Biocontrol of V. cholerae using bacteriophage

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This dissertation is dedicated to my beloved wife and loving son for their unwavering support and understanding throughout the work. Especially for my wife's resoluteness and her staunch backing in all the difficult times we went through together.

ABSTRACT

Cholera is a persistent threat to public health and is endemic in many countries. Of late, there is an emergence of antibiotic resistance in Vibrio cholerae and treatment is effective only if given early, thus there is a need for rapid and more effective treatment of cholera. One such treatment could be the use of bacteriophages. During infection, V. cholerae adheres to the surface of enterocytes but does not invade the host. They are therefore not protected from bacteriophage infection. The study presented in this dissertation evaluates the potential of bacteriophage being used as a biocontrol for V. cholerae. The aim of this project was isolation and in vitro characterisation of bacteriophages, selection of a candidate bacteriophage for biocontrol and its use in an infant rabbit model to assess its therapeutic efficacy.

Seven phages were isolated in China, attempts to isolate in the UK environments were unsuccessful and five more phages were obtained from various sources. In total twelve phages were characterised for the one step growth curves following their host strain growth curves, their lytic spectra, electron microscopy, PFGE, restriction analysis and annotation of sequenced genomes. These in vitro characterisations could help in selecting the candidate bacteriophage for in vivo phage therapy trials. Amongst the phages studied, the phage Φ1 most nearly fitted the selection criteria. Its burst size was 43 ± 5.5; while the latent period was 12 ± 0.0 and it had broad host range as it could lyse 67 % of the total 91 strains; while its genome did not show any undesirable genes associated with lysogeny/antibacterial resistance or any cholera toxin genes upon genome annotation. In therapeutic trials using an infant rabbit model, Φ1 reduced the bacterial numbers significantly (4.7 $\log_{10}$ reduction with $P < 0.001$) and treated animals showed no symptoms of disease.

DECLARATION

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Nottingham.

The work is original and has not been submitted for any other degree at the University of Nottingham or elsewhere.

Name: Sudhakar Ganapati Bhandare

Signature:

Date: 15/06/2015

CONFERENCE

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LIST OF COMMON ABBREVIATIONS

ACT: Artemis Comparison Tool

AMP: Adenine monophosphate

APW: Alkaline peptone water

ATP: Adenine triphosphate

BIM: Bacteriophage insensitive mutants

BLAST: Basic Local Alignment Search Tool

bp: Basepair

BRIG: BLAST Ring Image Generator

$CaCl_2$: Calcium chloride

Cas: CRISPR-associated genes

CDS: Coding sequence

CFR: Crude Fatality Rate

CFU: Colony forming units

cm: Centimetre

CRISPR: Clustered regularly interspaced short palindromic repeats

CsCl: Caesium chloride

CT: Cholera toxin

DNA: Deoxyribonucleic acid

ds: Double stranded

dsDNA: Double stranded DNA

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

FAR: Fluid accumulation ratio

g/RCF: G-Force/Relative centrifugal force

g: Gram

GDP: Guanosine diphosphate

GTP: Guanosine Tri Phosphate

h: Hour

HHpred: Homology detection & structure prediction

ICTV: International committee on taxonomy of viruses

Kb: Kilobase

KCl: Potassium chloride

kDa: KiloDalton

kV: Kilovolt

LB broth: Luria bertani broth

LPS: Lipopolysachharide

m: Minute

M: Molar

Mb: Megabase

mg: Milligram

$MgCl_2$: Magnesium chloride

MgSO4.7H2O: Magnesium sulphate hexahydrate

$MgSO_4$: Magnesium sulphate

ml: Millilitre

mM: Millimolar

MOI: Multiplicity of infection

MRD: Maximum recovery diluent

NaCl: Sodium chloride

NAD: Nicotinamide adenine dinucleotide

NaOH: Sodium hydroxide

NCBI: National Center for Biotechnology Information

ng: Nanogram

nm: Nanometre

OD_{600}: Optical density 600

ORF: Open reading frames

PAM: Proto spacer associated motif

PEG: Polyethylene glycol

PFGE: Pulsed-field gel electrophoresis

PFU: Plaque forming units

Phage: Bacteriophage

PHAST: Phage search tool

PHE: Public Health England, erstwhile HPA i.e. Health Protection Agency

RAST: Rapid annotation using subsystem technology

RFLP: Restriction fragment length polymorphism

RIL: Rabbit ileal loop

RITARD: Removable intestinal tie-adult rabbit diarrhoea

RM: Restriction modification

RNA: Ribonucleic acid

RO: Reverse osmosis

RPM: Revolutions per minute

s: Seconds

SAM: Sealed adult mice

SD: Standard deviation

SEM: Scanning electron microscope

SEM: Standard error of mean

SM buffer: Salt magnesium buffer

SmR: Streptomycin resistant

SNP: Single Nucleotide Polymorphism

SXT: Sulfamethoxazole–trimethoprim

TAE buffer: Tris-acetate-EDTA

TCBS: Thiosulphate citrate bile salt

TCP: Toxin Co-regulated Pilus

TE buffer: Tris-EDTA buffer

TEM: Transmission electron microscope

tRNA: Transfer RNA

TSA: Tryptone soya agar

UK: the United Kingdom

V. cholerae: Vibrio cholerae

w/v: Weight/volume

WHO: World Health Organisation

%: Percentage

°C: Degree Celsius

µl: Microlitre

µm: Micrometer

INTRODUCTION

1. INTRODUCTION

1.1 Cholera the disease

Cholera is a historically important disease causing many deaths globally and remains a very significant public health concern in several developing countries. Along with other old world bacterial diseases such as tuberculosis, typhoid, plague etc that have costed millions of lives cholera is even today a considerable risk to public health. There were 1.5 million deaths for tuberculosis, 161 thousand deaths for typhoid and 126 deaths for plague in the year 2013 (WHO 2013). Though the number of deaths for tuberculosis seems higher they were coupled with HIV (Human Immunodeficiency Virus) disease, while typhoid shows similar deaths and impact as like cholera but doesn't take pandemic proportion. Thus, cholera still remains to be a bacterial disease with pandemic potential. The world has witnessed several cholera pandemics with the major focus being the Indian subcontinent and with the current pandemic affecting Central and South America with rapid epidemic spread (Roy, Zinck et al. 2014). The disease is endemic in Africa, Asia and South America, with the occurrence of outbreaks usually coinciding with and/or resulting from conflict and natural disasters (Martinez, Megli et al. 2010). The disease shows seasonality in South Asia with most outbreaks being reported just before and after the monsoon i.e. April and September to December, respectively, with case numbers peaking during the summer season i.e. from January to February. In South America and in the African countries the outbreaks occur following summer rains or floods (Emch, Feldacker et al. 2008). The incidence of cholera cases and deaths in recent years is shown in the Figure 1. The sudden surge of cases in the year 2011 is attributed to the Haiti epidemic following a devastating earthquake. Haiti still continues to contribute most of the cholera cases reported globally and it was estimated that 45,000 cases to be reported for the year

2014 (UNO 2014). The most recent epidemic outbreak occurred in South Sudan with 1,812 cholera cases reported and 38 deaths (WHO 2014). Thus, in spite of extensive efforts by Governmental and non-Governmental agencies over the decades, cholera has not been eradicated as a major cause of epidemic disease and death.

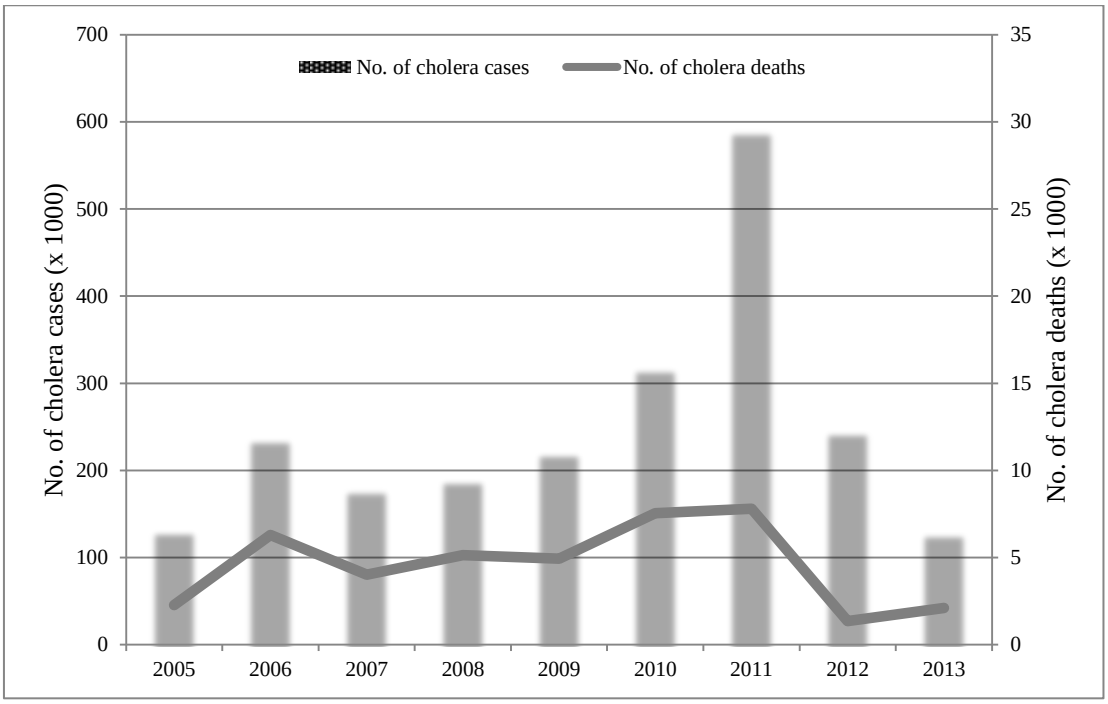

Figure 1: The cholera cases and deaths globally (2005-2013)

(Source: WHO statistics data base, http://apps.who.int/ghodata/# accessed on 18/03/2015)

The CFR (Crude Fatality Rate i.e. percentage of people who die after being diagnosed positive for cholera) can be as high as 50% without treatment (Sack, Sack et al. 2004). Moreover, many cholera deaths remain unreported, owing to the remoteness of the communities in developing countries and a lack of communication and reporting infrastructure. Developed countries in Europe and North America usually have imported cases (Fig. 2), reported from travellers visiting disease-prone areas and returning back with the disease (Morger, Steffen et al. 1983). With appropriate sanitation measures in place, the developed countries are able to prevent cholera but

there is a real need to address the possible measures for prevention and effective cholera treatment in developing countries.

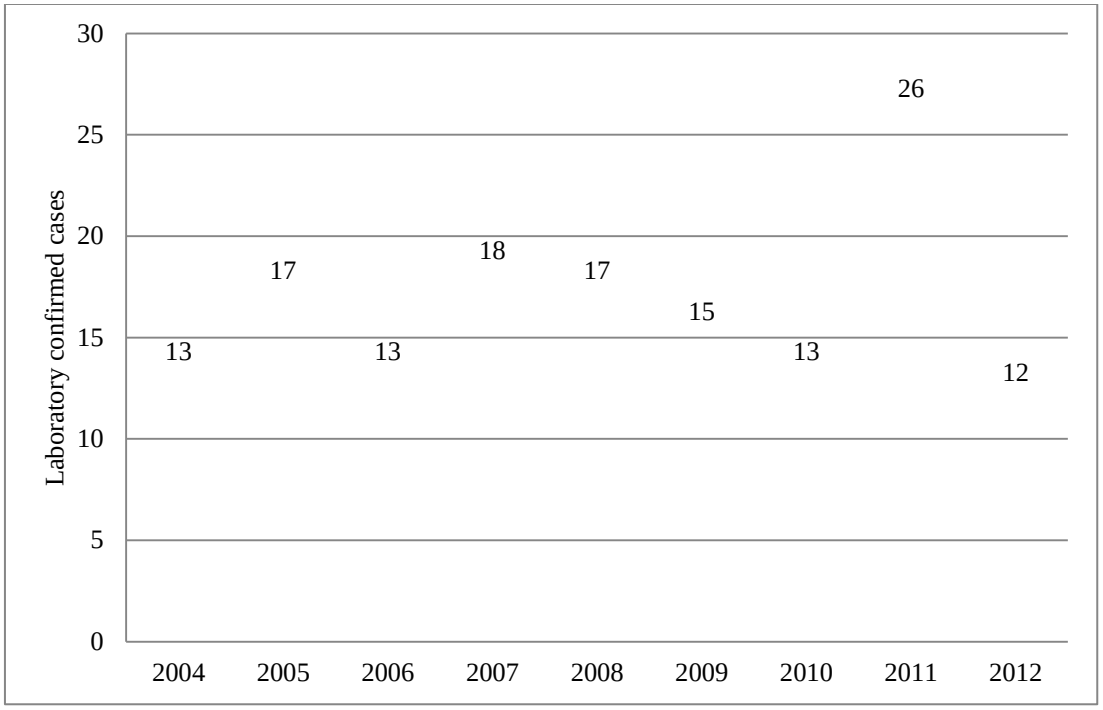

Figure 2: Reports of imported cholera cases in the UK (2004–2012)

(Source:http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/TravelHealth/EpidemiologicalData/GastrointestinalInfections/travCholera/ accessed on 18/03/2015)

1.2 Pathogenesis of cholera

V. cholerae is an aetiological agent of cholera which is contracted through consumption of water or food contaminated with the organism mainly arising directly or indirectly from human faeces. V. cholerae produces an enterotoxin (CTX) which leads to acute, copious, watery diarrhoea with as much as 20 litres of fluid loss per day and death due to circulatory failure owing to dehydration, if not treated in time. V. cholerae is a curved and motile Gram negative rod shaped organism with a single polar flagellum. The strains of disease-causing V. cholerae are grouped into two serogroups O1 and O139, based on their lipopolysachharide (LPS) O antigens.

Serogroup O1 has three serotypes viz. Inaba, Ogawa and Hikojima; while two biotypes viz. classical and El Tor (Salyers and Whitt 2002). The earlier pandemics were caused by members of the O1 serogroup, but the eighth pandemic was reported to be caused by O139 which originated from the Bay of Bengal (Swerdlow and Ries 1993).

The major virulence genes needed for pathogenesis are clustered as two genetic elements viz. the genes encoded by the lysogenic filamentous phage CTXphi for cholera toxin (Levin and Tauxe 1996, Waldor and Mekalanos 1996, Davis and Waldor 2003) and the genes encoded for the Toxin Co-regulated Pilus (TCP), a colonisation factor situated next to the Vibrio Pathogenicity Island (VPI) (Faruque, Albert et al. 1998, Faruque and Mekalanos 2003). Cholera toxin (CT) and TCP are controlled by a regulatory protein, ToxR which co-regulates their expression. Intestinal colonisation by V. cholerae is mediated by fimbriae, which are filamentous protein structures e.g. TCP, Type B and Type C fimbriae (Hall, Vial et al. 1988). TCP is essential for the colonisation process. TCP attaches to receptors present on the mucosa of upper small intestine and helps in colonisation (Attridge, Voss et al. 1993). Other fimbriae (i.e. Type B and Type C) are non-adhesive and do not play a role in colonisation of V. cholerae (Nakasone, Yamashiro et al. 1994). After attachment the organism produces CT which causes secretory diarrhoea. The CT causes increased chloride ion secretion and net water flow in to the gut lumen and decreased sodium ion absorption into the tissues via the blood stream (Fig 3). As a result there is rapid loss of water into the lumen along with chloride ions causing massive diarrhoea and electrolyte imbalance (Salyers and Whitt 2002).

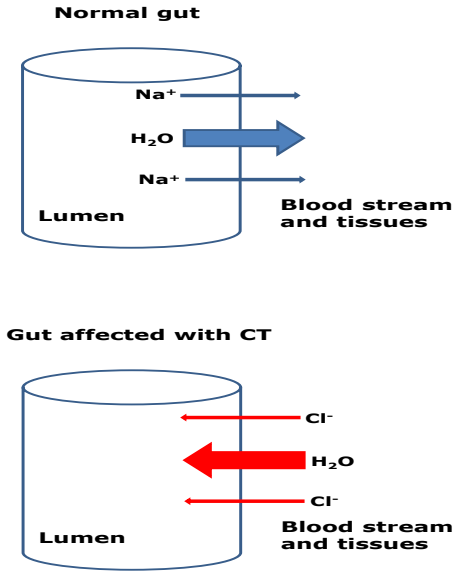

Figure 3: Movement of ions and water across the normal and affected gut

The sodium and chloride ion channels are altered by high levels of cAMP (cyclic AMP) which is a mediator in intracellular signalling pathways and cAMP synthesis is catalysed by an enzyme adenyl cyclase. Activity of adenyl cyclase is regulated by G proteins which are intracellular signalling proteins (Ghose 2011). The cholera toxin keeps adenyl cyclase activated continuously through active G proteins. In normal gut the G proteins are in an active form when they are GTP bound and when GTP is hydrolysed by GTPase they become inactive (Alberts, Johnson et al. 2008). In the gut affected with CT, a toxin stops GTP hydrolysis and thus the G protein remains in an active state all the time (Salyers and Whitt 2002). Eventually, it increases the levels of cAMP which alter ion channels causing a huge efflux of chloride ions as well as water into the gut producing severe diarrhoea.

In early stages of the disease, symptoms include muscle as well as stomach cramps accompanied by vomiting and fever. The disease later progresses into the severe diarrhoea exhibiting ‘rice water stools’, which has appearance similar to water after

washing rice. The skin turns black and blue while the eyes and cheeks become sunken due to rapid dehydration (Anonymous 2015).

1.3 Treatment/prophylaxis of cholera

1.3.1 Rehydration therapy

Rehydration therapy is effective if replacement of fluids lost due to severe diarrhoea are compensated as quckly as they are lost (Sack, Sack et al. 2004). WHO recommends that oral rehydration salts (ORS) (WHO 2002) which come in the standard sachets are useful but for severely dehydrated patients intravenous fluid replacements are needed.

1.3.2 Antibiotic therapy

Though rehydration therapy is the mainstay of treatment for cholera, oral antibiotics are given to dehydrated patients as soon as possible after vomiting stops (Sack, Sack et al. 2004). They are given to shorten the duration of illness and also to reduce the diarrhoeal fluid output (Lindenbaum, Greenough et al. 1967). The combination of antibiotics gives a synergistic effect in treatment of cholera (Mandal, Pal et al. 2009). The different types of antibiotics used and antibiotic resistance in V. cholerae are discussed further in the next section (§ 1.4).

1.3.3 Vaccination

Soon after discovery of V. cholerae as an aetiology of cholera, injectable parenteral vaccines (killed whole cell) were developed but these provided short lived immunity of just 6 months and frequently involved painful local inflammatory reactions (Mosley, Aziz et al. 1972). Later oral whole-cell/recombinant-B-subunit cholera vaccines (e.g. Dukoral) were developed which provide protection for up to 2 years (WHO 2010) for cholera and they also provide cross protection for enterotoxigenic E.

coli (ETEC) for up to 6 months (Peltola, Siitonen et al. 1991) and are recommended by WHO. Though recent oral cholera vaccines are better than earlier parenteral vaccines they do not confer 100 % protection but they reduce the risk by 80 % and the immunity can be overcome by a high inoculum of infective organisms (Sack, Sack et al. 2004). The oral cholera vaccines should not be taken as sole preventive measures in control of cholera disease in isolation from other measures (Lopez, Gonzales et al. 2014).

1.4 Antibiotic resistance in V. cholerae

As with many other bacterial pathogens, V. cholerae is rapidly developing resistance to several antibiotics used for treatment of cholera (Kitaoka, Miyata et al. 2011). A report on 'Global surveillance of antibiotic sensitivity of Vibrio cholerae' in the year 1976 revealed that out of 1156 strains (single colony isolates from pure culture) examined, 27 were resistant to ampicillin, chloramphenicol, tetracycline, furazolidone and three different sulphonamides; while 2 were resistant to chloramphenicol only (O'Grady, Lewis et al. 1976). Recently, the reports of multiple drug resistant V. cholerae strains from different countries around the world have increased substantially viz. Bangladesh (Rashed, Mannan et al. 2012), Cameroon (Akoachere, Masalla et al. 2013), China (Li, Tan et al. 2011), Ethiopia (Abera, Bezabih et al. 2010), Haiti (Sjolund-Karlsson, Reimer et al. 2011), India (Panda, Patra et al. 2012), Indonesia (Tjaniadi, Lesmana et al. 2003), Iran (Ranjbar, Rahmani et al. 2010), Madagascar (Rakoto Alson, Dromigny et al. 2001), Mozambique (Mandomando, Espasa et al. 2007), Namibia (Smith, Keddy et al. 2008), Nepal (Karki, Bhatta et al. 2010), Pakistan (Jabeen, Zafar et al. 2008), Thailand (Chomvarin, Jumroenjit et al. 2012), Vietnam (Tran, Alam et al. 2012), Zimbawe (Islam, Midzi et al. 2009) etc.

The antibiotics for which resistance has been reported in the last decade are amoxicillin, ampicillin, chloramphenicol, cotrimoxazole, ciprofloxacin, doxycycline, erythromycin, fluoroquinolone, furazolidone, gentamicin, kanamycin, nalidixic acid, neomycin, norfloxacin, polymyxin B, quinolone, streptomycin, spectinomycin, SXT (sulfamethoxazole–trimethoprim), sulphonamides, tetracycline, trimethoprim and vancomycin (Kitaoka, Miyata et al. 2011). In the 1980s, the first antibiotics to which V. cholerae showed resistance were tetracycline, ampicillin and sulphonamides followed by nalidixic acid, cotrimoxazole and recently fluoroquinolones which led to emergence of multiple antibiotic-resistant V. cholerae (MARV) (Mandal, Dinoop et al. 2012). There are varied mechanisms whereby V. cholerae become resistant including efflux pumps, spontaneous mutations, conjugative exchange of extra chromosomal elements such as plasmids, transposons/SXT elements and integrons. V. cholerae is able to export the tetracycline, chloramphenicol, norfloxacin, ciprofloxacin or nalidixic acid molecules out of the cell using multidrug efflux pumps as they are chemically and structurally unrelated (Paulsen, Brown et al. 1996). Spontaneous chromosomal mutations confer resistance to the antibiotics like alafosfalin and β lactam antibiotics (amoxicillin, ampicillin, cephalexin) which act by inhibiting cell wall biosynthesis (Allen, Atherton et al. 1979) and to quinolones which act by inhibiting DNA replication (Baranwal, Dey et al. 2002). Resistance is also conferred by horizontal gene transfer via extra chromosomal elements like plasmids (tetracycline, ampicillin, kanamycin, streptomycin, gentamicin and trimethoprim) (Kitaoka, Miyata et al. 2011), integrating transposable conjugative elements i.e. SXT elements (sulfamethoxazole, trimethoprim, streptomycin and furazolidone) (Waldor, Tschape et al. 1996) and integrons (trimethoprim) (Mazel 2006) by conjugation.

Antibiotic resistance is a major problem in V. cholerae with no new antibiotic classes being developed (Braine 2011) and thus there is a growing need for an alternative therapy. One such alternative treatment is the biological control of V. cholerae using host-specific bacteriophage (Barrow 2001, Matsuzaki, Uchiyama et al. 2014). Unlike broad-spectrum antibiotics which also kill beneficial intestinal bacteria, phages are species and strain specific and kill only the targeted bacteria. Lytic phages are responsible for limiting bacterial numbers in an aquatic environment with up to 80 % of mortality evidenced in the bacterial population (Weinbauer 2004). There is evidence for V. cholerae in the environment being controlled by lytic phages (Faruque, Islam et al. 2005, Jensen, Faruque et al. 2006, Nelson, Chowdhury et al. 2008). Earlier reports on the use of phage therapy for cholera indicated partial success (Asheshov, Saranjam et al. 1930, Morison 1932, Monsur, Rahman et al. 1970, Marcuk, Nikiforov et al. 1971). There were no reports based on animal studies at that time. Thus, a systematic study based on in vivo animal experiments may give positive outcomes for disease control using this alternative approach.

1.5 Bacteriophage

Bacteriophages are viruses that infect bacteria. They are obligate intracellular parasites which rely on the host bacterium in order to replicate. Bacteriophages enclose their nucleic acid in a protein coat (capsid), which may be further surrounded by a lipid layer (Nicklin, Graeme-Cook et al. 1999). In addition to the capsid (head), tailed bacteriophage (members of the Caudovirales order), possess a tail which may either be contractile (e.g. T4 phage) or non-contractile (e.g. phage λ). They may also possess additional structures such as a collar, basal plate, spikes and tail fibres, which

are involved in attachment to the bacterium and injection of the nucleic acid into the cell (Fig. 4).

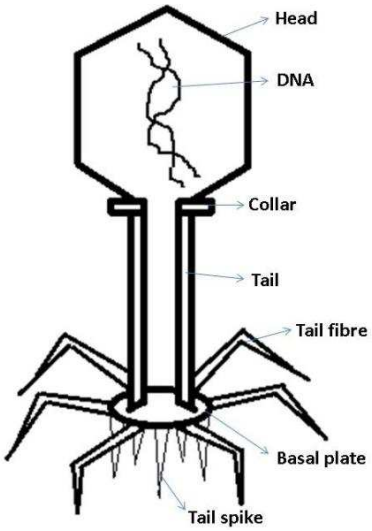

Figure 4: Schematic representation of bacteriophage

Phages are ubiquitous on earth and are found in large numbers in the environment (i.e. water, soil, sewage etc.), wherever their hosts are present (Guttman, Raya et al. 2003). Phages can remain viable under adverse conditions (Jonczyk, Klak et al. 2011). Phages exhibit three different lifestyles based on their survival strategies viz. lytic, lysogenic and pseudolysogenic (Miller and Day 2008). In a lytic life cycle, the phage infects the cell, multiplies and progeny phages burst from the cell killing the bacterium. In a lysogenic life cycle, the phage does not replicate but its genome goes into a quiescent condition where it is called prophage and where it is usually integrated into the host genome or alternatively it may be maintained as an extrachromosomal plasmid (Guttman, Raya et al. 2003). There are some phages which can enter either the lytic cycle or the lysogenic cycle and they are called the temperate phages, while some phages are strictly lytic. In a pseudolysogenic life cycle, the phage does not undergo lysogeny nor does it show a lytic response but it remains in a non-active state (Ripp and Miller 1997). Pseudolysogeny occurs during starvation

conditions and when nutrient supplies are available again the phage can either enter the lysogenic or lytic life cycle. Apart from these three generally described life cycles, a carrier state life cycle (CSLC) is reported (Siringan, Connerton et al. 2014) as an alternative phage life cycle in which bacteria and phages are in an equilibrium state with some bacteria resistant to phage but some of them sensitive to phage and thus allowing both of them to sustain.

For phage therapy purposes, lytic phages are utilised and thus only the lytic life cycle of phage is explained further. In the lytic life cycle (Fig. 5), the phage adsorbs on the surface of the bacterium at receptor structures such as lipopolysaccharides or membrane proteins in the outer membrane of the bacterium (Kutter, Raya et al. 2003) or pili (Roncero, Darzins et al. 1990). For adsorption in T4 like phages the first step is a reversible binding of long tail fibres to their specific receptors as mentioned above. The initial energy for this process is provided by the baseplate. Once three or more long tail fibres position the baseplate parallel to the host surface, the six short tail fibres or spikes bind irreversibly to a secondary receptor i.e. heptose residue of the LPS inner core (Crawford and Goldberg 1980, Montag, Hashemolhosseini et al. 1990). In order to cross the peptidoglycan layer, the baseplate protein utilises a lysozyme enzyme which is located in the gp5 protein at its tip (Kanamaru, Leiman et al. 2002). When gp5 protein comes in contact with with phosphatidyl glycerol of the inner membrane, the signal for release of DNA is given and DNA is transported by electrochemical potential (Goldberg, Grinius et al. 1994). After injection of genetic material, the replication of phage DNA occurs. Later, synthesis of various phage components is carried out and further to it the assembly of all phage particles in to new progeny phages is done. The new virions burst out of the cell by the action of

holin and endolysin proteins. Holins form a pore in the inner membrane through which a peptidoglycan degrading enzyme (endolysin) accesses the cell wall, eventually causing it to rupture and release the bacteriophage progeny (Wang, Smith et al. 2000).

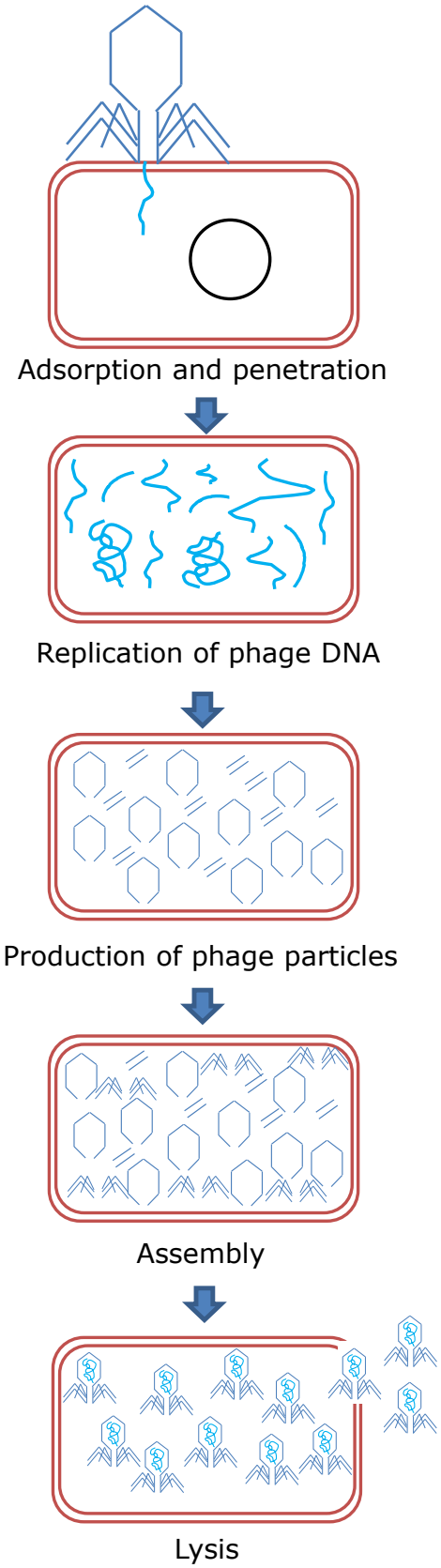

Figure 5: Schematic representation of lytic life cycle of bacteriophage

The different stages of the lytic life cycle are studied by the one step growth curve which was originally performed by Ellis and Delbrück (Ellis and Delbruck 1939). The one step growth curve was designed to determine the kinetics of phage multiplication and the number of phage particles that result from infection of a cell by a single phage. As depicted in Figure 6, after adsorption, the phage particles can not be isolated for some time (Doermann 1953) due to uncoating of phage and thus only naked phage DNA is present during this period of time which is called the eclipse phase. Chloroform-induced lysis and subsequent plating allows us to identify the eclipse period by enumerating intracellular phages which will be lower than the intial phage numbers until the time they start increasing again. The latent period is the minimum length of time from adsorption until release of newly formed phages extracellularly. The replication of nucleic acid and proteins formation occurs during the latent period. Later the phage particles undergo maturation i.e. the nucleic acid and proteins are assembled into mature phages. After maturation the phages are released by enzymatic lysis of the host bacterium. Burst size is defined as the average yield of phage particles per cell (§ 2.4.12). The latent period as well as burst size are characteristic of different phage strains but may vary according to the propagating host, media and temperature used (Guttman, Raya et al. 2003). With most phages a single round of multiplication is completed within 30-60 m (Madigan, Martinko et al. 2003).

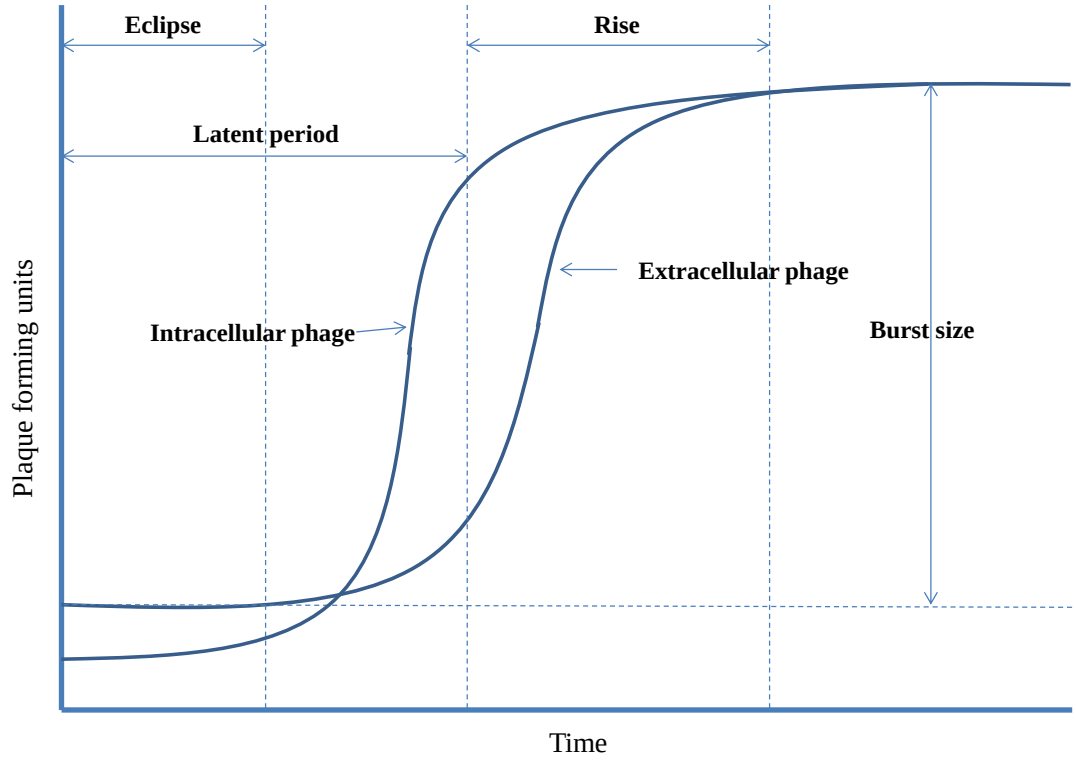

Figure 6: Schematic representation of phage one-step growth curve

1.6 Phage therapy

1.6.1 History of phage therapy

The first antibacterial activity of phage was noticed against V. cholerae by a British bacteriologist Hankin in the year 1896 (Hankin 1896), when he filtered water from the rivers (Ganga and Yamuna) in India (Summers 2001) to find that a substance which was heat labile could cause lysis of V. cholerae and he thought that it could have limited the cholera epidemic. Later, bacteriophages were discovered independently by Twort (Twort 1915) and d'Herelle (d'Herelle 1917). The term bacteriophage meaning bacteria eater was coined by d'Herelle. As reviewed by Summers (Summers 2005), the therapeutic potential of phages to treat bacterial infections was particularly appreciated by d'Herelle and he successfully carried out experiments in chickens for treating Bacillus (Salmonella) gallinarum that causes avian typhosis. He also

conducted experiments with claims of effectiveness against Salmonella pullorum and Pasteurella infections of animals. In 1926 d'Herelle self administered phage preparations and gave them to his colleagues and family members to ascertain their safety before administering them to human patients in treating bacillary dysentery. After he treated four patients of bubonic plague in Egypt, the British Government invited him to the Haffkine Institute, Bombay, India for Asiatic cholera treatment studies (these studies are discussed later in this chapter). Later, phages were commercially expoited by pharmaceutical companies for some time with exaggerated claims and marketing e.g. the Enterophagos preparation was marketed for treatment of herpes infections, urticaria and eczema (Barrow and Soothill 1997) which was clearly not possible. By the end of the 1940's, the advent of antibiotics accompanied by the poor quality of some of the scientific studies arising from an inadequate understanding of the phage-host interactions led to phage therapy being discontinued in the western world. Nevertheless, phage therapy continued to be used in Russia and Eastern European countries (Chanishvili, Chanishvili et al. 2001, Ryan, Gorman et al. 2011).

1.6.2 Reappraisal of phage therapy in the west

Phage therapy was again revived during the 1980's after rigorous clinical phage therapy experiments were carried out by H. Williams Smith and his colleagues on oral infections of enterotoxigenic Escherichia coli (ETEC) diseases of neonatal animals and on systemic infection caused by E. coli in mice (Smith and Huggins 1982, Smith and Huggins 1983, Smith, Huggins et al. 1987). They successfully treated experimental systemic E. coli infection in mice using bacteriophage comparing the effect with antibiotics (Smith and Huggins 1982). They tested 15 phages isolated from

sewage, out of which 9 were anti-K1 (K1 is an antigen which is an important surface virulence factor of E. coli strain O18ac:K1:H7ColV^{+}). Administered intramuscularly, the most effective phage was also the one found to be most rapidly lytic in vitro. Phage prevented death and illness in mice inoculated intramuscularly (in a different muscle) or intracranially with the bacterium. There was evidence of phage multiplication in vivo and a single dose of phage was more effective than 8 doses of streptomycin. Some resistant mutants arose but these were largely K1-negative mutants and thus of reduced virulence. Similar studies have been performed with E. coli septicaemia and meningitis in chickens and colostrum-deprived calves (Barrow, Lovell et al. 1998). As evidenced to be effective for treatement of meningitis, the phage therapy can be used in central nervous system diseases as phages can cross the blood brain barrier (Ritchie, Wagner et al. 2003). Smith's studies on enteritis involved enterotoxigenic E. coli infection in neonatal calves, pigs and sheep. The pathogenesis of ETEC is virtually identical to that of cholera with adhesion to the small intestinal mucosa and production of a toxin affecting cAMP levels. In these cases, phages were sought which would attach to the surface virulence determinants K88 and K99, but without success, and phages attaching to LPS were used. Phages were used singly and in combination, the latter to ensure ability to control phage resistant mutants that arose against single phage use. Phages were used successfully prophylactically and also therapeutically such that administration could be delayed until the onset of diarrhoea. They could also be used to spray bedding which was also effective in preventing clinical disease after administration of the pathogen. Although, the interest in phage therapy in the West was reinvigorated by these very rigorous experiments, this approach had not taken hold as might have been expected given its experimental

success but there is now a resurgent interest owing to the emergence of antibiotic resistance in recent years (Chopra, Hodgson et al. 1997, Sack 2001)

In recent years phage therapy was attempted for several other bacterial species viz. Salmonella spp. (Berchieri, Lovell et al. 1991, Goode, Allen et al. 2003, Atterbury, Van Bergen et al. 2007), Campylobacter spp. (Atterbury, Connerton et al. 2003, Loc Carrillo, Atterbury et al. 2005, Wagenaar, Van Bergen et al. 2005, Carvalho, Susano et al. 2010, Carvalho, Gannon et al. 2010, Connerton, Timms et al. 2011), Listeria monocytogenes (Leverentz, Conway et al. 2003, Leverentz, Conway et al. 2004), P. aeruginosa (McVay, Velasquez et al. 2007, Vinodkumar, Kalsurmath et al. 2008, Harper and Enright 2011, Morello, Saussereau et al. 2011, Krylov 2014), Staphylococcus aureus (Wills, Kerrigan et al. 2005, Gill, Pacan et al. 2006, Capparelli, Parlato et al. 2007, Sunagar, Patil et al. 2010, VinodKumar, Srinivasa et al. 2011), Klebsiella pneumonia (Vinodkumar, Neelagund et al. 2005, Kumari, Harjai et al. 2009), Clostridium difficile (Ramesh, Fralick et al. 1999), Enterococcus faecalis (Biswas, Adhya et al. 2002, Yoong, Schuch et al. 2004, Uchiyama, Rashel et al. 2008) etc. There are increasing reports on the therapeutic utility of phages leading to development of a substantial knowledge bank for harnessing the true potential of phages in curing bacterial diseases.

1.6.3 Phage therapy of cholera

Fluid replacement therapy is often effective for cholera, if given in the earlier stages (§ 1.2). However, due to poor infrastructure and facilities in many countries where cholera is endemic, many patients during cholera outbreaks present with advanced stages of the disease (§ 1.2). In these cases the case fatality rate is usually high and

also there are limitations on the use of vaccines for cholera (§ 1.3.3) to prevent such situations. In addition, fluid replacement therapy results in extensive shedding of the pathogens during treatment and simultaneous use of antibiotics such as tetracycline can increase the risk of development of resistance. Thus, there is a need for a rapid and more effective alternative treatment for cholera and that could be the use of phage therapy.

1.6.3.1 Phage therapy of cholera in humans

The first study for phage therapy against cholera disease was reported by Felix d'Herelle. During this study when the cholera patients were treated with oral doses of bacteriophage the mortality rate was 8.1 %, while in the controls i.e. patients treated with other medicines it was 62.9 %. The mortality rate in the phage treated group was zero if treatment occurred within 6 hours of appearance of the first symptoms (d'Herelle, Malone et al. 1930). It was also reported that between 1928 and 1931 phage was used successfully to treat cholera cases in the North Eastern region of India viz. Shillong, Goalpara, Jakrem, Naogaon and Habibganj (Morison 1932). During the same period of time Asheshov and colleagues (Asheshov, Saranjam et al. 1930) reported successful treatment of patients in one location although their treatment with phage was unsuccessful in another location. The authors mentioned that although the phage was able to arrest the progress of disease it was more effective used as a prophylactic rather than a therapeutic. In the years 1958 and 1960, animal passaged phage preparations (§ 1.6.3.3) were successfully used in treating cholera patients in Afghanistan (Sayamov 1963). An initial intravenous or intramuscular phage administration with saline followed by oral administration for three days gave satisfactory results.

The WHO reported the studies which assessed the effectiveness of phage therapy for cholera (Monsur, Rahman et al. 1970, Marcuk, Nikiforov et al. 1971). Monsur and coworkers (Monsur, Rahman et al. 1970) treated eight patients of cholera with large doses of bacteriophage with high titre phage (10^{12} PFU/ml). They compared their results with 50 patients as control group treated with intravenous fluid alone and also with 18 patients treated with tetracycline. Four out of eight phage treated patients showed a rapid decline of V. cholerae numbers in stool samples as observed by means of dark field microscopy. The same four patients also showed appreciable reduction in the stool output and duration of diarrhoea compared to the control group. In another four patients treated with phage, V. cholerae numbers declined more slowly and the volume of the stool as well as the duration of diarrhoea was greater than the control group. Overall, the treatment with bacteriophage was partly (50%) effective based on the rate of decline of bacterial numbers, the stool output and duration of diarrhoea but was not as effective as the treatment with tetracycline.

Marcuk and colleagues (Marcuk, Nikiforov et al. 1971) performed studies using phage preparations of between 10^8 and 10^9 PFU/ml given both orally and intramuscularly to adult as well as paediatric patients. They also compared their results with tetracycline treatment and with a placebo control. The post-treatment stool output (9.3 & 2.4 litres in phage treated; while 1.7 & 1.2 litres in tetracycline treated for adults and children, respectively), the duration of diarrhoea (76 & 52 h in phage treated; while 34 & 39 h in tetracycline treated for adults and children, respectively) and the duration of positive culture from stool samples (4.3 & 3.2 days in phage treated; while 0.7 & 0.8 days in tetracycline treated for adults and children, respectively) were all significantly

less in the tetracycline treated group as compared to the phage treated and placebo groups. The authors mentioned that although phage therapy was promising, it did not work in their clinical trial. They reasoned that this might be due to relatively low dosage used in their trial compared to the earlier work of Monsur et al. (1970).

