ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES AGAINST *Shigella flexneri*

By

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ABSTRACT

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Soo Zheng May

Bacteriophages are viruses that parasitise on bacteria. Bacteriophage is studied widely to improve the safety of foods and prevent food borne diseases of bacterial aetiology, as well as to reduce the use of antibiotics in livestock. Besides, therapeutic applications of bacteriophage in human and the usage of phages in veterinary medicine and agriculture are also highly assessed. Hence, the present study aimed to isolate and to characterise *Shigella flexneri* specific bacteriophage. Bacteriophages were isolated from various environmental water samples. These environmental phages were then selected based on the size and clarity of their formed plaques. The selected bacteriophage particles were further enriched and purified via PEG precipitation method. Host range of these bacteriophages was determined by spot test on selected bacteria panel. Lastly, the genomes of these bacteriophages were characterised through restriction enzyme digestion analysis. The 8 isolated bacteriophages were found to be able to lyse the *Shigella flexneri*. However, among the 8 isolates, there was 1 isolate designated as D2, was found to demonstrate lytic activity against ETEC and EPEC. Two bacteriophages, C2 and D1, were also found to have lysogenic effects towards *E. coli*. Based on

restriction enzyme digestion analysis using *Eco*RI, it was found that B1, D1, D2, E1 and E2 phages showed similar restriction patterns, thus, they are most probably belonged to the same strain. D2 phage was selected for further restriction enzyme characterisation. After digestion with *Bam*HI, *Not*I, *Sal*l, *Xba*I and *Sac*I enzymes, only *Sac*I, *SaI*I and *Bam*HI enzymes able to produce the desired fragment size of 600 bp to 1000 bp suitable for cloning. Further identification and characterisation of the isolated bacteriophages should be carried out to determine the suitability of these bacteriophages for therapeutic application.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

SOO ZHENG MAY

APPROVAL SHEET

This project report entitled **"ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES AGAINST *Shigella flexneri*"** was prepared by SOO ZHENG MAY and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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Supervisor

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FACULTY OF SCIENCE

Date: _____

SUBMISSION OF FINAL YEAR PROJECT

It is hereby certified that **SOO ZHENG MAY (ID No: 09ADB03712)** has completed this final year project entitled **"ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES AGAINST *Shigella flexneri*"** under the supervision of **DR. TAN GIM CHEONG** (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my final year project in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(SOO ZHENG MAY)

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMBL-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
HCI	hydrochloric acid
ICTV	International Committee on the Taxonomy of Viruses
LB	Luria-Bertani
NaCI	Sodium chloride
NaOH	sodium hydroxide
OD	optical density
PAIs	pathogenicity islands
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	potential of hydrogen
RNA	ribonucleic acid
SRL	*Shigella* resistance locus

ssDNA	single stranded deoxyribonucleic acid
ssRNA	single stranded ribonucleic acid
TBE	Tris-Borate-EDTA
TTP	tail tube protein
UV	ultraviolet
WHO	World Health Organisation
µg	microgramme
µL	microlitre
µM	micrometre
bp	base pair
kbp	kilobase pair
L	litre
M	molar
nm	nanometre
rpm	revolutions per minute
x*g*	relative centrifugal force

CHAPTER 1

INTRODUCTION

Bacteriophages are viruses that specifically target and infect bacterial cells. They are the most abundant living entities on earth. They are found predominantly in area where their hosts live, for example, sewage, soil, deep thermal vents and natural bodies of water. They play an important role in regulating the microbial balance in every ecosystem due to their high level of specificity, long-term survivability and ability to reproduce rapidly in suitable host (Guttman et al., 2005). Like all viruses, bacteriophages are obligate intracellular parasite that absolute depend on specific host bacterium for reproduction. Each phage particle contains lipoprotein coat or capsid that encloses the nucleic acid genome. Although they carry all the genetic information for their replication in a susceptible host, they have no machinery to generate energy and no ribosomes to make proteins. Therefore, they replicate inside a host cell with the aid of host biosynthetic machinery (Carlton 1999).

Shigella flexneri is a human intestinal pathogen that is transmitted by the faecal-oral route. Consumption of food contaminated with these bacteria causes shigellosis within 12-48 hours (Anany et al., 2011). Upon infection, an individual will develop severe abdominal cramps, fever and diarrhoea, which turn to bacillary dysentery or shigellosis after a period of time. *S. flexneri* causes

infection by invading and replicating within the colonic epithelium, which leads to severe inflammation and epithelial destruction (Jennison and Verma 2004). According to The European Bioinformatics Institute (EMBL-EBI), shigellosis causes 1.5 million death and over 165 million episodes of cases each year worldwide. This infection most commonly occurs in children of developing nations due to poor sanitation and inadequate hygiene practice.

Bacteriophages have been extensively used in therapeutic application to prevent and treat bacterial infections in humans (Sulakvelidze and Kutter 2005). They have raised interest in various agricultural settings to improve the safety of foods and to control food borne diseases of bacterial aetiology, as well as to reduce the use of antibiotics in livestock. Phage therapy has shown success in treating infections in livestock, plants, aqua-cultured fish and human (Sulakvelidze and Kutter 2005). Recently, bacteriophages have received much attention as tool for DNA vaccination and as cloning and expression vectors in the field of genetic engineering (Verheust et al., 2010). Besides, bacteriophages provide a more convenient and inexpensive alternative technique for environmental monitoring and epidemiological surveillance by using phage typing method (Faruque et al., 2003).

Bacteriophages are found wherever the host bacteria are present and important in regulating bacterial population densities in aquatic environments. Study done by Kokjohn et al. (1991) indicated that phages are likely to affect microbial ecology

significantly in freshwater ecosystems. It has been found that the bacteriophage population exceeded bacteria and the probable ratio of bacteriophage to bacteria in water is 10 to 1 (Mahaffy 2006). Phages in aquatic ecosystems have a high probability of encountering host bacteria due to continual movement in water (Kokjohn et al., 1991). Previous work has shown that the number and behaviour of phages in water environment is influenced by the densities of both host bacteria and phages; the association of phages and bacteria with solids; the presence of organic matter; temperature; pH; ultraviolet and visible light; the concentration and type of ions; and the metabolic activities of microorganisms (Goyal et al., 1987). Bacteriophages can be easily isolated from ocean, river, lake or any other water sources where their hosts exist.

Hence, this study mainly focused on isolation and characterisation of *Shigella flexneri* bacteriophages that have potential to be used for therapeutic application.

The objectives for this project were:

1) To isolate bacteriophages capable of infecting *Shigella flexneri* from environmental water samples.
2) To determine the host specificity of the isolated bacteriophages.
3) To study the genotypic characteristics of isolated bacteriophages via molecular analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Bacteriophage Discovery

Bacteriophage was first discovered by a British bacteriologist, Ernest Hankin in 1896. He reported the presence of bactericidal activity against *Vibrio cholerae* in the water of the Ganges and Jumna rivers in India. He proposed that a heat labile unknown substance which passed through fine porcelain filters was responsible for preventing the spread of cholera epidemics. Two years later, Gamaleya, the Russian bacteriologist observed similar phenomenon while working with *Bacillus subtilis*. Almost 20 years after Hankin's observation, Frederick Twort, a medically trained bacteriologist from England reported similar cases and hypothesised that it was most probably caused by virus. However, Twort did not pursue his discovery due to various reasons such as financial difficulties. Two years later of Twort discovery, Felix d' Herelle, a French Canadian microbiologist at the Pasteur institute in Paris reported the same observation. Felix d' Herelle proposed that it was virus that parasitised bacteria based on the appearance of small, clear zones in the lawn of bacterial culture and the lysis in liquid culture. He was initially named it as *taches,* then *taches vierges* and later *plaques*. Felix d' Herelle officially named it as "bacteriophage" that formed from "bacteria" and "phagein" which means phages "eat" or "devour" bacteria (Sulakvelidze et al., 2001). He called phages as "exogenous agents of immunity" due to their function

as therapeutic and prophylactic agents in eradicating various types of infectious disease (Sulakvelidze et al., 2001; Summers 2005).

2.2 Bacteriophage in General

2.2.1 Morphology of Bacteriophages

Basically, bacteriophages are classified into 1 order, 13 families and 31 genera (Ackermann 2005). The International Committee on the Taxonomy of Viruses (ICTV) classifies the phages into 13 families according to the nature of phage nucleic acid and overall virion morphology traits (Figure 2.1). Most of the characterised phages (95%) are in the order of *Caudovirales* or tailed dsDNA phages. The three main families comprising the *Caudovirales* are distinguished by their distinct tail morphologies: 60% of the phages with long and flexible tails are *Siphoviridae*; 25% with double-layered, contractile tails is known as *Myoviridae*; and 15% with short, stubby tails is called *Podoviridae* (Guttman et al., 2005). Polyhedral, filamentous and pleomorphic phages only comprise less than 4% of the studied phages. They are grouped into 10 families and sometimes include only a single genus and species (Ackermann 2005).

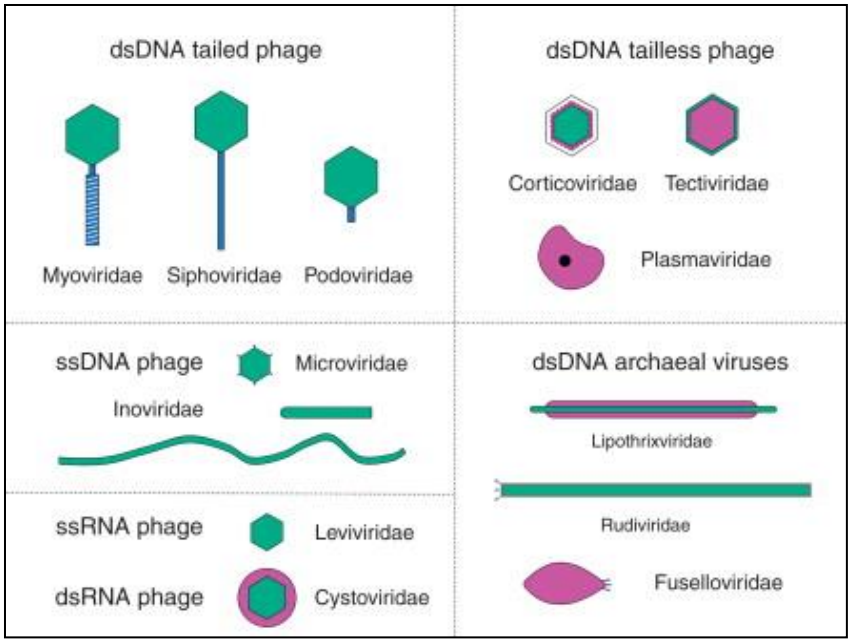

Figure 2.1: Basic morphologies of different families of prokaryote viruses (Adapted from Hyman and Abedon 2009).

