 5.8 gram Nacl 2 gram MgSO4•7H2O 50 ml Tris-cl (1M, pH 7.4) 5 ml Gelatin (2% w/v)

Appendix – III

Instruments

Autoclave	Wisd Laboratory Instruments Made in Korea
Electronic Balance	Model: WTB 200 RADWAG Wagi ELEktroniczne
Incubator	Model: DSI 3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Microcentrifuge	Model: MC-12 Benchmark Scientific
Refrigerated microcentrifuge	Model: ScanSpeed 1730R Labogene
Refrigerator (40C)	Model: 0636 Samsung
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea
Spectrophotometer	Model: UVmini-1240 UV-VIS spectrophotometer SHIMADZU Corp
Syringe filter	MS® MCE Syringe Filter Membrane Solutions, LLC
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Water Bath WiseBath®	Wisd Laboratory Instruments DAIHAN Scientific Co., Ltd Made in Korea