Until Smith's group carried out his animal studies, there was very little known about the phage host interactions. Smith suggested that the phages of highest virulence in vitro should be used for in vivo studies. Previously, phage therapy experiments were poorly designed and in some cases phages were poured in to drinking water wells for control of cholera (Morison 1932). Also, the use of phage prophylaxis led to neglect of basic hygiene measures and thus, when antibiotics were successful in treating cholera, phage investigations were discontinued (Pollitzer, Swaroop et al. 1959). To understand better the nature of phage host interactions in vivo as suggested by Smith, a good animal model of cholera phage therapy is required.

1.6.3.2 Animal models for cholera phage therapy

Several animal models may be utilised for the study of pathogenesis of the V. cholerae and also for biological assays of their toxins. The first animal model developed was the adult rabbit (De and Chatterje 1953, Richardson 1968, Finkelstein and LoSpalluto 1969, Nelson, Clements et al. 1976, Spira, Sack et al. 1981, Spira and Sack 1982, Pierce, Kaper et al. 1985, Russell, Tall et al. 1992), followed soon afterwards by the infant rabbit (Dutta and Habbu 1955, Dutta, Panse et al. 1959, Jenkin and Rowley 1959, Oza and Dutta 1963, Finkelstein, Norris et al. 1964, Kasai and Burrows 1966, Smith 1972, Madden, Nematollahi et al. 1981, Ritchie, Rui et al. 2010); mice (Ujiiye and Kobari 1970, Chaicumpa and Rowley 1972, Baselski, Briggs et al. 1977,

Angelichio, Spector et al. 1999, Krebs and Taylor 2011); rat (Aziz, Mohsin et al. 1968); gnotobiotic/germfree mice (Sack and Miller 1969); sealed adult mice [SAM] (Richardson, Giles et al. 1984); dog (Sack, Carpenter et al. 1966, Carpenter, Sack et al. 1968) and non-human primates (Northrup and Chisari 1972).

It was in the early 1950s that the use of animal models for cholera came to be assessed to improve understanding of the mechanism of pathogenesis of V. cholerae. Since then many animal models have been used but until today no animal model replicates completely all the essential features of the cholera as exhibited in humans (Richardson 1994). The earliest animal model developed for cholera was adult rabbit model (De and Chatterje 1953) for investigation of the permeability changes in the capillaries due to V. cholerae after introducing them into ligated intestinal (ileal) loops of the small intestine. Further, Richardson (Richardson 1968) studied an ion translocase system of the rabbit intestinal mucosa which is inhibited by V. cholerae culture filtrates, while Finkelstein and Lospalluto (Finkelstein and LoSpalluto 1969) studied the biological activity of choleragen i.e. cholera toxin administered into ligated intestinal loops of adult rabbits. In 1981, Spira and colleagues (Spira, Sack et al. 1981) developed an adult rabbit model for enteric infection by V. cholerae and enterotoxigenic E. coli using a slip knot tie of the small intestine for challenge by vibrios which was removed after 2 hours and they designated this model as RITARD (removable intestinal tie-adult rabbit diarrhoea) model. With this improvisation, they were able to produce massive and usually fatal cholera-like diarrhoea in adult animals. They further (Spira and Sack 1982) studied the kinetics of cholera infection in their RITARD model with production of overt diarrhea after 11 hours postchallenge. The RITARD model had also been used for investigating the pathogenesis of non-O1 V. cholerae (Russell, Tall

et al. 1992). Pierce et al (Pierce, Kaper et al. 1985) investigated the role of cholera toxin in enteric colonisation by vibrios in rabbits ileal loop (RIL) model. They noticed that fully toxigenic strains varied appreciably in their ability to colonise rabbit intestinal mucosa and colonisation was dose dependent.

Dutta and Habbu (Dutta and Habbu 1955) were the first researchers to use the infant rabbit cholera model. They used ten day old rabbits weighing 100-200 g inoculated orally. Susceptibility to the disease was dependent on the age of the animals and all animals showed clinical signs and mortality up to 16 days of age. Despite the similarities with the human disease the signs differed in several aspects viz. absence of vomiting, minimal pathological picture, marked congestion of small intestine and distension of the large intestine with fluid. The signs of cholera could also be reproduced in infant rabbits by oral administration of the cell free products (Dutta, Panse et al. 1959, Oza and Dutta 1963) confirming the role of cholera toxin in the pathogenesis of cholera. Finkelstein and colleagues (Finkelstein, Norris et al. 1964) also confirmed the usefulness of the infant rabbits as experimental cholera models. They suggested that in spite of the drawback that immaturity of animals makes immunological studies difficult, there are certain advantages in that their intestinal tract remains unobstructed and even oral administration of cell free material produces cholera symptoms. They found that an atraumatic preparatory gastric lavage was necessary to avoid gastric acidity and they used catheter-mediated per os introduction of organisms into stomach but also used laparotomy for intraintestinal introduction of organisms. They observed the inhibition of gastric emptying due to a potential role of cholera toxin in the intraintestinally infected animals. It was noted that some strains of cholera or El Tor vibrios were unable to cause cholera in infant rabbits. Smith

demonstrated the production of diarrhoea in infant rabbits of 6-9 days old by oral administration of cholera toxin (Smith 1972). Madden and coworkers (Madden, Nematollahi et al. 1981) studied virulence of non O1 V. cholerae in infant rabbit assays. In this case, for pathogenicity assays, infant rabbits were inoculated intraintestinally by unspecified method and all animals showed a positive reaction. Fluid accumulated in the intestines and the animals either died or became sick with diarrhoea. Recently, a nonsurgical model of cholera gravis in infant rabbits was reported (Ritchie, Rui et al. 2010). Three-day-old rabbits pre-treated with cimetidine as antacid were inoculated with V. cholerae orogastrically and all the animals developed lethal watery diarrhoea. Non-toxigenic V. cholerae which do not produce cholera toxin (CT) and toxin-coregulated pilus (TCP) did not induce cholera-like disease in the infant rabbits. They also highlighted the association of V. cholerae with the host intestine for their attachment and exit from the intestinal tract is significantly influenced by CT-dependent mucin secretion.

If we compare the adult rabbit model with the infant rabbit, the former are resistant to experimental cholera infection and for adult rabbit cholera model to be effective the prevention of peristalsis through intestinal ligation is required (Finkelstein 1996). Adult animals, especially rabbits, need surgical modification of their intestinal tract in order to create intestinal loops RIL or RITARD. Thus except by surgical modification adult animals do not produce cholera symptoms as susceptibility to the disease is also dependent on the age of the animals as animals showed mortality only up to the sixteenth day of life (Dutta and Habbu 1955). There may also be an inhibition of the V. cholerae in the adult animals by the gut flora which may therefore require clearance by using antibiotics prior to infection (Rhine and Taylor 1994). In the infant

rabbits the intestinal tract remains open and clear and thus oral administration of cell free material is also able to produce cholera signs (Finkelstein, Norris et al. 1964). The colonisation of intestinal villi was observed at a faster rate in the infant rabbits than in the adult rabbits and V. cholerae evenly covered the intestinal surface of infant rabbits while they occurred in patches in the adults (Nelson, Clements et al. 1976). This might be the reason that infant rabbits are more susceptible to experimental infection in the patent gut than the adult rabbits.

Infant mice also show fatal infection after oral administration of vibrios (Chaicumpa and Rowley 1972). Ujiiye and colleagues (Ujiiye and Kobari 1970) first developed this model for cholera in which they demonstrated that infant mice responded well to oral infection by V. cholerae with diarrhoea and death. They observed that experimental cholera was produced only in the infant mice less than 10 days of age. Mice were isolated from their mothers after 5 or 6 days of suckling and were starved for 1 day before infecting them. Diarrhoea and deaths were recorded during the 5 days following challenge. Chaicumpa and Rowley (Chaicumpa and Rowley 1972) confirmed these findings With diarrhoea developing by 18 h while and occurring between 24 and 48 h of infection. Infection resembled human cholera as up to 10-fold more vibrios were recovered from the gastrointestinal tract after initial challenge. Baselski and coworkers (Baselski, Briggs et al. 1977) used infant mice to study intestinal fluid accumulation induced by oral challenge with V. cholerae and delivered the challenge dose into the stomach per os using a tuberculin syringe. Evans blue dye was also introduced in the stomach which was seen through the abdominal wall indicating the proper delivery of the inoculum. The Fluid accumulation (FA) ratio, which is the gut weight/remaining body weight, was quantified to see the diarrhoeal

response of mice to oral challenge with live vibrios or enterotoxin. The FA ratio was both time and dose dependent. The onset of FA occurred by 8 h post-challenge and was at its peak by 10 h.

There are two obvious choices of animal models for phage therapy of V. cholerae viz. infant rabbits and infant mice. Amongst them, the infant rabbits produce the signs resembling human cholera more closely than do infant mice. V. cholerae colonizes rabbits and humans by mechanisms that are similar, though rabbits are not the natural hosts for V. cholerae (Pierce, Kaper et al. 1985). Though the infant mice produce signs of cholera as in infant rabbits, inhibition of gastric emptying was noticed in infant mice challenged orally or subcutaneously with cholera toxin (Ujiiye and Kobari 1970). Infant rabbits do not require starving as is the case with infant mice. One other practical advantage of infant rabbits over the mice is that they are larger and their handling is easier during in vivo studies.

1.6.3.3 Phage therapy of cholera using animal models

As discussed earlier (§ 1.6.3.2), the choice of animal model for phage therapy of V. cholerae is very critical owing to many factors which play a crucial role in making phage therapy either successful or not. Some of the studies on phage therapy of V. cholerae showed a lack of success possibly either due to the inability of V. cholerae to produce disease in the animal models or due to inappropriate selection of phage which were not capable of lysing the bacteria. Though Koch discovered V. cholerae (comma bacillus at his time) as the causative organism of cholera in the year 1883, later he failed to prove his third postulate (i.e. pure culture of micro-organism will produce disease in susceptible animal when injected) as he could not get suitable animal model

to prove this (Salyers and Whitt 2002). Much later on some animal models were developed to study pathogenesis of cholera as described in preceding section but none of the model could produce complete array of symptoms typical to cholera disease. Very few studies were done for phage therapy of cholera using animal models as discussed further in this section and they indicate that phage therapy did not work or was not as effective as antibiotics and thus it is possible that inspite of proper selection of animal model and appropriate phage selection the phage therapy may not work.

Sayamov (Sayamov 1963) devised a method of passaging phages into the small intestine of guinea pigs for enhancing the lytic abilities of phages. After animal passaging of the phages, they successfully used them in human patients (§ 1.6.3.1). During this animal passaging the segments of small intestine of guinea pigs were isolated by ligatures. These intestinal loops were inoculated with V. cholerae (10^7 CFU/ml) and simultaneously introducted with 0.1 ml of unknown titre phages. They used nine animals in control group and nine in the treatment group. After 24hrs it was observed that phages had completely lysed the bacteria and no bacteria were recovered or were present in very small numbers. In this study, though guinea pigs were used for phage passaging and phages effectively reduced the bacterial numbers during passaging, there are no further reports of guinea pigs being used either for cholera pathogenesis or for phage therapy.

Sarkar and colleagues (Sarkar, Chakrabarti et al. 1996) used the adult rabbit ileal loop (RIL) model (§ 1.6.3.2) for examining the potential of V. cholerae typing phages to reduce in vivo numbers of organisms and fluid accumulation. Ten typing phages (ATCC 51352 B1-B10) were utilised for this study with the phage (10^{11} PFU/ml)

inoculated into the segments of ileal loop either alone or in combination with 10^8 CFU/ml V. cholerae O1 biotype ElTor Ogawa MAK 757. They have not provided the number of control and treatment animals used in this study. These phages did not reduce the number of challenge bacteria nor the fluid accumulation ratios. The production of cholera toxin was not inhibited by the challenge of phages and additional factors in the intestinal milieu may have inhibited phage activity. However, Sulakvelidze and Barrow (Sulakvelidze and Barrow 2005) pointed out that the lytic ability of these phages was not specifically examined in vitro but it was likely that at least some phages could have had in vitro lytic ability against the challenge strain. Also they suggested that the negative outcome of the above study could be due to the use of the RIL model, which is not an optimal model for evaluating the efficacy of phages for therapy against naturally occurring cholera The negative outcome of the RIL model prompted Bhowmick and coworkers (Bhowmick, Koley et al. 2009) to perform this study in the RITARD model (§ 1.6.3.2). They challenged with 10^9 CFU/ml of V. cholerae MAK 757 (animal passaged earlier in adult mice to increase its virulence) in each of the six control and phage treated rabbits. The diarrhoea of variable severity was developed. In the phage-treated rabbits they gave 10^9 CFU/ml V. cholerae MAK 757 with 10^8 PFU/ml cocktail of phages which developed mild diarrhoea. Fewer pathological changes in the intestine were observed after phage treatment. The authors claimed that their study is the first direct evidence of phage multiplication in an open system such as the intestine infected by a V. cholerae O1 serogroup strain. Further to this, their group (Jaiswal, Koley et al. 2013) performed oral cocktail phage therapy with five lytic V. cholerae specific phages to combat orally inoculated V. cholerae O1 in adult rabbits. They administered 1×10^8 PFU of phage cocktail, 6 h and 12 h prior as well as post-infection. For administration of

phages 6 h prior and after infection they used two rabbits, while one rabbit was used as a bacterial control and one more as a phage only control. Similar number of rabbits and controls were used for administration of phages12 h prior and after infection. The post infection application of phages recovered 2 $\log_{10}$ lower bacterial numbers than the bacterial control. Recently, the adult mouse model was used to test oral phage cocktail therapy of cholera (Jaiswal, Koley et al. 2014). The efficacy of a cocktail of phages administered at the MOI of 0.1 was compared to that of ciprofloxacin antibiotic. They used sixteen mice, four each in phage treated, antibiotic treated, ORS trated and control groups. In phage treated animals the bacterial numbers were reduced by 3 $\log_{10}$ ($p < 0.05$) while ciprofloxacin showed 5 $\log_{10}$ ($p < 0.05$) reductions in bacterial numbers compared to control group animals.

The data presented above indicates that it is a logical choice to use the infant rabbit model for this project.

1.6.4 Bacterial resistance to bacteriophage

The emergence of phage resistant mutants was observed soon after the discovery of phage and this phenomenon was thought to be a potential problem to phage therapy (Summers 2001). Smith and Huggins (Smith and Huggins 1983) noticed very few phage resistant mutants in their study of phage therapy for E. coli diarrhoea in calves, piglets and lambs using mixture of two phages but these mutants tended to be less virulent than the original strains, while use of single phage did not produce any phage resistant mutants. In most of the Gram-negative pathogens bacteriophage resistance is due to the changes in phage receptors on the bacteria (Matsuzaki, Rashel et al. 2005). For example T even phages recognise outer membrane proteins (Omp) of E. coli as

receptors. If one Omp is changed by bacterial mutation, the phage may mutate to use another Omp and if that is also changed then phage again may mutate to use one more Omp or even to use lipopolysaccharide as receptor (Montag, Riede et al. 1987, Drexler, Riede et al. 1989).

Bacteria produce restriction enzymes which cleave phage DNA at specific sites and thereby restrict the phage from infecting them (Roberts 2005). Bacteria protect their own DNA from these enzymes by modifying or adding a methyl group to their adenine or cytosine bases using the enzyme methylase. Not all bacteria contain this Restriction – Modification (RM) system but for those which have such systems, phages have also evolved an anti-restriction system. In this activity phages undergo mutations to erase the sites in their genome which are the recognition sites for endonuclease (Labrie, Samson et al. 2010). Mutation is one possible way of getting around this, although it seems unlikely that all of the sites will be removed in one round (or even multiple rounds) of replication. It is likely that if a large number of bacteriophages infect a single bacterium at one time, some of the phage will be able to replicate despite the presence of RM enzymes. If that is the case, then the DNA of the bacteriophage may be acted on by the methylases, rendering daughter phage resistant to restriction (Atterbury 2003).

Many bacteria also use the CRISPR-cas system which is an immunity system that targets phage nucleic acid. It is a locus of genes having clustered regularly interspaced short palindromic repeats (CRISPR) as well as CRISPR associated (cas) genes. It contains 21–48 bp repeats interspaced by 26–72 bp non-repetitive spacers and these are generally flanked by variable number of cas genes (usually 4 to 20 in numbers)

(Labrie, Samson et al. 2010). When lytic phages attack sensitive bacterial strains, a small number of Bacteriophage Insensitive Mutants (BIMs) arise which contain the repeat spacer unit identical to the short conserved nucleotide motif called Proto spacer Associated Motif (PAM) on the phage genome. When these BIMs are attacked by phage carrying a proto-spacer which is identical to its repeat spacer the phage will be inactivated. Phages again undergo mutations in their PAM regions to circumvent this resistance (Andersson and Banfield 2008). If we compare this resistance to antibiotic resistance, the rate of developing resistance to phage is up to 10 times lower than to antibiotics (Carlton 1999). The use of several phages in one preparation i.e. a phage cocktail which are virulent against different targeted pathogen strains, helps to overcome the resistance synergistically as with antibiotics (Sulakvelidze, Alavidze et al. 2001).

1.6.5 Ideal properties of phage therapy candidates

In order to maximise the potential success of bacteriophage therapy, the phages selected should fulfil a number of criteria. First, the phage should have the ability to infect a wide range of susceptible host bacteria (broad host range/lytic profile) and replicate only through the lytic life cycle. In addition, the phage should have a short latent period and high burst size. The phage should not carry genes which encode virulence factors or are involved in antibiotic resistance. Thus, temperate phages which possess many of such characteristics should be avoided for phage therapy (Gill and Hyman 2010). Phages should ideally be selected which attach to specific surface virulence deteminants such that any resistant mutants that emerge would show reduced virulence or avirulence (Smith and Huggins 1982).

1.7 Characterisation of bacteriophage

1.7.1 Biological characterisation

To identify the ideal properties of phage for phage therapy, the host range, latent period and burst size of the candidate phage are needed. The host range profile of the phage is defined by lysis of a range of bacterial host strains. The host growth curve provides a mid exponential phase and generation time (§ 2.3) which is further utilized to devise a protocol for phage one step growth curve which will then provide an estimation of the burst size and latent period (§ 1.5).

1.7.2 Physical characterisation

Although bacteriophage can be seen under ordinary and phase microscope (Hofer 1947), an electron microscopy is used and can magnify the image up to 400,000 times (Douglas 1974). Electron microscopy allows the morphological classification of bacteriophage.

As per the International Committee on Taxonomy of Viruses (ICTV), the phages are classified as one order, ten families and forty genera (ICTV 2011) (Table 1). The symmetries of the phage classified are binary (having two divisions), cubical, helical or pleomorphic. Most of the phage genome may have double stranded DNA but a few may contain single stranded DNA and even double or single stranded RNA as their genome. A few phage may have a lipid envelope surrounding the capsid. Most of the phage are classified in to the order Caudovirales which have binary symmetry and are tailed phage. There are three phylogenetically related families in the order Caudovirales viz. the contractile-tailed Myoviridae, the long non-contractile tailed Siphoviridae and the short tailed Podoviridae (Ackermann 2006).

Order	Family	Genome	Genome size (Kb)	Envelope	Morphology	Virion size
Caudovirales	Myoviridae	dsDNA linear	31-317	No	Icosahedral head with tail	Icosahedral heads: 60-45 nm; Elongated heads: 80-110 nm; Tail: 16-20 × 80-455 nm
Caudovirales	Podoviridae	dsDNA linear	16-78	No	Icosahedral head with short tail	Icosahedral heads: 60-70 nm; Tail: 10-20 nm
Caudovirales	Siphoviridae	dsDNA linear	21-134	No	Icosahedral head with tail	Icosahedral heads: 40-80 nm; Tail: 5-10 × 100-210 nm
Unassigned	Corticoviridae	dsDNA circular supercoiled	10	No	Icosahedral	60 nm
Unassigned	Plasmaviridae	dsDNA circular supercoiled	12	Yes	Quasi-spherical, pleomorphic	50-125 nm
Unassigned	Tectiviridae	dsDNA linear	15	No	Icosahedral	66 nm
Unassigned	Inoviridae	ssDNA (+) circular	Inoviruses: 5.8-12.4 Plectroviruses: 4.5-8.2	No	Inoviruses: filamentous Plectroviruses: rod shaped	Inoviruses: 7 × 700-3500 nm; Plectroviruses: 15 × 200-400 nm
Unassigned	Microviridae	ssDNA (+) circular	4.4-6.1	No	Icosahedral	25-27 nm
Unassigned	Cystoviridae	dsRNA three linear segments	6.4-7.1; 3.6-4.7; 2.6-3.2	Yes	Spherical	85 nm
Unassigned	Leviviridae	ssRNA (+)	3.5-4.3	No	Icosahedral	26 nm

Table 1: Classification of phages as per ICTV.

1.7.3 Genomic characterisation

In order to differentiate phages, the genome size is estimated by pulsed field gel electrophoresis (PFGE) and restriction analysis is done using different restriction enzymes (Gill and Hyman 2010). Using genome sequencing, the potential phage therapy candidates can be screened for harmful genes associated with virulence, antimicrobial resistance or lysogeny-related genes (Skurnik, Pajunen et al. 2007). Pulse field gel electrophoresis is useful for sizing large DNA fragments; in this technique, the direction of an electric field is periodically switched which helps in separation of DNA fragments of up to 5Mb (Carle and Olson 1984, Schwartz and Cantor 1984).

The sequencing of microbial genomes has become more efficient and cost-effective over the past decade, resulting in a significant increase in the number of published genomes in online databases such as Gen bank. Notwithstanding these advances, the number of bacteriophage genomes available in such databases is approximately 750, as compared with over 5,000 individual phages which have been isolated (Hatfull and Hendrix 2011). High throughput sequencing platforms have been introduced since 2005. After next generation sequencing, genome annotation is done to identify predicted open reading frames (ORFs) or CDSs (CoDing Sequences) which can code for particular proteins (Kropinski, Borodovsky et al. 2009). By assigning the functions to different phage genes through CDSs provides an insight in the phage type which we are dealing with so that we can decide whether to use it for phage therapy purpose or not.

1.8 Aims

It is clear from the above considerations that there is little definitive work demonstrating efficacy of lytic phages against experimental cholera using an appropriate animal model. The main reason for this could be inadequate assessment of phages in vitro prior to their evaluation in vivo combined with the use of a model which is less than optimal. Drawing on the expertise acquired by Smith and his colleagues with well controlled animal experiments using a comparable enteric infection of neontal animals and in which the phages were assessed thoroughly in vitro beforehand, we decided to collect a small panel of phages from various sources and to evaluate these in vitro followed by assessment in experimental infection in the infant rabbit model.

The main aim of this project is thus to explore the use of bacteriophage for biocontrol of V. cholerae with the following activities to be undertaken:

1. Isolation of bacteriophages which could be used as bio-control agents for V. cholerae.
2. In vitro characterisation of the bacteriophages and selection of candidate bacteriophages for biocontrol.
3. Determination of the efficacy of biocontrol by candidate bacteriophage in therapeutic experiments using the infant rabbit model.

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Preparation of media and buffers

2.1.1 Media for growth of V. cholerae

2.1.1.1 Alkaline Peptone Water (APW) broth:

For enrichment of V. cholerae Alkaline Peptone Water (APW) broth [CM 1117, Oxoid limited, Basingstoke, Hampshire, UK] was prepared according to manufacturer's instruction. For this, 40 g of APW powder was added to 1 litre of Reverse Osmosis (RO) water. After dispensing in to smaller volumes it was autoclaved at 121 °C for 15-20 m. Sterile APW was stored at room temperature until used. Concentrated (five times) APW broth was prepared by adding 40 g of APW powder to 200 ml of RO water.

2.1.1.2 Luria Bertani (LB) broth:

For routine culturing of the V. cholerae strains, Luria Bertani (LB) broth [L3152 Fluka, LB broth, Miller, Sigma Aldrich Ltd., Gillingham, Dorset, UK] was used. For preparing the broth, 25 g LB broth powder was added to 1 litre of RO water and autoclaved 121 °C for 15-20 m. Sterile broth was stored at room temperature until used.

2.1.1.3 Thiosulphate Citrate Bile Salt (TCBS) agar:

Thiosulphate Citrate Bile Salt (TCBS) agar [CM 0333, Oxoid limited, Basingstoke, Hampshire, UK] is a selective differential medium for isolation and culturing of halo-tolerant V. cholerae. It was prepared according to the manufacturer's instruction i.e. 88 g of TCBS agar powder was added to 1 litre of RO water which was boiled without autoclaving, cooled down to below 50 °C and poured into the petri plates. The plates

were dried in to the drying cabinet overnight before shifting them to the cold room (4 °C) and used within few weeks.

2.1.1.4 Maximum Recovery Diluent (MRD):

Maximum Recovery Diluent (Oxoid CM0733) was used for serial dilutions of bacterial cultures and also for sample collection during isolation of V. cholerae. It was prepared as per the manufacturer's direction. For this 9.5 g of MRD powder was dissolved in 1 litre of distilled water and sterilized by autoclaving at 121 °C for 15 m. It was stored at room temperature till further use.

2.1.2 Media and buffers for phage isolation and characterisation

2.1.2.1 Salt Magnesium (SM) buffer:

Salt Magnesium (SM) buffer is used for storage and dilution of phages. For preparing SM buffer, 5.8 g/L sodium chloride (NaCl); 2 g/L of magnesium sulphate hexahydrate (MgSO4.7H2O); 5 ml/L of 2 % w/v Gelatin solution and 6 g/L of 1M Tris-Cl/TRIS was added to one litre of the RO water and pH was adjusted to 7.5. After autoclaving at 121 °C for 15-20 m, it was stored at room temperature till used.

2.1.2.2 Luria Bertani (LB) base medium and top agar:

LB base medium (1 %):

LB base medium was prepared by adding 1 g Sigma select agar per 100 ml of LB broth (§ 2.1.1.2) and then autoclaved at 121 °C for 15-20 m. The agar was cooled to below 50 °C and then poured into petri plates. The plates were stored in to cold room at 4 °C after they were dried in a drying cabinet.

Suplemented LB base medium (1 %):

LB base medium (1 %) was prepared as mentioned in the preceding paragraph and was supplemented with 0.3 % Glucose, 0.075 mM CaCl2 and 2 mM MgSO4 (Sambrook and Russell 2001). The Glucose reduces phage loss by adsorption to bacterial debris; calcium helps to reduce loss by reabsorption, while magnesium is essential for attachment of the phage to the receptor structures on bacteria.

LB top agar (0.5 %) overlay:

Top agar was prepared for overlaying it on the 1 % base agar while making the bacterial lawns. For this 0.5 g Sigma select agar was added per 100 ml of LB broth (§ 2.1.1.2) and autoclaved at 121 °C for 15-20 m. The top agar was stored at room temperature until required.

2.1.2.3 TE buffer:

10× TE buffer was prepared by adding 12.1 g/L Trizma base and 3.7 g/L EDTA to 1 litre of RO water. 10× TE buffer was diluted 1:9 with RO water for 1× TE buffer.

2.1.2.4 Lysis buffer for PFGE:

Lysis buffer was prepared by adding 50mM Tris-HCl (pH 8.0), 50mM EDTA, 1% Lauryl Sarcosine i.e. Sarcosyl detergent and 100 µg/ml Proteinase K to 20 ml of sterile RO water. The buffer was used fresh for one PFGE run.

2.1.2.5 Wash buffer for PFGE:

Wash buffer was prepared by adding 50mM Tris-HCl (pH 8.0) and 50mM EDTA to 20 ml of sterile RO water. The buffer was used fresh for each PFGE run.

2.1.2.6 TAE buffer for Gel electrophoresis:

The 1L of 50× TAE buffer (pH 8.0) was prepared by adding 242 g Tris base, 5.7 ml Glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0). For 1 L of 1× TAE buffer, 20 ml of 50× TAE buffer was added in 980 ml of RO water.

2.1.2.7 EDTA (0.5 M, pH 8.0):

For this 186.1 g of disodium EDTA $2H_2O$ was added 800 ml of RO water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH and sterilized by autoclaving.

2.1.2.8 $MgCl_2$ (1M):

$MgCl_2$ (1M) was prepared by dissolving 203.3 g of $MgCl_2.6H_2O$ into 800 ml of RO water and then adjusting volume to 1 L by RO water. Sterilization was done by autoclaving and stored at room temperature.

2.1.2.9 Tris-EDTA i.e. TE (pH 8.0):

This was prepared by using Tris-Cl (pH 8.0) 100 mM and EDTA (pH 8.0) 10 mM in an appropriate quantity of RO water and sterilised by autoclaving.

2.1.2.10 Ammonium Acetate (10M):

A stock solution of Ammonium Acetate (10M) was prepared by dissolving 77 g of Ammonium Acetate to 70 ml of RO water and then adjusting volume to 100 ml by RO water. Sterilization was done by filtering through 0.22 µm syringe filter and stored at 4 °C.

2.2 Bacteria and bacteriophages

2.2.1 V. cholerae

A total of 91 V. cholerae strains were used for this project (Table 2) and they were obtained from four sources as mentioned below:

1. London School of Hygiene and Tropical Medicine, London, UK:

Professor Brendan Wren and Mr. Muhammad Ali of London School of Hygiene and Tropical Medicine kindly provided 86 V. cholerae strains of which 71 were V. cholerae O1 El Tor Ogawa while the remaining 15 were V. cholerae non-O1 and non-O139.

2. University of Bristol, UK:

Two V. cholerae isolates O395NT and M14 were obtained from Dr. Neil Williams, University of Bristol. M14 was V. cholerae O1 El Tor Inaba, while O395NT is non-toxin producing V. cholerae O1 El Tor Ogawa.

3. Felix d'Herelle Reference Centre for Bacterial Viruses, Canada:

V. cholerae 1051, which was a classical O1 serotype was obtained from the Felix d'Herelle Reference Centre for Bacterial Viruses, Québec, Canada.

4. China Agricultural University, Beijing, China:

The strains 2095 and 2134 were obtained from Dr. Jingliang Su, China Agricultural University, Beijing, China. Strain 2095 was V.cholerae 01 El Tor Inaba (NT) and 2134 was V. cholerae O139 (NT).

Seri-al No.	Bacteria	Strain	Seri-al No.	Bacteria	Strain
1	V.cholerae non-O1/non-O139	238	47	V.cholerae 01 El Tor Ogawa	D-1
2	V.cholerae 01 El Tor Ogawa	257	48	V.cholerae non-O1/non-O139	D-13
3	Vibrio spp. but not V. cholera	404	49	V.cholerae non-O1/non-O139	D-15
4	Vibrio spp. but not V. cholera	406	50	V.cholerae 01 El Tor Ogawa	D-25
5	V.cholerae 01 El Tor Ogawa	709	51	V.cholerae 01 El Tor Ogawa	D-30
6	V.cholerae 01 El Tor Ogawa	719	52	V.cholerae 01 El Tor Ogawa	D-56
7	V.cholerae 01 El Tor Ogawa	722	53	V.cholerae 01 El Tor Ogawa	D-59
8	V.cholerae 01 El Tor Ogawa	729	54	V.cholerae 01 El Tor Ogawa	DN-4
9	V.cholerae 01 El Tor Ogawa	732	55	V.cholerae 01 El Tor Ogawa	F-5
10	V.cholerae 01 El Tor Ogawa	736	56	V.cholerae 01 El Tor Ogawa	F-6
11	V.cholerae 01 El Tor Ogawa	739	57	V.cholerae non-O1/non-O139	FB-01
12	V.cholerae 01 El Tor Ogawa	742	58	V.cholerae non-O1/non-O139	FN-2

13	V.cholerae 01 El Tor Ogawa	750	59	V.cholerae 01 El Tor Ogawa	FN-4
14	V.cholerae 01 El Tor Ogawa	751	60	V.cholerae non-O1/non-O139	FN-5
15	V.cholerae 01 El Tor Ogawa	752	61	V.cholerae 01 El Tor Ogawa	GB-39
16	V.cholerae 01 El Tor Ogawa	753	62	V.cholerae 01 El Tor Ogawa	HH-1
17	V.cholerae 01 El Tor Ogawa	754	63	V.cholerae 01 El Tor Ogawa	HH-14
18	V.cholerae 01 El Tor Ogawa	755	64	V.cholerae 01 El Tor Ogawa	HH-15
19	V.cholerae 01 El Tor Ogawa	756	65	V.cholerae 01 El Tor Ogawa	HH-4
20	V.cholerae 01 El Tor Ogawa	758	66	V.cholerae non-O1/non-O139	Ht-10
21	V.cholerae 01 El Tor Ogawa	759	67	V.cholerae 01 El Tor Ogawa	Ht-10A
22	V.cholerae 01 El Tor Ogawa	760	68	V.cholerae non-O1/non-O139	J-1
23	V.cholerae 01 El Tor Ogawa	761	69	V.cholerae 01 El Tor Ogawa	KCH-18
24	V.cholerae 01 El Tor Ogawa	762	70	V.cholerae 01 El Tor Ogawa	KPD-3
25	V.cholerae 01 El Tor Ogawa	763	71	V.cholerae non-O1/non-O139	KtH-4
26	V.cholerae 01 El Tor	764	72	V.cholerae 01 El Tor	KTH-7

	Ogawa			Ogawa	
27	V.cholerae 01 El Tor Ogawa	765	73	V.cholerae 01 El Tor Ogawa	N-10
28	V.cholerae 01 El Tor Ogawa	767	74	V.cholerae 01 El Tor Ogawa	N-5
29	V.cholerae 01 El Tor Ogawa	768	75	V.cholerae 01 El Tor Ogawa	N-7
30	V.cholerae 01 El Tor Ogawa	769	76	V.cholerae 01 El Tor Ogawa	NP-14
31	V.cholerae 01 El Tor Ogawa	770	77	V.cholerae 01 El Tor Ogawa	NP-3
32	V.cholerae 01 El Tor Ogawa	771	78	V.cholerae 01 El Tor Ogawa	NP-5
33	V.cholerae 01 El Tor Ogawa	772	79	V.cholerae 01 El Tor Ogawa	NP-6
34	V.cholerae 01 El Tor Ogawa	773	80	V.cholerae 01 El Tor Ogawa	NP-7
35	V.cholerae 01 El Tor Ogawa	774	81	V.cholerae non-O1/non-O139	O5
36	V.cholerae 01 El Tor Ogawa	775	82	V.cholerae non-O1/non-O139	P-1
37	V.cholerae 01 El Tor Ogawa	776	83	V.cholerae 01 El Tor Ogawa	PS-18
38	V.cholerae non-O1/non-O139	10	84	V.cholerae 01 El Tor Ogawa	PS-25
39	V.cholerae 01 El Tor Ogawa	A-4	85	V.cholerae 01 El Tor Ogawa	PS-7

40	V.cholerae 01 El Tor Ogawa	BW-5	86	V.cholerae 01 El Tor Ogawa	RG-6
41	V.cholerae 01 El Tor Ogawa	CS-1	87	V.cholerae 01 El Tor Inaba	M14
42	V.cholerae 01 El Tor Ogawa	CS-12	88	V.cholerae 01 El Tor Ogawa	O395NT
43	V.cholerae 01 El Tor Ogawa	CS-15	89	V.cholerae O1 strain	1051
44	V.cholerae 01 El Tor Ogawa	CS-16	90	V.cholerae 01 El Tor Inaba (NT)	2095
45	V.cholerae 01 El Tor Ogawa	CS-18	91	V.cholerae O139 (NT)	2134
46	V.cholerae non-O1/non-O139	CW-1			

Table 2: V. cholerae strains used for this project.

Serial No. 1-86: Obtained from London School of Hygiene and Tropical Medicine, London, UK; Serial No. 87-88: Obtained from the University of Bristol, UK; Serial No. 89: Obtained from Felix d'Herelle Reference Centre for Bacterial Viruses, Canada; Serial No. 90-91: Obtained from China Agricultural University, China; NT: Non-toxigenic.

2.2.1.1 Freezing of V. cholerae strains:

A single colony from a freshly cultured TCBS plate was grown in 10 ml LB broth up to mid-exponential phase i.e. approx. 4 hours at 37 °C. An aliquot of this culture (660 µl) was added to a sterile 2 ml graduated skirted Cryo tube (Starlabs, Milton Keynes, UK) to which 1 ml of sterile 50 % glycerol was added. The tube was vortexed briefly to mix the contents thoroughly and immediately frozen at – 80 °C.

2.2.2 Bacteriophages

2.2.2.1 Phages from Public Health England (PHE):

Four phage isolates (Φ1, Φ2, Φ3 and Φ4) were obtained from Dr. Tom Cheasty [Former Head, Gastrointestinal Infections Reference Unit, Public Health England (PHE)] without their host strains.

2.2.2.2 Phages from Canadian Culture collection:

Two phages, 24 and X29, were purchased from the Felix d'Herelle Reference Centre for Bacterial Viruses (Canada) together with the host strain V. cholerae 1051.

2.2.2.3 Phage isolation in China:

Seven more V. cholerae specific phages were isolated from China using Vibrio cholerae 2095 which is an O1 Inaba strain. The locations of the sample collection from South Eastern China (Table 3) were as below:

Sr. No.	Sample location	No. of samples collected	Sample codes
1	Beixiaohe sewage treatment plant, Beijing	1	BS
2	Beijing river	4	BR1-4
3	Qing He river Beijing and Cui Jia Yao river 50 miles away	9	QH, CJY, NSH1-3, SZ1-4
4	Fu Jia Wan, Ye Zhi Hu and Nan Hu lakes of Wuhan, Hubei	3	H1, H2, H3
5	Jiangxi, Nanchang, Yudai He river	4	J1, J2, J3, J4
6	Henan province, Zhengzhou city	4	Z1, Z2, Z3, Z4
	Total	25	

Table 3: The locations of the sample collection from South Eastern China.

A total of 25 samples were collected from six different locations from south eastern China. The sampling locations based on the Cholera prediction map (Xu, Cao et al. 2013) are given in the Figure 7. The location maps and images of sampling sites from where phages were isolated are shown in the Figures 8 to 11.

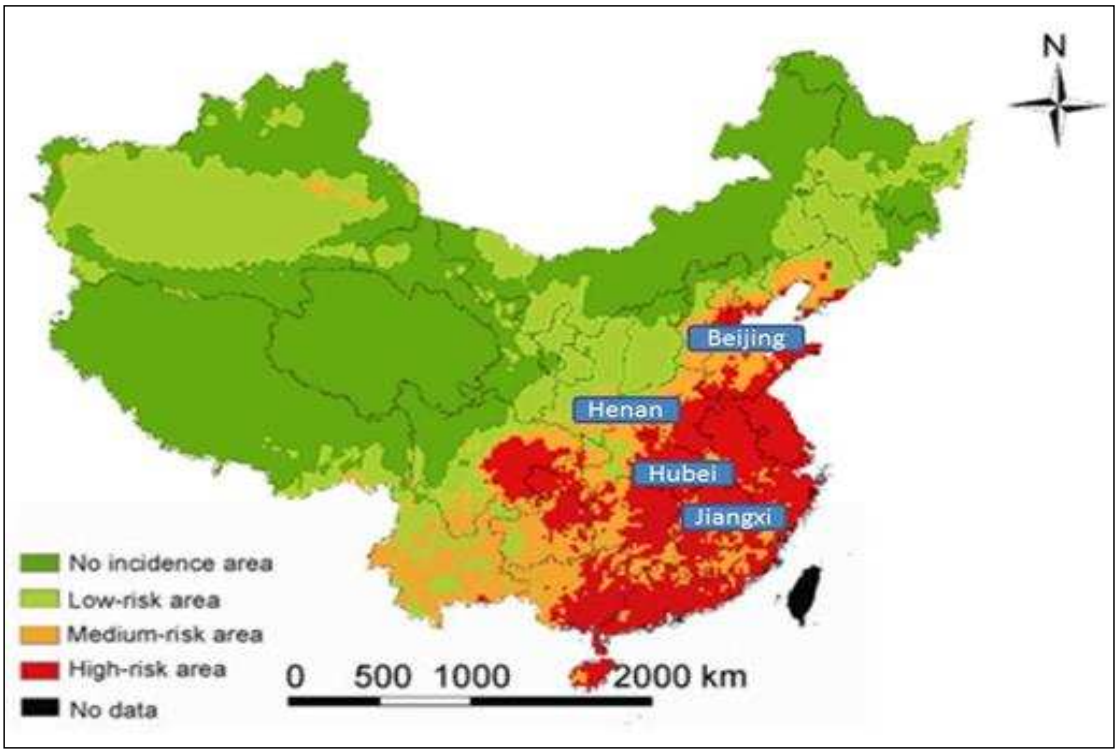

Figure 7: Cholera prediction map, based on which sampling plan was derived is shown in this figure. The high risk areas for cholera disease as indicated by red colour were chosen for sample collection.

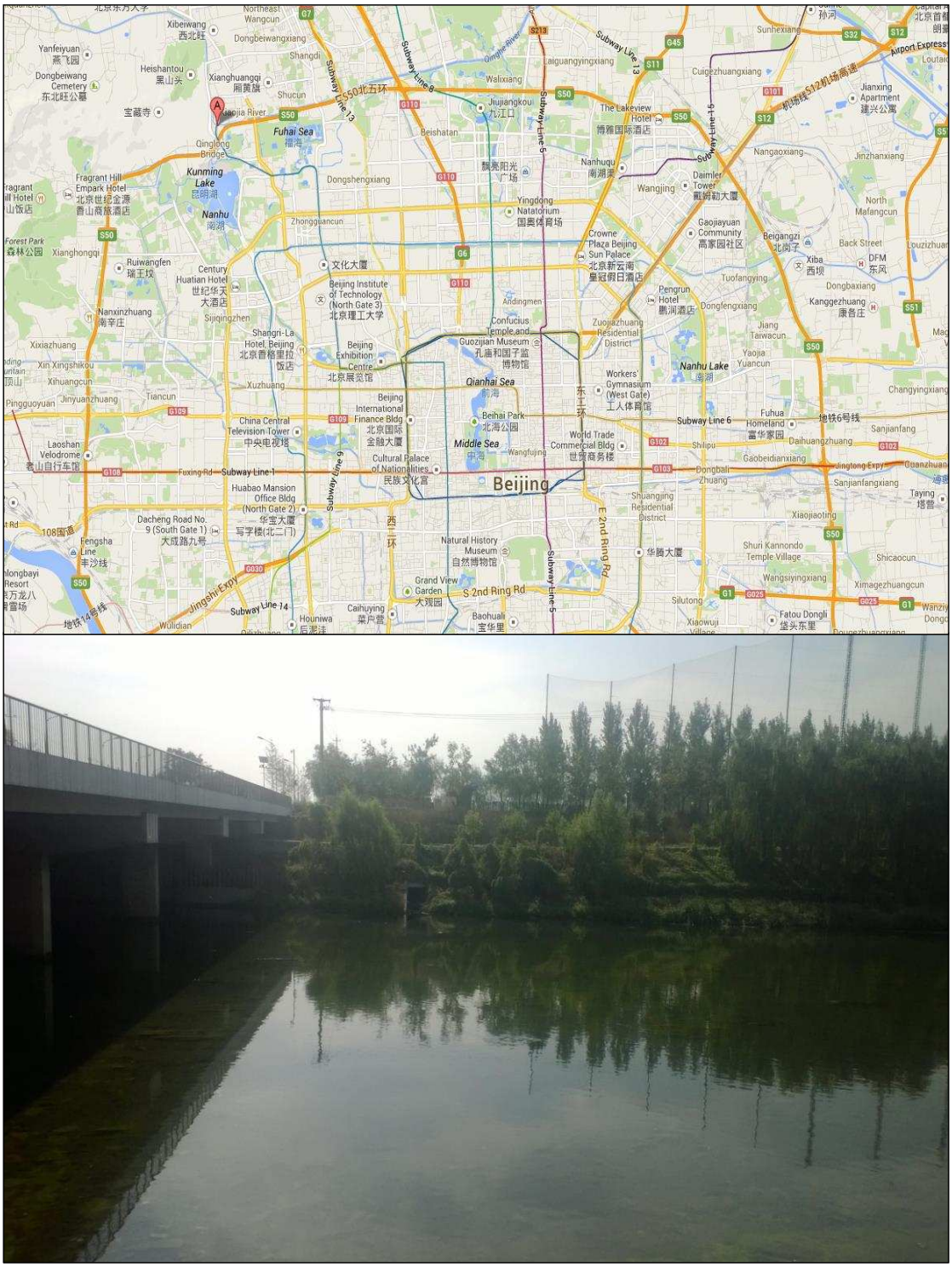

Figure 8: Sample location map and site image for Qing He river sampling site (QH). The location on the map at the top is indicated by 'A' and picture below is a sampling site image.