All *Caudovirales* have heads with icosahedral (20 sides/12 vertices) symmetry or elongated derivatives that are assembled from multiple copies of specific protein. The size of the phage heads varies in diameter between 45 and 100 nm, depending on the size of phage genome packaged during head assembly. The corners of the icosahedral head are made up of pentamers of capsid proteins, and the remaining sides are made up of hexamers of the same or a similar protein (Guttman et al., 2005).

The portal protein connecting the head and tail structures is important for the infection cycle. This connector which is located at the vertex of the phage head has a homo-oligomeric structure that participates in the morphogenesis of new progeny. This protein controls the entry of the DNA and assembly of the tail to

the immature head. During infection, it undergoes conformational change and allows the penetration of DNA from the viral core into the bacterial cell (Valpuesta et al., 2000).

The tail, base plates, spikes or terminal fibers play an important role in the attachment of host and delivery of the phage DNA from the head into the host cell cytoplasm. Long phage tails may be contractile, as seen in the phage T2 of *Myoviridae* family, or non-contractile, as shown in the phage λ of *Siphoviridae* family. During infection, the overall shape of the non-contractile tail remains unchanged while for contractile tails, the tail sheath contracts, allowing the central tail tube protein (TTP) to penetrate both the outer cell membrane and cell wall (Leiman et al., 2004). The short-tailed *Podoviridae*, for example T7 phage, form an extensible tail from the ejected internal core proteins, providing a channel for the translocation of phage genome into the cell (Molineux 2001). Figure 2.2 shows the morphologies of the three families of *Caudovirales*.

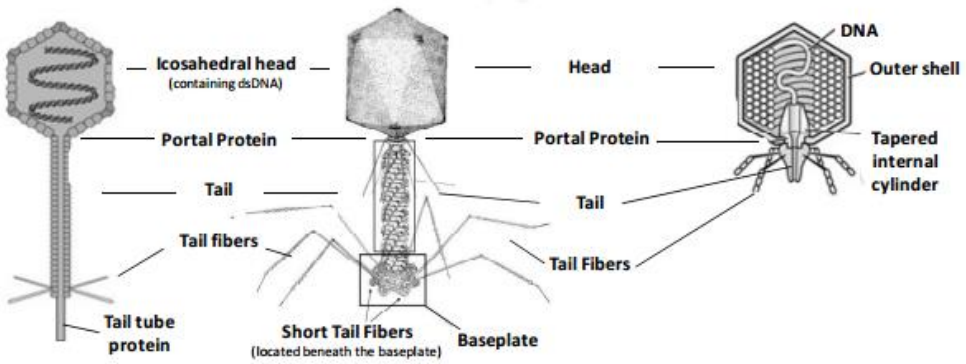

Figure 2.2: Basic morphologies of the three families of *Caudovirales*. From left to right, schematic drawings of *Siphoviridae*, *Myoviridae* and *Podoviridae* (Adapted from Ceyssens 2009).

2.2.2 Genome of Bacteriophages

Majority of the phages contain dsDNA, but there are also small proportion phage groups with ssDNA, ssRNA or dsRNA (Ackermann 2005). Studies done by Abedon (2011) on the differentiation of bacteriophages into four genome size categories: very small (single stranded RNA phages), small (single-stranded DNA phages), medium (lipid-containing, double-stranded DNA, tailless phages) and large (double-stranded DNA, tailed phages). As shown in Figure 2.3, very small phages belong to the members of family *Leviridae* with genome size that range in 3.5 to 4 kb. Small phages, have genomes that are slightly larger than the members of family *Leviridae*, which range from 4.5 kb up to about 9 kb. Medium-sized phages have genomes that range in size from approximately 9 to 15 kb. At last, the larger genome tailed phages, members of virus order *Caudovirales*, have genomes with 16 kb or greater. Phages with larger genome size have higher gene number, greater infectivity and virion sophistication, higher possibility of gene acquisition through horizontal gene transfer as well as additional genetic redundancy both within and between genomes (Abedon 2011).

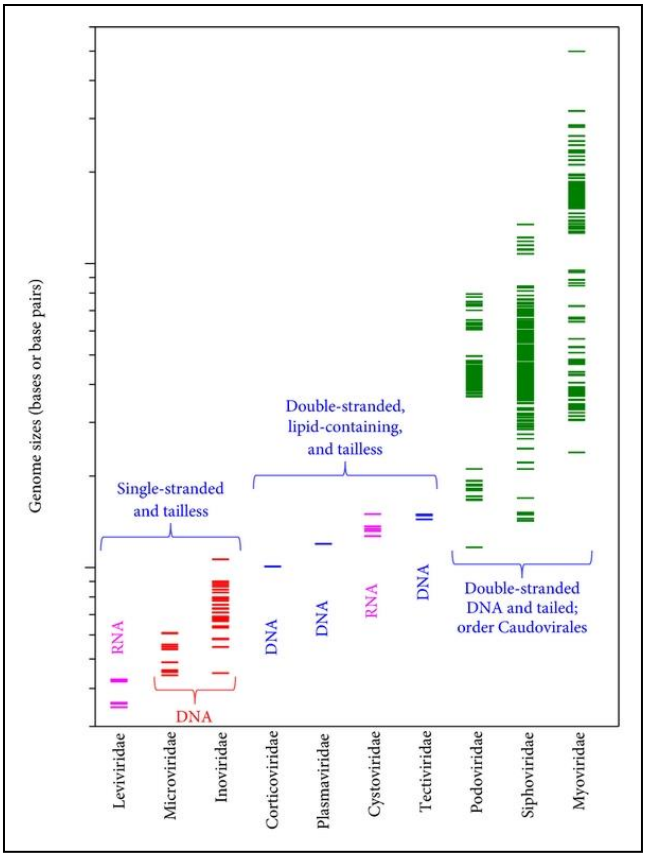

Figure 2.3: Phage morphologies and genome sizes (Adapted from Hyman and Abedon 2012).

2.2.3 Life Cycle of Bacteriophages

Bacteriophages may undergo lytic (virulent) cycle or lysogenic (temperate) cycle. Lysis of the infected host bacterial cell is caused by two mechanisms: lysis from within and lysis from without (European Food Safety Authority [EFSA], 2009). For the mechanism of lysis from within, lysis of the host cell is generally due to the phage replication. The phage virion adsorbs to the surface of a host bacterial cell and inserts its genetic material into the host cell by injection with contractile tails or forming pore on the cell wall. When it has successfully entered the cell,

the phage undergoes replication process with the aid of host biosynthetic machinery. The new copies of phage progeny are then liberated with the help of holin, a hydrophobic polypeptide that creates pores in the cell membrane, which causes lysis to the host cell. For lysis from without, the host cell lysed without phage replication process. This occurs through changes in the membrane electric potential and activity of wall degrading enzymes with large number of phage particles attach to the cell (EFSA, 2009).

Another alternative method of bacteriophage propagation is lysogenic cycle. In this process, progeny phages are not produced and the bacterial host cell is not lysed. This is because the phage is able to produce a repressor protein that stops the synthesis of phage enzymes and proteins needed in lytic cycle. If the repressor protein stops producing or is inactivated, an enzyme encoded by the prophage will excise the viral DNA from bacterial chromosome. The excised DNA will enter the lytic cycle that causes death in bacterial cell (Todar 2012). Additionally, the repressor also protects the host bacterium from infection by other phages (Guttman et al., 2005). In the presence of repressor protein, bacteriophage DNA is incorporated into the host chromosome via site-specific recombination but sometimes maintained as a plasmid. This prophage remains in this condition indefinitely and replicates together with host chromosome whenever the cell duplicates its chromosomal DNA during normal cell division (Guttman et al., 2005; Todar 2012). Lysogenic conversion frequently occurred in lysogenised cell where the host cell may exhibit new characteristics such as virulence factors and

antibiotic resistance when the temperate bacteriophages harbour other genes that are expressed during lysogeny (Guttman et al., 2005; EFSA, 2009). Previous studies showed that phage gamma and C_1 encode the virulence factors of *Corynebacterium diphtheria* and *Clostridium botulinum*, causing the host to possess pathogenic phenotype (Freeman 1951; Barksdale and Arden 1974). Figure 2.4 shows the replication cycles of lytic and lysogenic bacteriophages.

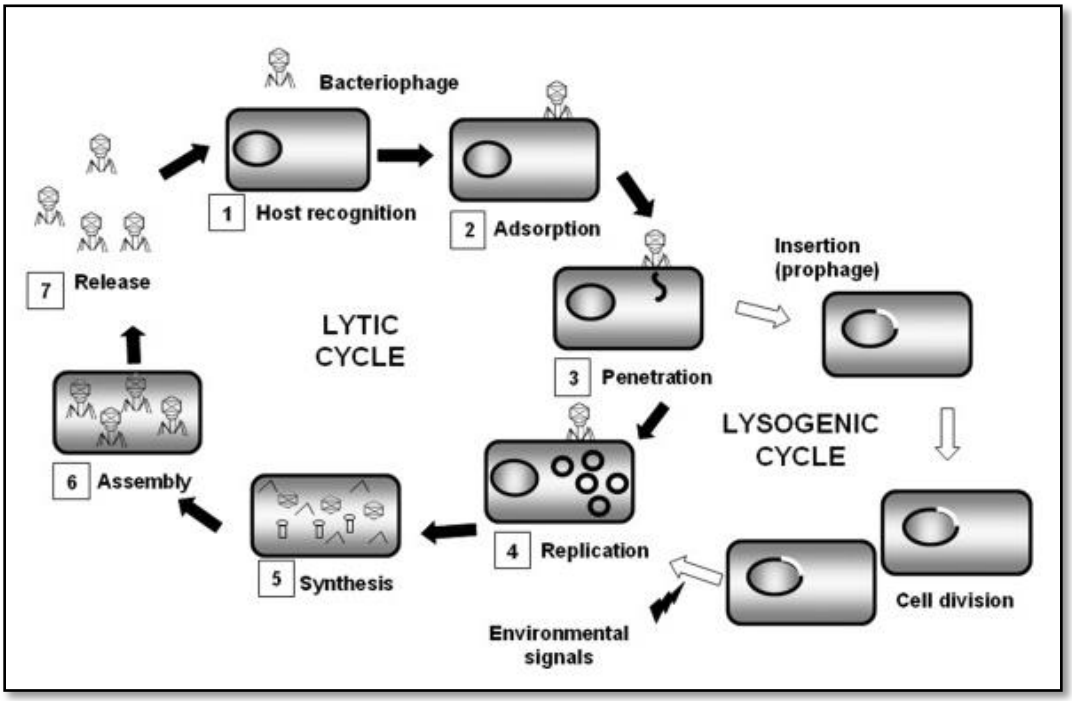

Figure 2.4: Replication cycles of lytic and lysogenic bacteriophages (Adapted from García et al., 2010).