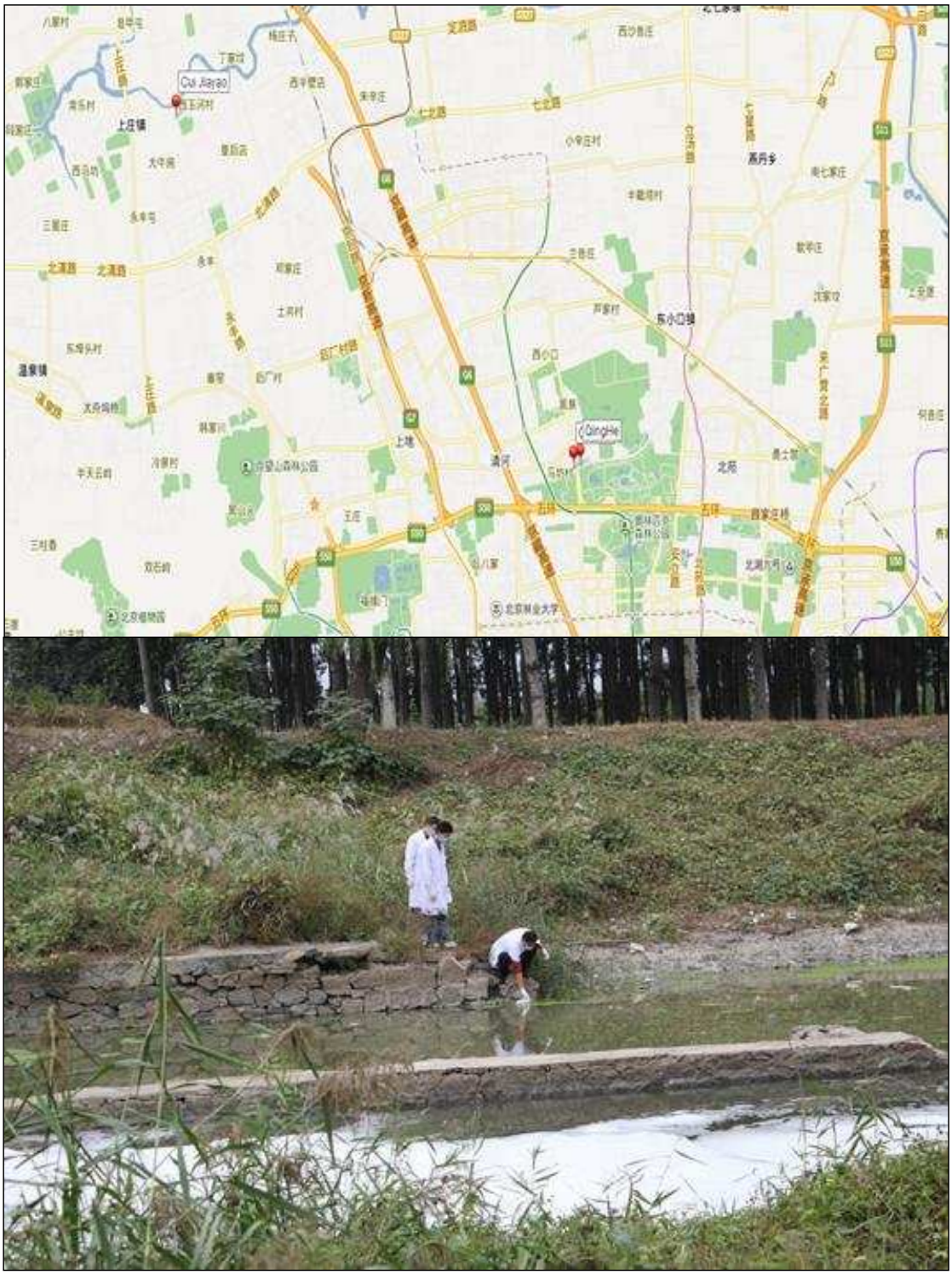

Figure 9: Sample location map and site image for Cui Jia Yao river sampling site (CJY). The distance between Cui Jia Yao and Qing He river (QH) sample locations, which is approx. 50 miles can be seen on the map at the top and picture below is a site photograph during sample collection.

Figure 10: Sample locations map at top and sampling site images below for Fu Jia Wan (H1), Ye Zhi Hu (H2) and Nan Hu (H3) lakes from Wuhan, Hubei.

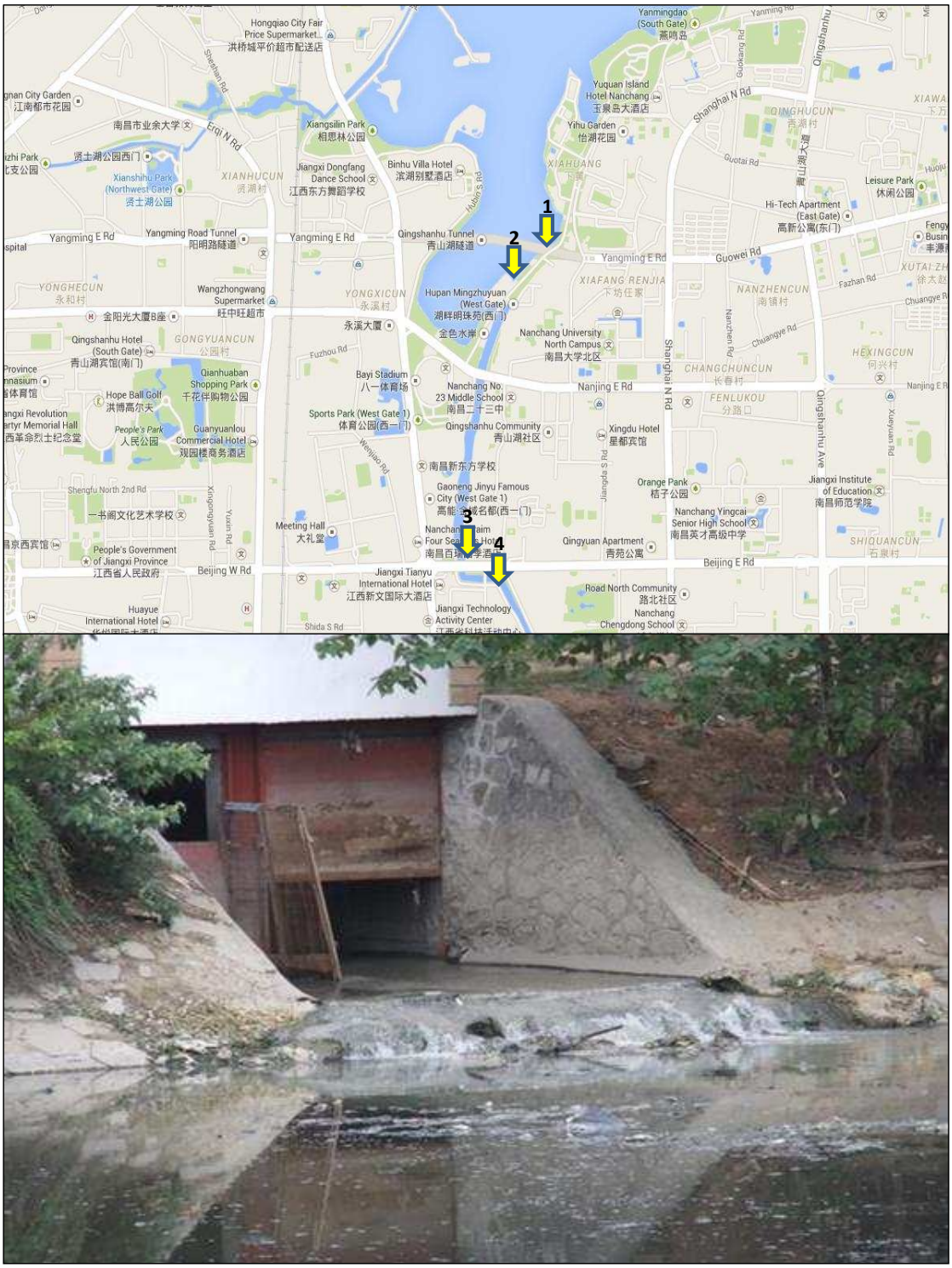

Figure 11: Sample locations map at top for Jiangxi, Nanchang, Yudai He river samples wherein 1 is J1; 2 is J2; 3 is J3 and 4 is J4. The image below is of J2 sample collection site.

2.2.2.4 Phage isolation in the UK:

Bacteriophage isolation was attempted twice from sewage samples collected from a local wastewater treatment plant i.e. Severn Trent Sewage Works, Raynesway, Derby (Figure 12) which deals with the sewage from approx. 165,000 people in the region. The reason for choosing this site was the fact that cholera is a human diarrhoeal disease and imported cases occur within the UK annually.

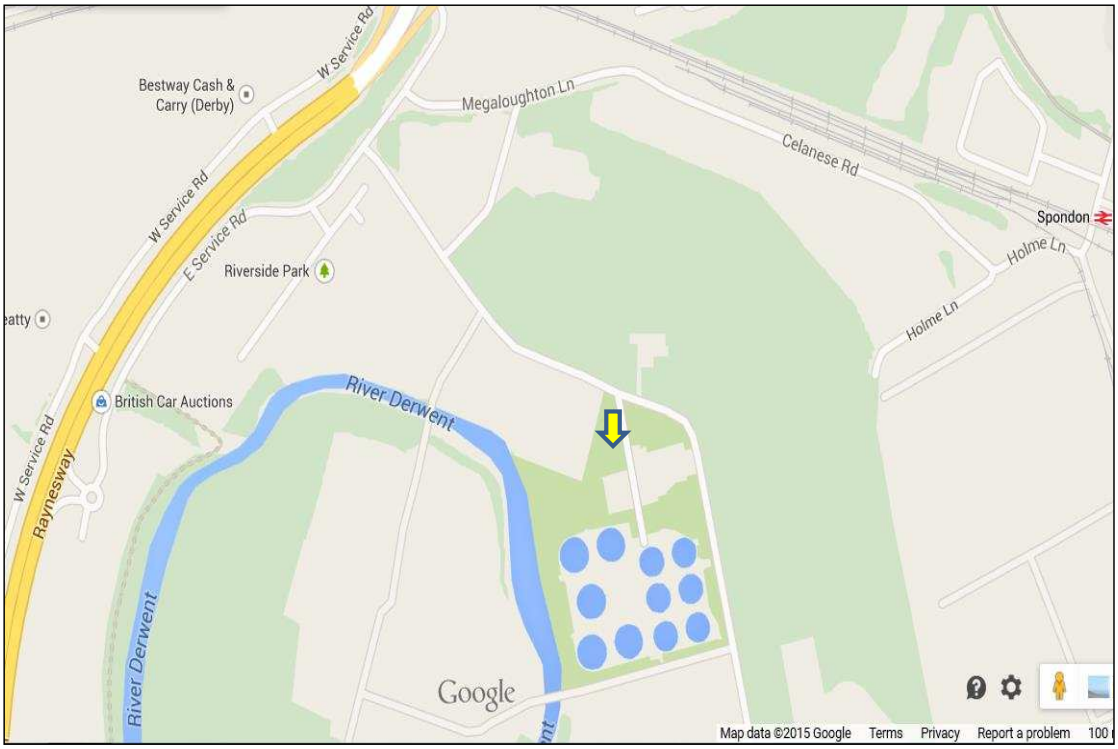

Figure 12: Severn Trent Sewage Works, Raynesway, Derby as indicated by arrow on the map.

2.2.2.5 Protocol used for isolation of phages:

For V. cholerae phage isolation from sewage, river, or lake samples, the method of Van Twest and Kropinski, 2009 (Van Twest and Kropinski 2009) was used with following modifications:

a) Filter units (0.45 µm) were used instead of 0.22 µm filter units as the large sized phage may not have been able to pass through 0.22 µm filter units.

b) The filtrate was not stored in the presence of chloroform as some phage may have been sensitive to the chloroform.

c) APW broth was used instead of LB broth as it is a selective broth for V. cholerae.

Sewage from settlement tanks or river or lake water samples (500 ml) were collected. After bringing the sample to the lab it was clarified by centrifugation at 10,000 × g for 30 m and filtered through 0.45 µm syringe filter units (Millipore, Millex). The 30 ml of filtrate was mixed with 9 ml of 5x APW, 1 ml of 1 M $CaCl_2$ (i.e. 20 mM in 50 ml) and 10 ml of V. cholerae overnight culture. This reaction mixture was incubated in a 250 ml screw capped flask at 37 °C for 48 h shaking at 50 RPM.

The culture was clarified by centrifugation at 10,000 × g for 10 min and the supernatant was filtered with 0.45 µm filter to remove the unwanted bacterial cells. An aliquot (10 µl) of filtrate was spot inoculated on the lawn of the target V. cholerae strain used for isolation and incubated at 37 °C overnight. Next day the lawns were checked for zones of lysis or individual plaques.

2.2.2.6 Plaque purification

Individual plaques were further purified using a streak plating method described by phage hunting protocols (Phagesdb.org 2013). An isolated single plaque was picked by touching the centre of the plaque once with a pipette tip and aseptically releasing the attached phages in to 100 µl of SM buffer. Then, a new sterile tip was swirled in to

a buffer with picked phages and streaked as per the Figure 13. The streaked plate was brought to room temperature; a top agar containing the host strain was poured carefully at the most diluted spot on the plate and slightly tilted for top agar to spread slowly across the plate. After the top agar was solidified, the plate was incubated at 37 °C for 24 h. After incubation, a single plaque was identified and again picked and streak plate purification was repeated two more times.

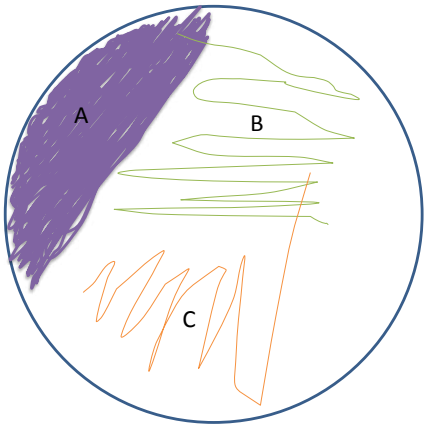

Figure 13: Schematic representation of streaking pattern for streak plate plaque purification.

A: Picked phages were streaked confluently on to a fresh cold LB plate back and forth across 20 % of the top plate.; B: With a new tip starting out of the confluently streaked area, the 40 % more area was streaked without overlapping the strokes at the corner.; C: Again with a new tip starting out of the earlier 40 % streaked area, the remaining 40 % was streaked without overlapping the strokes at the corner.

2.3 Growth kinetics of V. cholerae host strains

For the growth curve study the frozen stock of V. cholerae strain was streaked on three TCBS agar plates and incubated at 37 °C for 24 h. Next day a single colony from each plate was inoculated into 10 ml LB broth and incubated at 37 °C, shaking at 150 rpm for 3 h. At the same time 50 ml LB broth was added to three 250 ml screw capped flasks and incubated at 37 °C for three hours. After 3 h 0.5 ml of a mid-

exponential culture (time for mid exponential phase derived from the a pilot experiment) from 10 ml LB universals was added to 50 ml pre-warmed broth in flasks and incubated at 37 °C shaking at 150 rpm. This point is considered to be zero hour for taking first sample for plating (i.e. after diluting the culture up to the desired dilutions as decided by pilot experiment) the 100 µl was spread plated on 1 % LB agar medium in duplicate and the OD_{600} readings were taken on the spectrophotometer (Biochem, Libra S22).

Plating and OD readings were done every 2 h until 12 h, and the plates incubated at 37 °C overnight. Data was pooled from three trials and analysed after converting bacterial counts to $\log_{10}$ values and determining the standard deviation. The mid exponential phase was identified and the generation time at this point was calculated as per the formula in Pelzar et al (Pelczar, E.C.S. et al. 1993). The generation/doubling time is the time taken by the number of cells in the culture to double. It is calculated from the counts taken from the log phase of growth as the cells would be in the uniform phase of growth. The formula used was as below:

$$G = t/n$$

Where G = Generation time

t = the log phase time interval in minutes

n = number of generations

The total population of bacterial culture that begins with one cell is expressed as:

$$b = B - 2^n$$

Where B = number of bacteria at the beginning of time interval

b = number of bacteria at the end of time interval

n = number of generations

The above equation is rearranged by using Log_{10} values as below:

$$\log_{10} b = \log_{10} B + n \log_{10} 2$$

$$n = \log_{10} b - \log_{10} B / \log_{10} 2$$

$$n = \log_{10} b - \log_{10} B / 0.301$$

$$n = \log_{10} b - \log_{10} B \times 3.3$$

This equation is incorporated in to original equation as below:

$$\mathbf{G = t/3.3 (\log_{10} b - B)}$$

2.4 Characterisation of bacteriophage isolates

2.4.1 Liquid lysates of phage

A single colony of a *V. cholerae* host strain was picked from a fresh TCBS plate and inoculated in to 10 ml of LB broth and incubated at 37 °C with shaking at 150 RPM. At mid-exponential phase the culture was inoculated with 0.1 ml of phage stock and incubated overnight at 37 °C. Next day the culture was centrifuged at 10,000 × g for 10 m and then filtered through a 0.45 µm syringe filter. The filtrate was stored at 4 °C after determining its titre.

2.4.2 Preparation of bacterial lawn

The *V. cholerae* culture, 350 µl at mid-exponential phase was added to 5 ml of soft/top agar (0.5 % LB media) and overlaid on a 1 % LB media plate (§ 2.1.2.2). The top agar was maintained at 50 °C in water bath before overlaying. The bacterial culture was mixed in to the soft/top agar by gentle swirling and then poured. The lawn was dried up to 30 m and then used for phage spotting.

2.4.3 Plate lysates of phage

Phage stock was diluted to a dilution which gave just semi-confluent plaques touching each other with visible bacterial growth and gauzy webbing of plaques. A 100 µl aliquot of the chosen dilution of phage was mixed with 350 µl of V. cholerae culture taken from a mid-exponential phase culture. To this bacteria-phage mixture 5 ml of molten 0.5 % LB top agar was added, mixed and overlaid on 1% supplemented LB agar plates (§ 2.1.2.2) which were freshly prepared. The plates were incubated without the normal inversion (Sambrook and Russell 2001) at 37 °C for not more than 10-12 h (Kutter, Raya et al. 2003, Carlson 2004). Plates were not inverted to prevent the top agar from slipping and also to encourage sweating of fluid on the surface of the agar.

The top agar was removed with a disposable 'L' shaped spreader and was transfered into a 250 ml centrifuge tube along with 5 ml of SM buffer which had been used to wash the scraped plate. Phages were eluted from the agar by incubating the centrifuge tubes at 4 °C overnight with gentle shaking. Next day the tubes were centrifuged at 4000 × g for 10 m at 4 °C. The supernatant was removed and centrifuged again at 4000 × g for 10 min at 4 °C. After the second centrifugation the supernatant was filtered through a 0.45 µm syringe filter. The filtrate was diluted serially (tenfold) and spot-inoculated onto V. cholerae lawns on 1% LB agar plates The plates were incubated inverted at 37 °C for at least 8 h to see the titre of the phage (§ 2.4.2.1) recovered through plate lysate.

2.4.3.1 Titration of phage

Tenfold serial dilutions of the phage were done in the SM buffer. Volumes (10 µl) of each dilution were spotted onto lawns of the propagating host V. cholerae strain (§

2.4.2). After the spots were dried in to the agar, the plates were incubated in an inverted position at 37 °C for 24 h and examined for plaques. Plaques were counted on the lowest dilution. The titre was calculated by multiplying the number of plaques with the dilution factor of the serial dilution taking into account the aliquot plated.

2.4.4 Concentration of phage stocks

The protocol followed was modified from a standard protocol (Sambrook and Russell 2001), with centrifugation speed modified from 110,000 × g to 40,000 × g. The Beckman J2-21 centrifuge was pre-cooled to 4 °C before use (Beckman, High Wycombe, UK). Polypropylene centrifuge tubes of 50 ml capacity were filled with phage lysate. The tubes were balanced and then centrifuged at 40,000 × g for 2 h at 4 °C in a Beckman JA25 rotor using aforesaid centrifuge machine. After centrifugation, the supernatant was discarded carefully and the glassy pellet was resuspended in 1 ml of SM buffer and left overnight at 4 °C on a rocking platform to elute phages into the buffer. The following day, the phage yield was determined by spotting decimal dilutions of the phage on to lawns of the appropriate V. cholerae host strain.

2.4.5 Polyethylene Glycol (PEG) precipitation

The protocol followed was modified from a standard protocol (Sambrook and Russell 2001). A single colony of the V. cholerae host strain was inoculated into 10 ml of LB broth and incubated overnight at 37 °C. The following day, 1 ml of overnight culture was inoculated into each of four 500 ml LB broth in 2 litre screw cap flasks and incubated at 37 °C shaking at 150 rpm. On reaching mid-exponential phase all four flasks were inoculated with 1 ml of > 10^7 phage and incubated at 37 °C for 24 h. After 24 h cultures were brought to room temperature and 10 ml of chloroform was added to

each flask, mixed and again incubated for further 10 m at 37 °C and then brought to room temperature again.

To each 500 ml culture 29.2 g of NaCl (final concentration 1M) was added and mixed using a magnetic stirrer until it dissolved completely. The cultures were stored on ice for 1 h. Bacterial debris was removed by centrifugation at 11,000 × g for 10 mins at 4 °C. The supernatants from four cultures were combined in a 2 litre flask and to it PEG 6000 was added to a final concentration of 10% w/v. The PEG was dissolved by slow stirring with a magnetic stirrer at room temperature for 1 h. The suspension was transferred to polypropylene 250 ml centrifuge tubes and placed on ice for 1 h to precipitate the phage particles.

The precipitated phage particles were recovered by centrifugation at 11000 × g for 10 m at 4 °C. The supernatant was discarded, any residual fluid was removed by pipette and bottles were allowed to stand inverted in a tilted position for 5 m to allow all the remaining fluid to drain away from the pellet. A wide bore pipette was used to resuspend the phage pellet gently in SM buffer (8 ml each for earlier 500 ml of suspension). Then centrifuge tubes were placed on their sides at room temperature for 1 h so that SM covered the pellets. The walls of the centrifuge tubes were washed gently with SM buffer to recover any phages stuck to the walls.

The PEG and cell debris from the phage suspension were extracted by adding an equal volume of chloroform. The contents were mixed thoroughly by gentle shaking. The organic and aqueous phases were separated by centrifugation at 3000 × g for 15 m at 4

°C. The aqueous phase at the top was recovered. The titre of the recovered phages was determined.

2.4.6 Snap freezing of phage

In a 2 ml screw cap Nunc sterile cryo-vial, 0.5 ml of phage lysate was mixed with 0.5 ml of sterile glycerol. The cryo-vial was placed in liquid nitrogen. After 5 m, the vials were transferred to a freezing container (Mr. Frosty, Thermo Scientific) and then transfered to -80 °C for long term storage.

2.4.7 Host range of the phage

The host range of the phage was determined based upon their ability to form plaques on the lawns of all available isolates of V. cholerae. For this, 10 ml aliquots of LB broth were inoculated with single colonies of each V. cholerae isolate from TCBS plates and incubated overnight at 37°C. Next day lawns (§ 2.4.2) were prepared from each V. cholerae isolate. A 10 µl aliquot of each phage to be tested was spot-inoculated onto each V. cholerae lawn. After the spots were dried the plates were incubated in an inverted position for 24 h at 37 °C and next day they were checked for plaques.

2.4.8 Pulsed field gel electrophoresis

Aliquots of 50 µl of phage suspension with a count of 10^8 PFU/ml of phage were mixed with 10 µl of proteinase K (10 mg/ml, Sigma Aldrich, UK) and then to it was added an equal volume of molten 1.2 % agarose in TE buffer (§ 2.1.2.3). This was mixed well and dispensed into moulds and allowed to solidify. The solidified agarose

plugs were carefully removed from the moulds and transferred to Eppendorf tubes containing 1 ml lysis buffer (§ 2.1.2.4) and incubated for 18 h at 55 °C.

The next day the lysis buffer was discarded and 1 ml of sterile water was added and incubated for 15 mins at 55 °C. The plugs were then washed 3 times for 18 m at 55 °C in wash buffer and stored at 4 °C until used. A 1.2 % agarose gel, made by adding 0.6 g PFGE grade agarose (Bio Rad, USA) to 50 ml TE buffer (pH 8), was cast with a 10 well comb in the gel casting tray and allowed to set. After the gel was set the comb was removed. The plugs were sliced to 2 mm width and inserted into the wells. For comparison of the phage DNA size with a genome marker, the Pulse Marker 50-1000 Kb (Sigma Aldrich, UK) was used, which was sliced with a clean sharp blade up to two small graduations of syringe.

The gel was transferred to a PFGE gel tank containing 1 litre of 1 x TAE (see 2.1.2.6; 1× TAE buffer was prepared 2 litres i.e. 40 ml of 50 × TAE was added to 1960 ml of RO water and stored at 4 °C until required). The PFGE was run using the CHEF DRII system (Bio Rad, USA) for 17 h at 6 V with switch time of 10-30 s. After PFGE was complete the gel was stained in running buffer (100 ml) containing 20 µl of ethidium bromide for 30 m. The image was taken on an automated imaging system called Gel Doc (Bio Rad, USA). The approximate genome size was calculated by plotting a standard curve of distance migrated by the phage DNA.

2.4.9 CsCl purification of phages for electron microscopy

The phage particles were purified for electron microscopy by using CsCl density gradient centrifugation (Sambrook and Russell 2001). CsCl was added to high titre

phage lysate (> 10^9) to provide a concentration of 0.75 g/ml and dissolved. This mixture was transferred to 3.5 ml Beckman Ultra centrifuge tube using 2.5 ml syringes with a 16 gauge needle and the tube was heat-sealed. This solution was centrifuged with a Beckman Ultra centrifuge (TL100) at 70,000 rpm for 24 h at 10 °C. The single band of concentrated phage particles visible as a faint blue band was extracted using a 20 bore needle attached to a tuberculin syringe.

In order to remove the residual CsCl, the yield was passed through the column of an Amicon Ultra-0.5, 30 kDa MWCO centrifugal filter unit, 500 µl, (Millipore Ltd). To do this, after transferring the extracted band to the filter unit, this was capped and placed in to centrifuge rotor, aligning the cap strap towards the centre of rotor and counter balanced with a similar unit and centrifuged for 10 mins at 14000 × g. To recover the concentrated solute, the filter device was inverted in a clean micro centrifuge tube which was then placed in the centrifuge rotor aligning the open cap towards the centre of the rotor. It was counter balanced with a similar device and centrifuged for 2 m at 1000 × g to transfer the concentrated sample from the device to tube. The ultra-filtrate was stored at 4 °C for further usage after the titre yielded had been determined.

2.4.10 Electron Microscopic imaging of the phages

Following CsCl purification of phages electron microscopic imaging was done. The processing of samples for imaging was done by late Stefan Hyman in the Electron Microscopy Laboratory at the University of Leicester. The grid used to hold phage suspensions was a carbon-coated Pioloform grid which was glow-discharged (ionisation of Argon gas in to plasma under vacuum which glows while discharging

upon the grid) to eliminate its hydrophobicity before adding the phage sample. A 5 µl aliquot of phage suspension (> 10^8 PFU/ml) was transferred to the grid with a Gilson pipette and negatively stained for 5 mins with 8 µl of 1 % w/v uranyl acetate. The excess stain was quickly removed by touching the filter paper to the edge of the grid at a right angle. After staining the grids were air dired for 20 m and images were captured with a JEOL (Japanese Electron Optic Ltd.) JEM-1400 EM transmission electron microscope which was set at 80 kV.

2.4.11 Adsorption curve of phage

A loopful of frozen stock culture was streaked to TCBS plate and incubated overnight at 37 °C overnight to obtain single colonies. One isolated colony was picked and transferred to 10 ml of LB broth in a 50 ml Falcon tube which was incubated at 37 °C with shaking at 150 rpm for 2 h in a water bath incubator. The OD_{600} was checked after 2 h, which was approx. 0.3, indicating that the bacterial count was approx. 3 × 10^7 CFU/ml (calculated from the bacterial host strain growth curve; § 2.3). At this stage 0.5 ml of bacterial culture was collected into an Eppendorf tube marked 'C' (Control without phage) which was placed on ice.

Immediately after collecting the uninfected control sample, a 1 ml aliquot of approximately 3 × 10^7 PFU/ml of phage stock was added and mixed well. Very quickly after that, 0.1 ml of the infected culture was collected in an Eppendorf tube marked '0' (for time zero) and which contained 0.9 ml of SM buffer. This sample was also placed on to ice. After time '0', at every minute a sample was taken in tubes marked from 1 to 10 (which contained 0.9 ml of SM buffer) and kept on the ice.

The uninfected control bacterial sample collected in tube 'C' was diluted tenfold down to 10^{-5} and the 0.1 ml quantity of the 10^{-4} and 10^{-5} dilutions were plated on the duplicate LB agar plates using the spread plate method. The plates were incubated inverted at 37 °C overnight. The samples from the tubes marked with 0 to 10 were diluted tenfold down to 10^{-4} and a 0.1 ml volume of 10^{-4} dilution was mixed into 5 ml of 0.5 % LB overlay agar on to the 1 % LB agar plates in duplicate. The plates were incubated inverted at 37 °C for overnight. Next day the bacterial numbers were estimated for the uninfected control sample to know the actual bacterial numbers at the time when phage was inoculated. The phage counts for infected samples were enumerated to plot a curve. Based on the curve the adsorption time was noted which is the time corresponding to the lowest point on the curve when the phage counts were reduced and after which the counts started to rise again.

2.4.12 One step growth curve of phage

The protocol used for the one step growth curve of phage was based on the protocol provided by Karin Carlson (Carlson 2004). Three 50 ml Falcon tubes labelled with G, G2 and G3 with 10 ml, 10 ml and 20 ml LB broth, respectively were placed in a water bath incubator. A single well-isolated colony from a fresh TCBS plate with the growth of V. cholerae host strain was inoculated into the tube labelled G, while G2 and G3 were kept uninfected but all three were incubated shaken at 150 RPM at 37 °C in a water bath. After 2 h, the OD_{600} was checked for tube G, which was approx. 0.3, indicating that the bacterial count was approx. 3×10^7 CFU/ml (calculated from the bacterial host strain growth curve; § 2.3). At this stage 0.5 ml of the bacterial culture was collected into an Eppendorf tube marked 'C' (Control without phage) which was placed on to ice.

Immediately after collecting the uninfected control sample, a 1 ml aliquot of approximately 3×10^7 PFU/ml of phage stock was added and mixed well. Very quickly after that, 0.1 ml of the infected culture was collected in an Eppendorf tube marked 'S0' (for sample at zero time) and which contained 0.9 ml of SM buffer. This sample was also placed on to ice. After collection of the S0 sample, the tube G was again incubated until time D, which is the adsorption time obtained from the adsorption curve studies (§ 2.4.11). At time D, 0.1 ml of the infected culture was transferred from tube G to tube G2 and mixed well which gave a 10^2 fold dilution. Immediately after that, 0.2 ml from G2 was transferred to G3 and mixed well which gave a 10^4 fold dilution. At this point, the G and G2 tubes were discarded and 0.1 ml of the culture from G3 was collected in an Eppendorf tube marked 'S1' which also contained 0.9 ml of SM buffer and placed on to ice. Tube G3 was again incubated and at 5 min S2 sample was collected i.e. 0.1 ml of the culture was collected in an Eppendorf tube marked 'S2' which contained 0.9 ml of SM buffer and placed on to ice. Similarly, at 5 m intervals the samples were collected and placed on ice until 90 mins with tubes labelled S3, S4, S5 and so on.

The samples collected from the G3 tube were already diluted down to 10^{-4} and thus an aliquot i.e. 0.1 ml (100 µl) was overlaid in to the lawns made from bacterial host strain on duplicate plates (§ 2.4.2 but in this case phage was overlaid together with bacteria in top agar instead of spotting it later). The plates were incubated at 37 °C inverted overnight. The sample in the tube labelled 'C' was diluted down to 10^{-5} in MRD as a diluent and then 10^{-5} dilution was spread-plated in duplicate on LB agar plates. The sample labelled 'S0', which was already diluted to 10^{-1} in SM buffer was further

diluted down to 10^{-4} and 100 µl of 10^{-4} dilution was overlaid in duplicate on LB agar plates. Next day the plaques and colonies were enumerated. The phage counts were plotted graphically to check whether they gave a standard phage growth curve. The bacteriophage burst size was determined by using following formula:

Burst size = P- x/I-x

where:

P = maximum phage titre after lysis complete (PFU/ml)

x = number of phage from the original inoculum which did not adsorb (PFU/ml)

I = titre of the original inoculum (PFU/ml)

The latent period was noted from the phage growth curve which corresponded to the time period until the rise of phage numbers after the initial decline.

2.4.13 Phage genomic DNA extraction

The phage genomic DNA was extracted using modified Promega Wizard method adopted from the Center for Phage Technology, Texas A&M University, USA (Summer 2009).

A 10-20 ml volume of phage lysate with a density of greater than 10^9 PFU/ml was placed in the sterile centrifuge tube and 0.5 µl/ml of nuclease solution (DNase I, 20 mg/ml and RNase A, 20 mg/ml) was added to get rid of free bacterial DNA/RNA and incubated at 37 ºC for 30 min. The precipitant solution (30% v/v PEG-8000 and 3M NaCl) was added to the lysate at a rate of 1:2 precipitant:lysate. This was mixed gently by inversion and incubated at 4 ºC overnight. The precipitated phage lysate was centrifuged at 10,000 x g and 4 ºC for 10 min. The supernatant was carefully poured off and the pellet, which usually spread up the side of the tube, was retained.

The pellet was resuspended in 500 µl of resuspension buffer ($MgSO_4$, 5 mM) by pipetting gently up and down and by rinsing down the sides of the tube. The resuspended phage was transferred to a labelled 1.5 ml microcentrifuge tube and centrifuged for 5-10 s to pellet any insoluble particles. The supernatant was transferred to a new 2 ml microcentrifuge tube. To expose the DNA, phage capsids were broken by addition of proteinase K (Sigma Aldrich, UK). To each 500 µl aliquot of resuspended phage, 10 µl of 0.5 M EDTA pH 8 was added and proteinase K was also added to a final concentration of 100 µg/ml. The mixture was incubated at 50 ºC for 30 m and allowed to cool down to room temperature.

The purification resin from the Promega Wizard DNA Cleanup kit (cat# A7280) was swirled gently without shaking and 1 ml of it was added to the phage suspension (Figure 14). The content was mixed by inverting 5-6 times. The new 1.5 ml microcentrifuge tube was placed in a tube rack and a Wizard minicolumn (Silicon carbide) was placed into the tube. The plunger was removed from a 3 ml syringe and placed on a clean paper towel on the bench. The syringe barrel was attached to the minicolumn in the tube rack and left standing in the tube rack. The resin/lysate mix was pipetted into the syringe. Holding the syringe over a waste beaker, the syringe barrel was inserted and the resin was pushed into the minicolumn until all the liquid was forced through the resin. The minicolumn was detached from the syringe and placed back into its microcentrifuge tube. The plunger was removed from the syringe and then the syringe barrel was reattached to the minicolumn. The column was washed by adding 2 ml of 80% isopropanol to the syringe and holding the syringe over a waste beaker, the syringe barrel was pushed to force the isopropanol through

the minicolumn. The syringe was then removed from the minicolumn and discarded. The Isopropanol increases the affinity of phage DNA to the column and without this DNA yield will be low.

The lid of a 1.5 ml microcentrifuge tube was cut off and labelled. The minicolumn was placed into this tube and centrifuged for 2 min at 13,000 x g at room temperature to dry the resin. The lid of a new 1.5 ml microcentrifuge tube was cut off and labelled. The minicolumn with dry resin was placed into this tube and 100 µl of water, preheated to 80 ºC, was aliquoted to the top of each column and immediately centrifuged at 13,000 x g at room temperature for 1 min to elute the DNA. If the volume of eluate was much less than 100 µl then another 100 µl of heated water was added and centrifuged again. The eluted DNA was transferred into the new 1.5 ml microcentrifuge tube. The minicolumn was discarded and the extracted DNA was stored at -20 ºC.

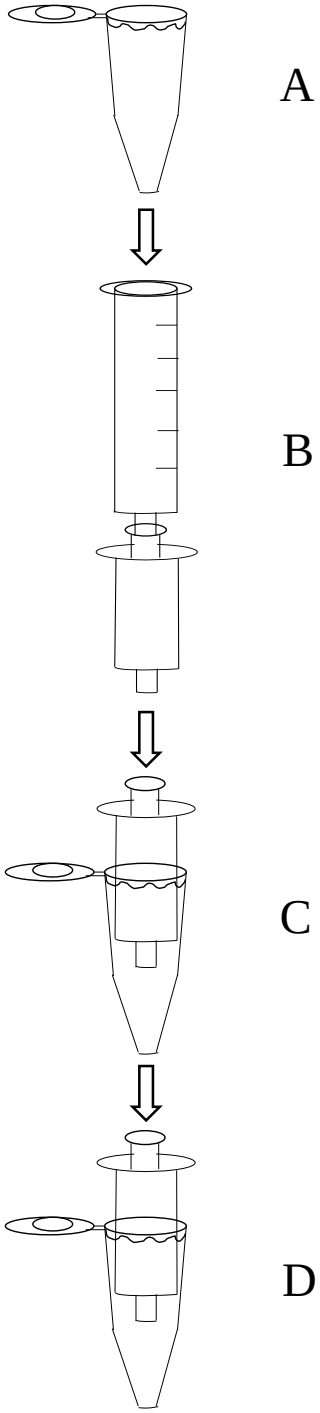

Figure 14: Schematic representation of phage genomic DNA extraction method.

A: DNA cleanup resin added to sample and mixed by inversion; B: Syringe barrel attached to minicolumn, resin/DNA mixture pipetted and pushed with plunger in to minicolumn; C: Minicolumn transferred to microcentrifuge tube and centrifuged; D: Minicolumn transferred to new microcentrifuge tube, prewarmed water/TE added and centrifuged to elute DNA

2.4.14 Ethanol precipitation of phage DNA

The protocol used for ethanol precipitation was adopted from the method of Green and Sambrook (Green and Sambrook 2012).

Ammonium acetate (§ 2.1.2.10) was added to the extracted DNA to a final concentration of 2.5M and was mixed well. Ice cold ethanol (95%) was added in twice the volume of the ammonium acetate-DNA solution mix, mixed well and then 1M $MgCl_2$ (2.1.2.8) was added to it. This solution mix was stored at -20 °C for overnight to enable precipitation.

Next day the DNA was recovered by centrifugation at 0 °C at 20000 RPM speed for 30 m. The supernatant was carefully removed without disturbing the pellet which was invisible. The microcentrifuge tubes with the pellet were filled half the way with 70 % ethanol (at room temperature) and recentrifuged at 20000 RPM speed for 2 m at 4 °C. Again, the supernatant was carefully removed without disturbing the pellet. The tubes with DNA pellets were stored open at room temperature for approx. 15 m until the last traces of fluid had evaporated.

The DNA pellet was dissolved in 10 µl of TE buffer pH 8.0 (§ 2.1.2.9) by carefully rinsing the walls of the tube with buffer. After quantification of the DNA yield using a NanoDrop-8000 spectrophotometer (Thermo Scientific, USA), the DNA was stored at -20 °C until further use.

2.4.15 Restriction analysis of phage DNA

The restriction enzymes to be used for restriction analysis were identified by using NEBcutter (Vincze, Posfai et al. 2003). The protocol available on the Promega website (http://www.promega.com/~/media/files/resources/protocols/technical%20manuals/101/restriction%20enzymes%20protocol.pdf?la=en) was used. The digestion was done in 20 µl final volume which contained 16.3 µl of sterile deionised water, 2 µl of an appropriate RE 10 × Buffer, 0.2 µl of acetylated BSA (10 µg/µl) and 1 µl of DNA (1 µg/µl) which was mixed by pipetting. To this mixture 0.5 µl of the Restriction enzyme (10 u/ µl) was added, again mixed gently by pipetting and centrifuged for few seconds in a micro centrifuge. This digestion mixture was then incubated at 37 °C for up to 4 h in the heating block which was pre-heated to 37 °C. The loading buffer (6 ×) was added, 4 µl to each of the digest before gel analysis. The gel electrophoresis was done as per the protocol given in the next section.

2.4.16 Gel electrophoresis

To prepare a small sized gel, 1 g of agarose (Sigma Aldrich, UK) was dispensed in 100 ml 1 × TAE Buffer (§ 2.1.2.6) in a 250 ml conical flask. The gel was dissolved by placing it in a microwave for approximately 3 m on a high setting with intermittent mixing. The agarose gel was cooled to around 50 °C before adding 2 µl per 50ml of NANCY-520 (Sigma Aldrich, UK) as a safer alternative to ethidium bromide which is mutagenic, followed by mixing. The gel was poured into the minigel cast (Anachem, UK) trying to avoid the formation of air bubbles. The comb (1.5 mm size) was placed into position and allowed to set for 30 m. Once the gel was completely set the comb

was carefully removed by pulling it straight upwards. The gel was placed into an electrophoresis tank filled with 1 × TAE Buffer.