2.3 Phage Ecology

Phages are the most abundant life form on earth and estimated to be in the range of 10^{30} to 10^{32} (Brüssow and Kutter 2005). A wide variety of phages can be found

in water environments (Goyal et al., 1987). Random sequencing of viral DNA had estimated 400 to 7000 various phage types to be contained in two uncultured 100-litre marine water (Breitbart et al., 2002). Besides, phages have been isolated from mammalian faeces (O'Flynn et al., 2004) and environmental sources including soil (Ashelford et al., 2003), water (Bergh et al., 1989) and sewage sludge (Carey-Smith et al., 2006; Oliveira et al., 2009).

The production and distribution of phage are dependent on host concentration. The phage populations can grow faster when there is greater density of susceptible bacteria. Generally, the virus-to-bacterium ratio falls between 3 and 10, depending on the nutrient level (Brüssow and Kutter 2005). The consequence of phage lysis not only reduces the productivity of bacterial populations, but also delays the ecosystem nutrient cycling and energy flow (Abedon 2006).

Studies by Jensen et al. (2006) showed the correlation of populations of bacteria and phage with cholera outbreak. They proposed that the severity of the outbreak is determined by the density of phage in the reservoir. Increase of phage predation will reduce the bacterial density, which ultimately ends the outbreak by returning the bacterial populations to pre-outbreak stage. Therefore, outbreak occurs when there is reduction of phage density. In short, introduction of vibriophage can reduce the duration and severity of cholera outbreak.

The 4 key steps in the phage lytic life cycle are correlated to the phage ecology. Firstly, the extracellular search for host bacteria is limited by diffusion rates and determined by the density of the host populations. Secondly, a phage adsorption step occurs after phage-host collision, which involves reversible phage binding, irreversible attachment and transfer of genome into the host. Thirdly, the infection step where the phage genome is replicated and phage particles are synthesised, resulting the production of phage progeny. Lastly, for temperate phage, the phage DNA is integrated into the host chromosome and replicates with it or replicates synchronously as a plasmid (Brüssow and Kutter 2005).

2.4 Applications of Bacteriophages

2.4.1 Phage display

The development of phage display enables the synthesis of novel polypeptides with high binding affinity to a particular substrate. In this system, the DNA that encodes the polypeptide is cloned as gene fusions with the phage coat protein genes. The desired protein is then incorporated into the mature phage particle and expressed on the surface of the phage (Rees and Loessner 2005). Libraries of phage particles can also be used for the screening and identification of clones expressing peptides that are highly specific and possess desired binding characteristics (Rees and Loessner 2005; Haq 2012).

This approach has been extensively used for medical or pharmaceutical applications. Phage display has led to the revolution of phage antibody technology where phages were used to display antibody molecule with specific antigen-binding domain that can be used for affinity selection process (Rader and Barbas 1997). This antibody-derived peptide has been successfully developed as therapeutic agent that serves the function of agonist and antagonist in receptor-ligand interaction. Research work done by Dickerson et al. (2005) had showed the effectiveness of this method in the treatment of cocaine addiction where bacteriophage displaying cocaine-sequestering antibodies was used to block the action of cocaine in the brain.

Furthermore, phage display can be applied for detection assay to detect for the biological threat agents. This assay utilises phage-borne peptide as diagnostic detector or probe that specifically binds to bacterium, spore, virus and toxin. This technique has proved to be successful in the identification of various types of viruses (Petrenko and Vodyanoy 2003), detection of *Bacillus* spores (Zhou et al., 2002) and differentiation of *Candida* species in clinical samples (Bliss et al., 2003).

2.4.2 Phage Typing

Phage typing is a procedure for characterising and identifying bacterial strains by their reaction (susceptibility or resistance) to various known strains of phages. It

is a relatively rapid, simple and inexpensive method for the typing of bacteria (Hagens and Loessner 2007). The host specificity of phages is a useful tool for the classification of bacteria and the detection of pathogenic bacteria (Clark and March 2006). Many phages are highly specific for the receptors on host cell surface and only receptors with similar structure and configuration can interact with the respective phage.

Phage typing is easy and convenient to be performed with large number of bacterial isolates can be analysed at the same time. For example, *Escherichia coli* O157:H7 has been successfully subdivided into 66 different phage types (Frost et al., 1993). Phages are not only specific towards species of bacteria, but also strains of bacteria, permitting typing beyond the level of species (Welkos et al., 1974). According to the work done by Pruneda and Farmer (1977), phage typing method appears to be more sensitive than colicin typing and antibiograms in differentiating bacterial strains.

Susceptibility to infection by a particular phage enables the phenotypic differentiation of strains and identification of the strain that causes an outbreak of disease. This property can also be employed for epidemiological investigations to trace the causative agent responsible for the infection. Faruque et al. (2003) had successfully isolated phage SF-9 that has epidemiological applications in tracing and monitoring the presence of *Shigella dysenteriae* type 1 from environmental waters in Bangladesh. This method has proved to be useful in predicting

outbreaks and the spread of shigellosis, which occurs as epidemics in many developing countries. Besides, this system provides reliable, sensitive and fast results for epidemiologists in the surveillance of outbreaks (Pruneda and Farmer 1977).

2.4.3 Phage Therapy

Phage therapy is the therapeutic use of bacteriophages to treat pathogenic bacterial infections. Lytic phages are preferred for the biocontrol of pathogenic bacteria since they are highly specific and very effective in lysing targeted pathogenic bacteria. Furthermore, lytic phages do not contain integrase genes on their genomes, therefore, they are unable to coexist with the host and carry virulent genes from one host to another (Brüssow 2005).

Phage therapy is an alternative for the antibiotics treatment of bacterial infection. Unlike broad-spectrum antibiotics, phages are specific to their prokaryotic host cell and less commonly illicit resistance in non-host cells (Sulakvelidze and Kutter 2005). Additionally, phages are not susceptible to the onset of bacterial resistance due to its ability to evolve with the host (Sulakvelidze and Kutter 2005). Moreover, phage therapy is not affected by antibiotic resistance of bacteria where it is effective for the treatment of multidrug resistant strains infection. It was showed that *Shigella* phages were successfully used as a prophylaxis of bacterial dysentery (Babalova et al., 1968). Anpilov and Prokudin (1984) reported

that the incidence of dysentery in the phage-treated group was ten fold less than that occurring in phage-untreated group.

Phage therapy has more advantages compared to antibiotics that are in clinical use due to its specificity towards targeted bacteria. In addition, this therapy is harmless to the eukaryotic host undergoing therapy and beneficial bacteria such as normal flora in gut, so, reduce the chances of opportunistic infections (Carlton 1999). Another advantage is that bacteriophage usually replicates at the site of infection and they are self-limiting. They are only able to self-replicate with the presence of susceptible bacterial pathogens (Carlton 1999).

Humans are continuously exposed to bacteriophage because phages are the simplest and most abundant organisms on earth that present as part of both gastrointestinal and environmental ecosystems (Carlton 1999; Sulakvelidze et al., 2001). Hence, it is suggested that phage therapy is well tolerated by humans, providing little or no side effects to human (Hausler 2007; Sulakvelidze et al., 2001).

Phages having lytic activity against a wide range of bacterial strains are preferred to be used as therapeutic agent (Verma et al., 2013). These wide host range bacteriophages are potential candidate for phage therapy due to their broad antimicrobial range which could potentially treat a wide range of infections. However, precautions need to be taken to avoid cross-interaction and lysis of

normal microbiota. Besides, amplification of bacteriophages in non-pathogenic alternative host bacteria improves the safety of bacteriophage application in treatment of infections (Bielke et al, 2007).

2.5 *Shigella flexneri*

Shigella flexneri is highly infectious and causes communicable bacterial diarrhoeas, shigellosis. This disease is commonly transmitted via faecal-oral route. Dose response studies done at the University of Maryland have shown that *S. flexneri* needs as few as 100 cells to cause disease in adult volunteers (DuPont 1989). This phenomenon is associated with the ability of *S. flexneri* to express acid resistance genes that enable this enteric pathogen to survive the acidic environment of the human stomach (Small et al., 1994; Jennison and Verma 2007).

When *S. flexneri* invades the colonic mucosa of humans, it begins to multiply intracellularly, spread from cell to cell and disseminate throughout the mucosal epithelial cells. The inflammatory response of the host subsequently destroys the colonic epithelial layer, causing abscesses and ulceration in the superficial layer of the colonic mucosa. Damage of the epithelial layer produces the clinical symptoms of shigellosis ranging from watery diarrhoea to classic dysentery characterised by fever, violent intestinal cramps and eventually discharge of

mucopurulent and bloody stools characteristic of bacillary dysentery (Philpott et al., 2000).

Numerous virulence genes have been identified in *Shigella flexneri*, which termed as pathogenicity islands (PAIs). PAIs are only present in pathogenic strains and absent or limited distribution in less pathogenic strains. Three chromosomal PAIs have been located on the *S. flexneri* chromosome, participate in the pathogenic process directly or contribute to survival in the environments encountered during infection. These three PAIs are involved in virulence such as enterotoxin, aerobactin operon and *Shigella* resistance locus (SRL) encoding resistance determinants to streptomycin, ampicillin, chloramphenicol and tetracycline (Moss 1999; Al-Hasani et al., 2001; Turner 2001). The genome of *S. flexneri* serotype 2a, the most prevalent serotype that causes bacillary dysentery, has found to contain a number of bacteriophage-related genes conferred by temperate bacteriophages, which responsible for the O-antigen modification in *S. flexneri* (Allison and Verma 2000).

Study done by Mandal et al. (2012) highlighted the increasing antimicrobial resistance in *Shigella flexneri* in recent years. Ciprofloxacin has been recommended by WHO as the drug of choice for effective treatment of multidrug-resistant strains of *Shigella*. However, as shown in Figure 2.5, *S. flexneri* is gaining resistance towards this drug. Unfortunately, the alternative drugs such as pivmecillinam, ceftriaxone and azithromycin are limited by their high cost, rapid

development of resistance, their formulation and limited data on efficacy (World Health Organization [WHO], 2005). There is also no vaccine available that can protect human from the many different serotypes of *Shigella* (Wei et al., 2003). Hence, the emergence of anti-microbial resistance has limited the options for the therapy.