Phage DNA samples, either pure DNA or DNA digested with restriction enzymes, were mixed with loading buffer (bromophenol blue, 0.01% w/v, glycerol, 50% v/v, EDTA, 0.025 M pH 8.0) at the ratio of 4:1 (sample:buffer) before loading into the wells. The DNA ladder marker, if needed, was loaded without DNA loading dye. After loading the samples and ladder, the lid of the electrophoresis tank was closed and attached to a power pack. The electrophoresis was run at an appropriate voltage of 80 V for 2 h until the dye front was 2 cm from the end of the gel. The power was switched off and the electrophoresis tank was disconnected from the power pack. The gel was carefully removed from the caster and imaging was done using the Gel doc imaging system (Bio Rad, USA).

2.4.17 DNA sequencing, assembly and annotation of phage genome

After phage genomic DNA was extracted using the Promega DNA extraction kit (A7280 Wizard DNA Clean-Up System) [§ 2.4.13] and after further purification by ethanol precipitation [§ 2.4.14]. Next generation sequencing was performed by Source Biosciences, UK and NU-OMICS, Northumbria University, UK using the Illumina Miseq platform, 2 x 250-bp paired-end run. The sequence data was assembled by the University of Nottingham's Advanced Data Analysis Centre (ADAC) using de novo assembly and single cotigs for the phages were generated using the SPAdes v. 3.1.0 assembler (Bankevich, Nurk et al. 2012) with 120× coverage. Quality of the data was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and reads were quality trimmed/filtered; while the depth of assembly also ensured that

sequencing errors were minimised. Owing to above quality checks and due to shared SNPs; the SNPs identified were real and not artefacts of the method. Genome annotation was carried out with the help of Andrew Warry of ADAC using RAST (Aziz, Bartels et al. 2008) and Geneious (V6.1.7, Biomatters, Auckland, NZ) software with some manual curation, which provided the translated sequences of protein coding regions. These sequences were then used to interrogate the NCBI database using BLASTP (Altschul, Gish et al. 1990). Conserved protein motifs were identified using a HHpred search of the pfam database (Punta, Coggill et al. 2012). In case of BLASTP search for protein motifs atleast 90% identity was sought to be conserved within the motif to assign a protein to a gene sequence (Kropinski, Borodovsky et al. 2009). This cutoff value for homology detection instilled a confidence in annotation. The tRNA annotation was done using tRNAscan-SE (Schattner, Brooks et al. 2005) and ARAGORN (Laslett and Canback 2004). Post annotation the genomes were submitted to the GenBank using the online BankIt (http://www.ncbi.nlm.nih.gov/WebSub/?form=login&tool=genbank) pathway after following the instructions available on the NCBI website.

2.5 Phage therapy experiments

2.5.1 The phage and host strain

Phage Φ1 was selected for therapeutic purposes owing to its suitability in terms of its broad host range and lytic ability pertaining to burst size and latent period as discussed in chapter 3 and also its genomic traits showing a strictly lytic nature as discussed in the chapter 4. The phage was concentrated up to 10^9 PFU/ml by plate lysates (§ 2.4.2) and stored at 4°C until used.

The host strain for phage Φ1, V. cholerae 1051 is a classical O1 serotype and was used as a streptomycin resistant (SmR) mutant generated by Dr. J. Ritchie (JR) at the University of Surrey. The bacterial inoculum was prepared by harvesting overnight broth cultures incubated at 37°C by centrifuging and then resuspending the residual pellet at a final concentration of ~2 × 10^9 CFU/ml in a sodium bicarbonate solution (2.5 g in 100 ml with pH 9) as an antacid for oral inoculation.

2.5.2 Animal model used for phage therapy

The infant rabbit cholera model (§ 1.5.3) developed by Dr. Jenny Ritchie (JR) was used (Ritchie, Rui et al. 2010). All the laboratory work was done by author while samples (§ 2.5.5) were collected by JR. The New Zealand white rabbit pregnant does were obtained from Harlan Laboratories (Harlan laboratories, UK Ltd, Shaw's farm, Blackthorn, Bicester, Oxon, England). The work was carried out under the HO project licence number PPL 70/7495.

2.5.3 Number of animals and their dosing

The control and treatment group litters were housed separately along with their mothers (litter size varied from five to nine kittens with average of eight). The control group contained 18 infant rabbits from three litters born on three separate occasions used for experiments 1, 2 and 3; while the treatment group involved 15 infant rabbits from two litters born on two separate occasions used for experiments 2 and 3. The control and treatment groups were dosed on different days within an experiment owing the cross contamination of phage from phage treated animals to control group animals which was noticed in a pilot study. The number of rabbits per litter, the bacterial dose and the phage dose used for treatment were as per Table 4. Prior to

infection with bacteria, animals were given Ranitidine (a histamine H2-receptor antagonist that inhibits stomach acid production) at the dose rate of 5mg/100g body weight by the intraperitoneal route.

For the control group, two day old infant rabbits were infected with 0.5 ml of V. cholerae 1051 SmR only (not treated with phage). To each of the treated animals 1 ml of phage Φ1 was administered between 6-8 h post-bacterial infection. Size 5 French i.e. 1.6 mm diameter polyurethane catheters (Product code PR-05041, Arrow International, USA) of 50 cm length were used for oral inoculation using separate catheters for administration of phage and bacterial cultures to avoid cross contamination.

Experiment No *	Gouping of animals	No. of rabbits per litter	Bacterial dose (CFU/ml) [Π]	Phage dose (PFU/ml)	Phage MOI
1[♯]	Control group	5	1×10^8	-	-
	Treatment group	-	-	-	-
2	Control group	6	3×10^8	-	-
	Treatment group	9	5×10^8	1×10^9	2
3	Control group	7	5×10^6	-	-
	Treatment group	6	4×10^8	1×10^9	5

Table 4: The litter size, the bacterial dose, the phage dose and phage MOI (multiplicity of infection) during therapeutic experiments.
* Experiments were separated by several weeks; [♯] For experiment 1 only control group was done in order to check the colonisation of the SmR V. cholerae 1051; [Π] Bacterial dose was varied due to separate overnight cultures and thus affected by differences in conditions.

As per the clinical signs observed the animals were categorised in the following groups based on the measured clinical signs which were uniform across studies in the literature (Ritchie and Waldor 2005, Ritchie, Rui et al. 2010) by using the descriptors mentioned below:

Normal – rabbit free from faecal contamination on its body and upon handling produced hard, dark green, formed faecal pellets (if any); on dissection the intestines looked normal (no significant fluid accumulation in caecum; hard, formed digesta/stool in the lower colon).

Mild diarrhoea – small smears or faecal material evident on body and/or upon handling the rabbit produced soft, usually yellow coloured, faecal material (loose stool); on dissection the intestines also contained fluid above what would normally be expected to be seen and this was in the caecum and lower small intestine; any digesta (i.e. pellets in the lower colon) was soft to touch.

Severe diarrhoea – rabbit fur around anus had clear signs of faecal or watery/faecal contamination (i.e. watery diarrhoea), usually this was evident by staining on the fur; on dissection the intestines looked abnormal i.e. swollen and contained fluid (caecum and/or small intestine); no formed digesta was present in the lower colon.

2.5.4 Calculation of Multiplicity of Infection (MOI):

It was calculated as a ratio of number of phage (volume in ml × PFU/ml) to the number of host bacteria (volume in ml × CFU/ml).

2.5.5 Samples collected

Based on the infant rabbit model of cholera (Ritchie, Rui et al. 2010), after 24 h of infection, animals were euthanized by inhalation of Isoflurane (Isoflo®, 100% Isoflorane, Abbott, Maidenhead, UK) until non-responsive to toe pinch and then

euthanized by intracardiac KCl (MercuryPharma, City West Business Park, Co Dublin, Ireland), 15 % w/v solution given as overdose at 2.5 ml per 100 g of rabbit body weight.

After euthanization samples were collected post mortem in pre-weighed Eppendorf tubes containing 2 ml of sterile phosphate buffered saline (PBS). The tissue samples, 1 square cm in size and approx. 0.02 g, were collected from the upper, middle and lower small intestine and mid colon by dissection. Caecal fluid was collected from the caecum by gravity and approx. 0.4-0.8 ml fluid was drained.

2.5.6 Processing of the samples

After collection of the samples (both tissues and caecal fluid), all the Eppendorf tubes were reweighed and the tissue samples were homogenised between sterile microscopic glass slides in 2 ml sterile PBS and the homogenate was used for serial dilutions.

The samples collected were processed as below:

a) Enumeration of the bacterial counts:

Following homogenisation, the samples were decimal-diluted down to 10^{-6}. A 0.1ml aliquot of the following dilutions (as shaded in table 5 below) were spread-plated in duplicate onto TSA (Tryptone Soya Agar, Oxoid 0131) with streptomycin (200 ug/ml) plates using glass beads/L shaped spreaders. The bacterial counts recovered were corrected for tissue/fluid weights.

Segment	Dilutions plated						
	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Upper small intestine							
Mid small intestine							
Lower small intestine							
Caecal fluid							
Mid colon							

Table 5: Dilutions used for bacterial enumeration.

b) Enumeration of phage:

The sample homogenates of tissue samples and the caecal fluids after using for dilutions of bacterial enumeration were centrifuged at 10000 × g for 5 m in a table top micro-centrifuge and filtered through a 0.45 µm syringe filter. After filtration the samples were tenfold diluted down to 10^{-8} and 5 µl of each dilution was spotted on to the lawns of host strain (§ 2.4.3). The phage counts recovered were corrected for tissue/fluid weights.

c) Fluid accumulation ratio (FAR):

Fluid Accumulation Ratio (FAR), was calculated as the ratio of the weight of caecal fluid drained to the weight of caecal tissue (Ritchie, Rui et al. 2010).

2.5.7 Statistical analysis

The bacterial counts for control and treatment groups were compared statistically. There was no phage-only control and thus the phage counts recovered from the treatment group could not be compared statistically with this particular control. Geometric means were calculated by Log_{10} transformation of bacterial counts/ml before statistical analysis which was done using GraphPad Prism statistical software (version 6.05, GraphPad Prism, San Diego, CA). As the bacterial count data was continuous and unpaired but not normally distributed (confirmed by checking for the Gaussian distribution), the non-parametric Mann Whitney U test was conducted to

obtain a probability/significance value as we were comparing only two groups (control and treatment).

ISOLATION OF PHAGES AND THEIR BIOLOGICAL AND PHYSICAL CHARACTERISATION

3 ISOLATION OF PHAGES AND THEIR BIOLOGICAL AND PHYSICAL CHARACTERISATION

3.1 Introduction

Bacteriophages are quite abundant in the fresh/ocean waters and their concentration is as high as 10^7 per millilitre (Rohwer 2003, Van Twest and Kropinski 2009). Sewage samples are always ideal for isolation of phages for enteric bacteria as they contain diverse enteric bacterial hosts. Additional promising sampling locations include surface water such as lakes, rivers and canals since these frequently receive faecal material from animals. The isolation protocols vary slightly according to the type of sample collected and have been discussed in the preceding chapter. Once the phages are isolated, they need precise characterisation and a battery of these phages should be available for choosing a few of them for further therapeutic evaluation. In order to know the host range, latent period and burst size of the candidate phage its biological characterisation pertaining to host range profile and host as well as phage growth curve is necessary. The mid-exponential phase and generation time derived from the host growth curve are necessary to devise a protocol for the one step growth curve of each phage. The one step growth curve provides us with an estimation of the burst size and latent period both of which have been explained in chapter 1 (§ 1.5). A short latent period and higher burst size increases the effectiveness of phage therapy (Mateus, Costa et al. 2014) as a greater number of progeny phages are produced in a shorter time. Determination of the host range of the isolated phages is also required in order to select the candidates with a broad host range for phage therapy trials. Electron microscopy is desirable for morphometry and classification of phages so that it is known what type of phage being used therapeutically. Thus, isolation and biological

as well as physical characterisation of phages are important elements for recruiting the right candidate for phage therapy studies.

3.2 Results

3.2.1.1 Isolation of phages in UK

Isolation of bacteriophages specific to O1 strain of V. cholerae from sewage (Derby sewage treatment plant), a lake (Nottingham University park lake) and river water (Kegworth river) samples from UK was unsuccessful. All the samples were negative for any phages specific to V. cholerae O1 strains.

3.2.1.2 Isolation of phages in China

Seven phages were isolated from China and the locations from which the positive samples were collected are as per Table 6. Of seven positive sample locations two were in Beijing, isolated from the Qing He river and Cui Jia Yao rivers, three in Wuhan, Hubei, isolated from the Fu Jia Wan, Ye Zhi Hu and Nan Hu lakes, while two more were isolated from Nanchang, Jiangxi province from the Yudai He river. The plaque morphologies of the phages isolated in China were as per the Table 6. Six of the seven phages were 0.5 - 1 mm and clear while the seventh, phage J3, was pinpoint in size. All of them had regular margins except for phage QH. The plaques obtained during isolation were relatively pure but three rounds of plaque purification was done to purify them further by picking a plaque with streak plate purification (§ 2.2.2.6).

Serial No.	Phage	Sample location	Plaque morphology
1	QH	Qing He (QH) river Beijing	0.5 - 1 mm, clear with irregular margin
2	CJY	Cui Jia Yao (CJY) river 50 miles away from Beijing	0.5 - 1 mm, clear with regular margin
3	H1	Fu Jia Wan lake in Wuhan, Hubei (H1)	0 - 0.5 mm, clear with regular margin
4	H2	Ye Zhi Hu lake in Wuhan, Hubei (H2)	0 - 0.5 mm, clear with regular margin
5	H3	Nan Hu lake in Wuhan, Hubei (H3)	0.5 - 1 mm, clear with regular margin
6	J2	Yudai He river, Nanchang, Jiangxi (J2)	0.5 - 1 mm, clear with regular margin
7	J3	Yudai He river, Nanchang, Jiangxi (J3)	Pinpoint, clear with regular margin

Table 6: Locations for positive samples and their plaque morphology.

3.2.2 Characterisation of V. cholerae

3.2.2.1 Growth kinetics of the V. cholerae

The growth kinetics, which involved the establishment of a growth curve for the V. cholerae host strains used for propagation of different phages were studied to ascertain the mid-exponential phase and the generation time which in turn were useful for phage growth curve studies. The host strains used in this study were V. cholerae F5, 1051, 2095, C6706 and 238.

The generation time and mid-exponential phase for various V. cholerae host strains were as per the Table 7. Figure 15 depicts a representative growth curve of V. cholerae 1051 and the raw data is presented in Appendix A. The mid-exponential phase for these V. cholerae host strains ranged between 132 ± 0.00 m for C6706 to 188 ± 18.33 m for 2095. The generation time for V. cholerae F5 was small i.e. 18.68 ± 0.14 m; while the largest generation time amongst studied host strains was of strain 2095 i.e. 43.35 ± 13.6 m.

Host strain	Mid-exponential phase (m ± SD)	Generation time (m ± SD)
F5	172 ± 3.46	18.68 ± 0.14
1051	152 ± 18.33	37.70 ± 8.59
2095	188 ± 18.33	43.35 ± 13.6
C6706	132 ± 0.00	36.46 ± 1.95
238	156 ± 20.78	29.64 ± 2.50

Table 7: The mid-exponential phase and generation time for various V. cholerae host strains.

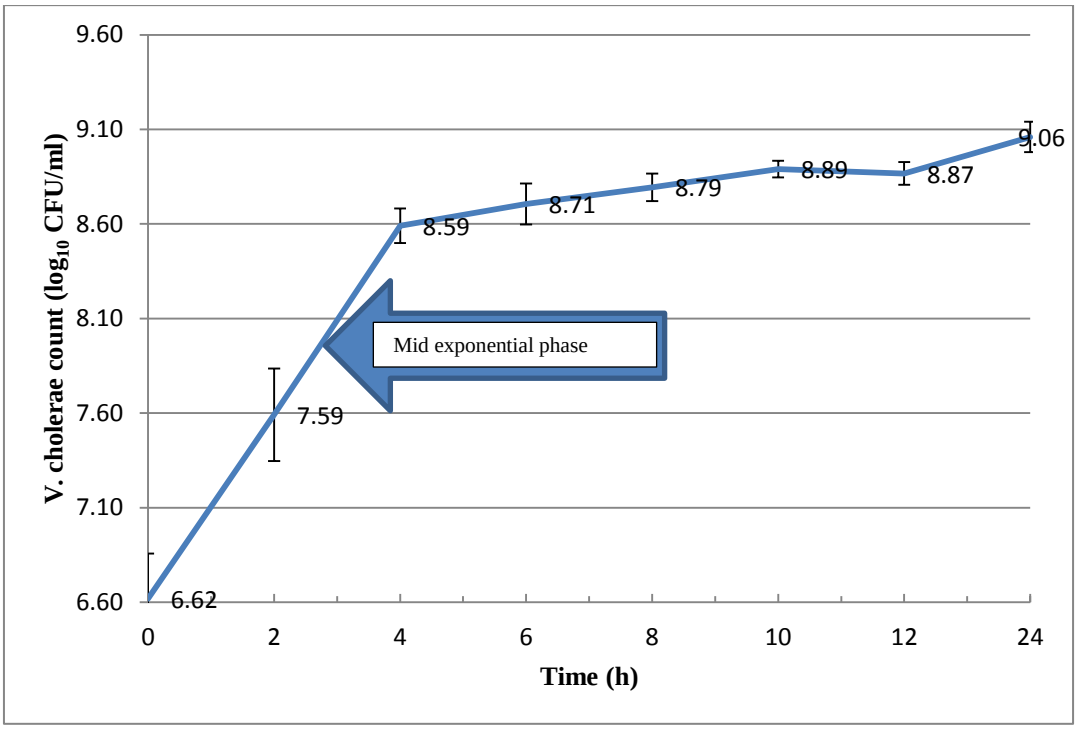

Figure 15: Representative growth curve of V. cholerae strain 1051.

3.2.3 Biological Characterisation of phages

3.2.3.1 One step growth curve of phages

Phage growth curves were done to determine the latent period and burst size. The latent period and burst size for different phages under study are as per Table 8. Figure 16 shows a representative one step growth curve of Φ1 and the raw data tables are in Appendix B. The latent periods of the phages under study ranged between 4 to 15 m and the burst sizes of these phages were between 6 to 664 PFU/cell.

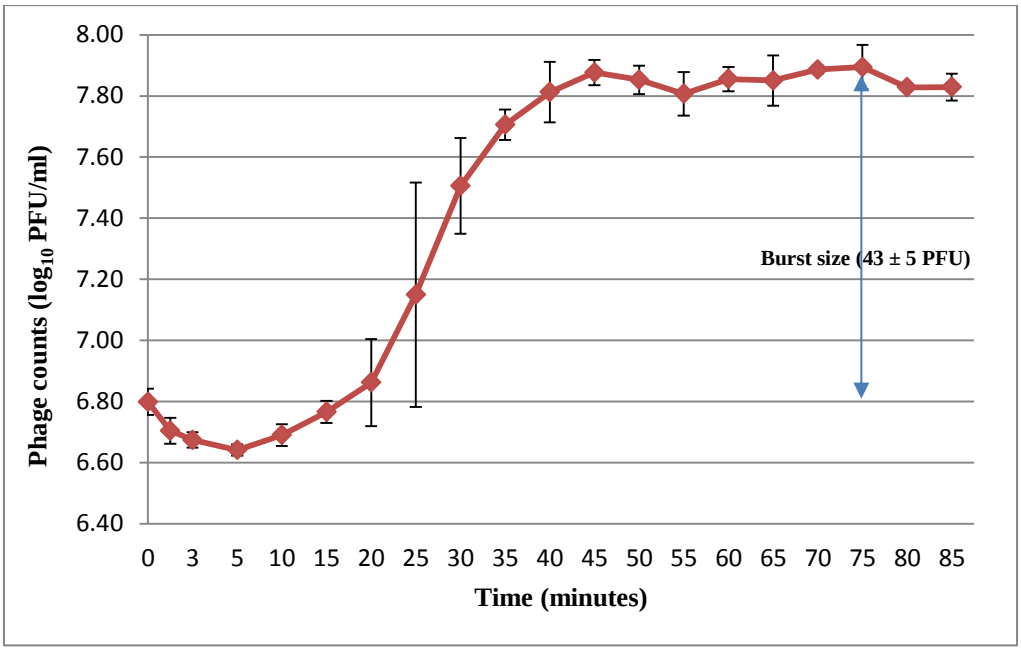

Figure 16: Representative one step growth curve of V. cholerae phage Φ1.

V. cholerae Phage	V. cholerae host strain	Latent period (m)	Burst size (PFU/cell)
Φ1	1051	12 ± 0.0	43 ± 05
Φ2	F5	14 ± 1.6	06 ± 01
Φ3	1051	13 ± 4.1	54 ± 26
Phage 24	1051	04 ± 0.0	87 ± 26
ΦX29	1051	07 ± 0.0	664 ± 50
ΦQH	238	12 ± 4.0	92 ± 09
ΦCJY	238	13 ± 4.3	182 ± 62
ΦH1	238	06 ± 2.3	89 ± 32
ΦH2	238	15 ± 1.6	63 ± 13
ΦH3	238	07 ± 3.5	126 ± 18
ΦJ2	238	05 ± 0.6	34 ± 13
ΦJ3	238	14 ± 1.5	56 ± 17

(n= 3*; ± Standard Error of Mean i.e. SEM; * For X29, H3 and J3 n= 2)

Table 8: The latent periods and burst sizes for different phages

3.2.3.2 Host range profiles

The host range profile of fourteen bacteriophages was determined. Broad spectrum phages are considered suitable candidates for phage therapy trials since they will be able to be used against a wide range of strains (Viertel, Ritter et al. 2014). The knowledge of host range could also help to identify the propagating bacterial host for producing large volumes of phage for downstream characterisation. The host strain on which a particular phage could produce a confluent clear lysis was used as the propagating bacterial host e.g. Φ2 could only produce confluent clear lysis with the V. cholerae F5 strain and therefore this host strain was used. The host ranges are given in Table 9. The zones of lysis were visible upon the lawns prepared from the susceptible host strains. The broader host ranges were displayed for phage Φ1, Φ3, ΦQH, ΦH3 and ΦJ3 which were able to lyse 67.0 %, 62.6 %, 84.6 %, 70.3 % and 76.9 % of the total 91 strains, respectively.

Vibrio strain	Bacteriophages													
	Φ 1	Φ 2	Φ 3	Φ 4	Φ 24	Φ X29	JA1	ΦQH	ΦCJY	ΦH1	ΦH2	ΦH3	ΦJ2	ΦJ3
238	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++
757	+	-	+	-	-	-	-	++	-	-	-	-	-	-
404	-	-	-	-	-	-	-	-	-	-	-	-	-	-
406	-	-	-	-	-	-	-	+	+	+	+	+	+	-
709	+	-	+	-	-	-	-	++	-	+	-	+++	-	+++
719	+	-	+	-	-	-	-	++	-	-	-	+++	-	+++
722	+	-	-	-	-	-	-	+++	-	+	-	++	-	+++
729	+++		+		-	-	-	+++	-	-	-	+	-	+++
732	+++	-	-	-	-	-	-	+++	-	+	-	++	-	+++
736	+	-	+	-	-	-	-	-	-	-	-	-	-	-
739	-	-	-	-	-	-	-	++	-	+	-	++	-	+++
742	+++	-	-	-	-	-	-	+++	+	++	-	+	-	++
750	-	-	-	-	-	-	-	+++	-	++	-	++	-	+++
751	+++	-	-	-	-	-	-	+++	-	+	-	++	-	+++
752	++	-	+	-	-	-	-	+	-	-	-	+	-	++
753	+++	-	+	-	-	-	-	++	-	+	-	++	-	+++
754	++	-	+	-	-	-	-	+	-	-	-	-	-	++
755	+	-	+	-	-	-	-	+	-	-	-	-	-	++
756	+	-	+	-	-	-	-	+	-	-	-	+	-	++
758	+	-	+	-	-	-	-	+	-	-	-	++	-	+++
759	+	-	+	-	-	-	-	+	-	-	-	+	-	++
760	-	-	-	-	-	-	-	-	+	-	-	-	+	-
761	+	+	+	-	-	-	-	++	-	-	-	+	-	+++

-, no lysis; +, opalescent lysis; ++ semi confluent lysis; +++, confluent clear lysis; non O1/O139 strain; O139 strain

Table to be continued …

Vibrio strain	Bacteriophages													
	Φ 1	Φ 2	Φ 3	Φ 4	Φ 24	Φ X29	JA1	ΦQH	ΦCJY	ΦH1	ΦH2	ΦH3	ΦJ2	ΦJ3
762	++	-	+	-	-	-	-	+	-	-	-	+	-	++
763	-	-	-	-	-	-	-	-	-	-	-	-	-	-
764	+	+	+	-	-	-	-	+	-	-	-	+	-	+
765	-	-	-	-	-	-	-	+	+	-	+	-	+	+
767	+	-	+	-	-	-	-	+	-	-	-	++	-	+++
768	++	-	+	-	-	-	-	+	-	-	-	++	-	+++
769	+++	+	+	-	-	-	-	+++	-	+	-	++	-	++
770	+	-	+	-	-	-	-	+	-	-	-	-	-	++
771	+	-	+	-	-	-	-	++	-	-	-	+	-	++
772	+	-	+	-	-	-	-	+	-	-	-	+	-	+++
773	+	-	-	-	-	-	-	+	-	-	-	-	-	+
774	+	-	+	-	-	-	-	++	-	-	-	+	-	+++
775	-	-	-	-	-	-	-	+	-	-	-	-	-	-
776	-	-	-	-	-	-	-	+	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A-4	+++	-	+	-	-	-	-	+++	-	+	-	+++	+	+++
BW-5	+	-	+	-	-	-	-	+	-	-	-	+	-	++
CS-1	+	-	-	-	-	-	-	+	+	-	-	++	-	++
CS-12	+	-	+	-	-	-	-	+	-	-	-	+	-	++
CS-15	++	-	+	-	-	-	-	+	-	-	-	-	-	+
CS-16	+	-	+	-	-	-	-	++	-	-	-	+	-	++
CS-18	+	-	+	-	-	-	-	+	-	-	-	-	-	+
CW-1	-	-	-	-	-	-	-	+	+	+	+	+	+	+

-, no lysis; +, opalescent lysis; ++ semi confluent lysis; +++, confluent clear lysis; non O1/O139 strain; O139 strain

Table to be continued …

Vibrio strain	Bacteriophages													
	Φ 1	Φ 2	Φ 3	Φ 4	Φ 24	Φ X29	JA1	ΦQH	ΦCJY	ΦH1	ΦH2	ΦH3	ΦJ2	ΦJ3
D-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-15	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-25	+++	-	+	-	-	-	-	+	-	-	-	+	-	+++
D-30	++	-	+	-	-	-	-	+	-	+	-	+	-	+
D-56	++	-	+	-	-	-	-	++	-	-	-	++	-	+++
D-59	++	-	++	-	-	-	-	+	-	-	-	++	-	+++
DN-4	++	-	+	-	-	-	-	+	-	-	-	++	-	+++
F-5	+	+++	+	-	-	-	-	+	-	-	-	++	-	+++
F-6	++	-	+	-	-	-	-	+	-	-	-	+	-	+
FB-01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FN-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FN-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FN-5	-	-	-	-	-	-	-	+	++	+	++	-	++	-
GB-39	++	-	+	-	-	-	-	+++	-	+	-	++	-	+++
HH-1	++	-	+	-	-	-	-	++	+	+	+	+	+	++
HH-14	+	-	+	-	-	-	-	++	-	+	-	+	-	++
HH-15	++	-	+	-	-	-	-	++	-	+	+	++	-	++
HH-4	+	-	+	-	-	-	-	++	-	+	+	++	-	++
Ht-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ht-10A	-	-	-	-	-	-	-	+	-	-	-	-	-	-
J-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KCH-18	++	-	+	-	-	-	-	+	-	+	+	+	-	++

-, no lysis; +, opalescent lysis; ++ semi confluent lysis; +++, confluent clear lysis; non O1/O139 strain; O139 strain

Table to be continued …

Vibrio strain	Bacteriophages													
	Φ 1	Φ 2	Φ 3	Φ 4	Φ 24	Φ X29	JA1	ΦQH	ΦCJY	ΦH1	ΦH2	ΦH3	ΦJ2	ΦJ3
KPD-3	++	-	+	-	-	-	-	+	-	+	-	+	-	++
KtH-4	+	-	+	-	-	-	-	+	-	+	-	+	-	++
KTH-7	++	-	+	-	-	-	-	+	-	+	+	+	-	++
N-10	-	-	-	-	-	-	-	+	-	+	-	+	-	++
N-5	++	-	++	-	-	-	-	++	-	-	-	++	-	++
N-7	++	-	+	-	-	-	-	+	-	-	-	++	-	++
NP-14	-	-	-	-	-	-	-	+	+	++	++	++	+	+
NP-3	++	-	+	-	-	-	-	+++	-	+	-	++	-	++
NP-5	++	-	+	-	-	-	-	+	-	-	-	++	-	+++
NP-6	-	-	+	-	-	-	-	+	-	+	+	++	+	+++
NP-7	++	-	+	-	-	-	-	+	-	-	-	++	-	+++
O5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P-1	-	-	-	-	-	-	-	+++	++	+++	+++	+++	++	+++
PS-18	-	-	-	-	-	-	-	+	++	++	++	++	++	++
PS-25	++	-	+	-	-	-	-	+	-	-	-	+	-	++
PS-7	+++	-	++	-	-	-	-	+	-	-	-	+	-	+++
RG-6	++	-	+	-	-	-	-	+	-	+	-	++	-	+++
M14	+++	-	+++	-	-	-	-	+++	-	-	-	-	-	-
O395NT	+++	-	+++	-	-	++	-	+++	+	+	+	+	+	++
1051	+++	-	+++	-	+++	+++	-	+++	-	-	-	-	-	-
2095	-	-	+	-	-	-	-	+++	+++	+++	+++	+++	+++	+++
2134	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++

-, no lysis; +, opalescent lysis; ++ semi confluent lysis; +++, confluent clear lysis; non O1/O139 strain; O139 strain

Table 9: Host range profile of the V. cholerae phages.

All non highlighted Vibrio strains are V. cholerae O1 strains.

3.2.4 Physical characterisation by electron microscopy

Electron micrographs of phages were obtained in order to determine their morphological features and to classify them as per the International Committee on Taxonomy of Viruses (ICTV) classification (§ 1.7.1 and table 1). The morphometry and classification of phages under study are as per the Table 10. Out of 12 phages under study, three belonged to the Myoviridae family (Figure 17) viz. phage Φ2, phage 24 and phage X29; one belonged to the Siphoviridae family (Figure 18) phage Φ3, and remaining eight phages were of the Podoviridae family (Figure 19). Two of the Myoviridae phages had a similar head diameter viz. 64 nm for phage 24 and X29, while Φ2 had longer tail of 118 nm than the other two. Amongst the Podoviridae phages Φ1 had smaller head with head diameter 34 nm, while the rest (all Chinese Podoviridae phages) had head diameter ranging between 51 to 57 nm.

Phage	Head diameter (nm ± SD)	Neck (nm ± SD)	Tail length (nm ± SD)	Classification
Φ2	53 ± 0.16	8 ± 0.04	118 ± 0.36	Myoviridae
Phage 24	64 ± 0.06	8 ± 0.04	69 ± 0.14	Myoviridae
ΦX29	64 ± 0.05	16 ± 0.11	95 ± 0.32	Myoviridae
Φ3	75 ± 0.07	-	156 ± 0.23	Siphoviridae
Φ1	34 ± 0.18	-	13 ± 0.08	Podoviridae
ΦQH	51 ± 0.07	-	12 ± 0.02	Podoviridae
ΦCJY	54 ± 0.07	-	10 ± 0.04	Podoviridae
ΦH1	56 ± 0.07	-	11 ± 0.04	Podoviridae
ΦH2	57 ± 0.08	-	12 ± 0.03	Podoviridae
ΦH3	55 ± 0.06	-	12 ± 0.03	Podoviridae
ΦJ2	54 ± 0.05	-	12 ± 0.03	Podoviridae
ΦJ3	52 ± 0.05	-	11 ± 0.02	Podoviridae

Table 10: Morphometry and classification of V. cholerae phages.

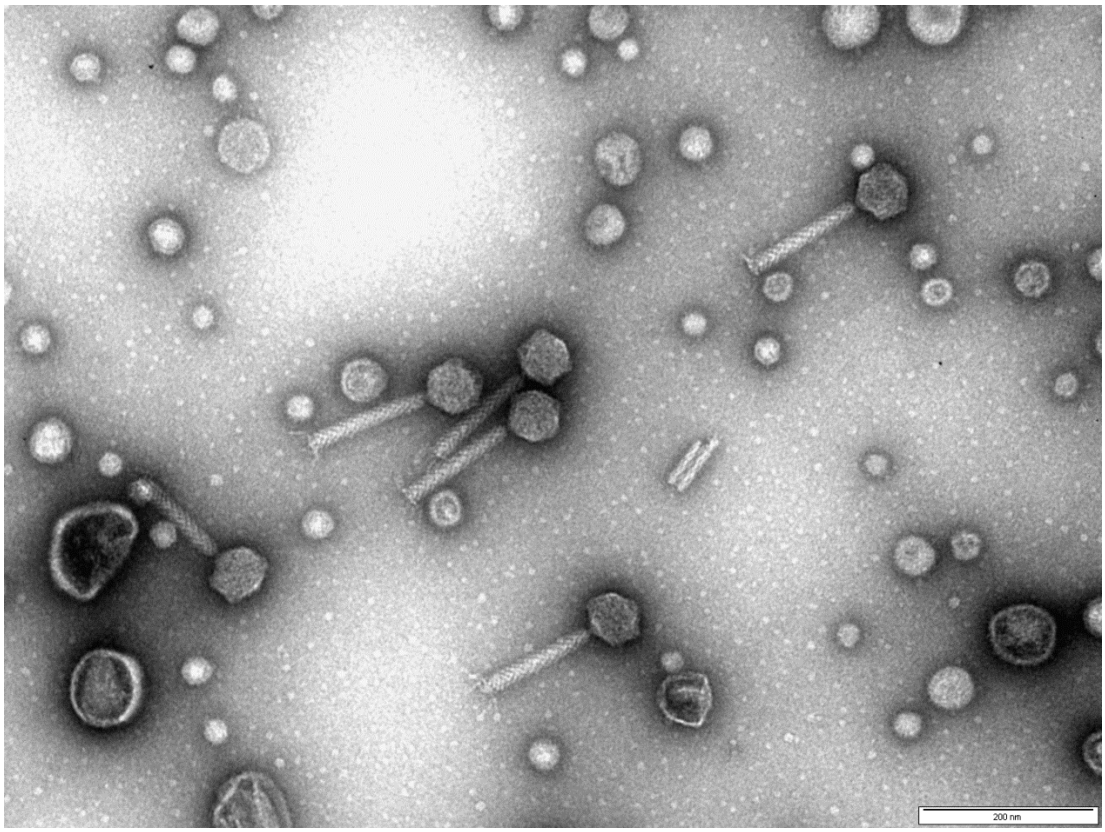

Figure 17: Representative electron micrograph of Myoviridae V. cholerae phage Φ2.

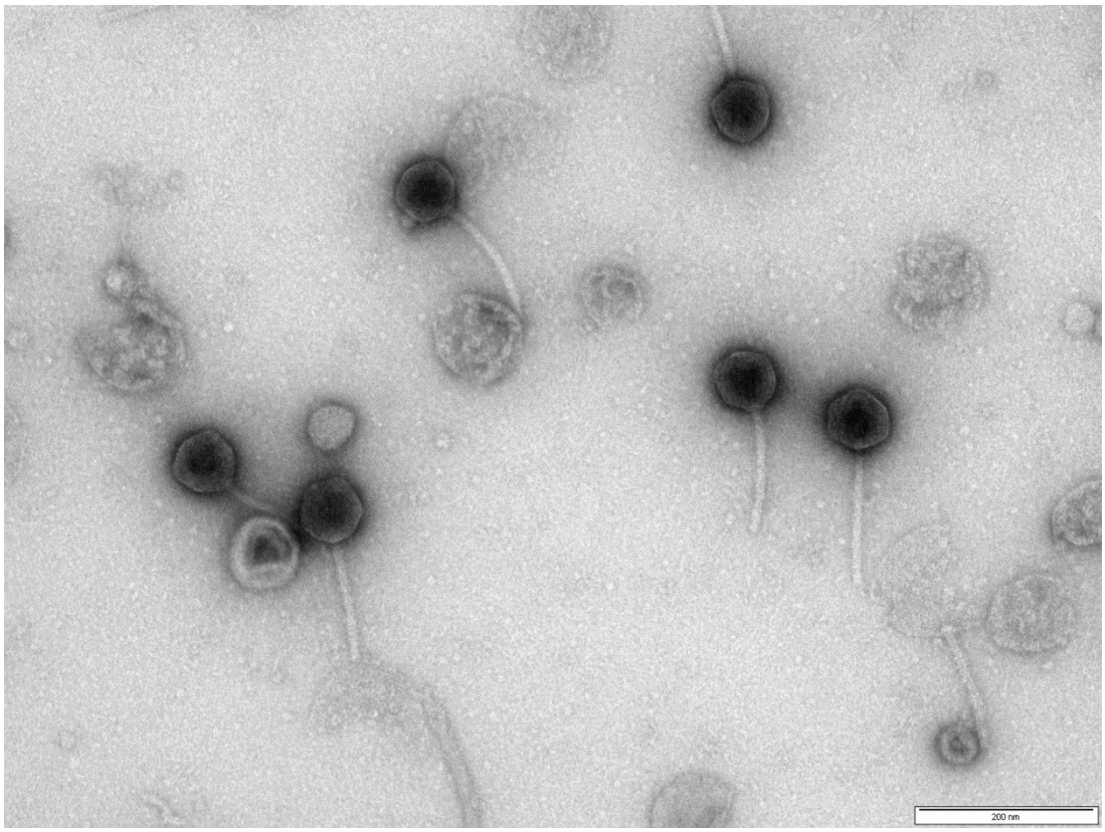

Figure 18: Representative electron micrograph of Siphoviridae V. cholerae phage Φ3.

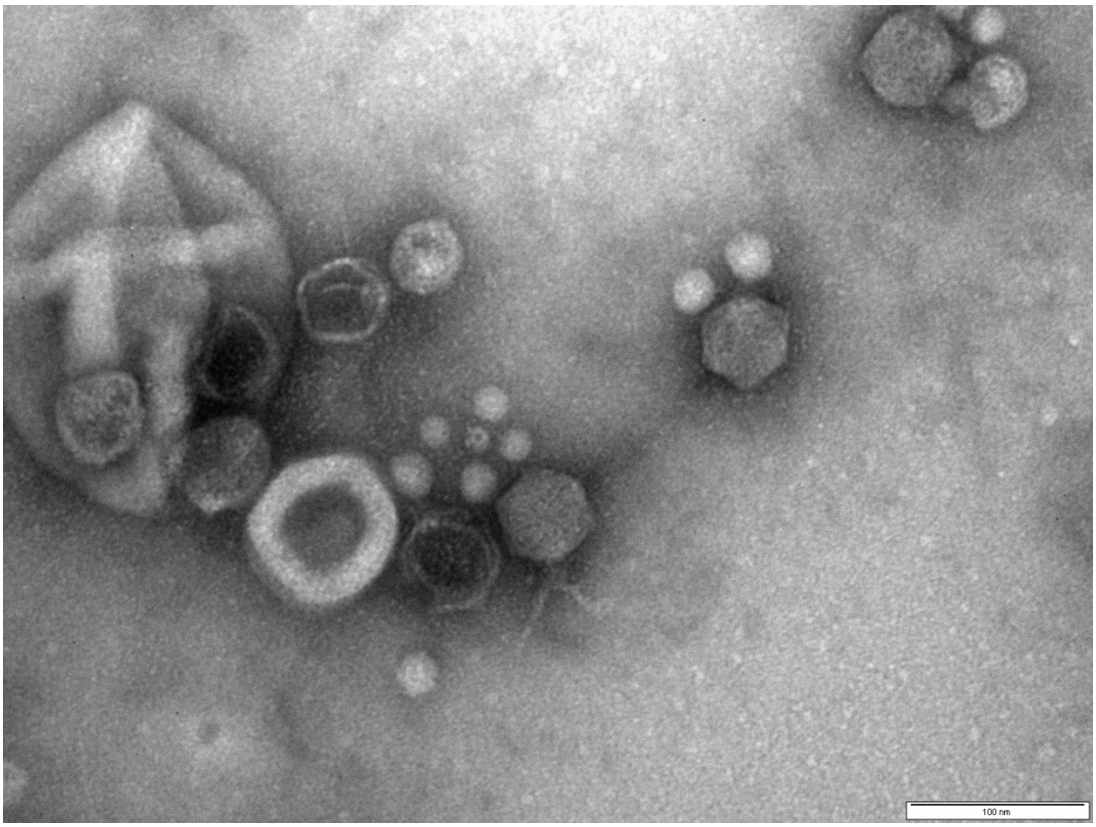

Figure 19: Representative electron micrograph of Podoviridae V. cholerae phage Φ H1.

3.3 Discussion

Isolation of V. cholerae O1 specific phages in the UK environment was not successful but seven phages could be isolated in China. The negative outcome in the UK could be due to the fact that the cholera is no longer an endemic disease in England and Wales (NHS 2014). Developed countries in Europe and North America usually have imported cases, reported from travellers visiting areas where the cholera is endemic, and returning back with the disease (Morger, Steffen et al. 1983, PHE 2013). Thus it might be expected that O1 cholera-specific phages might be isolated from sewage from large metropolitan areas. There were some reports of isolation of low numbers of V. cholerae both O1 and non O1 strains in the UK environment (i.e. water and aquatic birds) between 1976 and 1980 (Lee, Bashford et al. 1982, West and Lee 1982). The authors believed that aquatic birds i.e. gulls were acting as vectors. Later in 1997,

Little and colleagues (Little, Monsey et al. 1997) reported the presence of non-cholera Vibrio spp. in mollusc products in the UK. There were reports of non-O1/non-O139 V. cholerae from other European countries (Aldova, Laznicko.K et al. 1968, Visser, Vellema et al. 1999, Andersson and Ekdahl 2006, Huhulescu, Indra et al. 2007). Looking in to the aforesaid reports, although it has been possible to isolate phages specific to non-O1/non-O139 V. cholerae from the UK environment (Sarkar pers comm); this was of no value for this project which was to develop a treatment for cholera caused by O1 and or O139 serotypes of V. cholerae. It is also worth mentioning that our bacterial strain collection was biased with O1 serotypes as they were dominantly represented, while O139 serotypes were under represented owing to availability of only one O139 strain. The isolation of phages specific to O139 strains could have been possible if they were represented well in our strain collection.

It was thus imperative to isolate cholera phages from cholera endemic areas. China is a country where cholera is endemic and south eastern China is a predicted high risk area (Xu, Cao et al. 2013). The isolation of phage is an indicator of the presence of V. cholerae in the sampled environment (Leclerc, Edberg et al. 2000). Thus, the isolation of seven V. cholerae specific phages in the environmental samples from China might be an indicator of cholera prevalence in that country. Moreover, only two host strains were used to isolate phages in China. This might have limited the type of phages isolated as there might have been much wider range of phages in samples but not selected due to a very limited host strain range. Isolation of bacteriophages for Vibrio spp. other than V. cholerae have been reported earlier from China (Zhao, Wang et al. 2005, Lin, Chiu et al. 2012, Peng, Ding et al. 2013, Wang, Kan et al. 2013, Yu, Gong et al. 2013). Reports on the prevalence of cholera in China are also available (Wang,

Lou et al. 2012, Hu, Zheng et al. 2013, Liang, Zhao et al. 2014, Yue, Gong et al. 2014, Zhang, Li et al. 2014, Zhao, Zhang et al. 2014, Zhou, Zhao et al. 2014) with incidences reported from the seventh pandemic in China between 1961-2010 with the cases from south eastern china.

After isolation, for further characterisation of phages the knowledge of host strain growth parameters was essential because the host strain growth curve gave us the mid exponential phase and generation time, requisites for phage one step growth curve experiments (Carlson 2004, Viscardi, Perugini et al. 2008). The generation times of the host strains under study ranged between 18 to 43 m. These estimated generation times concur with the findings of previous studies which suggest the generation time ranging between <30 to 40 m for V. cholerae (Finkelstein 1996, Martinez, Megli et al. 2010, Microbewiki 2014).

Phage one step growth curves are affected by culture conditions and variations between phage species (Brussow and Kutter 2005, Hendon-Dunn 2011). The latent period is the time between the initial infection and release of the phage progeny (i.e. lysis). The latent period may be affected by the density of the bacterial host, and a lower density of host bacteria will give a longer latent period (Abedon, Herschler et al. 2001, Abedon, Hyman et al. 2003). In the present study the shortest latent period was 4 ± 0.0 m for phage 24 revealing a much shorter adsorption time involved in this specific infection cycle. As discussed earlier, there can be an impact of bacterial numbers on latent period and the bacterial hosts used for this study were in mid exponential phase and the phages showing shorter latent periods might be prolific propagators as evidenced by their burst sizes. According to the literature the shortest

latent periods of phages specific to Vibrio spp. were shown by the Vibrio phages P8D and pVp-1 which were 10 and 15 mins, respectively (Yu, Gong et al. 2013, Jun, Shin et al. 2014). The range of latent periods of phages specific to Vibrio spp. fall between 10 to 120 min; majority of them above 30 m (Phumkhachorn and Rattanachaikunsopon 2010, Cohen, Joseph Pollock et al. 2013, Yu, Gong et al. 2013, Jun, Shin et al. 2014, Lee, Choi et al. 2014, Mateus, Costa et al. 2014) but the range of latent periods for phages in this study are between 4 to 15 m which is in the lower range compared to the phages infecting the same species of host strains. The report on less than 10 min latent period i.e. 9 min was reported for Acinetobacter baumannii lytic phage (Jin, Li et al. 2012). Even the historical literature of Hershey and Chase experiments mentions less than 10 min latent period for phages (Konforti 2001). Though it is possible to have short latent periods which are less than 10 min for phages, an experimental error cannot be ruled out inspite of three independent trials showing similar results. In fact, these phages with shorter latent periods (SLPs) are likely to be specialists for propagation in high density bacterial host populations, owing to their faster adsorption and ability to exploit the bacteria growing at higher density (Abedon, Hyman et al. 2003).

The burst size is the number of progeny phages released by a single infected bacterial host cell. Larger burst sizes are more desirable in bacteriophages which are used to treat infections as they might eradicate bacterial infections quickly. The smallest burst size in the present study was of phage Φ2 with 6 ± 1 PFU per cell. Similar small burst sizes were revealed for Vibrio phages ΦH17-5c and Φ H17-9b with their respective burst sizes being 5.4 and 5.5 PFU/cell (Okano, Yoshikawa et al. 2007). The largest burst size in the present study was for phage X29 which yielded 664 ± 50 PFU/cell but

the range of phage burst sizes except for phage Φ2 were between 34 to 664. The range of burst sizes shown in the present study are in agreement with the literature on range of burst sizes shown for the Vibrio phages which were in between 23 to 500 (Baudoux, Hendrix et al. 2012, Cohen, Joseph Pollock et al. 2013, Yu, Gong et al. 2013, Jun, Shin et al. 2014, Lee, Choi et al. 2014, Mateus, Costa et al. 2014).

The phages studied in this project showed discriminatory lytic spectra and thus the diversity amongst them based on their host range was established. Lee and Furniss (Lee and Furniss 1981) studied the host range of 41 different phages against over 1500 Vibrio strains but only 14 of the phages could lyse some of the host strains. Similarly, in our study, phage Φ4, obtained from Public Health England (§ 2.2.2.1) which arrived without a host strain did not lyse any of the V. cholerae strains used for this study and thus it could not be characterised further. The present study involved 15 non O1/O139 V. cholerae strains in lytic spectra analysis and 6 out of 15 were lysed by different phages, predominantly those isolated in China. In the earlier study, phage ΦP15 was also able to lyse 2 out of 4 non O1/non O139 strains along with O1 strains (Talledo, Rivera et al. 2003). Payne and colleagues (Payne, Oakey et al. 2004) found that the Vibrio harveyi myovirus-like (VHML) bacteriophage was able to infect one out of 8 V. cholerae isolates. It was interesting to note that phage specific to V. harveyi could infect other Vibrio spp. but the host range was narrow for V. cholerae isolates in comparison. Various authors suggested that this ability of a phage to infect other Vibrio spp. could be due to common receptor proteins. Capsular O antigen was identified as the receptor for some Vibrio phages (Seed, Bodi et al. 2011, Fouts, Klumpp et al. 2013) whilst lipopolysaccharides (LPS) on the cell wall of host strains were demonstrated to be the phage receptors for others (Maiti and Choudhuri 1979,

Chatterjee and Chaudhuri 2003). Thus, the ability of many phages in the present study to infect both O1 and non O1 V. cholerae strains could be due to common receptor proteins or carbohydrates including O antigens or LPS. Conversely, Al-Fendi and colleagues (Al-Fendi, Shueb et al. 2014) checked the host range of VPUSM 4, VPUSM 7 and VPUSM 8 phages using various strains of Vibrio spp. and other common enteric pathogens but all these phages were able to infect and lyse only one strain V. cholerae O1 El Tor Inaba. One out of six Vibrio phages isolated by Alagappan and coworkers (Alagappan, Deivasigamani et al. 2010) was able to lyse all the eight strains of V. parahaemolyticus used during isolation, indicating its broad host range. None of the phages in our study were able to lyse 100% of the host strains. The present study used 91 host strains for lytic spectra analysis of phages and the broader host range phages Φ1, Φ3, ΦQH, ΦH3 and ΦJ3 could lyse up to 84% of them. Similarly, 125 host strains were used to determine the host range of phages uVh1, uVh2, uVh3, and uVh4 infecting V. harveyi and they could lyse 78 to 98% of the host strains (Thiyagarajan, Chrisolite et al. 2011). Surekhamol and colleagues (Surekhamol, Deepa et al. 2014) found the lytic spectra of six broad spectrum phages to be between 50 to 70 %.

All the phages under study belong to the order Caudovirales as they have binary symmetry and tails (Ackermann 2006) (§ 3.2.4). Out of twelve, three phages (phage Φ 2, phage 24 and phage X29) were classified in the Myoviridae family owing to their rigid tails. One phage (phage Φ 3) was classified in the Siphoviridae family as it had a long flexible tail. Eight more phages belonged to the Podoviridae family owing to their short tails, thought to be non-contractile. Although most of the phages in this study are Podoviridae family, the podoviruses are not very common amongst the

Vibrio phages, especially in V. parahaemolyticus viruses (VpVs) and in aquatic ecosystems (Comeau, Chan et al. 2006, Thiyagarajan, Chrisolite et al. 2011). Average head dimensions of the podoviruses studied in this project ranged between 34 – 57 nm and the tail length ranging between 11 – 13 nm which is at the lower range of the average dimensions noted earlier for Podoviridae Vibrio phages i.e. 50 to 72 nm for head and 15 to 27 nm for tail (DePaola, Motes et al. 1998, Comeau, Chan et al. 2006, Thiyagarajan, Chrisolite et al. 2011, Fouts, Klumpp et al. 2013). Of these few reports on podoviruses DePaola et al. (1998) reported an unusual podovirus with elongated capsid measuring 258 nm.

The typical dimensions of tailed phages range 30-160 nm for the capsid and 80-800 nm for tail length (Ackermann 2005). The Myoviridae phages studied in this project had an average range of 53-64 nm head size and 69-118 tail length, while only Siphovirus studied i.e. phage Φ 3 has 75 nm head and 156 nm tail length. Average dimensions of the phages studied are in agreement with the average dimensions reported earlier for Myoviridae phages infecting Vibrio spp. which have 43 to 107 nm heads and 85 to 221 nm tails (Sen and Ghosh 2005, Shivu, Rajeeva et al. 2007, Crothers-Stomps, Hoj et al. 2010, Surekhamol, Deepa et al. 2014). Similarly, the dimensions of Siphovirus studied falls under the average range of dimensions mentioned for Siphoviridae phages specific to Vibrio spp. which have shown their head sizes between 53 to 115 nm and tails lengths between 126 to 329 nm (Sen and Ghosh 2005, Shivu, Rajeeva et al. 2007, Crothers-Stomps, Hoj et al. 2010, Thiyagarajan, Chrisolite et al. 2011, Khemayan, Prachumwat et al. 2012, Yu, Gong et al. 2013, Surekhamol, Deepa et al. 2014). Apart from the phage particles other vesicle/membranous capsule like structures were visible in some of the electron

micrographs, likely to be bacterial outer membrane blebs after lysis of host cells (Young 1992).

The phages studied in this project were different from other Vibrio specific phages owing to their ability to infect non-O1 strains of V. cholerae. Chinese phages, apart from their infectivity to non-O1 V. cholerae strains were all classified as podoviruses with smaller heads compared to other Vibrio phages. Interestingly, amongst the podoviruses studied in this project, phage Φ 1 had smallest head size of 34 ± 0.18 nm.

Thus, overall this chapter discussed the isolation and biological as well as physical characterisation of phages. Seven phages were isolated in China, attempts to isolate in the UK environments were unsuccessful and five more phages were obtained from various sources (§ 2.2.2). In total twelve phages were characterised for the one step growth curves following their host strain growth curves, their lytic spectra and by electron microscopy. These characterisations could help in selecting the candidate bacteriophage for phage therapy trials as discussed in the further chapters in this dissertation. Based on the data produced in this chapter, the broader host range phages were Φ1, Φ3, ΦQH, ΦH3 and ΦJ3 which could lyse 67.0 %, 62.6 %, 84.6 %, 70.3 % and 76.9 % of host strains. The probable phage therapy candidate would be the one with broader host range with a priority for O1/O139 lytic one, owing to cholera being caused by these two serotypes. The phages X29, CJY and H3 had larger burst sizes of 664 ± 50, 182 ± 62 and 126 ± 18 PFU/cell, respectively and most of them had shorter latent period; thus the selection would be among these phages after further scrutiny of their genome characteristics which is discussed in the next chapter.

GENOMIC CHARACTERISATION OF PHAGES

4 GENOMIC CHARACTERISATION OF PHAGES

4.1 Introduction

When we consider phage for therapeutic purposes it is important to check for its genome characteristics as the phages to be used for phage therapy may possess harmful genes such as those associated with virulence, antimicrobial resistance and lysogeny (Barrow and Soothill 1997, Skurnik, Pajunen et al. 2007). The genome size and restriction profile helps for differentiating phages (Gill and Hyman 2010). The virulence of Shiga-Toxigenic E. coli (STEC) is encoded by Shiga toxin-encoding (Stx) phages (Herold, Karch et al. 2004, Allison 2007). Filamentous phages (CTXΦ) are responsible for genes encoding cholera toxin (Waldor and Mekalanos 1996, Wagner and Waldor 2002, Davis and Waldor 2003). Some phages are associated with transduction which can transfer antibiotic resistance between bacteria (Abedon, Kuhl et al. 2011). Such concerns regarding usage of phages with harmful genes mentioned above can be addressed by detailed genomic characterisation of the phages with genome sequencing, annotation of genome and determining the functions of genes. Having an idea about the gene functions helps us to decide whether the phage is suitable for therapy or not. Genome analysis for undesirable genes as per specifications of the US Food and Drug Administration (FDA) Code of Federal Regulations (CFR) in 40 CFR §725.421 (Abedon, Kuhl et al. 2011); listed in its section D (available at http://www.law.cornell.edu/cfr/text/40/725.421) is advisable. This USFDA CFR section specifically mentions that the genomes to be free from certain sequences which encode harmful toxins; for example in the context of this project the sequences for toxins affecting membrane function such as V. cholerae cholera toxin (choleragen) should not be present in the potential phage therapy candidate.

Few phage genomes have been sequenced to date (Ackermann and Kropinski 2007) even though the first sequenced genome was phage ΦX174 sequenced in 1976 (Air, Sanger et al. 1976, Sanger, Air et al. 1977, Sanger, Coulson et al. 1982). Very few references are available for the complete Vibrio phage genome sequences in the databases (Liu, Lin et al. 2014). In this chapter the characterisation of V. cholerae specific phages by PFGE, restriction profile and whole genome sequencing will be discussed in order to understand the phages in greater detail and determine the suitability of these phages for therapeutic application.

4.2 Results

4.2.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed to assess the DNA size of the phages studied. For PHE (Public Health England) phages the largest estimated genome size was for phage Φ3 i.e. 114 kb followed by phage Φ1 with 74 kb genome size and phage Φ2 which was less than 48.5 kb (Figure 20). The two Canadian phages as well as all the Chinese phages were below 48.5 kb (Figure 21-22).

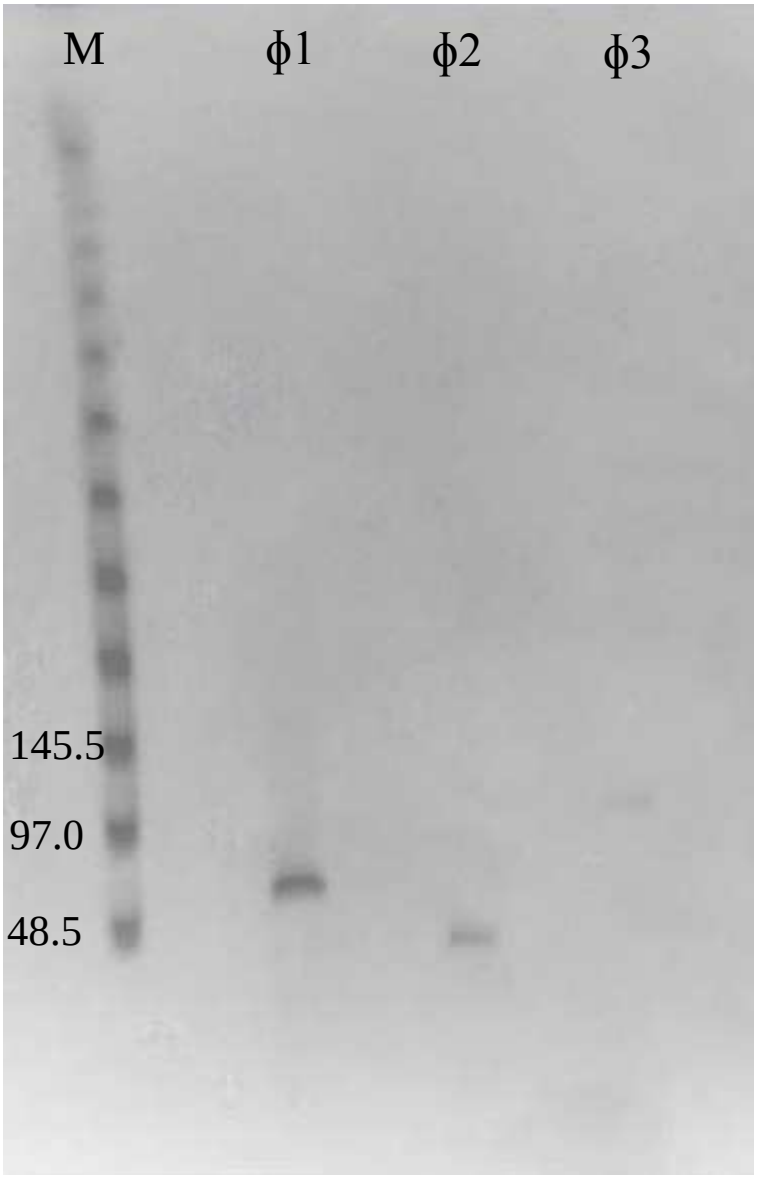

Figure 20: PFGE analysis of genomic DNA of PHE phages

Lane 1 marked M = 50-1000kb pulse marker (High marker λ concatemers of 48.5 kb increments)

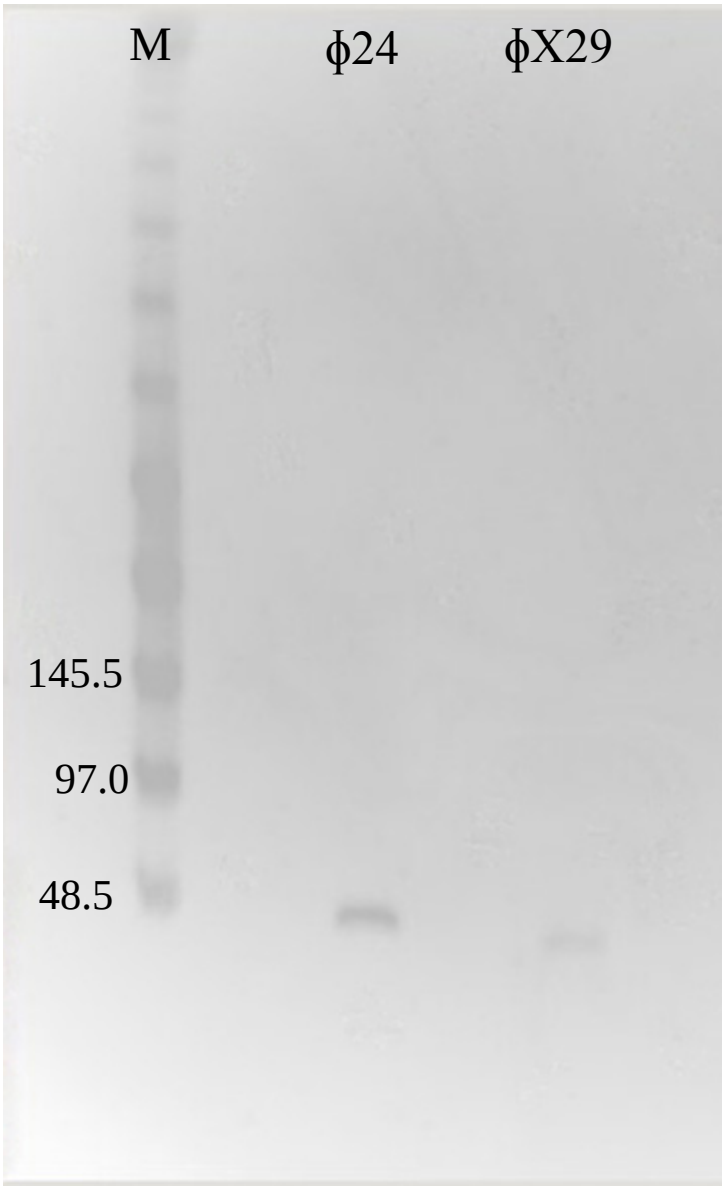

Figure 21: PFGE analysis of genomic DNA of Canadian phages

Lane 1 marked M = 50-1000kb pulse marker (High marker λ concatemers of 48.5 kb increments)

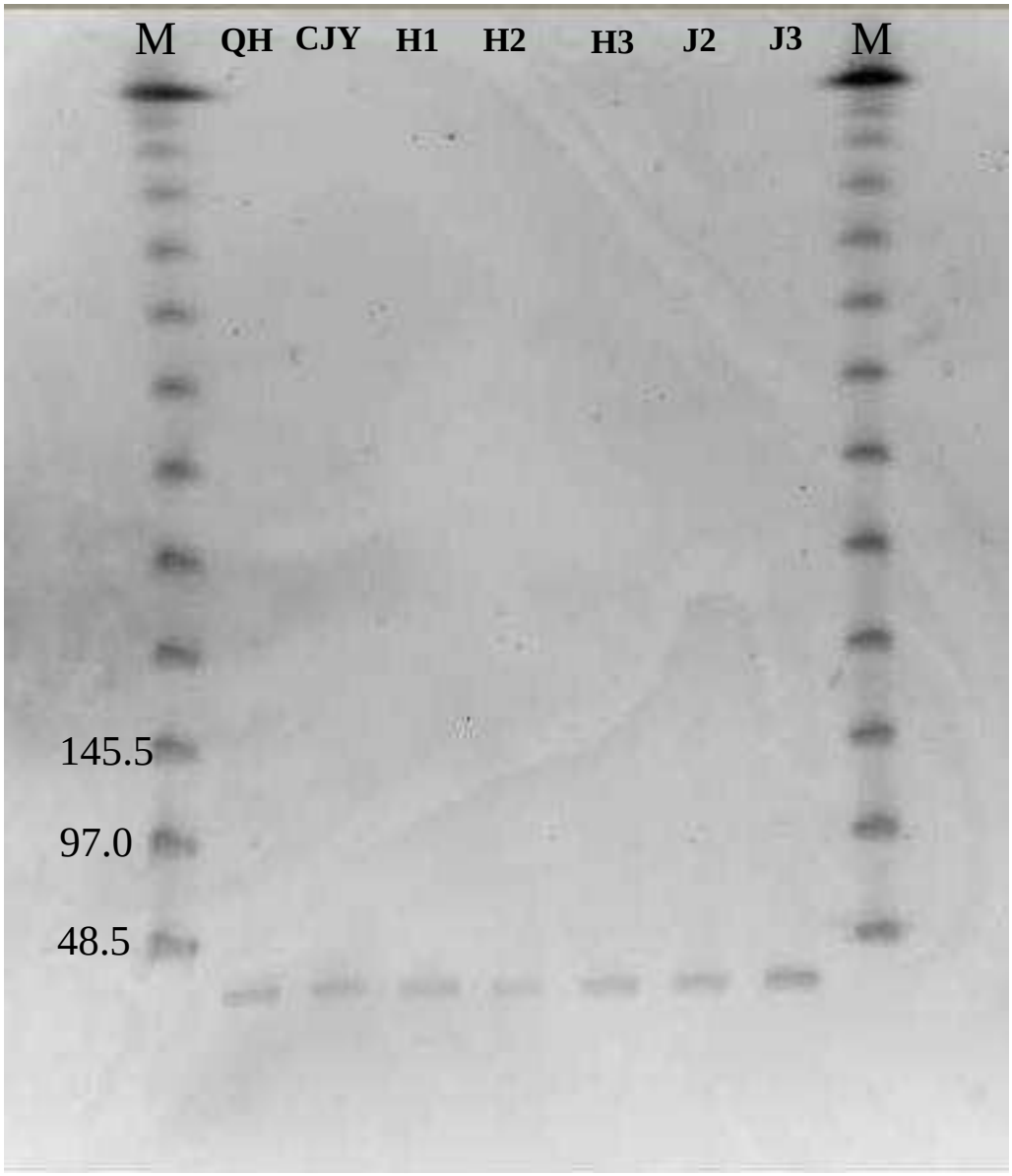

Figure 22: PFGE analysis of genomic DNA of Chinese phages

Lane 1 marked M = 50-1000kb pulse marker (High marker λ concatemers of 48.5 kb increments)

4.2.2 Phage DNA extraction

The amount of DNA extracted using Promega Wizard DNA extraction kit and further ethanol purification (§ 2.4.14) was as per the Table 11. The minimum DNA concentration required for sequencing purpose is 150 ng/µl and the extracted and

ethanol purified DNA yields for all the phages under study were beyond the minimum concentration required.

V. cholerae phage	Concentration of DNA extracted (ng/µl)
Φ1	453
Φ2	237
Φ3	521
24	345
X29	300
QH	215
CJY	287
H1	173
H2	305
H3	196
J2	250
J3	4043

Table 11: Concentration of the DNA extracted from V. cholerae phages

4.2.3 Restriction analysis of phage DNA

The macrorestriction profiles of the phages under study was performed (§ 2.4.15) by using the restriction endonuclease enzymes which cut the DNA at restriction site as per Table 12.

Endonuclease	Restriction site (5' to 3')
PstI	CTGCA/G
SacI	GAGCT/C
XbaI	T/CTAGA
EcoRI	G/AATTC
HpaII	C/CGG

Table 12: Endonucleases used for the restriction analysis of phage DNA

The DNA digest patterns for the phages under study are shown in the Figures 23 to 27. XbaI cut the DNA for phage Φ1 and gave distinct bands but the Φ1 DNA was resistant to digestion by PstI and SacI. Phage 24 was cut by the restriction enzyme PstI but was resistant to digestion by SacI and XbaI. Phages Φ2, Φ3 and X29 were cut by all three restriction enzymes PstI, SacI and XbaI. The seven Chinese phages produced a smeared pattern for PstI, SacI and XbaI. They were digested using EcoRI and HpaII. For EcoRI they again showed the smearing of DNA on the gel but all phages were distinctly cut by HpaII with the exception of phage QH which remained uncut without smearing (Figures 26-27).

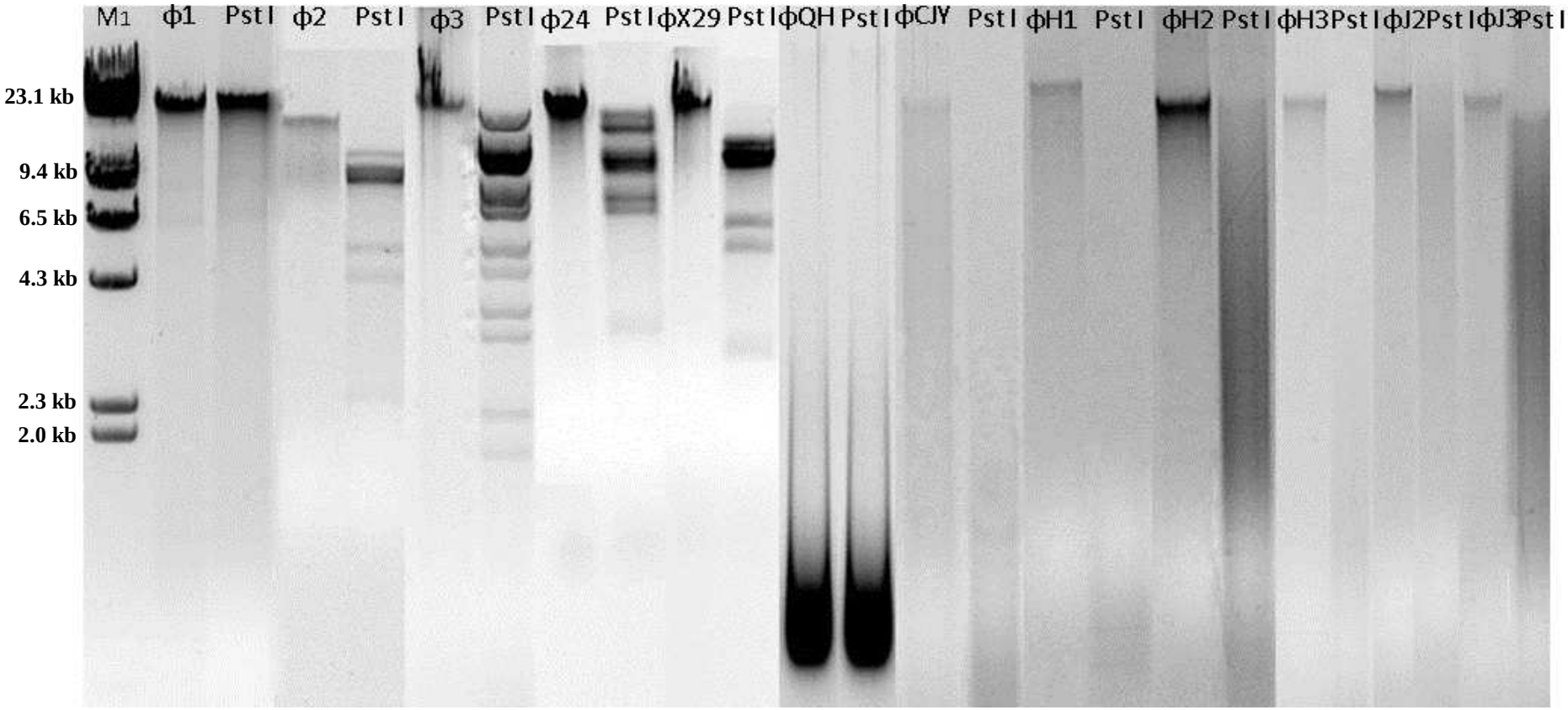

Figure 23: Restriction digests of phage genomes under study by using restriction enzyme PstI.

M1: lambda DNA ladder.

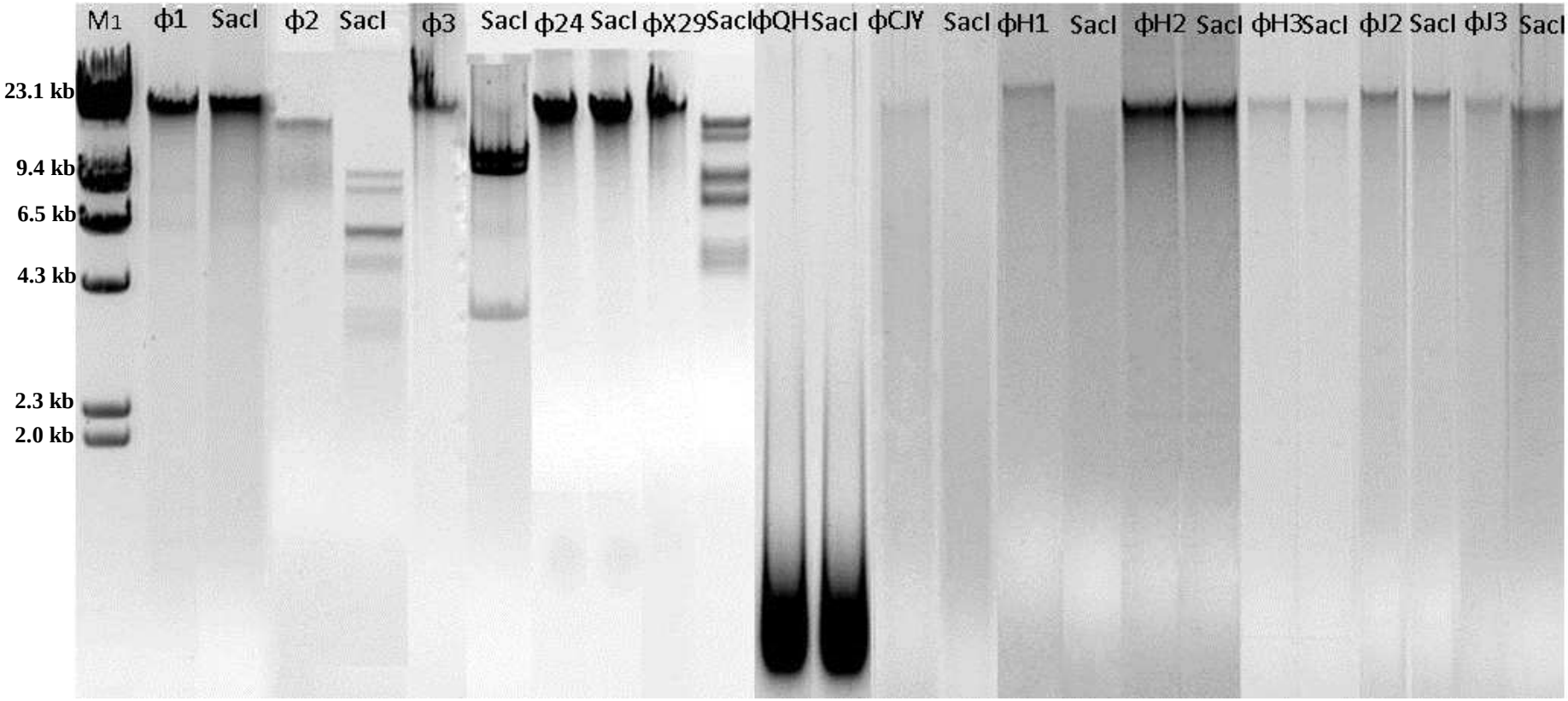

Figure 24: Restriction digests of phage genomes under study by using restriction enzyme SacI.

M1: lambda DNA ladder.

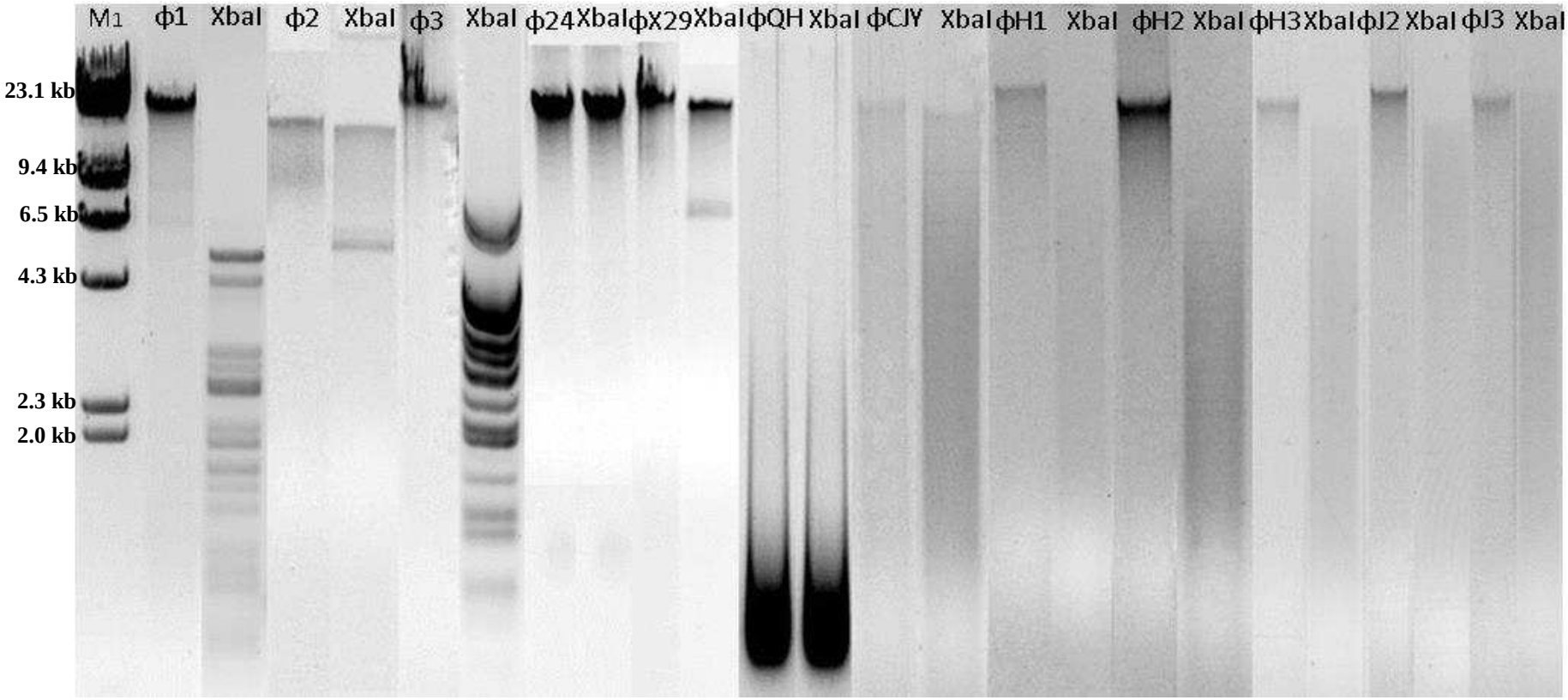

Figure 25: Restriction digests of phage genomes under study by using restriction enzyme XbaI.

M1: lambda DNA ladder.

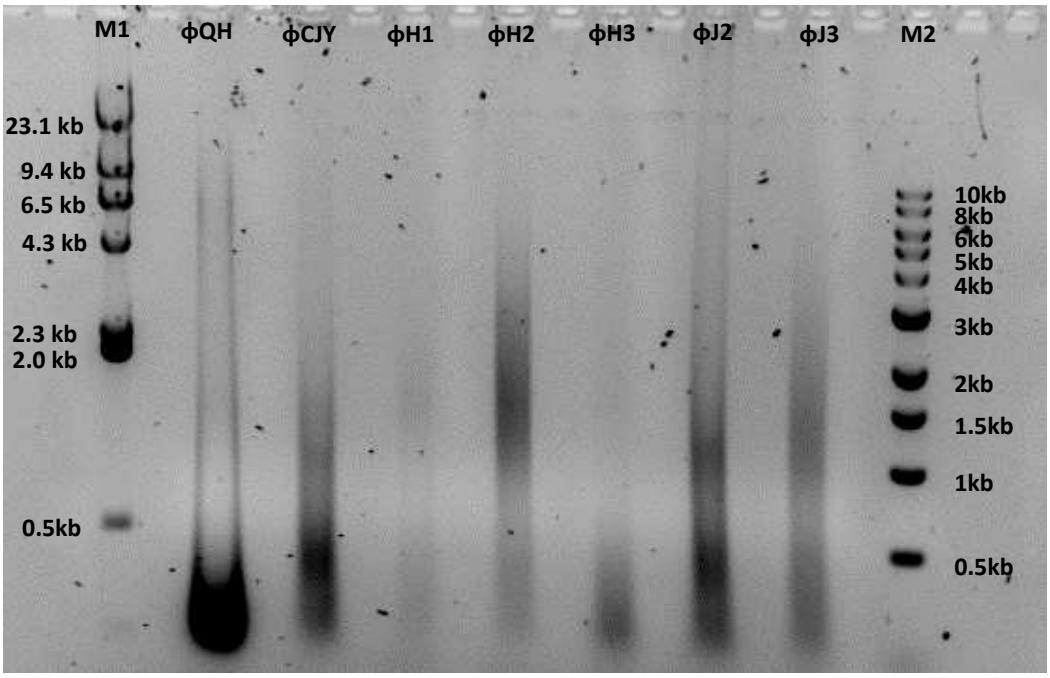

Figure 26: Restriction digests of Chinese phages by using restriction enzyme EcoRI. M1: lambda DNA ladder, M2: 1 kb DNA ladder

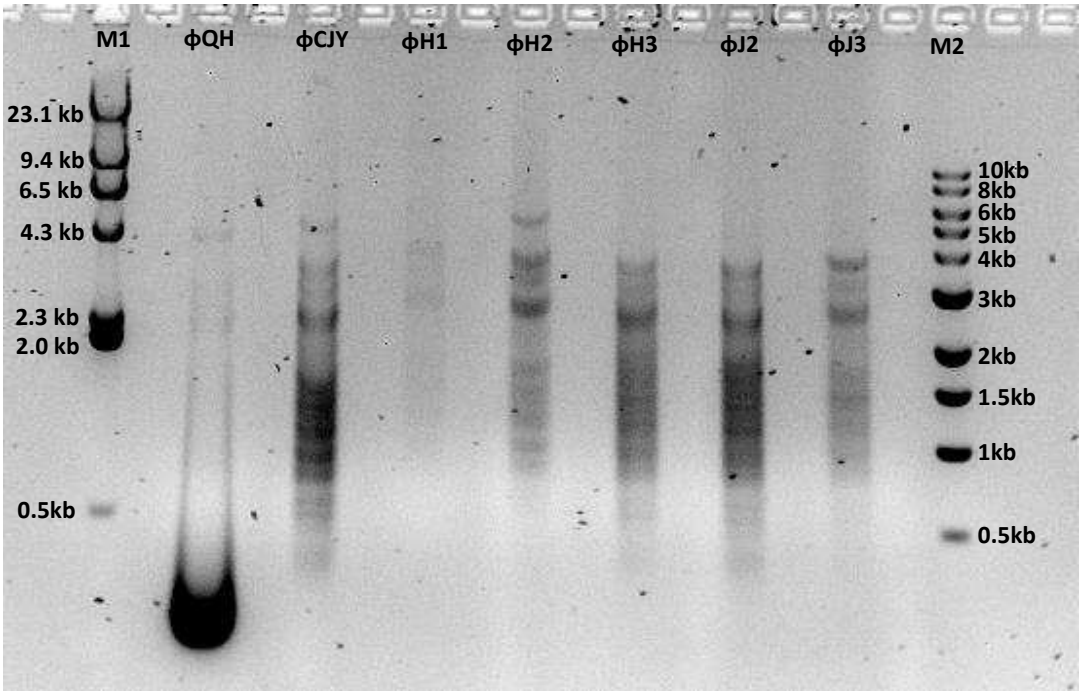

Figure 27: Restriction digests of Chinese phages by using restriction enzyme HpaII. M1: lambda DNA ladder, M2: 1 kb DNA ladder

4.2.4 Sequencing and annotation of phage genomes

Further sequencing of the genomic DNA and genome analysis helps us to know whether the candidate to be used for phage therapy harbours any genes that encode for antimicrobial resistance and or toxin/virulence genes. For this purpose the phage DNA were extracted using the Promega DNA extraction kit (§ 2.4.13) and further purified by Ethanol precipitation (§ 2.4.14). Next generation sequencing was performed on the Illumina Miseq platform (§ 2.4.17). The annotation of genome sequences revealed the precise genome size for the sequenced phages, no. of genes, coding sequences and percent G+C content (Table 13).

The details of the functional genes are highlighted in the Tables 14-19. In these tables the functional genes were grouped in to the early to mid genes and late genes as well as other groups depending on the functions of proteins encoded by them. The groups were as below:

1. Lysogeny related (Early to mid genes)
2. DNA metabolism and replication (Early to mid genes)
3. Head morphogenesis (Late genes)
4. Tail morphogenesis (Late genes)
5. Host cell lysis (Late genes)
6. Other conserved protein domains
7. Unknown function
8. Regulatory proteins

The numbers corresponding to these groupings were mentioned in the tables in parenthesis after the name of the protein encoded by these genes.

V. cholerae Phage	GenBank accession no.	Genome Length (bp)	No. of genes*	G+C (%)
Φ1	KP280062	66708	110 (20)	34.50
Φ2	KJ545483.2	41476	67 (27)	46.10
Φ3	KP280063	116138	156 (53)	42.83
Φ24	KJ572844.2	44395	71 (19)	45.40
ΦX29	KJ572845.2	41569	67 (34)	46.00
ΦQH	KM612259	39725	48 (9)	50.50
ΦCJY	KM612260	39542	48 (8)	50.56
ΦH1	KM612261	39530	48 (8)	50.54
ΦH2	KM612262	39530	48 (8)	50.55
ΦH3	KM612263	39530	48 (8)	50.54
ΦJ2	KM612264	39530	48 (7)	50.58
ΦJ3	KM612265	39782	49 (8)	50.55

Table 13: Details of the phage genomes under study post sequencing and genome analysis.
* Figures in parenthesis indicate no. of genes to which functions were assigned

The genome size of phage Φ1 was 66.7 kb and the numbers of genes identified were 110 but of which only 20 genes could be assigned functions. Most of the functional genes i.e. 12 out of 20 were early to middle genes associated with the DNA metabolism and replication. Six genes could be grouped in to the late genes associated with head morphogenesis (three genes) and host cell lysis (three genes). Two more genes encoded conserved bacteriophage proteins of unknown function (Table 14 and Figure 28). BlastN analysis of the Φ1 revealed that it is genetically similar to Vibrio

phages JA1 (GenBank: NC_021540.1) and VCO139 (GenBank: KC438283.1) with 97% pairwise identity. Comparison of the genome homology between the Φ1, JA1 and VCO139 is shown in Figure 29 and it depicts the zones of similarty as indicated by the red regions. The largest protein 'Viron-encapsulated RNA polymerase' is encoded by a gene SBVP1_0074 which was 9723 bp in length and was found to be common in all three genomes. The mean G+C content of Φ1 was 34.5% which is similar to JA1 and VCO139 as both of them have 34.6% G+C content. No tRNA was detected in the Φ1 genome in contrast to single tRNA each in JA1 and VCO139. No lysogeny related genes were detected for Φ1.