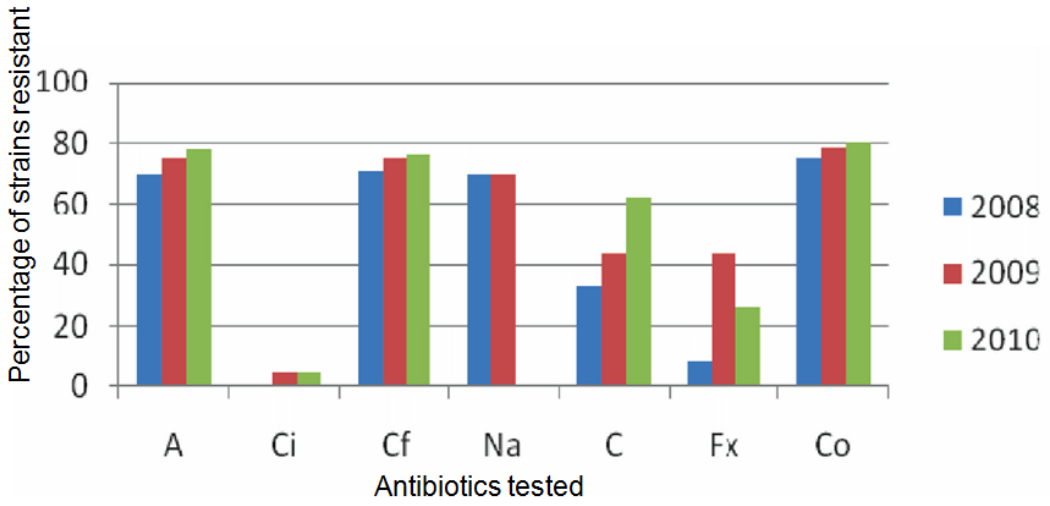

Figure 2.5: Resistance pattern in *Shigella flexneri* from 2008 to 2010. A-ampicillin, Ci-ceftriaxone, Cf-ciprofloxacin, Na-nalidixic acid, C-chloramphenicol, Fx-furoxone, Co-cotrimoxzole (Adapted from Mandal et al., 2012).

2.6 Epidemiological study of *Shigella flexneri* infection

World Health Organization (WHO) reported that the *Shigella* episodes occur in an estimated 164.7 million people throughout the world annually, where 163.2 million cases happen in developing countries (with 1.1 million cases leading to death) and the remaining 1.5 million occur in industrialised countries. 61% of all

deaths associated to shigellosis involved children less than 5 years of age (Kotloff et al., 1999). As shown in Figure 2.6, the four species of *Shigella* differ epidemiologically. *S. flexneri* is the most commonly detected strain in Asia-Africa, with *S. sonnei* being the next most common. Both *S. dysentriae* and *S. boydii* occur equally frequently. On the other hand, *S. sonnei* is seen most often in industrialised countries in Europe-America, followed by *S. flexneri*, *S. boydii* and lastly, *S. dysentriae* (Gu et al., 2012).

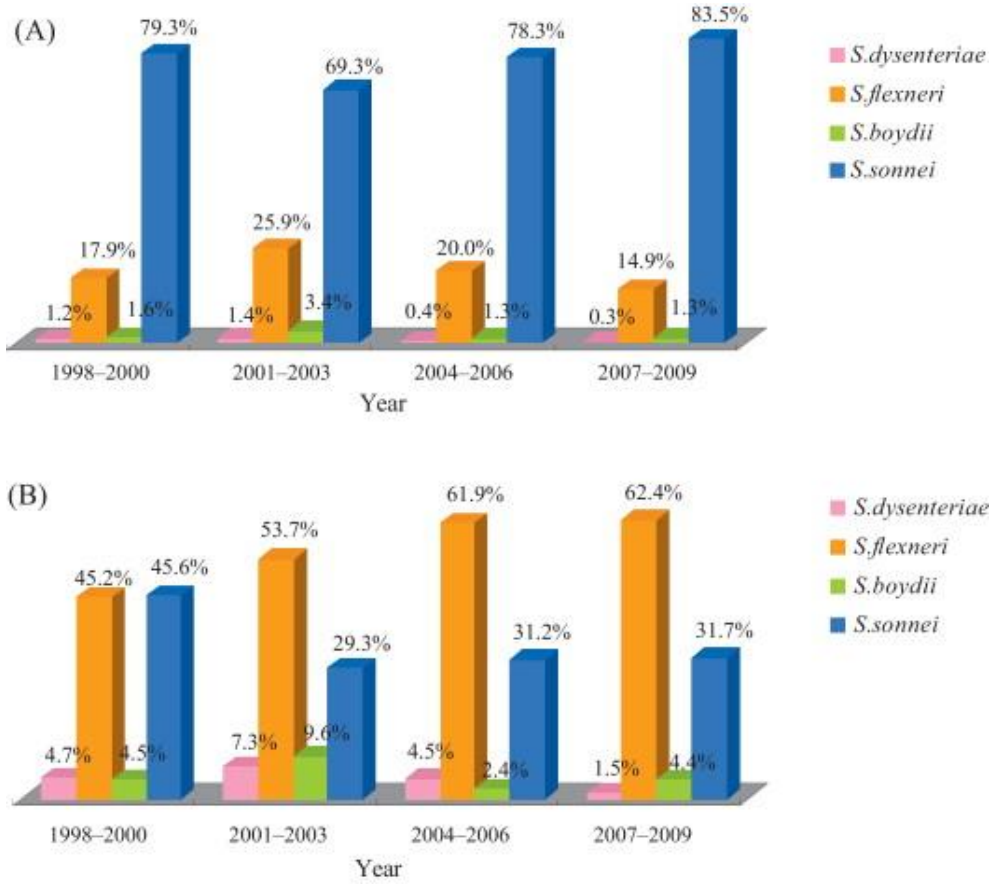

Figure 2.6: Incidence of different subtypes of *Shigella* from 1998 to 2009 in (A) Europe-America and (B) Asia-Africa (Adapted from Gu et al., 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

This study included 8 clinical strains of bacteria (*Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, enterotoxigenic and enteropathogenic *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia*). All of them were clinical isolates from Hospital Universiti Science Malaysia (HUSM). The bacteria were grown on Luria Bertani (LB) agar pH 7.0 by streaking and incubating at 37^{o}C for 16-18 h. The plates were stored at 4^{o}C after incubation. Exponential growing bacteria culture was used in all the tests in this study. This was done by inoculating one colony of bacteria in LB broth and incubated at 37^{o}C with constant agitation (200 rpm) until mid-log phase (OD_{600} = 0.4-0.6) was reached.

3.2 Chemicals, Reagents and Equipments

All the chemicals and reagents used in this study are listed in Table 3.1.

Table 3.1: List of chemicals and reagents.

Chemicals/Reagents	**Manufacturers**
100bp DNA ladder	SOLIS BIODYNE
1 kbp DNA ladder	NEW ENGLAND BioLabs Inc.
95% ethanol	Copers Scientific
100% isopropanol	Merck Millipore
Agarose powder	1st BASE
Blue 6X loading dye	Promega
Bovine Serum Albumin (BSA)	BIORON
Ethidium bromide	BIO BASIC INC.
Luria Bertani agar	Merck Millipore
Luria Bertani broth	CONDA
Phenol: chloroform: isoamyl alcohol (25:24:1)	AMRESCO
Phosphate Buffered Saline (PBS) tablets	MP Biomedicals
Polyethylene Glycol (PEG) 6000	BIO BASIC INC.
Restriction enzymes (*Bam*HI, *Eco*RI, *Not*I, *Sal*I, *Xba*I)	BIORON
*Sac*I restriction enzyme	Thermo Scientific
Sodium acetate	SYSTERM
Sodium chloride (NaCI)	Merck Millipore

All the equipments and laboratory wares used in this study are listed in Table 3.2.

Table 3.2: List of equipments and laboratory wares.

Instruments /Equipments	**Brand/Model**
Autoclave machine	HIRAYAMA
Cellulose acetate syringe filter (0.45 µM)	GVS Filter Technology
Cotton Swab	Premier Diagnostics
Microcentrifuge tubes	AXYGEN Scientific
Conical tube (50 mL and 15 mL)	BD Bioscience
Filter paper	Whatman
High Speed Benchtop Centrifuge	Sartorius Sigma
Incubator	MEMMERT
Laboratory oven	MEMMERT
Laminar flow cabinet	ESCO
Microcentrifuge	SIGMA
Micropipette	Labmate
Microwave	SANYO
Petri dishes	Greiner bio-one
Portable Bunsen burner	CAMPINGAZ® LABOGAZ 206
Refrigerator and Freezer	Haier
Media bottle	DURAN
Shaking incubator	Labnet International
Spectrophotometer	Bio-Rad

Table 3.2: List of equipments and laboratory wares (continued).

Instruments/Equipments	Brand/Model
Spectrophotometer cuvette	Bio-Rad
Syringe	TERUMO (PHILIPPINES) CORPORATION
UV transilluminator	SYNGENE
Water bath	MEMMERT
Weighing balance	Sartorius

3.3 Preparation of Culture Media, Reagents and Solutions

3.3.1 Preparation of LB agar

LB agar was prepared by weighing 37.0 g of LB agar powder and added into 1 L of media bottle and topped up to 1 L with deionised water. The mixture was mixed well and autoclaved at 121^{o}C for 15 min. Medium was then poured into sterile petri dish and left to solidify at room temperature.

3.3.2 Preparation of LB broth

LB broth was prepared by weighing 25 g of LB broth powder and added into 1 L of media bottle and topped up to 1 L with deionised water. The mixture was then

mixed well and autoclaved at 121^{o}C for 15 min. The medium was allowed to cool down at room temperature.

3.3.3 TBE buffer (pH 8.3)

Tris-Borate-EDTA buffer was prepared by mixing 108 g of Tris Base and 55 g of boric acid in 900 mL of deionised water. Then, 40 mL of EDTA (0.5 M) was poured into the mixture. The mixture was measured and adjusted to pH 8.3 with 1 M NaOH and 1 M HCI. The solution was subsequently topped up to 1 L to prepare the solution of 10X TBE buffer. To prepare working solution of 1X TBE buffer, 100 mL of TBE buffer was diluted with 900 mL of deionised water.

3.3.4 Agarose gel (1.0%)

0.20 g of agarose powder was dissolved in 20 mL of 1X TBE buffer by microwave heating. The melted agarose was poured into the cassett and left to solidify at room temperature.

3.3.5 Ethanol (70%)

737 mL of 95% ethanol was topped up to 1 L with deionised water to prepare 70% ethanol.

3.3.6 Mixture of 20% PEG 6000 and 10% NaCI

10 g of PEG 6000 and 5 g of NaCI were dissolved in 50 mL of deionised water. The mixture was then filter sterilised.

3.3.7 Phosphate Buffered Saline (PBS)

Phosphate buffered saline was prepared by dissolving 1 tablet of PBS in 100 mL of deionised water and sent to autoclaved at 121^{o}C for 15 min..

3.3.8 Sodium Acetate (3 M)

Sodium acetate was prepared by dissolving 12.3 g of sodium acetate powder in 50 mL of deionised water and sent to autoclaved at 121^{o}C for 15 min.

3.4 Methods

3.4.1 Overview of Research Methodology

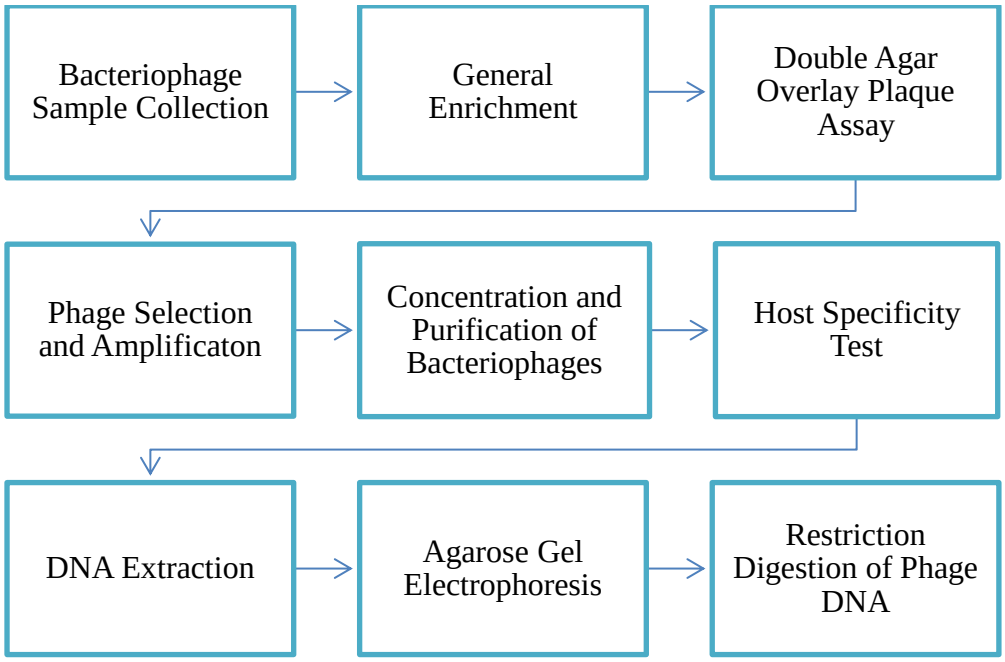

Figure 3.1: Overall work flow.

3.4.2 Isolation of Bacteriophages

3.4.2.1 Bacteriophage Sample Collection

Water samples were collected from Kampar area including rivers, drains, ponds and lakes. These samples were collected in sterile 50 mL conical tubes. The water at collection site was mixed thoroughly and the sediments were collected together with the overlying water.

3.4.2.2 General Enrichment

A procedure of bacteriophage enrichment method was performed on the collected samples (Twest and Kropinski 2009). The samples were centrifuged at 6000 rpm for 10 min to remove large particulates and bacteria. 40 mL of the supernatant were decanted into a new sterile conical tube. The tubes were inoculated with 1 mL of broth culture of *Shigella flexneri* host bacterium (OD_{600} = 0.4-0.6) and mixed thoroughly by inversion. The tubes were incubated at 37^{o}C in static condition to allow specific phage enrichment. After 24 h incubation, the contents of the tubes were centrifuged at 9,000 rpm for 10 min. The supernatant of the water sample was filtered slowly through the 0.45 µM cellulose acetate syringe filter to a 1.5 mL microcentrifuge tube. The filtered samples were then centrifuged at 14,000 x*g* for 15 min. The supernatant were carefully transferred to a new sterile tube and stored at 4^{o}C.

3.4.2.3 Double Agar Overlay Plaque Assay

This assay was adapted from a modified procedure of the double agar layered method (Kropinski et al., 2009). A row of 4 sterile microcentrifuge tubes was set up. Each of them was numbered with the appropriate sequential 100-fold dilutions from 100 X to 100 M. 990 µL of Luria Bertani (LB) broth that serves as diluents was added aseptically to each tube. 10 µL of undiluted phage was added to the first tube and mixed well. 10 µL of broth was then transferred from first tube to the second tube in the series. No phage was added to the control tube. Only the undiluted, third and fourth phage preparations (1 M and 100 M dilutions respectively) were tested for plaque assay. A plate of bacterial control without phage was prepared as negative control.

The *Shigella flexneri* host bacterium was grown in 5 mL of LB broth with shaking at 200 rpm, 37^{o}C until it reached log phase (OD_{600} = 0.4-0.6). LB bottom agar plates containing 1.5% agar were prepared.

Two millilitre of log phase *Shigella flexneri* was transferred into a sterile 15 mL conical tube. 200 µL of the selected dilution of phages was added immediately. The tubes were preincubated at 37^{o}C for 20 min to allow phage adsorption onto host bacterium. After preincubation, the tubes were added with 2 mL of molten (45^{o}C) soft agar (0.75%) that was previously prepared from 1.5% of LB agar. The tubes were gently mixed by inversion for a few times and the contents were poured on Petri dishes containing LB bottom agar. The plates were swirled in

circles to spread the mixture evenly over the plates. The plates were left at room temperature for 10-15 min until the soft agar has solidified and the Petri dishes were incubated at 37^{o}C for 16-18 h. The following day, the plates were checked and observed for the plaque formation. The plaques formed in each plate were enumerated.

3.4.3 Amplification and Purification of Bacteriophages

3.4.3.1 Phage Selection and Amplification

The exponential growing culture of *Shigella flexneri* was inoculated into a microcentrifuge tube. Phage plaques were selected based on size and clarity using a 100 µL pipette. The pipette tip was inserted carefully into centre of a discrete plaque and a plug of soft agar containing the bacteriophages was removed out. The phage-containing plug was added into the inoculated tube. The tubes were incubated at 37^{o}C for 24 h or more. The phage suspension was centrifuged at 14,000 x*g* for 15 min. The supernatant was transferred to fresh, sterile microcentrifuge tube for further analysis.

3.4.3.2 Concentration and Purification of Bacteriophages

The phages were concentrated and purified based on the precipitation with polyethylene glycol (PEG) 6000 (Boulanger 2009). The *Shigella flexneri* host bacterium was cultured in 50 mL of LB broth with constant agitation (200 rpm) at

37^{o}C until OD_{600} of 0.4–0.6. The isolated phage was then added into the bacteria culture and agitated overnight at 37^{o}C, 200 rpm. The bacteria-bacteriophage mixture was centrifuged at 9,000 rpm for 15 min to remove bacterial debris. 40 mL of phage-containing supernatant was transferred into a new 50 mL conical tube and 10 mL of the PEG/NaCI was added. The mixture was mixed by inversion and incubated on ice for at least 1 h in order to precipitate phage particles. The precipitated phage was collected by centrifugation at 9,000 rpm, 4^{o}C for 30 min and the supernatant was carefully discarded. The conical tube was turned over to drain away the remaining fluid from the pellet for 5 min. The phage pellet was resuspended in 500 µL phosphate buffered saline (PBS) and transferred to a sterile microcentrifuge tube. The phage particles were separated from co-precipitated bacterial debris by centrifugation at 11,600 x*g* for 10 min. The supernatant was then collected into a sterile microcentrifuge tube.

3.4.4 Host Specificity Test

Bacterial susceptibility to bacteriophage was assayed based on the spot test method (Raya and Hébert 2009) with modification. The host ranges of the isolated phages were determined using *Shigella sonnei*, *Shigella dysenteriae*, *Shigella flexneri*, enterotoxigenic and enteropathogenic *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia*. Briefly, the bacteria strains were cultured in LB broth at 37^{o}C with constant agitation (200 rpm). A sterile swab was moistened with the indicator cells and spread on the surface of LB agar.

The plate was previously marked with grids to allow identification of each inoculum. A sterile filter paper disk was placed on each grid and 10 µL of each phage suspension was spotted on the disk, dried and incubated for 16-18 h at 37^{o}C. The host range of phages was determined based on susceptibility of bacterial strains by observing lytic zone formed in inoculation area.

3.4.5 Molecular Characterisation

3.4.5.1 DNA Extraction

Extraction of nucleic acid was conducted according to the method of Sambrook and Russell (2001) with minor modifications. The DNA of phage particles were extracted by mixing 500 µL of the purified phage with 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1). The mixture was then centrifuged for 10 min at 14,000 x*g* to separate the phases. The top aqueous phase was transferred to a new fresh 1.5 mL microcentrifuge tube and 500 µL of 100% cold isopropanol and 50 µL of 3 M sodium acetate were added into it. The DNA was left to precipitate at room temperature for 20 min. After incubation at room temperature, they were centrifuged at 14,000 x*g* for 15 min at 4^{o}C. The DNA pellet was washed twice with 70% ethanol and air dried. The DNA was resuspended with 50 µL distilled water and stored at -20^{o}C.

3.4.5.2 Agarose Gel Electrophoresis

The extracted phage genomic DNA was analysed using agarose gel electrophoresis. Three microlitre of the DNA was mixed with 0.6 µL of Blue 6x Loading Dye and loaded into wells of a 1.0% agarose gel in 1X Tris-Borate-EDTA (TBE). The gel was subjected to electrophoresis at 80 V for 45 min until the dye front was near to the bottom of the gel. After electrophoresis, the gel was stained in 1 µg/mL of ethidium bromide for 10 min, followed by destaining with distilled water for a few seconds. The gel was then viewed using UV transilluminator to visualise the presence of DNA bands.

3.4.5.3 Restriction Digestion of Phage DNA

Six restriction enzymes were used to digest phage genomic DNA. The chosen enzymes were *Bam*HI, *Eco*RI, *Not*I, *Sac*I, *Sal*I and *Xba*I. The reaction mixture was prepared (Table 3.3) and mixed gently before allowed for incubation at 37^{o}C for 3 h. The restriction enzyme was then inactivated (Table 3.4) by incubating the reaction mixture in water bath. The digested DNA was separated by agarose gel electrophoresis process. The restriction patterns were visualised by transillumination with UV light after staining with ethidium bromide.

Table 3.3: Restriction enzyme digestion reaction for phage DNA.

Components	**per reaction**
Phage DNA	5.0 µL
Restriction enzyme	0.5 µL
Restriction enzyme buffer	1.0 µL
BSA	0.1 µL
PCR water	3.4 µL
Total	10.0 µL

Table 3.4: Heat inactivation of restriction enzymes used in this study (Adapted from Thermo Scientific, 2012; BIORON, 2013).