	Name of the protein encoded by gene	Start	End	Length	Direction
1	DNA-directed RNA polymerase RNAP1 (2)	4,427	5,368	942	forward
2	DNA-directed RNA polymerase RNAP2 (2)	6,918	7,703	786	forward
3	N-acetylmuramoyl-L-alanine amidase (5)	9,203	9,727	525	reverse
4	PF10947 family protein (7)	10,735	11,154	420	forward
5	Putative lipoprotein (5)	11,151	11,453	303	forward
6	Thymidylate synthase (2)	11,641	12,492	852	forward
7	PF11753 family protein (7)	16,553	16,846	294	forward
8	Metallopeptidase domain protein (5)	20,900	22,126	1,227	forward
9	DNA helicase (2)	22,698	23,858	1,161	forward
10	DNA polymerase (2)	24,424	25,992	1,569	forward
11	Putative HNH homing endonuclease (2)	27,085	27,618	534	forward
12	DNA polymerase (2)	27,878	28,735	858	forward
13	Phosphoribosyl-ATP diphosphatase (2)	29,469	29,918	450	forward
14	AAA domain protein (2)	33,399	34,136	738	forward
15	ssDNA binding protein (2)	34,198	34,920	723	forward
16	Crossover junction endodeoxyribonuclease RusA (2)	34,920	35,717	798	forward
17	Viron-encapsulated RNA polymerase (2)	36,190	45,912	9,723	reverse
18	N4-gp56 family major capsid protein (3)	50,947	52,260	1,314	reverse
19	Portal protein (3)	53,682	55,799	2,118	reverse
20	Terminase large subunit (3)	59,671	61,269	1,599	reverse

Table 14: Functional genes identified for Φ1

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

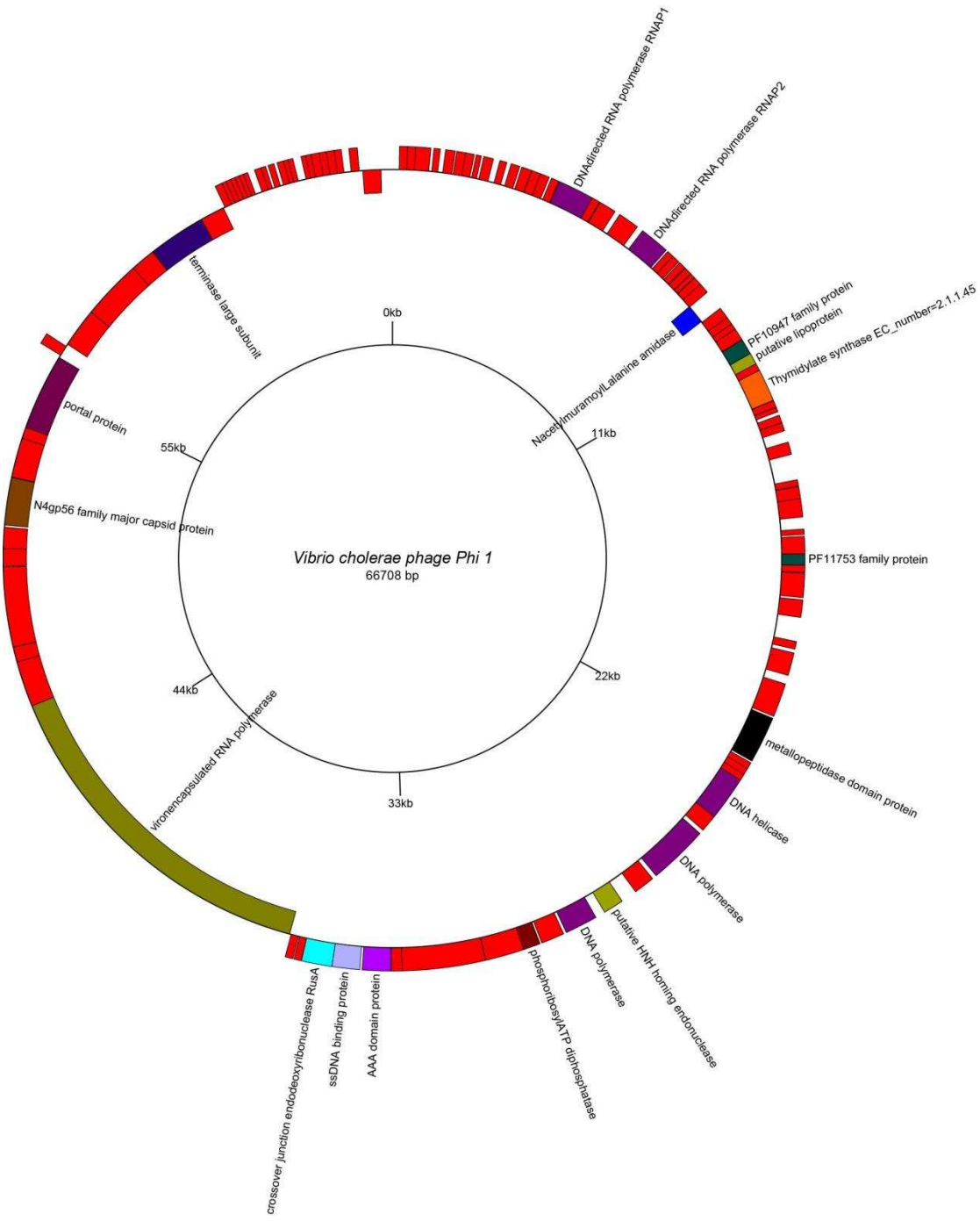

Figure 28: Genome map of Φ1 generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

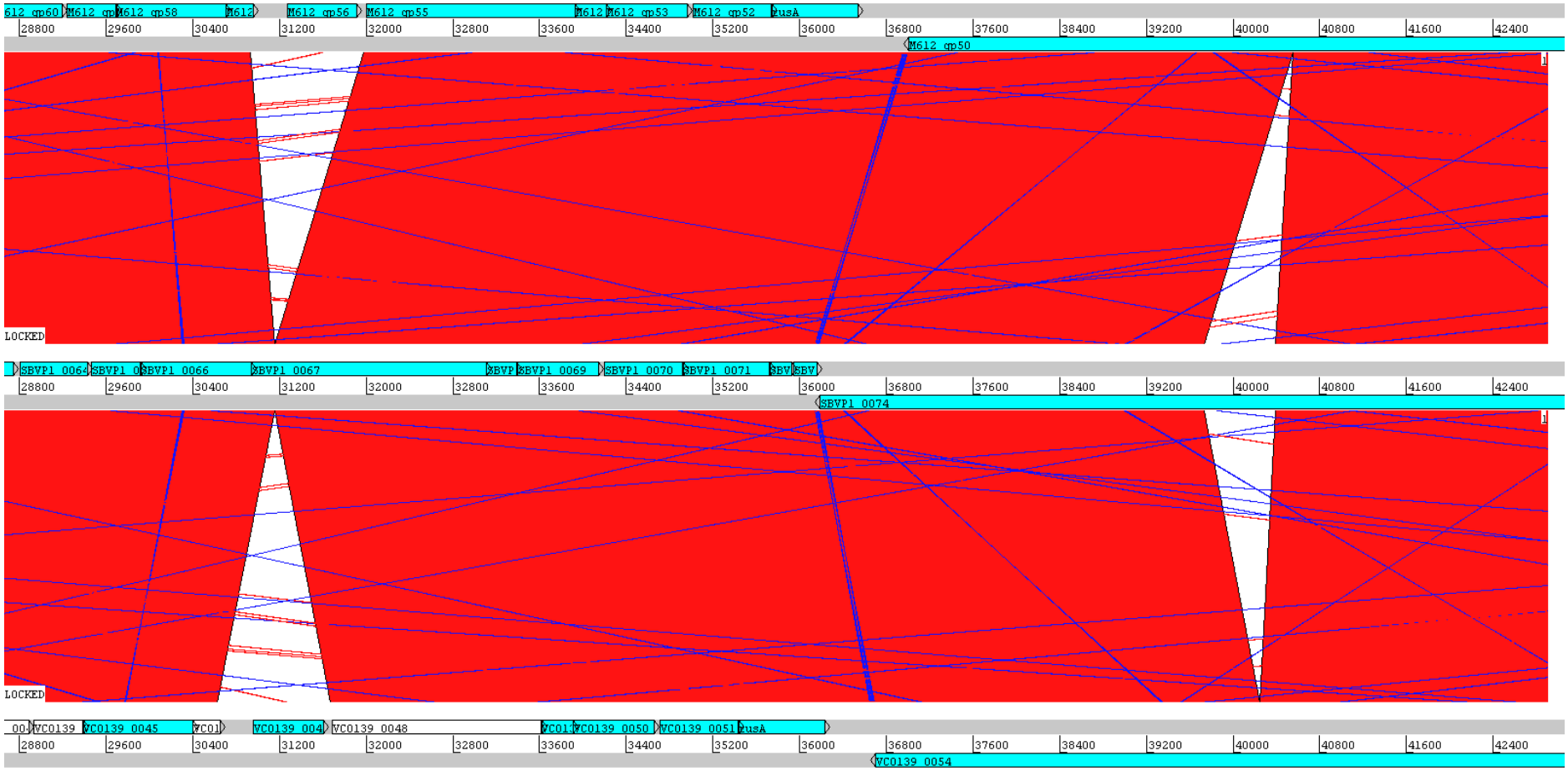

Figure 29: Artemis Comparison Tool (ACT) display of the genomes of JA1 (top), Φ1 (middle) and VCO139 (bottom). Zones of similarty are indicated by intensity of red colour.

Phage Φ2 had a genome size of 41.4 kb and 67 coding sequences were found; out of which functions could be assigned to 27 genes (Table 15 and Figure 30). The functional genes identified could be grouped in to early to mid and late genes. Eleven genes were classified as early to mid genes, of which 5 encoded lyogeny related proteins viz. Integrase family site specific recombinase, Putative excisionase family protein, Cro-like protein, Antirepressor protein and Intergrase; while 6 were related to DNA metabolism and replication. The late genes were associated with head morphogenesis (4), tail morphogenesis (9) and host cell lysis (1). One gene encoding VHS1122-like protein was of unknown function; while one more gene which encodes ATP-binding sugar transporter family protein was grouped in to the other conserved protein domain. Upon BlastN search Φ2 was found to be 99 % genetically identical to another Vibrio phage, ΦX29, which is being studied in this project. Homology of the sequences between Φ2 and ΦX29 were compared by ACT comparison tool as shown in Figure 31. Phage Φ2 revealed a ribosomal slippage (an error during translation due to addition or deletion of bases on mRNA causing shift i.e. slippage in the codon reading frame) at the coordinates 12760..13202 in the coding sequences upstream of the tape measure protein which was conserved in ΦX29 as well. The mean G+C content of Φ2 was 46.1% which was similar to that of phage X29 which contained 46 % of G+C regions.

	Name of the protein encoded by gene	Start	End	Length	Direction
1	DNA modification methylase (2)	140	745	606	Forward
2	Terminase large subunit (3)	1,375	3,225	1,851	Forward
3	VHS1122-like protein (7)	3,369	3,695	327	Reverse
4	Portal protein (3)	4,393	5,976	1,584	Forward
5	Clp protease-like protein (3)	5,963	7,066	1,104	Forward
6	Major capsid E family protein (3)	7,473	8,513	1,041	Forward
7	ATP-binding sugar transporter family protein (6)	8,824	9,153	330	Forward
8	Minor tail protein Z (4)	9,168	9,734	567	Forward
9	Tail protein (4)	10,502	11,971	1,470	Forward
10	Major tail tube protein (4)	11,986	12,507	522	Forward
11	Tail length tape-measure protein (4)	13,217	15,853	2,637	Forward
12	Late control D family protein (4)	16,056	17,084	1,029	Forward
13	Putative baseplate protein (4)	17,087	17,818	732	Forward
14	GpU-like protein (4)	17,827	18,213	387	Forward
15	Baseplate (4)	18,517	19,641	1,125	Forward
16	Tail protein I (4)	19,634	20,212	579	Forward
17	N-acetylmuramoyl-L-alanine amidase (5)	22,878	23,573	696	Forward
18	Integrase family site specific recombinase (1)	25,103	26,167	1,065	Forward
19	Methyltransferase (2)	26,239	26,742	504	Forward
20	Putative excisionase family protein (1)	27,786	27,989	204	Forward
21	Cro-like protein (1)	28,005	28,217	213	Forward
22	Antirepressor protein (1)	28,391	29,191	801	forward
23	Replication protein (2)	29,205	30,002	798	forward
24	DNA replication protein DnaC (2)	29,989	30,768	780	forward

25	Integrase (1)	30,768	31,967	1,200	forward
26	DNA recombination-dependent growth factor C (2)	32,053	32,964	912	forward
27	Single-stranded DNA-binding protein (2)	38,194	38,601	408	forward

Table 15: Functional genes identified for Φ2

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

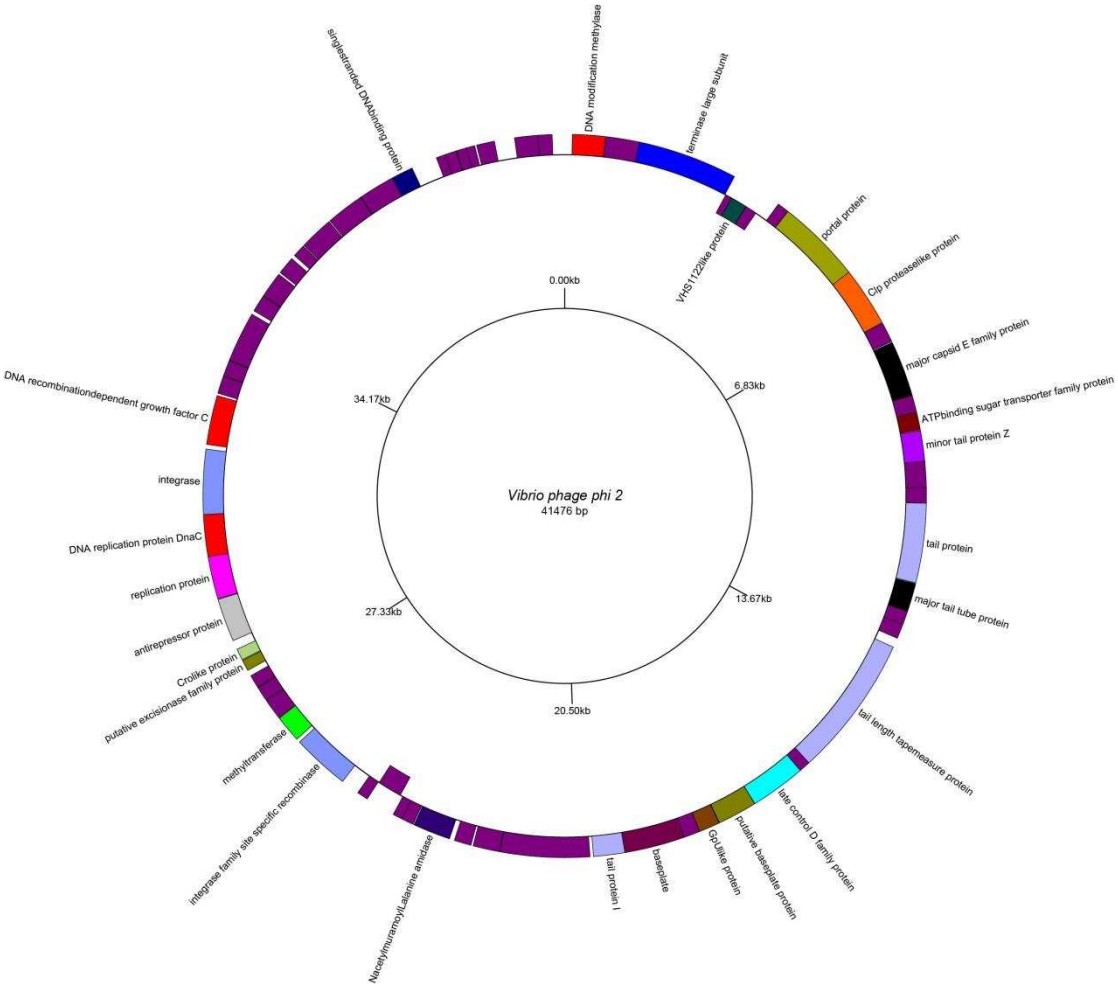

Figure 30: Genome map of Φ2 generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

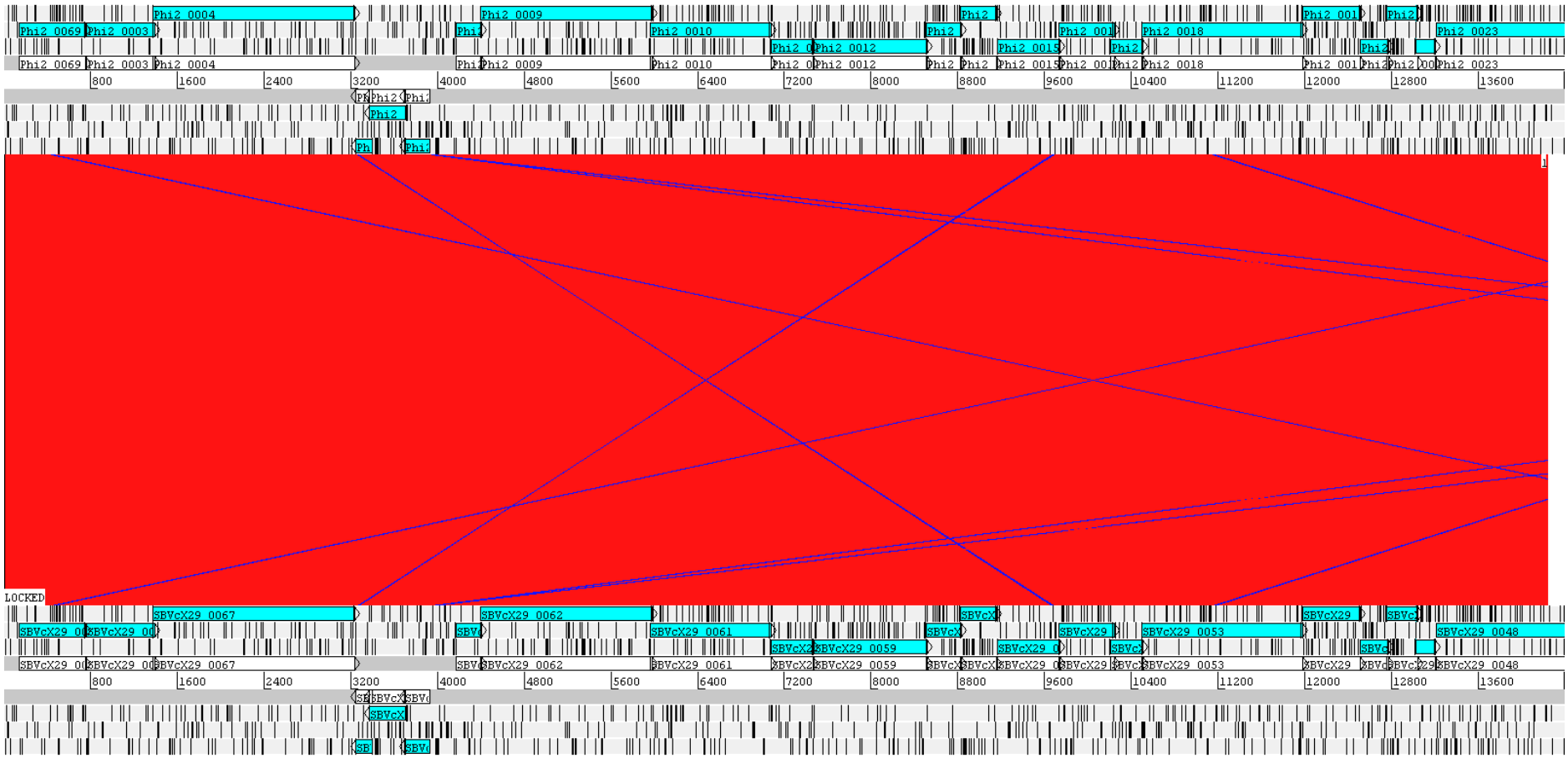

Figure 31: Artemis Comparison Tool (ACT) display of the genomes of Φ2 (top) and ΦX29 (bottom). Zones of similarty are indicated by intensity of red colour.

Phage Φ3 had the largest genome size of 116.1 kb among the phages under this study. The number of genes identified were also large i.e. 156 and out of which 53 genes could be assigned with functions (Table 16 and Figure 32). The early to mid genes for DNA metabolism and replication were 22 in number, while late genes involved in head morphogenesis, tail morphogenesis and host cell lysis were 6, 10 and 1 gene, respectively. There was one gene encoding protein of unknown function and five coded for other conserved protein domains. Phage Φ3 had two terminal repeats with left repeat spanning between 1 to 7769 and right terminal repeat between the cooridinates 108,370 and 116,138. Amongst all the phages under study only phage Φ3 had eight tRNAs viz. tRNA-Arg(tct), tRNA-His(gtg), tRNA-Arg(acg), tRNA-Phe(gaa), tRNA-Met(cat), tRNA-Ile(gat), tRNA-Val(tac) and tRNA-Arg(cct). The G+C content of Φ3 was 42.83 % and upon the BlastN search it could show similarity to Vibrio phage pVp-1 with 74 % pairwise identity. Both Φ3 and pVp-1 being T5 like phages, they were compared with T5 using the ACT comparison tool and light red colour bands show zones of similarity (Figure 33). The figure also shows that there is no left terminal repeat in pVp1, while Φ3 shows identical terminal repeats both on left as well as right sides. Phage Φ3 did not posses any lsogeny related genes.

	Name of the protein encoded by gene	Start	End	Length	Direction
1	Deoxynucleoside-5'-monophosphatase (2)	133	762	630	reverse
2	A1 protein (3)	1,184	2,896	1,713	reverse
3	A2 protein (2)	3,033	3,524	492	reverse
4	Nicotinate phosphoribosyltransferase (2)	16,112	17,725	1,614	reverse
5	Ribose-phosphate pyrophosphokinase (2)	18,265	19,170	906	reverse
6	Putative RNA ligase/tail attachment protein (4)	21,016	21,513	498	reverse
7	Calcineurin-like phosphoesterase family protein (2)	21,588	22,331	744	reverse
8	Peptidase M15 family protein (2)	26,904	27,287	384	reverse
9	Putative holin (5)	27,287	27,955	669	reverse
10	Tail protein (4)	29,866	30,195	330	reverse
11	Putative hydrolase (6)	30,316	30,969	654	reverse
12	tRNA-Arg(tct)	32,533	32,608	76	reverse
13	tRNA-His(gtg)	33,160	33,236	77	reverse
14	Dihydrofolate reductase (4)	36,190	36,645	456	reverse
15	Thymidylate synthase (4)	36,655	37,518	864	reverse

16	tRNA-Arg(acg)	38,491	38,566	76	reverse
17	tRNA-Phe(gaa)	38,573	38,648	76	reverse
18	tRNA-Met(cat)	38,949	39,025	77	reverse
19	tRNA-Ile(gat)	39,032	39,107	76	reverse
20	tRNA-Val(tac)	39,903	39,980	78	reverse
21	tRNA-Arg(cct)	40,663	40,739	77	reverse
22	Putative exodeoxyribonuclease (2)	43,036	43,728	693	reverse
23	Ribonuclease H (2)	45,334	45,810	477	reverse
24	PhoH-like protein (2)	46,523	47,176	654	reverse
25	Putative replication origin binding protein (2)	55,958	58,750	2,793	forward
26	D2 protein (6)	59,570	60,412	843	forward
27	D3 protein (7)	60,426	60,848	423	forward
28	NAD-dependent DNA ligase subunit A (2)	63,087	63,308	222	forward
29	NAD-dependent DNA ligase subunit B (2)	64,008	64,799	792	forward
30	D5 protein (6)	64,792	65,559	768	forward
31	Putative replicative DNA helicase (2)	65,615	67,039	1,425	forward
32	DNA primase (2)	67,026	67,916	891	forward
33	DNA polymerase (2)	68,005	70,587	2,583	forward
34	Putative ATP-dependent helicase (2)	71,173	72,531	1,359	forward

35	D11 protein (6)	73,017	73,820	804	forward
36	Putative recombination endonuclease subunit D12 (2)	74,237	75,220	984	forward
37	Putative exonuclease subunit (2)	75,210	77,081	1,872	forward
38	D14 protein (6)	77,074	77,553	480	Forward
39	Flap endonuclease (2)	77,550	78,446	897	Forward
40	Putative deoxyUTP pyrophosphatase (2)	78,433	78,885	453	Forward
41	Tail protein (4)	85,239	87,467	2,229	Reverse
43	Phage tail-3 (4)	87,464	90,319	2,856	Reverse
44	Pore-forming tail tip protein (4)	91,100	94,174	3,075	Reverse
45	Minor tail protein (4)	95,818	96,702	885	Reverse
46	Major tail protein (4)	96,709	98,172	1,464	Reverse
47	Major head protein precursor (3)	100,040	101,449	1,410	Reverse
48	Putative prohead protease (3)	101,453	102,064	612	Reverse
49	Portal protein (3)	102,296	103,516	1,221	Reverse
50	Terminase large subunit (3)	104,084	105,406	1,323	Reverse
51	Deoxynucleoside-5'-monophosphatase (2)	108,502	109,131	630	Reverse

52	A1 protein (3)	109,553	111,265	1,713	Reverse
53	A2 protein (2)	111,402	111,893	492	Reverse

Table 16: Functional genes identified for Φ3

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

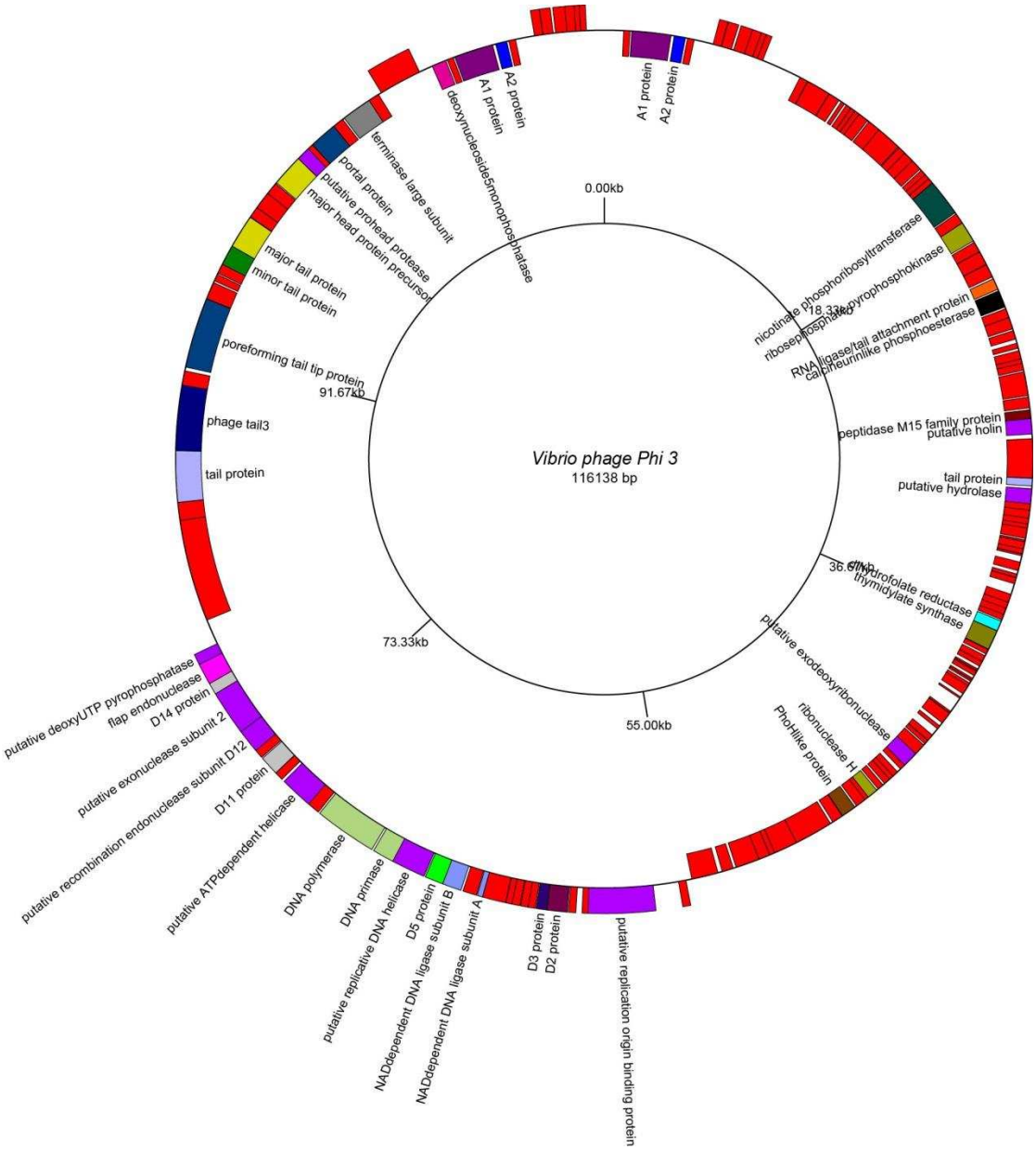

Figure 32: Genome map of Φ3 generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

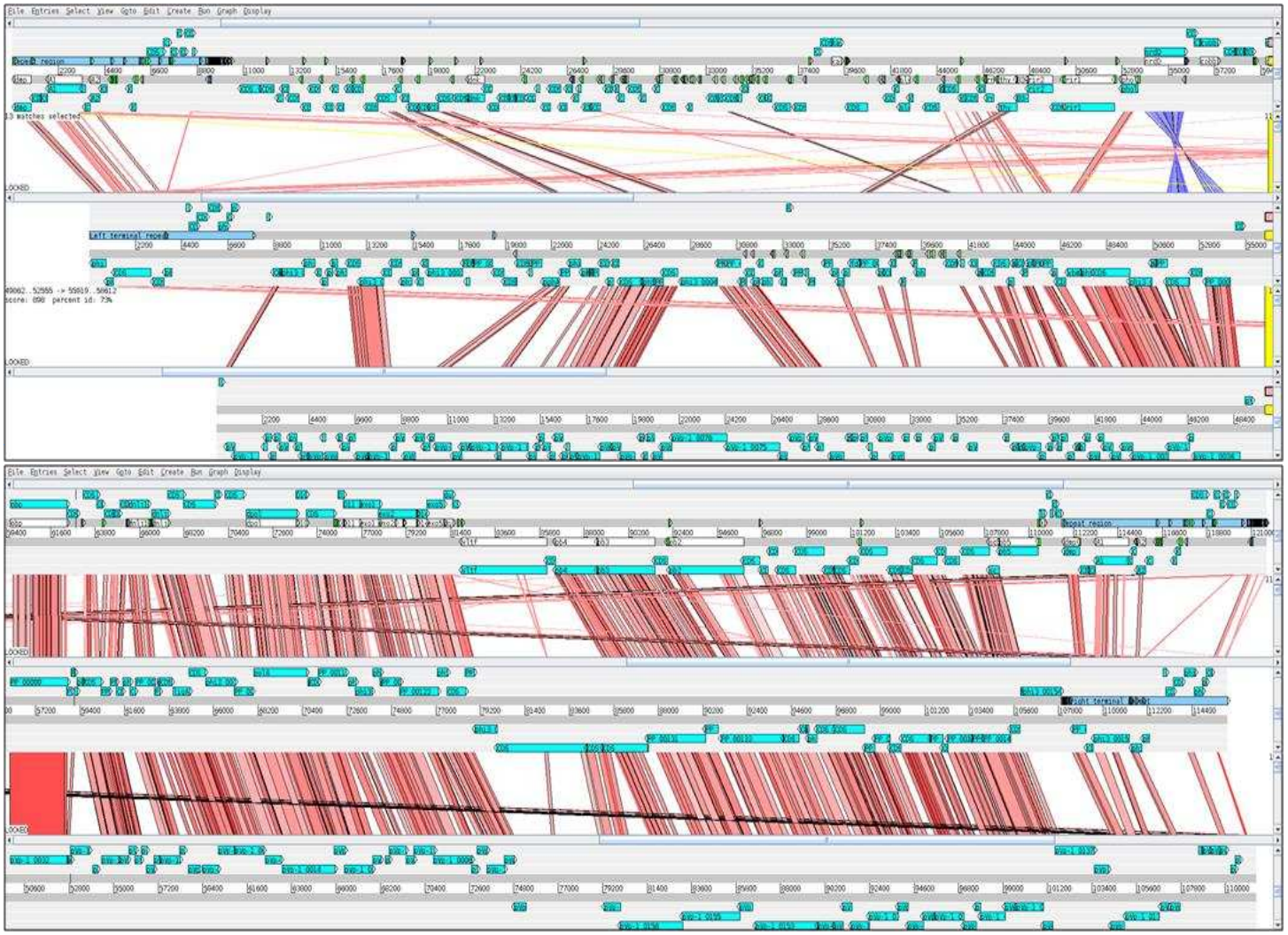

Figure 33: Artemis Comparison Tool (ACT) display of the genomes of phage T5 (top), Φ3 (middle) and pVp-1 (bottom). Zones of similarty are indicated by intensity of red colour. Blue band represents similarity on the opposite strand, while yellow represents highlighted region.

The genome size of phage 24 was 44.3 kb with mean G+C content as 45.4 %. Annotation of genome sequence revealed 71 genes/coding sequences of which 19 could be assigned with functions. Seven genes were related to early genes for DNA metabolism and replication, followed by late genes which were comprised of six head morphogenesis and four tail morhogenesis ones. Two more genes were grouped as encoding for other conserved protein domains (Table 17 and Figure 34). The phage 24 has 99 % identity with Vibrio phage CP-T1 (NC_019457.1) but has 75 bp in-frame deletion in the pentapeptide repeat-containing protein. The Figure 35 depicts the comparison of phage 24 and CP-T1 by ACT comparison with intense red colour indicating similar zones on their genomes with a strip of ClustalO (Sievers, Wilm et al. 2011) output at the bottom of the figure deciphering the in-frame deletion in the pentapeptide repeat-containing protein of phage 24. It was noticed that any lysogeny related genes were absent in phage 24.

	Name of the protein encoded by gene	Start	End	Length	Direction
1	Putative exonuclease (2)	189	722	534	Reverse
2	Putative DNA polymerase (2)	727	3,063	2,337	Reverse
3	Putative helicase (2)	6,842	9,823	2,982	Forward
4	DNA-cytosine methyltransferase (2)	10,320	11,621	1,302	Forward
5	Putative methylase (2)	11,618	12,319	702	Forward
6	Putative transcriptional regulator (6)	12,359	12,673	315	Forward
7	Pentapeptide repeat-containing protein (6)	12,900	13,322	423	Forward
8	Putative primase (2)	14,378	16,858	2,481	Forward
9	Putative small-subunit terminase (3)	17,762	18,394	633	Forward
10	Terminase large subunit (3)	18,384	19,844	1,461	Forward
11	Putative portal protein (3)	20,016	21,485	1,470	Forward
12	Putative head protein (3)	23,282	24,103	822	Forward
13	Putative helicase (2)	24,392	24,895	504	Reverse
14	Putative head protein	25,770	26,897	1,128	Forward

	(3)				
15	Putative major capsid protein (3)	27,409	28,422	1,014	Forward
16	Putative tail-fiber/lysozyme protein (4)	34,611	35,975	1,365	Forward
17	Putative baseplate protein (4)	38,110	38,808	699	Forward
18	Putative baseplate protein (4)	39,148	40,371	1,224	Forward
19	Putative tail-fiber protein (4)	41,013	42,044	1,032	Forward

Table 17: Functional genes identified for Φ24

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

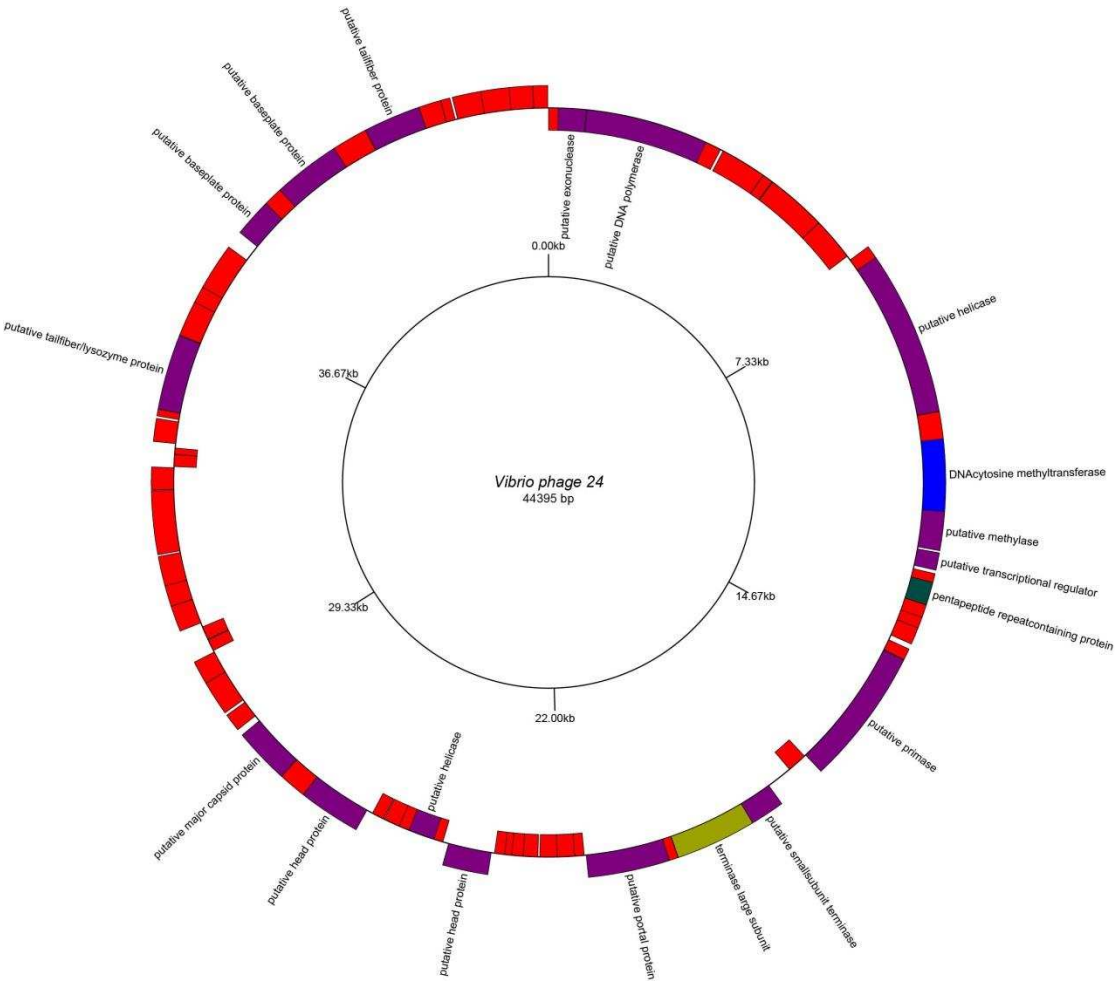

Figure 34: Genome map of phage 24 generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

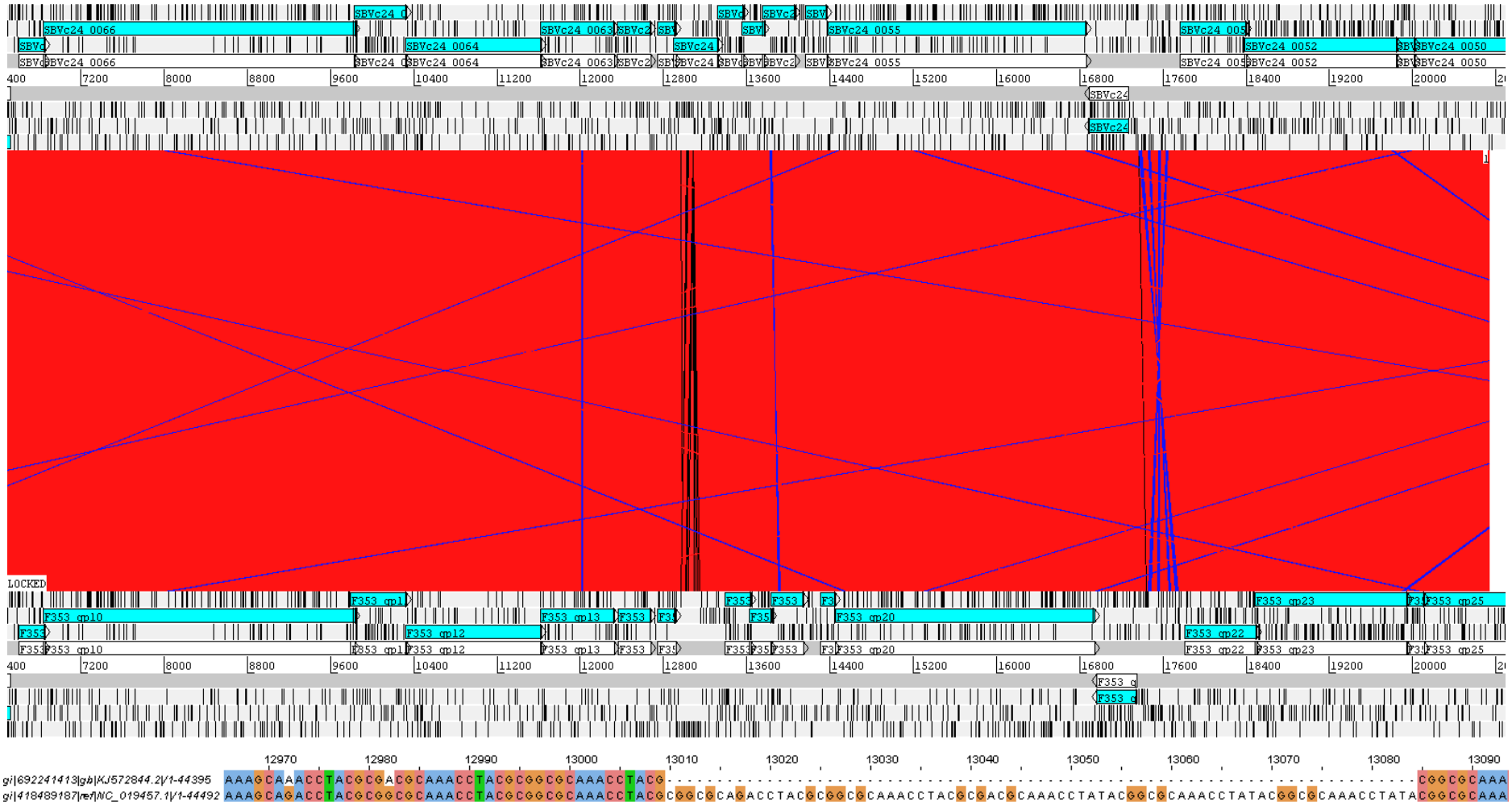

Figure 35: Artemis Comparison Tool (ACT) display of the genomes of phage 24 (top) and CP-T1 (bottom). Zones of similarty are indicated by intensity of red colour. ClustalO output at the bottom shows the in-frame deletion.