Restriction enzymes	**Temperature (^{o}C)**	**Time (min)**
*Bam*HI	80	20
*Eco*RI	65	20
*Not*I	65	20
*Sac*I	65	20
*Sal*I	65	20
*Xba*I	65	20

CHAPTER 4

RESULTS

4.1 Isolation of Bacteriophages

4.1.1 Sample Collection Sites

Sample A was collected from the river that was polluted by solid waste disposal which was flowing freely. The water sample collected was clear, with some sediment present. The river for sample collection site B was shallower as compared to site A and the river was flowing in slow motion. The water appeared to be clean and clear from sediments. The water sample from sample collection site C which was a drain was also clear with little sediment. The water in the drain was not flowing and was in static condition. Water sample D which was from a pond was greenish in colour with abundant amount of algae floating on the surface of water. The water was not flowing due to the confined area. The water in sampling site E which was from the lake was calm and stagnant. A large amount of algae was also present in this sample. The water in site E appeared to be the most turbid among all the samples collected. The sites of all the sample collection are shown in Figure 4.1.

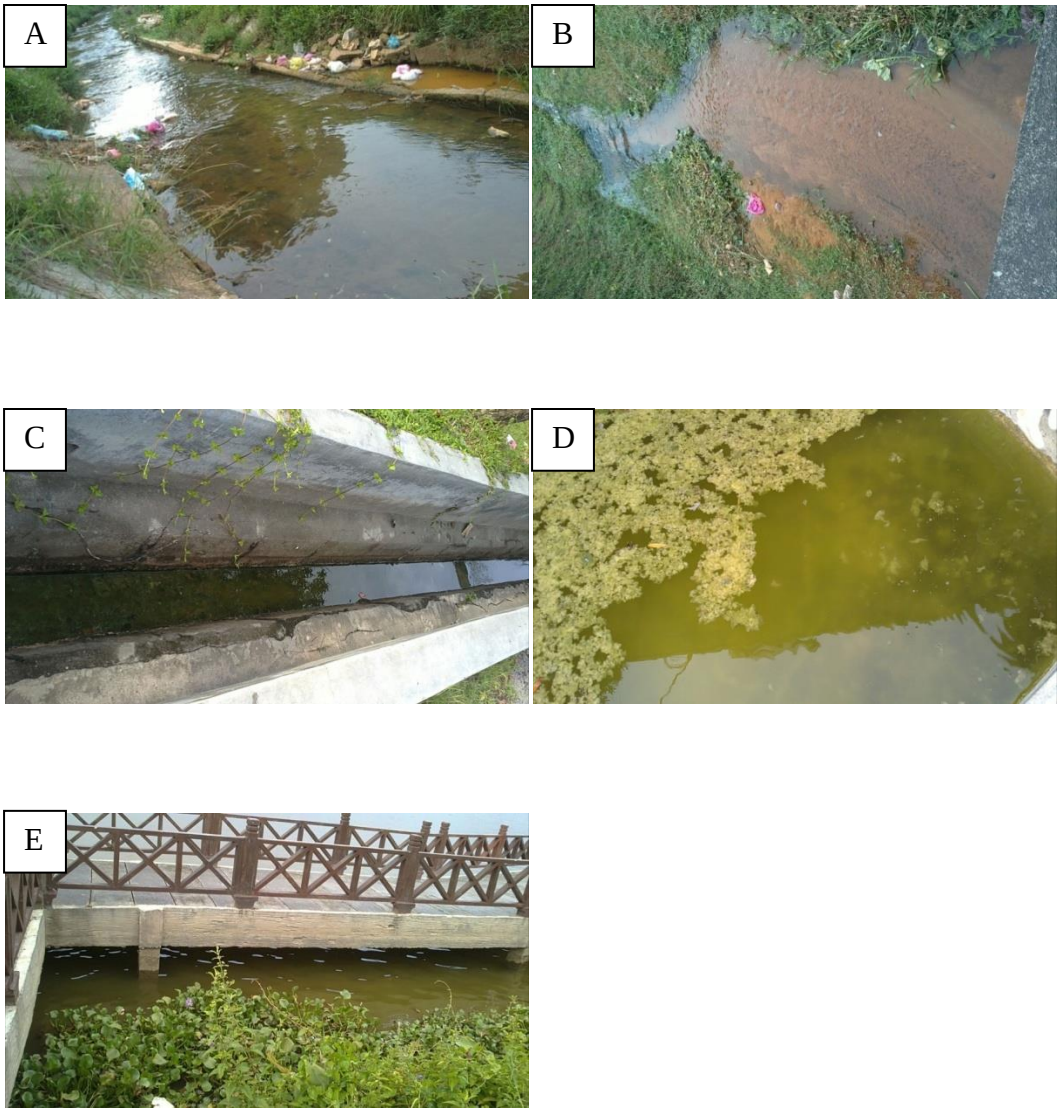

Figure 4.1: Sample collection sites in Kampar area. (A) River near to Old Town residential area, (B) River connecting Old Town and New Town, (C) Drain beside KTAR, (D) Pond outside Station A, and (E) Westlake.

4.1.2 Double Agar Overlay Plaque Assay

All the plates showed positive for the isolation of bacteriophages. Round, clear and transparent plaques were observed in all plates, indicating that the samples collected from different sites contained bacteriophages that were infectious against *Shigella flexneri*. The phages produced clear, medium-sized (1.0-3.0 mm in diameter) plaques with well-defined edges in bacterial lawn, showing that the isolated phages have lytic effect against *Shigella flexneri*. Different types of bacteriophages were found in each plate based on the formation of plaques with different degree of transparency and sizes. The plaque count in sample collection sites A and B were 22 and 17 respectively while the plaques produced in C, D and E were too numerous to be enumerated. These different morphologies of plaques were isolated from each plate for further study. Figure 4.2 shows the plaques formation for water samples A, B, C, D and E. Table 4.1 shows the size and morphology of the isolated plaques.

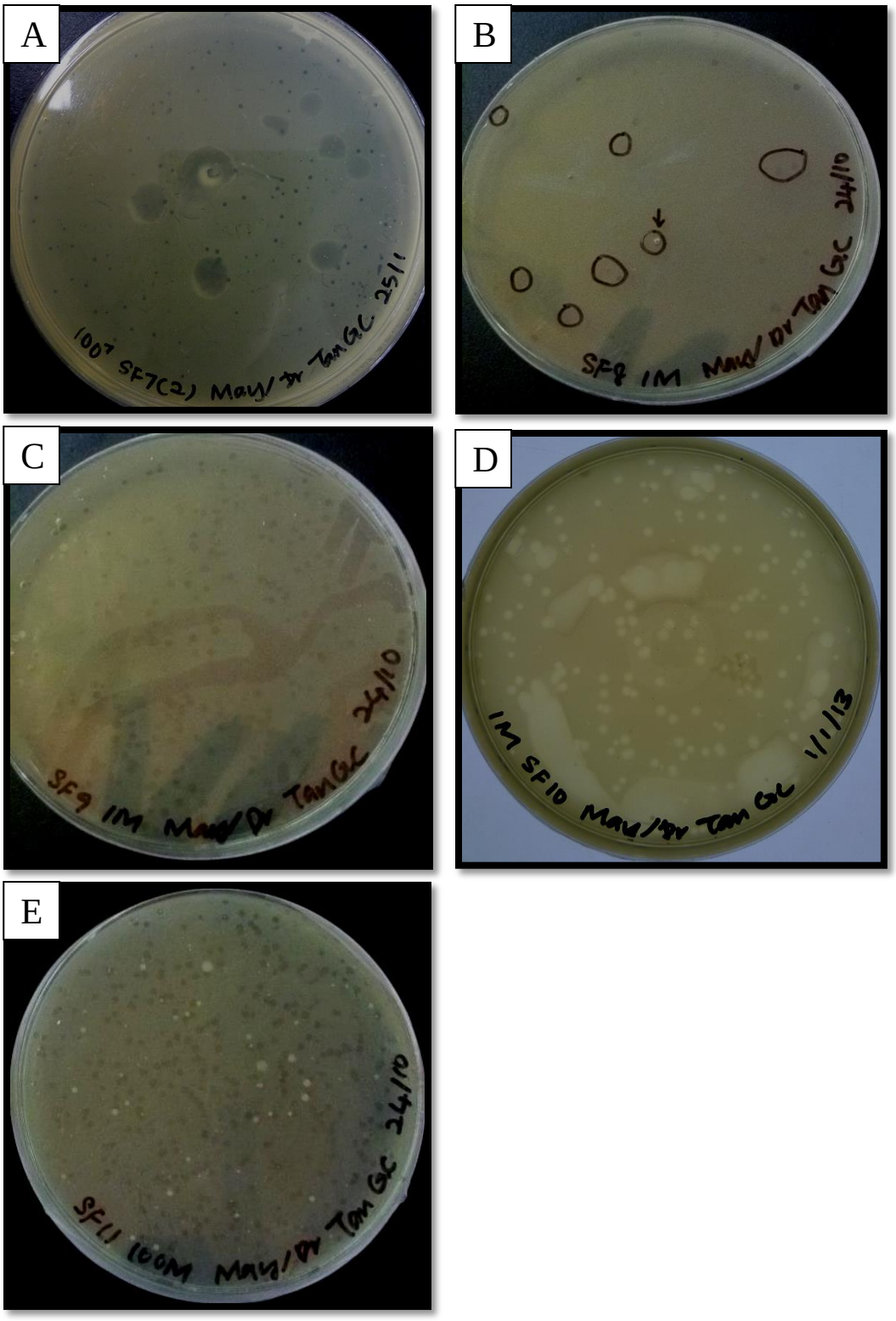

Figure 4.2: Plaques formation for water samples A, B, C, D and E.

Table 4.1: Morphology and size of the isolated plaques.

Bacteriophage isolates	Sampling site	Plaque Size (mm)	Plaque Morphology
A1	A	1.0	clear
B1	B	1.5	clear
C1	C	2.2	clear
C2	C	2.0	clear
D1	D	3.0	clear
D2	D	2.0	clear
E1	E	2.0	clear
E2	E	1.0	clear

4.2 Characterisation of Isolated Bacteriophages

4.2.1 Host Specificity Test

The host range of the 8 isolated bacteriophages were determined using *Shigella sonnei*, *Shigella dysenteriae*, enterotoxigenic and enteropathogenic *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* (Table 4.2). Only C2, D1 and D2 bacteriophages possessed broad host range. Other bacteriophages have narrow host ranges. D2 phage was lytic against 2 out of the 6 bacteria tested where clear zone was produced on the enteropathogenic and enterotoxigenic *E. coli* pathotypes tested. C2 and D1 phages produced faint clear zone in EPEC and ETEC bacterial lawn respectively. Meanwhile, other phages showed negative

result for these bacterial strains. This indicated that they were specifically infectious towards *Shigella flexneri*. As compared to other phage isolates, A1 phage showed faint zone in the inoculated area on the lawn formed by *Shigella flexneri*. The host range spectrums of the isolated bacteriophages are listed in Table 4.2. Figure 4.3 shows the spot test method used to determine the host range of the bacteriophage isolates.

Table 4.2: Host range spectrums of the isolated bacteriophages against different bacterial strains.

	Bacteriophages							
Bacteria	**A1**	**B1**	**C1**	**C2**	**D1**	**D2**	**E1**	**E2**
S. flexneri[a]	±	+	+	+	+	+	+	+
S. sonnei	-	-	-	-	-	-	-	-
S. dysenteriae	-	-	-	-	-	-	-	-
ETEC[b]	-	-	-	-	±	+	-	-
EPEC[c]	-	-	-	±	-	+	-	-
S.aureus	-	-	-	-	-	-	-	-
K. pneumonia	-	-	-	-	-	-	-	-

(+) clear zone of the inoculated area; (±) faint clear zone within the inoculated area; (-) no clear zone formation. [a]*Shigella flexneri* serves as the positive control; [b]Enterotoxigenic *Escherichia coli*; [c]Enteropathogenic *Escherichia coli*.

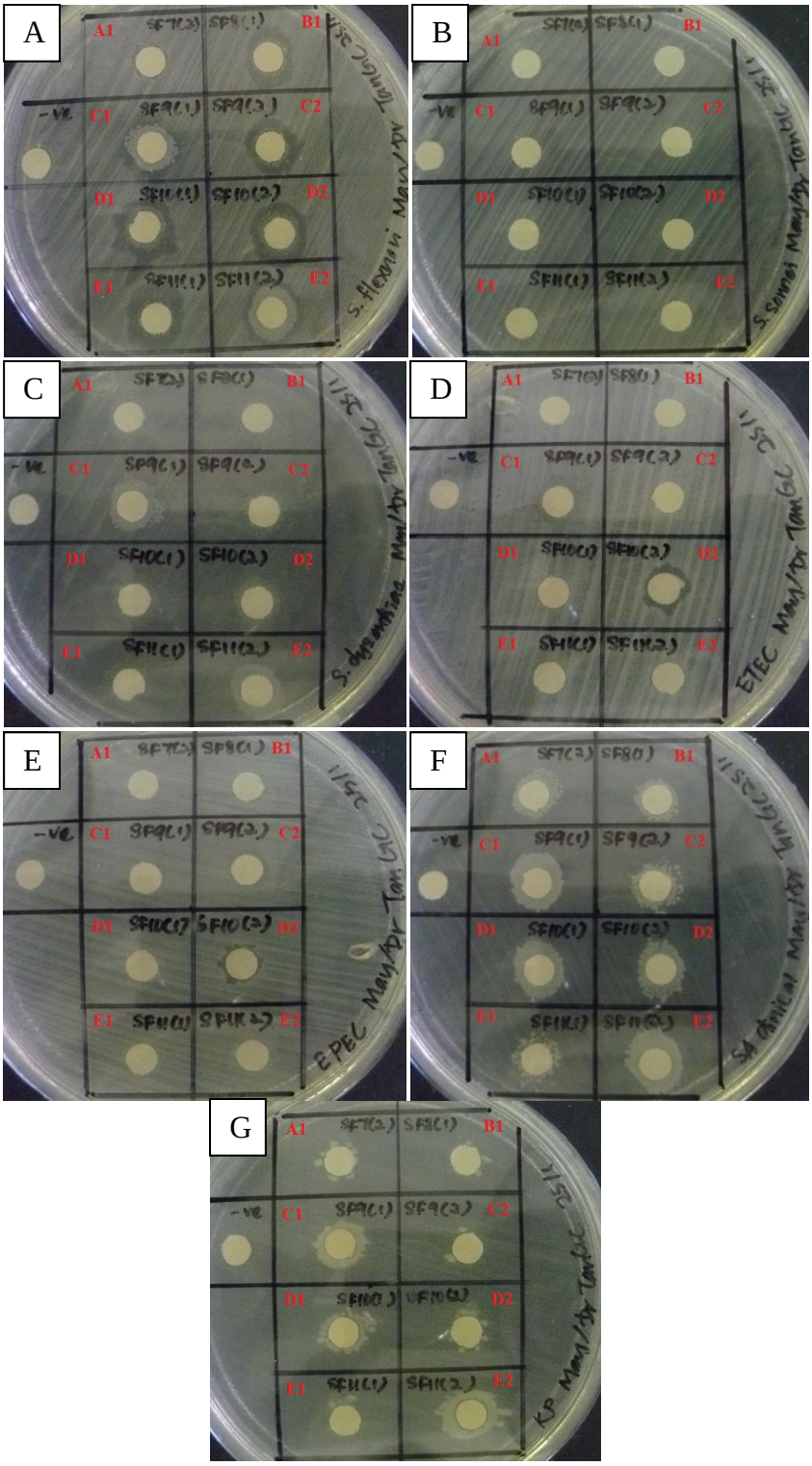

Figure 4.3: Host range determination of phages against (A) *Shigella flexneri* (positive control), (B) *Shigella sonnei*, (C) *Shigella dysenteriae*, (D) ETEC, (E) EPEC, (F) *Staphylococcus aureus* and (G) *Klebsiella pneumonia*.

4.2.2 Phage DNA Extraction

The phage genome was successfully extracted using phenol-chloroform method. As shown in Figure 4.4, all the phage genome sizes were above 3,000 bp. The bands for Lane 2, 6 and 8 were very clear, indicating higher concentration of phage DNA extracted from B1, D2 and E2 phages. The yield and purity of the phage DNA were high and there was no contamination of bacterial genomic DNA.

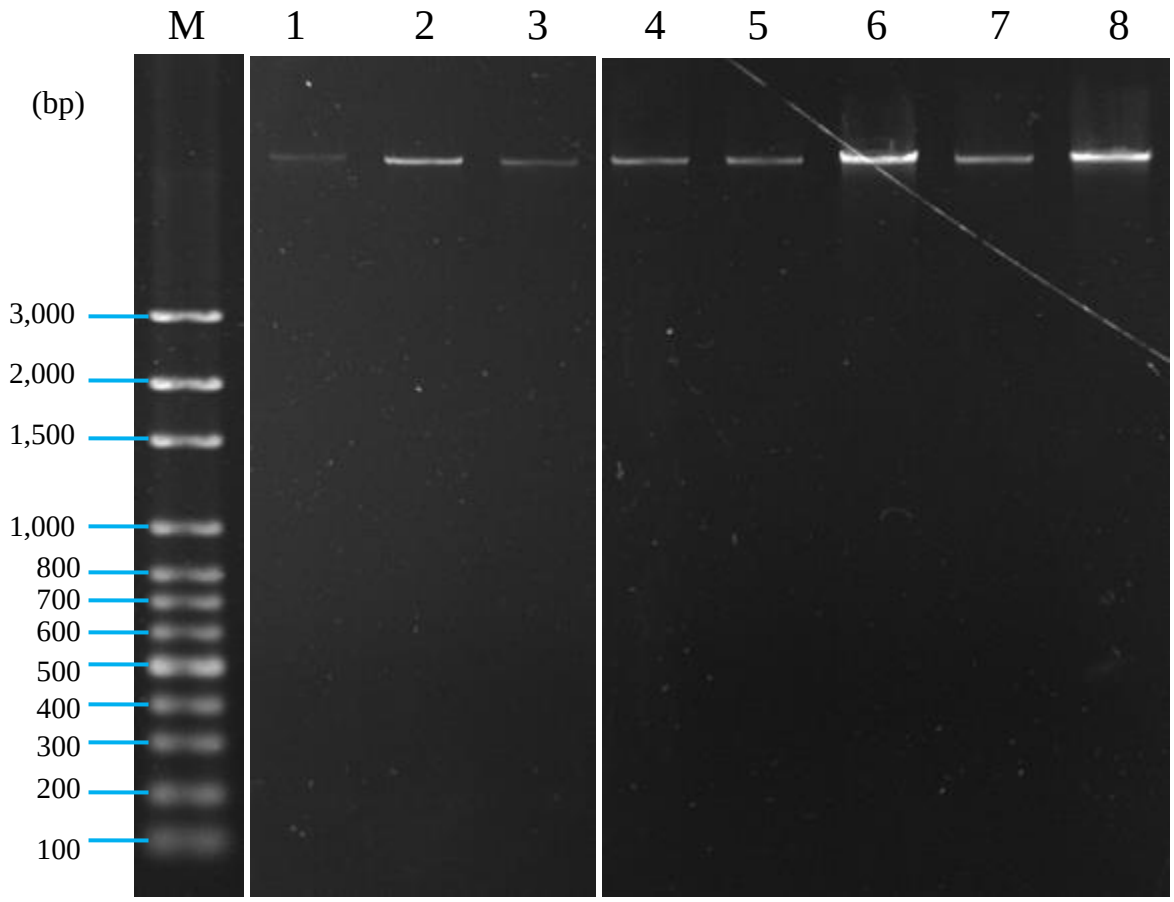

Figure 4.4: Phage DNA extracted using phenol-chloroform method. Lane M: 100 bp ladder; Lane 1: A1 phage; Lane 2: B1 phage; Lane 3: C1 phage; Lane 4: C2 phage; Lane 5: D1 phage; Lane 6: D2 phage; Lane 7: E1 phage; Lane 8: E2 phage.

4.2.3 Restriction Digestion of Phage DNA

Discriminatory bands were observed in each phage DNA restriction patterns for a given enzyme. After performing digestion with *Eco*RI, most of the phage DNA fragments were observed to be at 8-10 kbp and some were above 10 kbp. No band was observed in Lane 8. 2 faint bands with the size of 2 kbp can be seen in B1, D1, D2, E1 and E2 phages (Figure 4.5). Only D2 phage genome at Lane 3 was selected for further characterisation due to its higher concentration of phage DNA and broad lytic spectra. The D2 phage DNA was subjected to restriction by *Bam*HI, *Not*I, *Sal*I, *Xba*I and *Sac*I. The enzymes *Not*I and *Xba*I were not able to digest the D2 phage DNA (Figure 4.6b); on the other hand, *Bam*HI, *Sac*I and *Sal*I enzymes digested phage DNA into desired size (600 – 1000 bp) of DNA fragments which are suitable for cloning. Both *Sac*I and *SaI*I produced a DNA fragment with 800 bp (Figure 4.6a; Figure 4.7). Digestion with *BamHI* exhibited a DNA fragment of 1000 bp (Figure 4.6b).