The Canadian phage X29 had a genome length of 41.5 kb and 67 numbers of genes/coding sequences could be identified. Out of 67 genes 34 could be assigned with functions which more compared to the 27 number of genes which were identified with functions for similar phage Φ2 under this study (Tables 18 and 15; Figure 36). Eleven of these genes could be grouped in to early to mid genes with 2 genes for lysogeny which encoded the proteins integrase family site specific recombinase and an integrase; while 9 genes for DNA metabolism and replication. Out of remaining genes, 15 were belonging to late genes which had 4 genes for head morphogenesis, 9 for tail morphogenesis and 2 for host cell lysis. Interestingly phage X29 has shown 6 regulatory proteins of Helix-turn-helix domain and one protein each with unknown function and other conserved protein domain. As discussed earlier, the phage X29 has 99 % similarity with Φ2 and the ACT comparison is depicted in the Figure 30. The mean G+C content of phage X29 was 46.0 % which was slightly lower than Φ2 which contains 46.1 % G+C content.

	Name of the protein encoded by gene	Start	End	Length	Direction
1	DNA modification methylase (2)	140	745	606	forward
2	Terminase large subunit (3)	1,375	3,225	1,851	forward
3	Portal protein (3)	4,393	5,976	1,584	forward
4	Protease-like protein (3)	5,963	7,066	1,104	forward
5	Major capsid E family protein (3)	7,473	8,513	1,041	forward
6	Tail sheath protein (4)	10,502	11,971	1,470	forward
7	Tail tube FII family protein (4)	11,986	12,507	522	forward
8	Tail length tape-measure protein (4)	13,217	15,853	2,637	forward
9	Late control D family protein (4)	16,056	17,084	1,029	forward
10	Putative baseplate protein (4)	17,087	17,818	732	forward
11	Lysozyme (5)	18,213	18,524	312	forward
12	Baseplate J-like protein (4)	18,517	19,641	1,125	forward
13	Tail protein I (4)	19,634	20,212	579	forward
14	Tail fiber protein (4)	20,275	21,927	1,653	forward
15	Tail fiber assembly-like	21,938	22,429	492	forward

	protein (4)				
16	N-acetylmuramoyl-L-alanine amidase (5)	22,878	23,573	696	forward
17	Prokaryotic membrane lipoprotein lipid attachment site protein (6)	24,551	24,757	207	forward
18	Integrase family site specific recombinase (1)	25,103	26,167	1,065	forward
19	Methyltransferase (2)	26,239	26,742	504	forward
20	Helix-turn-helix domain protein (8)	27,149	27,421	273	forward
21	Helix-turn-helix domain protein (8)	27,786	27,989	204	forward
22	Helix-turn-helix domain protein (8)	28,005	28,217	213	forward
23	Antirepressor protein KilAC domain (2)	28,391	29,191	801	forward
24	Helix-turn-helix domain protein (8)	29,205	30,002	798	forward
25	IstB-like ATP binding protein (7)	29,989	30,768	780	forward
26	Integrase (1)	30,768	31,967	1,200	forward
27	DNA recombination-	32,053	32,964	912	forward

	dependent growth factor C (2)				
28	Exonuclease:ribonuclease T (2)	33,650	34,582	933	forward
29	Cro/C1-type HTH DNA-binding domain protein (8)	35,908	36,144	237	forward
30	DNA clamp protein (2)	36,162	36,791	630	forward
31	ASCH domain protein (2)	36,812	37,516	705	forward
32	DNA N-6-adenine-methyltransferase (2)	37,526	38,194	669	forward
33	Single-stranded DNA-binding protein (2)	38,194	38,694	501	forward
34	Helix-turn-helix domain protein (8)	39,753	39,908	156	forward

Table 18: Functional genes identified for ΦX29

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

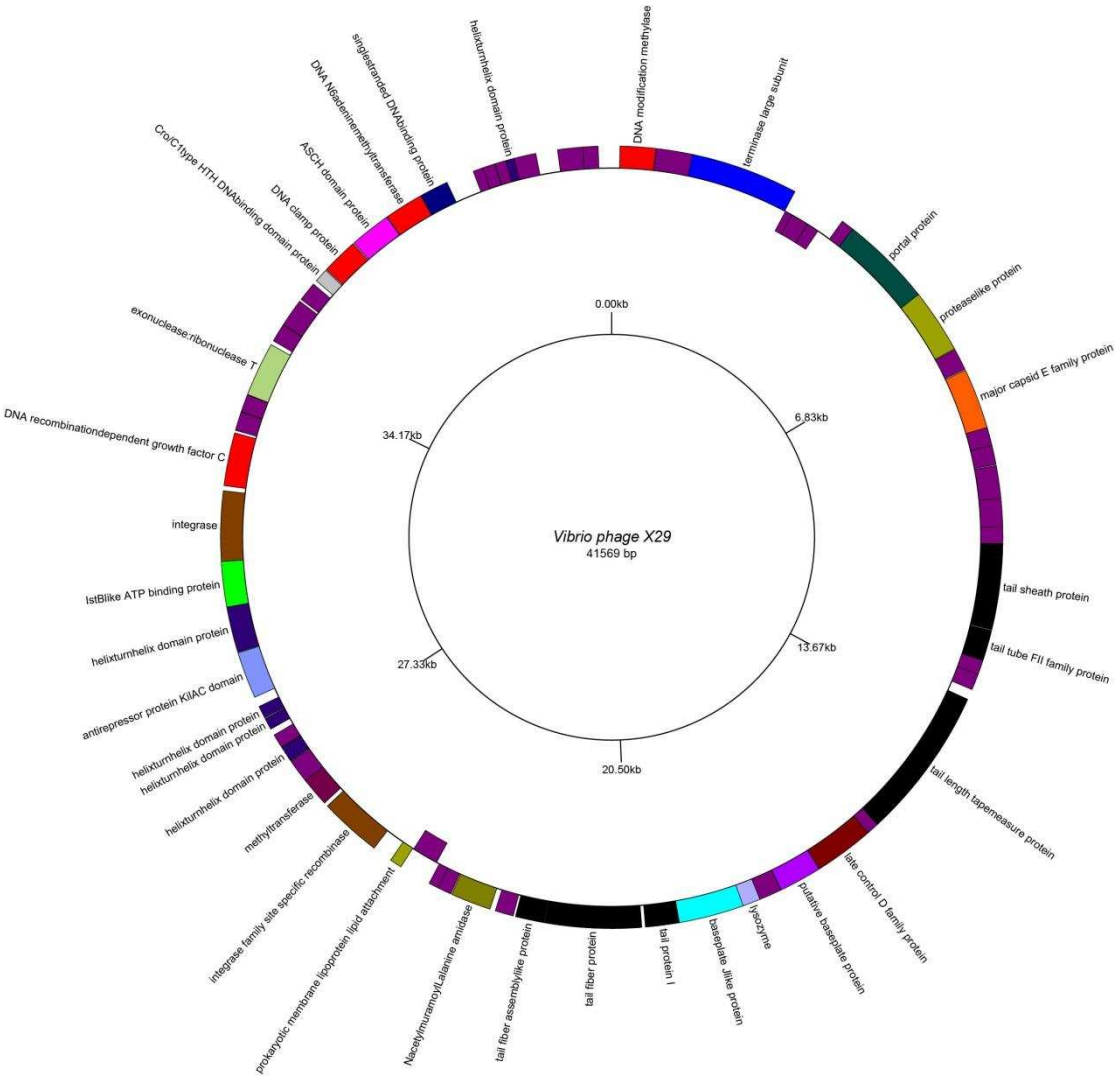

Figure 36: Genome map of phage X29 generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

Out of seven Chinese phages, the genome size of ΦQH and J3 were both 39.7 kb and rest all were 39.5 kb. Annotation of these genomes revealed that phage J3 possessed 49 genes and the other phages had 48 genes each (Table 13). The percent G+C content for Chinese phages was similar with 50.5 % for all of them (Table 13). The genes identified were assigned with functions and for ΦQH nine genes were given functions (Figure 37), while for phages CJY/H1/H2/H3/J3 eight genes each and for ΦJ2, seven genes were functionally identified (Table 19). All these phages had early to mid genes in common which contained one lysogeny related gene each encoding for Integrase and also four genes associated DNA metabolism and replication. Amongst the late genes the head morphogenesis related genes were two each but for tail morphogenesis some phages lacked few genes. Two genes encoding the tail components were possessed by ΦQH and phages CJY/H1/H2/H3/J3 had one each; while none was identified for ΦJ2.

BlastN search for all the Chinese phages revealed that they are closely related to Vibrio phages VP2 (NC_005879.1), VP5 (NC_005891.1) and phiVC8 (JF712866.1). Both VP2 and VP5 showed 97-98 % pairwise identity with all the Chinese phages; while phiVC8 showed 86-87 % identity. The Phages H1, H2, H3 and J2 being exactly the same length were also shown to be highly similar, differing only by a range of SNPs (Figure 38). The slightly more divergent CJY sequence could also be grouped with H1, H2, H3 and J2 with a 12 base insertion further differentiating it. Phages QH and J3 were more divergent and they shared an additional 79 aa CDS (QH_0045/J3_0046) of unknown function which was also found in VP2, VP5 and phiVC8 but absent in rest all Chinese phages under study. Similar to VP2, VP5 and phiVC8, all the Chinese phages had 53 aa CDS of unknown function (e.g. H1_0024)

except in phage QH. Phage QH had major tail subunit (QH_0014) which was absent in all other phages. They also share an enzyme adenylosuccinate synthase, a purine bio-synthesis enzyme with VP2, VP5 and phiVC08.

	Name of the protein encoded by gene	Direction	QH			CJY/H1/H2/H3/J3			J2		
			Start	End	Length	Start	End	Length	Start	End	Length
1	Terminase (3)	forward	1,135	2,841	1,707	1,137	2,843	1,707	1,137	2,843	1,707
2	Major tail subunit (4)	forward	9,330	11,561	2,232						
3	Tail fiber (4)	forward	19,362	20,435	1,074	19,367	20,440	1,074			
4	Outer capsid protein (3)	forward	20,437	21,819	1,383	20,442	21,824	1,383	20,442	21,824	1,383
5	Adenylosuccinate synthetase (2)	Reverse	23,448	24,479	1,032	23,604	24,635	1,032	23,604	24,635	1,032
6	Integrase (1)	Reverse	24,521	26,809	2,289	24,677	26,965	2,289	24,677	26,965	2,289
7	DNA polymerase I (2)	Reverse	26,799	28,700	1,902	26,955	28,856	1,902	26,955	28,856	1,902
8	Single stranded DNA-binding protein (2)	Reverse	28,709	29,185	477	28,865	29,341	477	28,865	29,341	477
9	Superfamily II DNA/RNA helicase (2)	Reverse	30,711	32,180	1,470	30,867	32,336	1,470	30,867	32,336	1,470

Table 19: Functional genes identified for Chinese phages

Figures in the parenthesis after protein name indicate 1. Lysogeny related; 2. DNA metabolism and replication; 3. Head morphogenesis; 4. Tail morphogenesis; 5. Host cell lysis; 6. Other conserved protein domains; 7. Unknown function; 8. Regulatory proteins

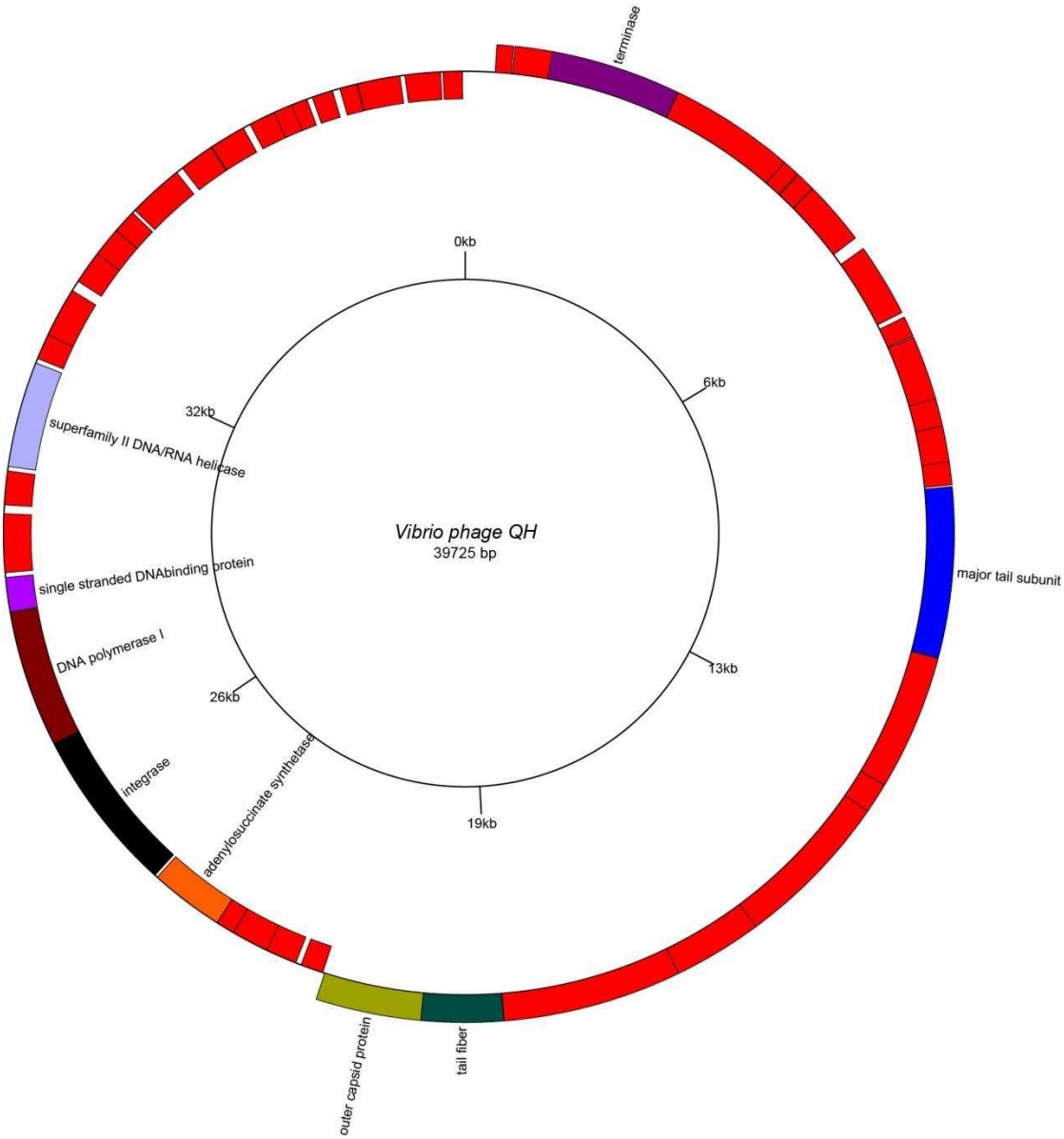

Figure 37: Genome map of Vibrio phage QH generated using GenomeVx available at http://wolfe.gen.tcd.ie/GenomeVx (Conant and Wolfe 2008). The unlabelled genes encode hypothetical proteins. Genes on outside are forward strand genes and genes inside are reverse strand genes.

Figure 38: Clustal omega alignments of Chinese phage genome sequences with SNP variations.

4.3 Discussion

In order to characterise the genomes of the phages under study the genome size was estimated using PFGE (§ 2.4.8). The predicted genome sizes revealed the largest genome i.e. 114 kb for phage Φ3, which happens to be a Siphoviridae type phage (§ 3.2.4). Similar genome sizes were observed by Comeau and colleagues (Comeau, Chan et al. 2006) for the Siphoviridae type phages of V. parahaemolyticus which showed larger sizes between 106 to 125 kb. The only Siphovirus (Φ3) studied under this project had larger genome size as the Siphoviruses infecting Vibrio spp. range between 44 to 94 kb size (Shivu, Rajeeva et al. 2007); while the Siphoviridae phage ΦVh4 genome was 107 kb in size (Thiyagarajan, Chrisolite et al. 2011). The Chinese phages (§ 3.2.4) were of relatively smaller genome sizes and all of them were below 48.5 kb which was similar to the size of between 44 to 46 kb reported by other workers (Comeau, Chan et al. 2006). One more Podovirus phage Φ1 in this study had comparatively larger genome size of 74 kb and the other report on Vibrio specific Podovirus phage ΦVh3 had genome size of 64 kb (Thiyagarajan, Chrisolite et al. 2011). The remaining three Myoviridae phages Φ2, phage 24 and phage X29 also had smaller genome sizes i.e. below 48.5 kb. Al-Fendi and colleagues (Al-Fendi, Shueb et al. 2014) noticed the similar genome size of 33.5 kb for Myoviridae phages specific to Vibrio spp. but the genome size of 83 kb was also reported by other researchers (Shivu, Rajeeva et al. 2007) and thus the Myoviridae phages under study seem to be small myoviruses.

The estimated genome sizes revealed through PFGE correspond well with the actual genome sizes of the phages determined through DNA sequencing of these phages. The PFGE genome size of phage Φ1 was 74 kb while the actual genome size after

sequencing was 66.7 kb. The PFGE estimate of the genome size for Φ3 was 114 kb while the actual size after sequencing was 116.1 kb. The sizes of the phages Φ2, the Canadian phages and all the Chinese phages were below 48.5 kb and it was confirmed by sequencing that they all fell below 48.5 kb. The precise size estimation of genomes under 48.5 kb through PFGE was difficult with 50-1000 kb pulse high marker and thus, low marker of 0.1 – 200 kb could have been useful but the DNA sequencing could resolve this issue by determining the actual size.

Further to genome size estimations the phage nucleic acids (DNA) were extracted (§ 2.4.14) for additional genomic characterisation. As mentioned in the section 4.2.2 the minimum requirement for the sequencing purpose was 150 ng/µl but with newest methods one can rapidly sequence the DNA containing phage genomes even from a single plaque (Kot, Vogensen et al. 2014).

Before sending the samples for sequencing the phage genomes were discriminated by restriction fragment length polymorphisms (RFLP). Looking in to the digestion patterns of all phages under study it revealed that there were genomic differences between them which gave different restriction profiles. Restriction endonuclease PstI could cut the phage 24 DNA in this study and in an earlier study the Vibrio harveyi phage PW2 was also digested by PstI ascertaining its double stranded nature and genome size estimate (Phumkhachorn and Rattanachaikunsopon 2010). Phage Φ1 genome was digested by XbaI and there was a report on digestion of the genomic DNA of four lytic phages infecting Vibrio harveyi by XbaI showing distinct restriction profiles (Thiyagarajan, Chrisolite et al. 2011). They used 12 different restriction enzymes but only two could digest the phage DNA while remaining 10 enzymes could

not cut the DNA. In our study, the restriction enzymes PstI and SacI could not cut phage Φ1, while phage 24 remained uncut by SacI and XbaI. This could be due to DNA methyltransferases encoded by these phages which catalyse the conversion of cytosine to hydroxymethyl cytosine protecting their DNA from digestion (Atterbury 2003). This was confirmed after genome sequencing by detection of the DNA methyltransferase in phage 24 genome at the coordinates 10,320..11,621 (Table 17). For Chinese phages the restriction enzymes PstI, SacI and XbaI could cut the genomes so much that there were not any distinct bands but smeared pattern was generated for all of them. Though there was smearing, the pattern of smearing remained different for each phage suggesting the difference amongst them but a clear discrimination was necessary so the new enzymes EcoRI and HpaII were used. The digestion pattern by HpaII could differentiate the Chinese phages but EcoRI showed smearing pattern for all the Chinese phage genomes. HpaII could digest DNA of all the phages to yield discriminatory patterns when it was used for restriction analysis of seven Vibrio harveyi bacteriophage DNAs (Shivu, Rajeeva et al. 2007).

After obtaining the discriminatory restriction fragment length polymorphism the phage DNA was sequenced, assembled and annotated (§ 2.4.17). Knowledge of functional genes through genome sequencing and further annotation helps to predict whether phage has a lysogenic life cycle e.g. the genes encoding lysogeny viz. Integrase family site specific recombinase, Putative excisionase family protein, Cro-like protein, Antirepressor protein and Intergrase as detected in Φ 2; while integrase family site specific recombinase and an integrase in Φ X29 and an integrase detected in all Chinese phages. As we need strictly lytic phages for phage therapy purpose, the

absence of genes associated with lysogeny and/or virulence/antimicrobial resistance genes is a crucial factor in deciding the potential phage therapy candidate.

The PHE phages Φ 1, Φ 2 and Φ 3 are three completely different phages morphologically as they fall in to three different classifications viz. Podoviridae, Myoviridae and Siphoviridae families, respectively (§ 3.2.4). All these three phages were of Russian origin as per the personal communication of Dr. Tom Cheasty, then Head of Gastrointestinal Infections Reference Unit at then Health Protection Agency now Public Health England. Vibrio phage Φ1 is N4 like i.e. Escherichia phage family and is genetically similar to Vibrio phages JA1 (GenBank: NC_021540.1) and VCO139 (GenBank: KC438283.1), both specific to Vibrio cholerae O139 (Fouts, Klumpp et al. 2013). Phage Φ1 is V. cholerae O1-specific though very similar to the other O139 specific phages. This could be due to the fact that phage Φ1 was isolated several years ago in Russia as it was obtained from the archives of PHE and the prevalent host strain at the time of isolation was V. cholerae O1. Later, the O139 serotype emerged as a pandemic strain (Swerdlow and Ries 1993) and the phages JA1 and VCO139 were isolated after the O139 serotype became prevalent. Phage Φ1 has a large protein, viron-encapsulated RNA polymerase with a molecular mass of about 360 kDa and was encoded by 9722 bp gene SBVP1_0074. The presence of this protein indicates that it belongs in the N4 like as it is a characteristic of this family (Falco, Zehring et al. 1980). All other genes assigned with functions for phage Φ1 did not reveal any lysogeny/antimicrobial resistance related as stated in the preceding paragraph and/or virulence/toxin related genes i.e. cholera toxin encoding genes (e.g. ctxAB, zot and ace); which would otherwise render it unsuitable for therapeutic use. Of the other two PHE phages phage Φ2 was identified as a Myoviridae phage (§

3.2.4) and was 99 % similar to ΦX29 at genetic level. Both these phages have a conserved translational frameshift which was analogous to the lambda gpG/gpGT proteins and was present in the coding sequences upstream of their tape measure protein (Xu, Hendrix et al. 2004). From a phage therapy perspective these two phages were not promising as their genomes contained integrases and Cro protein homologs which are lysogeny related proteins. The third PHE phage, Φ3, was the only T5 like phage classified in to Siphoviridae family (§ 3.2.4) having terminal repeats at both the ends similar to the T5 genome (Wang, Jiang et al. 2005). Amongst all the phages under study only phage Φ3 has eight tRNAs. Phage pVp-1 had 19 tRNAs (Kim, Jun et al. 2012). Phage pVp-1 proved to be therapeutically effective for the treatment of multiple antibiotic resistant Vibrio parahaemolyticus infection in a mouse model (Jun, Shin et al. 2014) and thus phage Φ3 could be a potential phage therapy candidate owing to the absence of any genes related to lysogeny/antimicrobial resistance and/or virulence/toxin as discussed above.

The Canadian phages in this study were of Myoviridae family and the phage X29 was a temperate phage as discussed in the preceding paragraph. The phage 24 has 99% identity with Vibrio phage CP-T1 (Comeau, Tremblay et al. 2012) but has 75 bp in-frame deletion in the pentapeptide repeat-containing protein of unknown function (Vetting, Hegde et al. 2006). There was not any gene related to lysogeny/antimicrobial resistance nor was any gene associated toxin production found. Thus, phage 24 was also considered to be suitable for therapeutic applications.

The Chinese phages isolated, sequenced and annotated in this study all belonged to the Podoviridae family (§ 3.2.4) and genome analysis showed that they clustered with

members of VP2-like phage group of the Podoviridae family (Lavigne, Seto et al. 2008). The other members of this VP2-like phage group were phiVC8 (JF712866.1), VP2 (NC_005879.1) and VP5 (NC_005891.1). All seven Chinese phages share an integrase protein with the members of the VP2-like phage group which indicates that they were temperate in nature. They also share an enzyme adenylosuccinate synthase, a purine bio-synthesis enzyme with all the VP2-like phages mentioned above.

Though most of the Chinese phages were of similar length, phages H1, H2, H3 and J2 were of exactly the same length and have 99 % sequence similarity. In spite of the high sequence similarity they are dissimilar as per the restriction analysis owing to different banding patterns though they were cut by one restriction enzyme HpaII except for phage QH (see 4.2.3), and moreover they were all isolated from independent locations. Phages H1, H2 and H3 were isolated from Fu Jia Wan, Ye Zhi Hu and Nan Hu lakes, respectively from Wuhan city of Hubei province and these water bodies were separated by land masses. J2 was isolated from the Yudai He river in a different city, Jiangxi, in Nanchang province. Phage CJY was differentiated by a 12 base insertion at the N-terminal region of CDS CJY_0035. VP2 and VP5 phages were lacking this insertion in to the equivalent CDS. Phages QH and J3 were more divergent and they share an additional 79 aa CDS (QH_0045/J3_0046) which is of unknown function and was also found in phiVC8, VP2 and VP5 but absent in the remaining Chinese phages. Similar to phiVC8, VP2 and VP5, all the Chinese phages had 53 aa CDS of unknown function (e.g. H1_0024) except in phage QH. Phage QH had a major tail subunit (QH_0014) which is absent in all other phages. None of the Chinese phages were considered to be suitable for phage therapy because of their temperate nature.

Overall, the genomic characterisation of all the phages under study revealed the relative suitability of these phages for biocontrol of V. cholerae. Considering the genomic features of all these phages only three phages viz. phage Φ1, Φ3 and phage 24 were found to be suitable for phage therapy. These three phages meet the criteria (§ 4.1) as they do not possess any undesirable genes associated with lysogeny/antibacterial resistance and also any cholera toxin genes. Thus, they are fit for use in therapeutic preparations either alone or in a cocktail. An assessment of their efficacy in the treatment of V. cholerae infection must be assessed in an appropriate animal model. This is covered by and discussed in chapter 5.

PHAGE THERAPY EXPERIMENTS

5 PHAGE THERAPY EXPERIMENTS

5.1 Introduction

Phages have been used for therapy since soon after their discovery in the early twentieth century. The Indian river waters of Ganges and Jamuna showed bactericidal activity against the cholera bacterium (Hankin 1896) but the exact nature of this bactericidal property was not known. Later, phages were discovered by Twort (1915) as well as d'Herrelle (1917) and discovery of phages allowed the use of phage for prophylaxis and treatment of bacterial diseases for the first time. Some initial reports were unclear on the effectiveness of phages in general and for treating cholera (Monsur, Rahman et al. 1970, Marcuk, Nikiforov et al. 1971) but later the effectiveness of phage therapy was demonstrated by the excellent and well documented clinical experiments of H. Williams Smith's group (Smith and Huggins 1983, Smith, Huggins et al. 1987). In one of their studies a single dose of phage was superior to multiple doses of prevalent antibiotics viz. tetracycline, ampicillin, chloramphenicol or trimethoprim plus sulphafurazole in treating enterotoxigenic E. coli enteritis.

Smith suggested that as the pathogenesis of cholera is very similar to that of enterotoxigenic E. coli enteritis, the phage treatment should also be successful with cholera (Smith, Huggins et al. 1987). For better understanding of phage host interactions in vivo, a good animal model of cholera phage therapy is required. The animal models which have been used for evaluation of phage therapy of V. cholerae are rabbits and mice (§ 1.6.3.2). Infant rabbits produce the clinical signs of disease similar to human cholera (Pierce, Kaper et al. 1985, Ritchie, Rui et al. 2010). The colonisation of intestinal villi by V. cholerae is at a faster rate in the infant rabbits than

in the adults (Nelson, Clements et al. 1976) owing to goblet cells not being fully functional in infants. In adult rabbits the secretion of mucus layer by goblet cells acts as a barrier to colonisation. Although V. cholerae readily colonises intestines of the infant mice, they do not develop clear clinical signs of cholera as occurs in infant rabbits (Ritchie, Rui et al. 2010). (Ujiiye and Kobari 1970) noticed inhibition of gastric emptying in infant mice challenged orally or subcutaneously with cholera toxin. Thus, the infant rabbit model was chosen for this project.

For therapeutic purposes either a single phage or a cocktail of phages could be used for treating cholera. A single phage gives the convenience of use and cost advantage pertaining to phage production; while cocktails of phages help in circumventing resistance development, if they are selected against different receptors. In a cocktail of phages, it is not known if the reduction in V. cholerae counts is due to a single phage in the cocktail or due to two or more phages working together. Thus, with a single phage one can scientifically and clearly test whether that particular phage is effective or not. The lytic phages with a broad host range are generally deemed most suitable for biological control purposes (§ 1.6.5). The characteristics of the phages under study pertaining to these criteria are discussed in the chapters 3 and 4. Amongst the phages studied, the phage which most closely fitted the criteria was phage Φ1. The burst size of Φ1 was 43 ± 5.5; while the latent period was 12 mins and it had a moderately broad host range as it could lyse 67 % of the total 91 strains in our collection. Phage Φ1 did not show any undesirable genes associated with lysogeny/antibacterial resistance or any cholera toxin genes upon genome annotation based on the present database knowledge.

In this chapter therefore phage Φ1 was tested in vivo using the baby rabbit model assessing its effects on clinical condition and V. cholerae numbers in the small intestines (upper, middle and lower), mid colon and caecal fluid. The control group animals were not administered with phage but only with V. cholerae; while the animals in the experimental group were given phage with multiplicity of infection (MOI) > 1 (§ 2.5.4). The clinical conditions and reduction in bacterial numbers were compared between control and treatment groups.

5.2 Results

5.2.1 Clinical findings

The control animals (n = 18) were infected orally with V. cholerae 1051 over three biological replicate experiments (Table 20) and were not treated with phage. They showed clinical signs of disease which varied from mild to severe diarrhoea. Some animals appeared normal (7 out of 18 animals) (Table 20), although they were still well colonised by V. cholerae as indicated by caecal fluid bacterial counts (Table 21 and 22).

All the treated animals (n = 15) were also orally infected with V. cholerae 1051 during two separate experiments and were treated with phage Φ1. None of the animals showed any signs of diarrhoea and were clinically normal at the time of euthanasia (Table 20).

Experiment No *	Gouping of animals	No. of rabbits per litter	Bacterial dose (CFU/ml)	Phage dose (PFU/ml)	No. of rabbits with clinical signs ¤		
					Normal	Mild diarrhoea	Severe diarrhoea
1♯	Control group	5	1×10^8	-	1	4	0
	Treatment group	0	-	-	0	0	0
2	Control group	6	3×10^8	-	2	3	1
	Treatment group	9	5×10^8	1×10^9	9	0	0
3	Control group	7	5×10^6	-	4	3	0
	Treatment group	6	4×10^8	1×10^9	6	0	0

Table 20: The number of therapeutic experiments, number of animals used and clinical signs of the disease noted after infection.
* Experiments were separated by several weeks. ♯ For experiment 1 only the control group was done in order to check the colonisation of the SmR V. cholerae 1051. ¤ Severity of clinical signs were scored as described in Materials and Methods (§ 2.5.3).

5.2.2 Recovery of V. cholerae and phages

The bacteria and phage of control and treatment groups were enumerated (§ 2.5.6). The mean bacterial counts for the control group in experiments 1, 2 and 3 were higher in the distal segments of the alimentary tract viz mid colon (MC) and caecal fluid (CF) than the proximal segments viz. upper small intestine (I1), middle small intestine (I2), lower small intestine (I3). Similarly, for experiments 2 and 3 reduction in the V. cholerae counts of the treatment groups in the distal segments of intestine i.e. caeca and mid colon was greater compared to the proximal segments (Figure 39 and 40). The mean bacterial counts for experiment 1 were 5.50 ± 1.09, 5.64 ± 1.25, 5.95 ± 0.96, 7.09 ± 1.16 and 7.31 ± 1.06 respectively for I1, I2, I3, MC and CF. Experiment 2 and 3 bacterial as well as phage counts are shown in tables 21 and 22.

Statistical analysis (2.5.7) revealed very signifiant reductions ($p<0.001$) for experiment 2 while experiment 3 also showed significant reductions ($p<0.005$) in V. cholerae counts for all five intestinal sites sampled in the phage-treated animals compared with the control group. For experiment 2, the V. cholerae counts of caecal fluid were highest in the control group i.e. 8.55 ± 0.65 $\log_{10}$ CFU/ml; while in the treatment group they were 3.80 ± 0.82 $\log_{10}$ CFU/ml indicating a mean reduction of 4.7 $\log_{10}$ CFU/ml. Similarly, the counts for mid colon were reduced by 3.9 $\log_{10}$ from 7.98 ± 0.36 to 4.07 ± 0.12 $\log_{10}$ CFU/g. Reduction in the counts of more proximal segments like lower, middle and upper small intestines were comparatively lower, as they showed 3.3, 2.2 and 2.0 $\log_{10}$ reductions, respectively but were still statistically very significant ($p<0.001$) (Figure 39; Table 21). For experiment 3, the V. cholerae counts of caecal fluid in the control group were 7.56 ± 1.53 $\log_{10}$ CFU/ml; while in the treatment group they were 3.44 ± 0.61 $\log_{10}$ CFU/ml with a mean reduction of 4.1

log_{10} CFU/ml. The counts for mid colon were reduced by 3.0 log_{10} CFU/g while reduction in the counts of lower, middle and upper small intestines were 2.4, 1.8 and 2.1 log_{10} reductions, respectively which were comparatively lower but were still statistically significant ($p<0.005$) (Figure 40; Table 22).

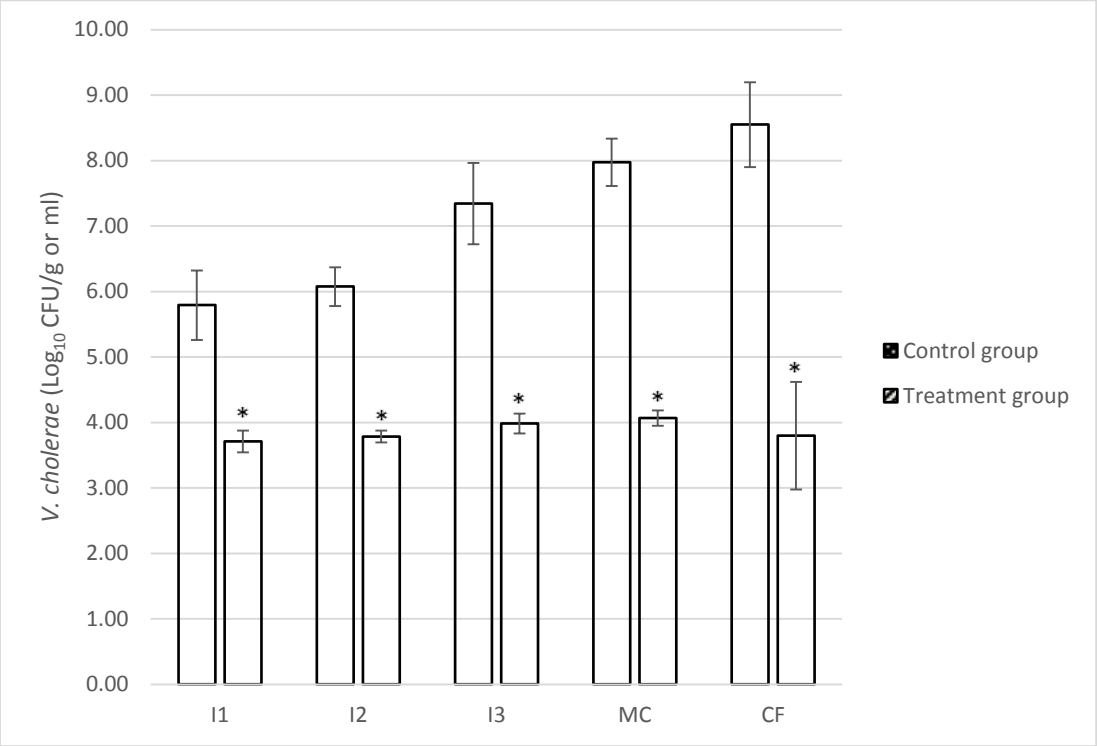

Figure 39: Experiment 2, comparison of mean V. cholerae counts (log_{10} CFU/g or ml ± SD) in groups of rabbits treated or not treated with bacteriophage.
The counts are given for five sections of intestine: I1=Upper small intestine; I2=Mid small intestine; I3=Lower small intestine; MC=mid colon; CF=caecal fluid. Bars marked with (*) indicate a significant reduction in bacterial counts between control and treated goups ($p<0.001$). Mean and standard deviation calculations were based on 18 and 15 biological replicates for control and treatment group, respectively.

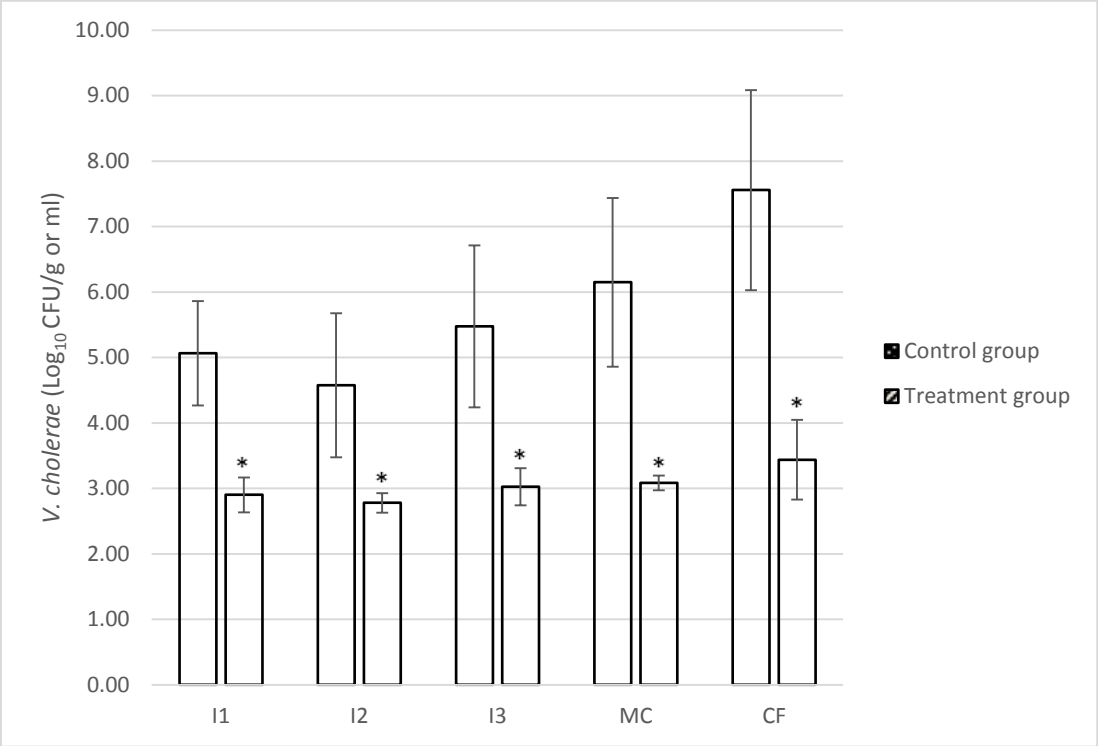

Figure 40: Experiment 3, comparison of mean V. cholerae counts (log_{10} CFU/g or ml ± SD) in groups of rabbits treated or not treated with bacteriophage.
The counts are given for five sections of intestine: I1=Upper small intestine; I2=Mid small intestine; I3=Lower small intestine; MC=mid colon; CF=caecal fluid. Bars marked with (*) indicate a significant reduction in bacterial counts between control and treated goups ($p<0.005$). Mean and standard deviation calculations were based on 18 and 15 biological replicates for control and treatment group, respectively.

Sample location	Control group bacterial counts (Mean Log_{10} CFU/g or ml ± SD)	Treatment group bacterial counts (Mean Log_{10} CFU/g or ml ± SD)	P value and significance for bacterial counts	Treatment group phage counts (Mean Log_{10} PFU/g or ml ± SD)
I1	5.79 ± 0.53	3.71 ± 0.17	0.0010 (***)	6.70 ± 0.17
I2	6.08 ± 0.30	3.79 ± 0.09	0.0004 (***)	6.70 ± 0.28
I3	7.34 ± 0.62	3.98 ± 0.15	0.0004 (***)	7.18 ± 0.28
MC	7.98 ± 0.36	4.07 ± 0.12	0.0010 (***)	7.26 ± 0.55
CF	8.55 ± 0.65	3.80 ± 0.82	0.0010 (***)	7.87 ± 0.71

Table 21: Experiment 2, mean V. cholerae counts (log_{10} CFU/g or ml ± SD) in groups of rabbits treated or not treated with bacteriophage, statistical significance and mean phage counts (Log_{10} PFU/g or ml ± SD) for treated group.
The counts are given for five sections of intestine: I1=Upper small intestine; I2=Mid small intestine; I3=Lower small intestine; MC=mid colon; CF=caecal fluid; ***=very significant

Sample location	Control group bacterial counts (Mean Log_{10} CFU/g or ml ± SD)	Treatment group bacterial counts (Mean Log_{10} CFU/g or ml ± SD)	P value and significance for bacterial counts	Treatment group phage counts (Mean Log_{10} PFU/g or ml ± SD)
I1	5.07 ± 0.80	2.90 ± 0.27	0.0012 (**)	6.59 ± 0.38
I2	4.58 ± 1.10	2.78 ± 0.15	0.0012 (**)	6.71 ± 0.68
I3	5.48 ± 1.24	3.03 ± 0.28	0.0012 (**)	6.61 ± 0.69
MC	6.15 ± 1.29	3.09 ± 0.11	0.0012 (**)	6.90 ± 0.76
CF	7.56 ± 1.53	3.44 ± 0.61	0.0025 (**)	8.77 ± 0.69

Table 22: Experiment 3, mean V. cholerae counts (log_{10} CFU/g or ml ± SD) in groups of rabbits treated or not treated with bacteriophage, statistical significance and mean phage counts (Log_{10} PFU/g or ml ± SD) for treated group.
The counts are given for five sections of intestine: I1=Upper small intestine; I2=Mid small intestine; I3=Lower small intestine; MC=mid colon; CF=caecal fluid; **=significant

The mean phage counts recovered for the treatment group in experiment 2 (n = 9) were higher in the distal regions of alimentary tract with 7.26 ± 0.55 in the mid colon and 7.87 ± 0.71 in the caecal fluid; while the proximal regions showed lower phage counts (Table 21 and 22). For experiment 3 (n = 6) also the more distal regions had higher phage counts viz. 6.90 ± 0.76 in the mid colon and 8.77 ± 0.69 in the caecal fluid; with lower phage counts for the the proximal regions. No phages could be detected in the samples from the control groups of both the experiments 2 and 3 as they were not administered with phage but only with bacterial dose. The presence of phages in control groups was tested to rule out any cross contamination and also to check if there were any resident/lysogenic phages already present in the guts of control animals.

The mean fluid accumulation ratio (a ratio of the weight of caecal fluid drained to the weight of caecal tissue) for the control groups in the experiments 1 (n = 4), 2 (n = 6) and 3 (n = 6) was 0.32 ± 0.11, 0.27 ± 0.20 and 0.10 ± 0.11, respectively; while for treatment groups in the experiments 2 (n = 9) and 3 (n = 6) it was 0.08 ± 0.04 and 0.05 ± 0.05, respectively. Statistical analysis revealed non significant differences between control and treatement groups of experiments 2 and 3 for fluid accumulation ratios.

As a pilot study, a single experiment was carried out for the comparison of clinical signs, colonisation and fluid accumulation ratio (FAR) of wildtype strain of V. cholerae 1051 to that of V. cholerae C6706 used in the original infant rabbit cholera model developed by Dr. Jenny Ritchie (Ritchie, Rui et al. 2010) (§ 2.5.2). It was noticed the wild type V. cholerae 1051 showed inconsistent clinical signs compared to

V. cholerae C6706. The colonisation was weak and also the FAR was less in the wildtype strain of V. cholerae 1051 compared to V. cholerae C6706 (Table 23).

	Mean bacterial counts (Log_{10} CFU/g or ml)						No. of rabbits with clinical signs [ɰ]		
	I1	I2	I3	MC	CF	FAR	Normal	Mild diarrhoea	Severe diarrhoea
Wildtype V. cholerae 1051	5.45	8.25	8.43	7.97	8.19	0.81	-	2	3
V. cholerae C6706	7.86	9.74	10.05	9.15	9.15	1.1	-	-	5

Table 23: Comparison of clinical signs, colonisation and FAR for wildtype strain of V. cholerae 1051 to that of V. cholerae C6706 used in the original infant rabbit cholera model.
I1=Upper small intestine; I2=Mid small intestine; I3=Lower small intestine; MC=mid c olon; CF=caecal fluid; FAR=fluid accumulation ratio. [ɰ] Severity of clinical signs were scored as described in Materials and Methods (§ 2.5.3).

5.3 Discussion

The experiments detailed in this chapter aimed to determine the efficacy of using host-specific bacteriophage to reduce the carriage of V. cholerae in an infant rabbit model of cholera. The results showed that V. cholerae numbers in phage treated animals were significantly lower than untreated control animals. As discussed in the introduction, Smith proposed that phage therapy for cholera could be effective owing to similar pathogenesis of V. cholerae to enterotoxigenic E. coli (ETEC) as both of them produce watery diarrhea induced by plasmid or phage encoded enterotoxins (Smith, Huggins et al. 1987). Though earlier reports on the use of phages against human cholera were not effective (Pollitzer, Swaroop et al. 1959), the recent studies on phage therapy of cholera using animal models viz. rabbits (Sarkar, Chakrabarti et al. 1996, Bhowmick, Koley et al. 2009, Jaiswal, Koley et al. 2013) and mice (Jaiswal, Koley et al. 2014) were more encouraging. The data presented in this chapter demonstrated that oral infection of infant rabbits with pathogenic V. cholerae O1 strain 1051 and subsequent oral administration of Vibrio phage Φ1 in phage treated group harboured significantly fewer V. cholerae than the control and no clinical signs of disease were evident. The reduction in V. cholerae numbers in the phage-treated group was greater than previous studies wherein 2 $\log_{10}$ reductions in bacterial counts were obtained (Jaiswal, Koley et al. 2013).

The animals were infected with bacteria after administration of antacid (§ 2.5.3) owing to the usual acid environment with low pH levels < 3.0 in the guts of rabbit species (Smith 1965). The antacid increases survival of Vibrio spp. and their phage (Koo, Marshall et al. 2001). Infected animals produced the clinical signs of disease. If the overt clinical signs in the control group were compared to the clinical signs

reported for original infant rabbit cholera model (Ritchie, Rui et al. 2010), the signs showed inconsistency with varied degree of diarrhoea in the control group. The diarrhoea score in the present study ranged from mild to severe, while some animals appeared normal (Table 20). This could be due to the fact that the V. cholerae strain 1051 used in this study was different to the V. cholerae strain C6706 used in the original infant rabbit cholera model. In fact the pilot study was done to compare the clinical signs produced by wild type strain of V. cholerae 1051 with V. cholerae C6706 and it was noticed the wild type V. cholerae 1051 showed inconsistent clinical signs compared to V. cholerae C6706 (Table 23). Though V. cholerae 1051 had inconsistent clinical signs, it was the only option for therapeutic experiments as V. cholerae C6706 was not susceptible to Φ1 which was chosen candidate for phage therapy. Also, the phages effective on V. cholerae C6706 were not able grow in the liquid cultures. Moreover, the streptomycin resistant (SmR) mutant of V. cholerae 1051 was used for this study instead of wild type strain to increase the selectivity for the strain on agar plates containing antibiotics. Streptomycin resistance is conferred by mutations in the ribosomal subunit S12 protein (Chen, Blumentritt et al. 2013) and it was noted that some of the antibiotic resistant mutants were less virulent than the wild type (Bjorkman, Hughes et al. 1998). Further to this the dosing of control and treatment animals on different days owing to cross contamination of phage from treatment animals to control animals and dosing of different numbers of bacteria owing to different culture conditions on different days (§ 2.5.3) were the limitations of the experimental design. Sample collection time at 24 h postinfection was appropriate owing to development of profuse diarrhoea between 12 to 18 h postinoculation and death of animals by 30 h for control groups (Ritchie, Rui et al. 2010). Thus any sample collection time beyond 30 h could not have improved the experimental design.

The fluid accumulation in the caeca was also variable owing to varying degree of clinical signs shown and fluid accumulation ratios (FAR) are directly correlated to the intensity of disease production. This was the reason for bigger standard deviation in the control group fluid accumulation ratios while the treatment group had fluid accumulation similar to normal uninfected animals. The fluid accumulation ratio in the treatment groups i.e. 0.08 ± 0.04 and 0.05 ± 0.05 for the experiments 2 and 3, respectively was in agreement with recorded fluid accumulation ratio of 0.05 ± 0.05 for non-infected animals (Ritchie, Rui et al. 2010) suggesting phage involvement in reducing symptoms of cholera. This result is important as the earlier report on fluid accumulation ratio in an adult rabbit ileal loop model for phage therapy of cholera did not show any difference in the FAR of the control and phage treated animals as they both showed the same FAR of 1.1-1.5 (Sarkar, Chakrabarti et al. 1996). The FAR is an indicator of the intensity of disease production which is directly correlated to the value of FAR.

The bacterial counts in the control group were in the normal range of colonisation with more distal segments of intestines and caecal fluid containing more V. cholerae (Figure 39 and 40) as observed for an infant rabbit model of cholera (Ritchie, Rui et al. 2010). The surface area available in the small intestines (~300-350 cm length) is much more than the surface area available in the large intestine (~140 cm length) and caeca (~50 cm length) in adult rabbits but is proportionate in infant rabbits (Lebas, Coudert et al. 1997) and thus it is clear that the bacterial numbers in the more distal parts of alimentary tract tissues are more concentrated than proximal segments. Concomitantly, reduction in the bacterial counts of the treatment group in the distal

segments of intestinal tissues was greater than that in the proximal segments (Figure 39 and 40) and this might be due to more colonisation and availability of the bacteria in this region for phage predation. The similar reductions in the bacterial counts with more reduced numbers in the distal segments of intestines were observed for oral phage therapy of E. coli infected calves (Smith and Huggins 1983). For uninfected two day old rabbits the caecal fluid is not possible to collect (Combes, Michelland et al. 2011) and hence in this study, the collection of caecal fluid and recovery of bacteria after infection with V. cholerae is due to disease production.

V. cholerae was not totally eliminated following phage therapy. This could be due to various factors viz. phage resistance and physical protection of bacteria from phage. The potential of phage to reduce bacterial numbers is heavily dependent on the population densities of phage and host. It relies on a proliferation threshold/replication threshold i.e. minimum bacterial density necessary for supporting phage growth; and inundation threshold i.e. minimum phage density necessary to prevent proliferation of bacteria. When the inundation threshold is exceeded by a phage concentration and also the proliferation threshold is exceeded by bacterial numbers then at this point the active replication of phage particles occurs. In contrast, if the inundation threshold is exceeded i.e. phages outnumber the bacteria with MOI of > 1 but the proliferation threshold is not reached then the bacterial reduction occurs by passive infection i.e. by lysis from without. In this case, once the phage population decreases below the inundation threshold any bacteria that are remaining will survive (Payne and Jansen 2001, Cairns, Timms et al. 2009). After initial application of phage with MOI of > 1, inside the gut the bacterial numbers might have increased and simultaneously the phage might have replicated at a

consistently higher rate than the bacteria. Thus, the mechanism of action of phage in this experiment could be a mixture of initial active replication and a passive inundation later. In the case of the latter scenario, when the phage concentration decreased below the inundation threshold any remaining bacteria might have survived. Apart from this there might be a possibility of already resistant subpopulations of bacteria that might have grown which was noticed for phage therapy of bacteria with a similar pathogenesis i.e. enterotoxigenic E. coli in oral infection of neonatal animals (Smith and Huggins 1983, Smith, Huggins et al. 1987). The physical protection of bacteria from phage results from their localisation in inaccessible sites like crypts and villi (Maura and Debarbieux 2012). There is evidence of V. cholerae localising in the inter-villous spaces and deep crypt-like structures along with epithelial surface (Ritchie, Rui et al. 2010). Such protected bacteria might not have come in contact with phage and moreover the V. cholerae moves actively within the intestine to colonise inaccessible areas (Butler and Camilli 2005) potentially escaping the passively moving phages.

The maximum phage recovery from the treatment group was 8.77 ± 0.69 $\log_{10}$ PFU/ml from caecal fluid in the experiment 3 after successful reduction in bacterial numbers. The dose of phage given was 1×10^9 PFU/ml but the recovery was less and this might be due to passive inundation as discussed in the preceding paragraph. During passive inundation phage numbers reduce below the inundation threshold and then the remaining bacteria begin multiplying (Payne and Jansen 2001). Probably, the passive inundation was in the proximal segments as the bacterial numbers were less there and active replication in the distal segments owing to more availability of bacteria (§ 5.2.2). Similar reduced recovery of phages was noticed by Jaiswal et al

(Jaiswal, Koley et al. 2013) who recovered 6×10^6 PFU/g of phages from faeces of phage treated adult rabbits post V. cholerae infection after first 3 hours of oral administration of phage at a dose of 1×10^8 PFU/ml which further decreased steadily until 12th hour and disaapeared by 24th hour.

The maximum of 4.7 $\log_{10}$ difference in the bacterial counts from 8.55 ± 0.65 $\log_{10}$ CFU/ml in control group to 3.80 ± 0.82 $\log_{10}$ CFU/ml for phage-treated group in caecal fluid samples of experiment 2 was a greater reduction than noted for rabbit model of cholera studied by Jaiswal et al (Jaiswal, Koley et al. 2013) as they recorded the maximum reduction of 2 $\log_{10}$ through faecal samples. The reduction recorded by Jaiswal and colleagues was for a phage cocktail administered 6 hours after bacterial challenge similar to our 6-8 hours post infection phage administration. One possible reason for this discrepancy is the use of an adult rabbit model in which the colonisation is slower than the infant rabbit model (Nelson, Clements et al. 1976). Even, using the RITARD (Removable Intestinal Tie-Adult Rabbit Diarrhoea) model for phage therapy of cholera only 2 $\log_{10}$ reduction in the V. cholerae counts was seen (Bhowmick, Koley et al. 2009). While, use of oral cocktail of phages could reduce the V. cholerae numbers by 3 $\log_{10}$ in an adult mice model (Jaiswal, Koley et al. 2014). Apart from V. cholerae there have been reports of successful phage therapy of other Vibrio spp. including the mouse model of V. vulnificus which showed a 4 log reduction in the bacterial counts in treated animals compared to control animals (Cerveny, DePaola et al. 2002). A report on the phage therapy of V. parahaemolyticus assessing the effect of individual as well as a cocktail of phages revealed that the cocktail was more effective than individual phages. The individual phage VP-1 reduced the bacterial count by 2.9 log, phage VP-2 by 3.6 log and phage VP-3 by 3.8

log but the cocktail of phages comprising VP-1/VP-2 could bring down the bacterial count by 4.0 log, while VP-1/VP-3 by 3.8 log; VP-2/VP-3 by 3.6 log and VP-1/VP-2/VP-3 by 4.2 log indicating that cocktails were slightly more effective (Mateus, Costa et al. 2014). Similarly, the mono-phage therapy for a multiple antibiotic resistant pandemic clinical strain of V. parahaemolyticus was successful using the phage pVp-1 and the phage protected mice infected with lethal oral as well as intraperitoneal doses of bacteria (Jun, Shin et al. 2014). The success of phage therapy for Vibrio spp. is well documented in aquatic settings where Vibrio anguillarum phages have protected against experimental vibriosis in salmon (Higuera, Bastias et al. 2013) and also in shrimp hatchery trials to protect Penaeus monodon larvae from Vibrio harveyi using cocktail of phages (Karunasagar, Shivu et al. 2007).

Some residual bacteria were recovered and it was an indication that phage resistant strains of bacteria could have emerged and thus more work is required on the recovered V. cholerae strains to study their resistance pattern. Smith and colleagues showed that the phage resistant mutants paid a fitness penalty and were usually less virulent than the parent strain (Smith and Huggins 1983, Smith, Huggins et al. 1987). In those studies the cocktail of phages took care of resistant mutants as a bacterium resistant to one phage is often susceptible to another phage in the cocktail or to the mutant phage derived from the phages active on parent strains (Smith, Huggins et al. 1987). The phages use bacterial surface receptors as their docking sites while infecting their hosts; while bacteria make structural changes in these surface receptors in order to become resistant to the phage (§ 1.6.4). During this resistance development bacteria pay a fitness penalty by losing the sites for the uptake of nutrients. The surface receptors which phages use for their adsorption are also the sites used by bacteria for

their nutrient uptake (Mizoguchi, Morita et al. 2003) but they have to conceal them for getting resistant. Thus, bacteria compromise themselves on their capacity to utilise nutirents and thereby loosing their virulence (Lenski 1988).

Thus, owing to the effectiveness of phage therapy using single phage in the present study, it is possible that a cocktail of phages will be more effective, if further studies are carried out. The most important aspect of this study is that the phage treated animals were clinically normal though V. cholerae could not be totally eliminated but maximum 4.7 $\log_{10}$ reduction in their counts could be therapeutically useful. This success has paved a way for further studies to develop human phage therapy for cholera disease.

GENERAL DISCUSSION

6 GENERAL DISCUSSION

The work presented in this thesis describes an investigation of the potential for biocontrol of V. cholerae using bacteriophage. At the outset, the isolation and characterisation of phages specific to V. cholerae was done and then the potential candidate for phage therapy was used for in vivo studies in an infant rabbit cholera model. The isolation/collection of phages from across the globe and their characterisation as evidenced in chapters 3 and 4 resulted in the creation of a cholera phage bank. Two attempts to isolate the phages specific to O1 strains of V. cholerae in the UK environment were unsuccessful. This was mainly due to the absence of cholera disease in the UK. Similar unsuccessful attempts have been made previously (Atterbury and Barrow, unpublished) to isolate phages specific to O1 strains of V. cholerae. Non-O1 V. cholerae strains have been isolated in the UK waters in the eighties (West and Lee 1982) and this supports the recent isolation of phages active against non-O1 V. cholerae strains (Sarkar, unpublished). Owing to non availability of phages specific to O1 strains of V. cholerae in the UK, seven new O1 specific phages were isolated in cholera endemic areas of south eastern China. Success in isolating V. cholerae specific phages from the environment confirms the endemicity of cholera disease in the predicted risk areas in China (Xu, Cao et al. 2013) and again supports the notion that phages can be used as indicators of the presence of particular pathogens (Madico, Checkley et al. 1996). Other phages used in this study had been isolated from cholera patients or from the environmental samples in the endemic areas and thus it implies that the phages originated from the human intestine or were present in the local environment. However, the use of phage therapy in the closed environment where patients excreta is collected and disposed of safely, as is the case

in cholera patients, the phage therapy can be effective so that the phage resistant bacteria are not circulated in the local environment.

Not all the phages isolated would necessarily be useful for phage therapy and as discussed in chapter 3 and 4 only selected phages fit the criteria of broad host range, large burst size (which is variable for individual phage but a larger burst is considered good for phage therapy), strictly lytic life cycle and absence of lysogeny related and bacterial virulence genes in their genomes. The host ranges of the Chinese phages QH, H3 and J3 were broader as they could lyse 84.6%, 70.3% and 76.9% of the 91 strains in the current collection, respectively. Also the burst sizes of the phages X29, CJY and H3 were 664 ± 50, 182 ± 62 and 126 ± 18 PFU/cell, respectively. Inspite of being broad in their host range and having good burst sizes, the above mentioned phages QH, H3, J3, X29 and CJY were not selected for therapeutic purposes as they possessed lysogeny related genes viz. Integrase family site specific recombinase, Putative excisionase family protein, Cro-like protein, Antirepressor protein and Intergrase identified by sequencing. On the other hand, out of twelve phages which were fully characterised, only three phages viz. phage Φ1, Φ3 and phage 24 were found to be suitable for phage therapy mainly based on their genomic features. They did not possess any undesirable genes associated with lysogeny/antibacterial resistance and also any cholera toxin genes. Although the host ranges of Φ1 and Φ3 phages were not the broadest they were nevertheless able to lyse 67.0% and 62.6% of phages of the 91 strains used in this study. Phage 24 did not lyse any of the strains other than its own host strain. The burst sizes of the Φ1, Φ3 and phage 24 were 43 ± 05, 54 ± 26 and 87 ± 26 PFU, respectively. However, although phage 24 showed a large burst size and was free of any deleterious genes; it did not have broad spectrum

activity and thus was not used as a candidate for phage therapy studies. As like Φ1, Φ3 was equally suitable for phage therapy and either Φ3 alone or in cocktail with Φ1 could have been effective but it won't be possible to know which phage in the cocktail actually works. Also owing to the huge costs involved in animal experiments only Φ1 was initially evaluated for its therapeutic potential.

Chapter 4 presents the genomic characterisation of phages. The genome annotaton provides functions to different genes based on the existing knowledge available in the various databases. The coding sequences for which existing databases do not have any information remain hypothetical. Therefore, sequencing of genomes do not reveal everything as most of the genes in all the phage genomes encode hypothetical proteins with no definite functions assigned to them. Even a slight difference (SNPs) in the genome at nucleotide level can significantly change the number of genes identified as it will rearrange the nucleotide sequence and thereby coding for different amino acids which inturn code for different functional proteins. For example phage X29 had more genes assigned with functions i.e. 34 out of 67 genes compared to Φ2 i.e. 27 out of 67 genes although they were 99 % similar to each other but had a minor difference in their genome sequences. Thus, some SNPs can be coding while some of them can be non coding ones. SNPs may also influence the latent period, burst size and host ranges as evidenced by differences shown for these parameters among the similar phages with SNP variations.

Phage Φ1 was shown to be a virulent phage which was subsequently used to reduce V. cholerae numbers in the intestinal tract of infant rabbits. An interesting feature of the Φ1 genome was its GC content. The V. cholerae GC content is approximately 47 %

(Dziejman, Balon et al. 2002) and Φ1 had significantly low GC content i.e. 34.5 %. The other N4 like family Vibrio phages also had considerably lower GC content than their bacterial hosts (Fouts, Klumpp et al. 2013). This deviation between the GC content of phage and host may be the characteristic feature of these N4 like family Vibrio phages and is maintained in them (Kwan, Liu et al. 2006). The virulent dsDNA phages have low GC content compared to their bacterial host genomes and bias towards AT enrichment in phages allow them to exploit the bacterial cell resources better during their replication (Rocha and Danchin 2002). AT rich regions aid replication of phage DNA as origin of replication (ori) having them adjacent to it for quick unwinding of DNA (Kutter, Raya et al. 2003). AT enriched phages are quick in transcription owing to more AT rich promoter elements (Carter, Demidenko et al. 2003) and also they require less energy as their base pairing contains two hydrogen bonds to be broken as against three in GC base pairing. Thus, Φ1 is capable of expoiting the bacterial cell resources and hence it might be more effective in phage therapy. The remaining two phages i.e. Φ3 and phage 24 were identified as potential phage therapy candidates and also have a lower GC content than the host strain viz. 42.83 and 45.40 %, respectively. It was also noted that even temperate phages are AT richer than their hosts but they have smaller AT deviation compared to virulent phages (Rocha and Danchin 2002) and it is true with all the temperate phages in this study.

The statistical difference in bacterial numbers of control and treatment groups may have been improved if the colonisation of V. cholerae 1051 had been better. As discussed in the chapter 5, the colonisation of SmR 1051 was low pathogenic and may partly be due to antibiotic resistance as some of the antibiotic resistant mutants are less virulent than wild type (Bjorkman, Hughes et al. 1998). Notwithstanding the relatively

low pathogenicity of the intestine by SmR 1051, rabbits dosed with bacteriophage Φ1 harboured significantly fewer V. cholerae than the control animals with difference of maximum 4.1 $\log_{10}$ CFU/g. It is conceivable that using a strain which colonised as well as V. cholerae C6706, the strain used in the original infant rabbit model could have been a better model with the availability of effective phage. Moreover, the original infant rabbit model had optimised the time points at which the overt clinical signs would be produced and also the euthanization at 24 h time point from animal welfare point of view. Thus, in future, alongwith the usage of a strain with better colonisation/pathogenicity; optimisation of the time points for wider time course might be necessary to see if more obvious clinical signs are noticed at later stages. Apart from bacterial numbers, the most significant result in this project is the fact that the treated animals no longer appeared to show symptoms of disease. As the distal portions might have had active replication (§ 5.3) further studies using lower phage titres for treatment may indicate whether there is an active multiplication of phages as evidenced by increase in phage numbers together with reduction in bacterial numbers. There was no 'bacteriophage only' control in the experiments carried out for this project and the kinetics of phage persistence were thus not known together with whether phage could be recovered in the absence of V. cholerae. Hence, it would be prudent to have 'bacteriophage only' control during any future studies based on this project.

In this study, the treatment of infant rabbits with bacteriophage resulted in a reduction in V. cholerae numbers of up to 4.1 $\log_{10}$ CFU/g, and marked amelioration of disease symptoms compared with untreated control animals. This reduction in bacterial numbers was greater than the reduction noted for an adult rabbit model of cholera

(Jaiswal, Koley et al. 2013) which achieved a 2 $\log_{10}$ reduction recovered from faecal samples using a cocktail of five phages. As discussed in the preceding paragraphs and in chapter 5, a cocktail of phages using Φ1 and Φ3 may improve the results for the infant rabbit cholera model used in this study and may address phage resistance in the target bacterium, if this becomes a major issue. Smith and colleagues (Smith and Huggins 1983) did not find any bacteriophage resistant bacteria after 24 h following phage therapy of lambs infected with E.coli S13 using a single phage. This study was based partly on their success of phage therapy to treat oral E. coli infections in neonatal animals calves, piglets and lambs (Smith and Huggins 1983, Smith, Huggins et al. 1987) and their suggestion for the effectiveness of phages against V. cholerae infection. Similar to V. cholerae, Enterotoxigenic E. coli (ETEC) causes secretary diarrhoea mediated by the heat labile (LT) enterotoxins which activate and produce high levels cAMP to alter the sodium and chloride ion channels causing massive efflux of chloride ions along with water in to the gut, resulting in secretary diarrhoea. Administering a dose of phage between 6-8 h was successful in controlling oral E. coli infection in calves (Smith, Huggins et al. 1987) and V. cholerae biocontrol by administering the phages in a similar timeframe was also successful. It is worth mentioning that though the phage therapy does not eliminate the pathogens but they are reduced to a level below which the disease does not occur and subsequently these low numbers of bacteria are cleared by the host immune system in an immunocompetent host. Moreover, it is known that cholera infection induces immunity to protect against subsequent infections (Nelson, Harris et al. 2009) in human beings.

In summary, this study has demonstrated that bacteriophage can be used to reduce the counts of V. cholerae in an established animal model of infection. This therapeutic trial gave promising results and suggests that further studies might improve on the results demonstrated here.

Future work:

The initial work of isolating and characterising the bank of phages is already done in this project and a monophage therapy for biocontrol of V. cholerae using bacteriophage in an infant rabbit model is also successful. After this initial success in therapeutic experiments in an infant rabbit cholera model, regulated human clinical experiments should be carried out to establish the safety and efficacy of phages for human phage therapy. The strength of this project lies in to the generation of a robust data and collaboration with different research groups within the UK and in cholera endemic region for fulfilling the objectives. Taking a clue from H. William Smith's pioneering work on phage therapy of enterotoxigenic E. coli (ETEC), where he suggested that phage therapy might work for V. cholerae due to similar pathogenesis; this project was designed for in vivo studies post in vitro characterisation. The characterisation involved not only the study of traditional biological parameters but also the advanced phage genomics to ensure that the potential therapy candidate is selected appropriately. The animal studies could give very significant reductions in the bacterial counts and most importantly the phage treated animals were clinically normal without any symptoms of disease.

Study had a few drawbacks pertaining to the subjective scoring for recording of clinical signs; involvement of variables like the bacterial dose and time of

inoculation/phage treatment; non-inclusion of phage only control and non-testing of residual bacteria for phage susceptibility. The Blinding of studies would take away the subjectivity of clinical scores. The variable infective bacterial dose and different days of dosing for the control and treatment groups might have bearing on the experimental outcome but two independent experiments (2 and 3) have shown significant reductions in bacterial colonisation across multiple GI sites and this strongly indicates that it is indeed the phage that is causing the effects. To be absolutely sure it is proposed that the further studies using this approach should have control and treatment groups isolated to prevent any phage cross contamination but both the groups be tested in parallel under identical conditions at the same time with the same bacterial and phage preparations. Few additional experiments with phage only control and resistance pattern studies would help in improving results. The following additional experiments would be needed to improve the results of this project:

1. The experiments with phage only control and with reduced dose of phage to determine active replication.
2. The resistance pattern study for bacterial strains isolated from the treatment group animals in this project.

APPENDIX ‘A’

APPENDIX ‘A’

Time (h)	**Trial 1 (log_{10} CFU/ml)**	**Trial 2 (log_{10} CFU/ml)**	**Trial 3 (log_{10} CFU/ml)**	**Mean (log_{10} CFU/ml)**	**Standard Deviation**
0	6.23	6.31	6.18	6.24	0.11
2	7.18	7.21	7.25	7.21	0.11
4	9.13	9.14	9.21	9.16	0.07
6	9.06	9.21	9.37	9.21	0.16
8	9.64	9.30	9.33	9.42	0.18
10	9.51	9.32	9.38	9.40	0.11
12	9.71	9.40	9.38	9.50	0.17
24	9.73	9.78	9.86	9.79	0.10

Table 24: V. cholerae F5 counts for growth curve

Time (h)	Trial 1 (log_{10} CFU/ml)	Trial 2 (log_{10} CFU/ml)	Trial 3 (log_{10} CFU/ml)	Mean (log_{10} CFU/ml)	Standard Deviation
0	6.34	6.68	6.83	6.62	0.24
2	7.35	7.80	7.63	7.59	0.24
4	8.54	8.57	8.66	8.59	0.09
6	8.72	8.66	8.73	8.71	0.11
8	8.84	8.75	8.79	8.79	0.07
10	8.88	8.88	8.91	8.89	0.04
12	8.84	8.84	8.92	8.87	0.06
24	9.13	8.97	9.08	9.06	0.08

Table 25: V. cholerae 1051 counts for growth curve

Time (h)	Trial 1 (log_{10} CFU/ml)	Trial 2 (log_{10} CFU/ml)	Trial 3 (log_{10} CFU/ml)	Mean (log_{10} CFU/ml)	Standard Deviation
0	7.02	6.94	7.01	6.99	0.04
2	7.65	8.04	8.11	7.93	0.25
4	8.84	8.91	8.74	8.83	0.08
6	9.22	9.33	9.29	9.28	0.05
8	9.45	9.62	9.74	9.60	0.14
10	9.63	9.83	10.05	9.84	0.21

Table 26: V. cholerae 2095 counts for growth curve

Time (h)	Trial 1 (log_{10} CFU/ml)	Trial 2 (log_{10} CFU/ml)	Trial 3 (log_{10} CFU/ml)	Mean (log_{10} CFU/ml)	Standard Deviation
0	7.12	7.08	6.97	7.06	0.08
2	8.15	8.14	8.07	8.12	0.05
4	9.09	9.16	9.11	9.12	0.04
6	9.13	9.19	9.22	9.18	0.05
8	9.39	9.30	9.22	9.31	0.08
10	9.33	9.31	9.26	9.30	0.04

Table 27: V. cholerae C6706 counts for growth curve

Time (h)	Trial 1 (log_{10} CFU/ml)	Trial 2 (log_{10} CFU/ml)	Trial 3 (log_{10} CFU/ml)	Mean (log_{10} CFU/ml)	Standard Deviation
0	6.48	6.61	6.51	6.54	0.07
2	7.87	7.42	7.68	7.66	0.22
4	9.15	8.54	8.98	8.89	0.31
6	9.22	8.95	9.19	9.12	0.14
8	9.47	9.23	9.34	9.35	0.12
10	9.55	9.37	9.24	9.39	0.15

Table 28: V. cholerae 238 counts for growth curve

APPENDIX ‘B’

APPENDIX 'B'

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.79	6.76	6.85	6.80	0.04
4	6.72	6.74	6.66	6.70	0.04
6	6.66	6.70	6.66	6.67	0.02
11	6.66	6.63	6.63	6.64	0.02
16	6.73	6.69	6.66	6.69	0.04
21	6.76	6.81	6.74	6.77	0.04
26	6.85	6.72	7.01	6.86	0.14
31	7.16	6.78	7.51	7.15	0.37
36	7.68	7.38	7.45	7.51	0.16
41	7.74	7.73	7.65	7.71	0.05
46	7.86	7.88	7.70	7.81	0.10
51	7.90	7.90	7.83	7.88	0.04
56	7.89	7.80	7.87	7.85	0.05
61	7.85	7.85	7.72	7.81	0.07
66	7.88	7.87	7.81	7.86	0.04
71	7.89	7.90	7.76	7.85	0.08
76	7.88	7.88	7.90	7.89	0.01
81	7.85	7.85	7.98	7.89	0.07
86	7.84	7.82	7.83	7.83	0.01
91	7.79	7.82	7.88	7.83	0.04

Table 29: V. cholerae phage Φ1 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.15	6.32	6.20	6.22	0.09
3	5.90	6.04	6.19	6.04	0.14
5	5.85	5.98	6.10	5.97	0.13
10	5.74	5.81	5.85	5.80	0.05
15	5.90	5.70	5.78	5.79	0.10
20	5.90	5.98	6.08	5.99	0.09
25	6.26	6.40	6.28	6.31	0.08
30	6.52	6.60	6.48	6.53	0.06
35	6.64	6.71	6.43	6.60	0.15
40	6.65	6.71	6.48	6.61	0.12
45	6.54	6.69	6.53	6.59	0.09
50	6.65	6.68	6.53	6.62	0.08
55	6.93	6.80	6.83	6.85	0.07
60	6.80	6.73	6.73	6.75	0.04
65	6.65	6.73	6.72	6.70	0.04
70	6.85	6.91	6.61	6.79	0.16
75	6.54	6.70	6.49	6.58	0.11
80	6.65	6.47	6.59	6.57	0.09
85	6.85	6.62	6.59	6.68	0.14
90	6.85	6.57	6.49	6.64	0.19

Table 30: V. cholerae phage Φ2 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.56	6.76	6.73	6.68	0.11
3	6.53	6.41	6.69	6.54	0.14
5	6.48	6.36	6.67	6.50	0.16
10	6.40	6.41	6.73	6.51	0.19
15	6.48	6.43	6.85	6.59	0.23
20	6.56	6.51	6.66	6.58	0.08
25	6.55	6.45	6.72	6.57	0.14
30	6.59	6.62	6.81	6.68	0.12
35	6.61	7.06	6.83	6.83	0.23
40	6.61	7.08	6.90	6.86	0.24
45	6.72	7.26	7.18	7.06	0.29
50	7.12	7.40	7.50	7.34	0.20
55	7.59	7.38	7.77	7.58	0.19
60	7.70	7.38	7.77	7.62	0.21
65	7.68	7.41	7.76	7.62	0.18
70	7.67	7.42	7.80	7.63	0.19
75	7.70	7.46	7.87	7.68	0.20
80	7.71	7.45	7.88	7.68	0.22
85	7.75	7.40	7.84	7.66	0.23
90	7.80	7.45	7.93	7.73	0.25

Table 31: V. cholerae phage Φ3 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	5.85	5.54	5.70	5.70	0.15
4	5.78	5.54	5.70	5.67	0.12
6	5.74	5.74	5.60	5.69	0.08
11	5.95	5.98	5.98	5.97	0.01
16	6.04	5.88	5.93	5.95	0.08
21	6.20	6.10	5.81	6.04	0.20
26	6.56	6.56	6.51	6.54	0.03
31	7.11	7.07	6.98	7.06	0.07
36	7.66	7.53	7.19	7.46	0.24
41	7.64	7.54	7.56	7.58	0.05
46	7.67	7.65	7.57	7.63	0.05
51	7.65	7.60	7.87	7.71	0.14
56	7.71	7.59	7.63	7.64	0.06
61	7.69	7.69	7.62	7.67	0.04
66	7.83	7.57	7.63	7.68	0.14
71	7.89	7.67	7.45	7.67	0.22
76	7.81	7.79	7.56	7.72	0.14
81	7.99	7.89	7.65	7.84	0.17
86	7.88	7.83	7.74	7.81	0.07
91	7.83	7.85	7.76	7.81	0.04

Table 32: V. cholerae phage X29 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.19	6.19	6.30	6.23	0.06
3	6.10	5.98	6.18	6.08	0.10
5	6.32	6.11	6.34	6.26	0.13
10	6.32	6.10	6.31	6.24	0.13
15	6.54	6.41	6.54	6.50	0.07
20	6.46	6.30	6.40	6.39	0.08
25	6.37	6.27	6.36	6.33	0.06
30	6.92	6.66	7.01	6.86	0.18
35	7.41	7.04	7.50	7.32	0.24
40	7.56	7.32	7.59	7.49	0.15
45	7.61	7.45	7.59	7.55	0.08
50	7.56	7.41	7.60	7.52	0.10
55	7.30	7.40	7.21	7.30	0.09
60	7.30	7.33	7.29	7.31	0.02
65	7.32	7.21	7.31	7.28	0.06
70	7.32	7.34	7.32	7.33	0.01
75	7.39	7.44	7.40	7.41	0.03

Table 33: V. cholerae phage 24 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	5.88	5.93	5.78	5.86	0.08
3	5.81	5.85	5.78	5.81	0.03
5	5.93	5.95	5.81	5.90	0.08
10	5.85	5.90	5.70	5.82	0.11
15	5.90	5.85	5.65	5.80	0.13
20	6.02	6.08	5.88	5.99	0.11
25	6.19	6.26	5.98	6.14	0.15
30	6.10	6.06	6.18	6.11	0.06
35	5.98	5.98	5.98	5.98	0.00
40	5.98	5.95	5.98	5.97	0.01
45	6.10	6.08	6.13	6.10	0.03
50	6.04	6.04	5.95	6.01	0.05
55	6.00	5.90	6.13	6.01	0.11
60	6.02	5.98	6.08	6.03	0.05
65	6.68	6.64	6.74	6.69	0.05
70	7.07	7.05	7.23	7.12	0.10
75	6.65	6.78	6.99	6.81	0.17

Table 34: V. cholerae phage QH counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	5.93	6.10	6.00	6.01	0.08
3	6.27	6.20	5.90	6.12	0.19
5	5.95	6.06	6.00	6.00	0.05
10	5.98	6.08	5.98	6.01	0.06
15	5.90	6.00	6.02	5.97	0.06
20	6.02	6.10	6.00	6.04	0.05
25	6.11	6.39	6.02	6.17	0.19
30	6.16	6.06	6.06	6.09	0.06
35	6.15	6.16	6.23	6.18	0.04
40	6.04	6.04	6.27	6.12	0.13
45	6.06	6.18	6.20	6.15	0.08
50	6.10	6.33	5.81	6.08	0.26
55	6.11	6.35	6.10	6.19	0.14
60	6.26	6.22	6.20	6.23	0.03
65	6.39	6.32	6.10	6.27	0.15
70	7.04	6.85	6.88	6.93	0.10
75	7.26	6.96	6.83	7.02	0.22
80		7.26	7.10	7.18	0.11
85		7.45	7.46	7.46	0.01
90		7.20	7.03	7.12	0.12

Counts were not done

Table 35: V. cholerae phage CJY counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	5.95	5.98	5.88	5.94	0.05
3	5.88	6.00	5.78	5.88	0.11
5	6.04	5.95	5.95	5.98	0.05
10	5.90	5.85	6.06	5.94	0.11
15	5.98	5.85	6.02	5.95	0.09
20	6.22	6.04	6.00	6.09	0.12
25	6.22	6.24	6.13	6.20	0.06
30	6.16	6.24	6.20	6.20	0.04
35	6.18	6.26	5.88	6.10	0.20
40	5.93	6.15	6.13	6.07	0.12
45	6.02	6.08	6.15	6.08	0.06
50	6.10	6.13	5.98	6.07	0.08
55	6.31	6.26	6.24	6.27	0.04
60	6.13	6.23	6.11	6.16	0.06
65	6.36	6.11	6.41	6.30	0.16
70	6.31	6.11	6.67	6.36	0.28
75	6.96	6.92	6.86	6.91	0.05
80		7.00	6.87	6.94	0.09
85		7.12	7.39	7.26	0.19
90		6.92	7.13	7.02	0.15

Counts were not done

Table 36: V. cholerae phage H1 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.32	6.22	6.18	6.24	0.08
3	6.20	6.00	6.11	6.11	0.10
5	6.19	6.15	6.16	6.17	0.02
10	5.98	6.02	6.08	6.03	0.05
15	6.29	5.81	6.04	6.05	0.24
20	6.27	6.00	6.28	6.18	0.16
25	6.42	6.11	6.23	6.26	0.16
30	6.29	6.19	6.41	6.30	0.11
35	6.19	6.26	6.41	6.29	0.12
40	6.35	6.24	6.31	6.30	0.06
45	6.38	6.22	6.23	6.28	0.09
50	6.43	5.90	6.36	6.23	0.29
55	6.57	6.35	6.48	6.47	0.11
60	6.49	6.13	6.30	6.31	0.18
65	6.57	6.31	6.35	6.41	0.14
70	6.67	6.41	6.60	6.56	0.13
75	7.21	6.80	7.26	7.09	0.25
80	7.18	7.53	7.22	7.31	0.19
85	7.11	7.68	7.59	7.46	0.30
90	7.60	7.59	7.45	7.54	0.09

Table 37: V. cholerae phage H2 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.04	5.95	6.13	6.04	0.09
3	6.13	5.70	6.08	5.97	0.24
5	6.32	5.90	6.16	6.13	0.21
10	6.27	5.78	6.06	6.04	0.25
15	6.27	5.98	6.16	6.14	0.15
20	6.34	5.98	6.20	6.17	0.18
25	6.20	6.31	6.30	6.27	0.06
30	6.18	6.08	6.19	6.15	0.06
35	6.26	6.08	6.23	6.19	0.10
40	6.44	5.95	6.24	6.21	0.24
45	6.37	6.13	6.28	6.26	0.12
50	6.37	6.13	6.26	6.25	0.12
55	6.43	6.42	6.40	6.42	0.02
60	6.28	6.23	6.28	6.26	0.03
65	6.53	6.53	6.50	6.52	0.02
70	6.77	6.47	6.67	6.64	0.15
75	7.11	7.33	6.95	7.13	0.19
80	7.40	7.63	7.22	7.41	0.20
85	7.49	7.43	7.33	7.42	0.08
90	7.51	7.13	7.48	7.37	0.21

Table 38: V. cholerae phage H3 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.19	5.85	5.93	5.99	0.18
3	5.85	5.60	5.78	5.74	0.13
5	5.54	5.85	5.65	5.68	0.15
10	5.98	5.81	5.74	5.84	0.12
15	5.88	5.81	5.70	5.80	0.09
20	5.85	5.90	5.90	5.88	0.03
25	6.19	6.19	6.15	6.18	0.03
30	6.08	6.08	5.93	6.03	0.09
35	6.02	6.15	5.90	6.02	0.12
40	6.00	6.08	5.85	5.97	0.12
45	6.16	5.88	5.98	6.00	0.15
50	6.00	5.98	5.93	5.97	0.04
55	6.42	6.23	6.22	6.29	0.12
60	6.26	6.15	6.10	6.17	0.08
65	6.16	6.50	6.16	6.27	0.19
70	6.44	6.46	6.39	6.43	0.04
75	7.10	7.29	6.91	7.10	0.19
80	7.32	7.06	7.04	7.14	0.16
85	7.16	7.05	7.00	7.07	0.08
90	7.14	7.10	6.96	7.07	0.10

Table 39: V. cholerae phage J2 counts for one step growth curve

Time (m)	Trial 1 (log_{10} PFU/ml)	Trial 2 (log_{10} PFU/ml)	Trial 3 (log_{10} PFU/ml)	Mean (log_{10} PFU/ml)	Standard Deviation
0	6.38	6.27	6.31	6.32	0.06
3	6.30	6.28	6.24	6.27	0.03
5	6.33	6.29	6.19	6.27	0.07
10	6.28	6.33	6.18	6.26	0.08
15	6.24	6.54	6.20	6.33	0.18
20	6.38	6.50	6.30	6.39	0.10
25	6.61	6.59	6.50	6.57	0.06
30	6.54	6.35	6.38	6.42	0.10
35	6.64	6.48	6.41	6.51	0.12
40	6.41	6.50	6.38	6.43	0.06
45	6.57	6.57	6.40	6.51	0.10
50	6.41	6.56	6.40	6.46	0.09
55	6.66	6.39	6.48	6.51	0.14
60	6.67	6.48	6.51	6.55	0.11
65	6.74	6.65	6.57	6.65	0.09
70	6.91	6.80	6.68	6.80	0.12
75	6.91	6.86	6.70	6.82	0.11
80	7.44	7.52	7.60	7.52	0.08
85	7.39	7.00	6.95	7.11	0.24
90	7.43	7.33	7.13	7.30	0.15

Table 40: V. cholerae phage J3 counts for one step growth curve

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