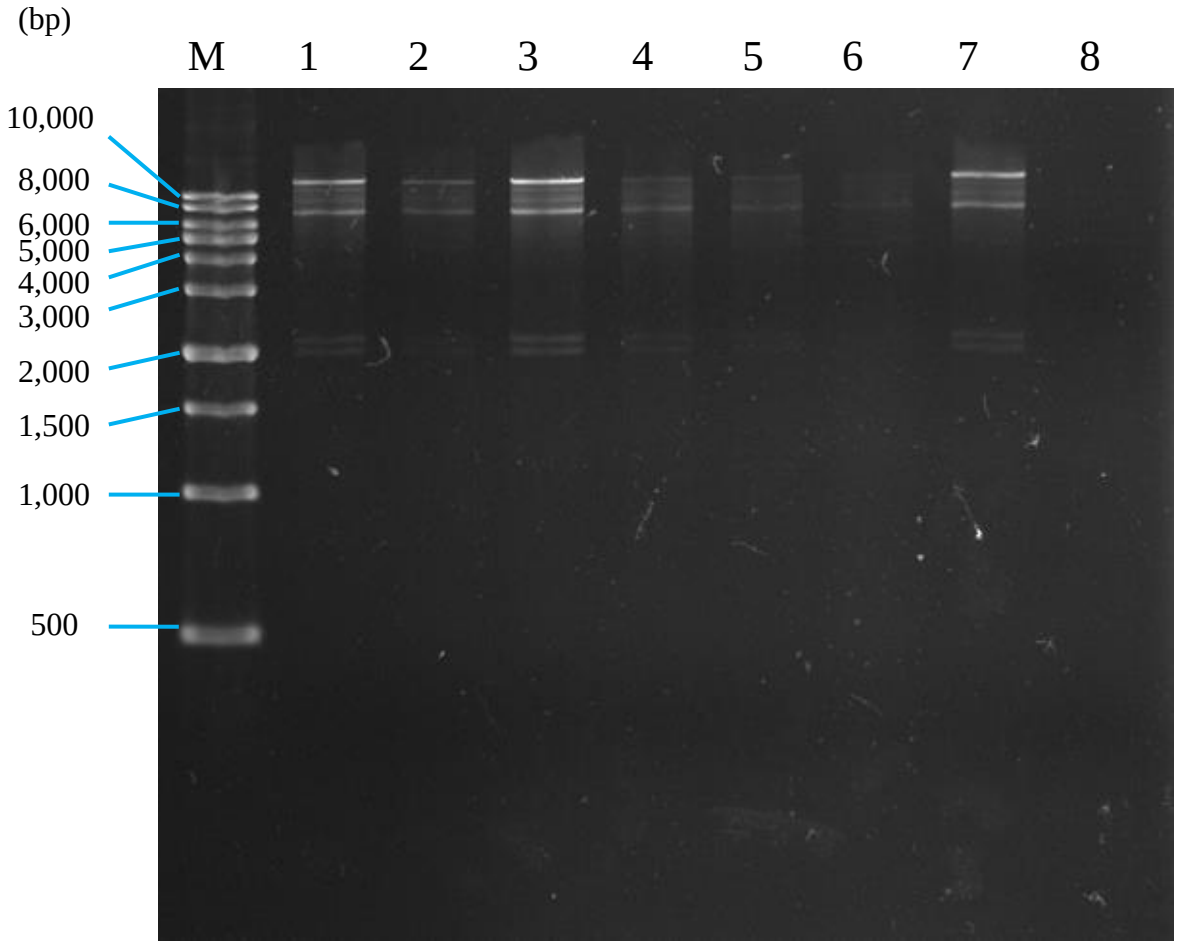

Figure 4.5: Restriction digestion profile of DNA digested with *Eco*RI. Lane M: 1 kbp DNA ladder; Lane 1: E2 phage; Lane 2: E1 phage; Lane 3: D2 phage; Lane 4: D1 phage; Lane 5: C2 phage; Lane 6: C1 phage; Lane 7: B1 phage; Lane 8: A1 phage.

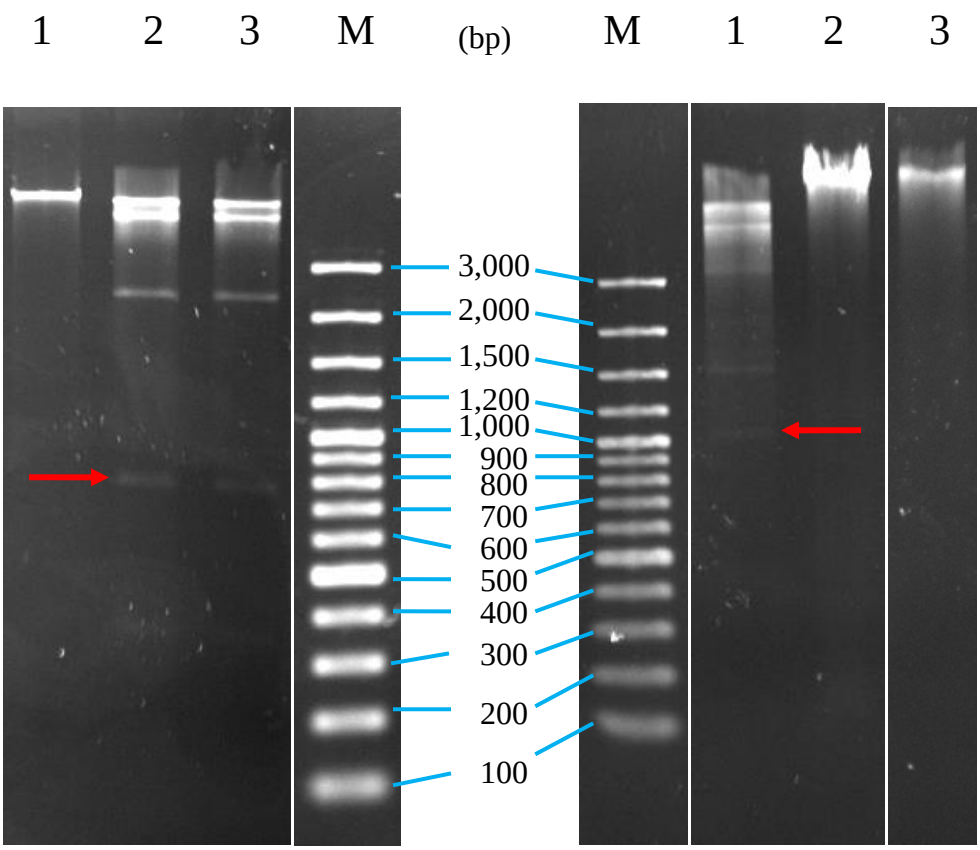

Figure 4.6: (a) Restriction digestion profile of D2 phage DNA with *Sal*I. Lane 1: undigested DNA; Lane 2-3: *Sal*I digested DNA; Lane M: 100 bp Plus DNA ladder. (b) Restriction digestion profile of D2 phage DNA with *Bam*HI, *Not*I and *Xba*I. Lane M: 100 bp Plus DNA ladder; Lane 1: *Bam*HI digested DNA; Lane 2: *Not*I digested DNA; Lane 3: *Xba*I digested DNA.

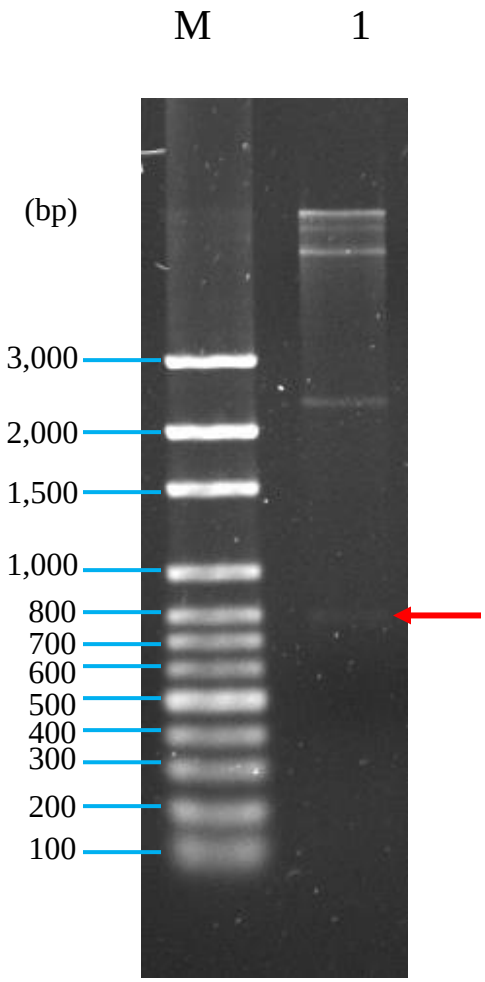

Figure 4.7: Restriction digestion profile of D2 phage DNA with *Sac*I. Lane M: 100 bp DNA ladder; Lane 1: *Sac*I digested DNA.

CHAPTER 5

DISCUSSION

5.1 Isolation of Bacteriophages

All the water samples were collected together with the sediment because virus present in higher number in bulk sediment compared to pore water (Drake et al., 1998). Most viruses will be adsorbed to the sediments in particle rich environment and only a small portion of them will be found in pore water. Once viruses are adsorbed to sediment, they are immobilised, which may lead to accumulation and concentration of viruses in the sediments (De Flora et al., 1975). Therefore, in order to isolate bacteriophage successfully, sediments which include both the particle-adsorbed virus and virus in pore water were collected in this study.

Many phages were isolated from sample collection sites C, D and E and the number of plaques formed were too numerous to be enumerated. Phages isolated from sample collection site A were observed to be lower in number (22 plaques), followed by B with the least number (17 plaques). Both the water samples A and B were collected from river. The flowing water in the river might constantly dilute the phage with fresh water source, hence, leads to lower number of phages isolated. In contrast, the water in lake, drain and pond remains stagnant, therefore, higher number of phages will be concentrated in this region. Apart from that, bacteria in fresh flowing water environments in sampling sites A and B may be

concentrated at the surface of solids rather than in the overlaying water, thereby lowering down the number of planktonic bacteria needed for replication of phages (Goyal et al., 1987). The number of phages will then be reduced. Furthermore, A and B sites may have insufficient food source or nutrient required for growth of *Shigella flexneri*. Abundant food source for bacteria propagation in water samples from sites C, D and E might serve as rich bacterial pool that provides the necessary host for the phage to grow.

Sunlight, specifically ultraviolet (UV) light damages viral genomic element beyond repair, thus, causes viral decay in aquatic environment (Kirchman 2012). Sunlight inactivation was significant down to a depth of 200 m. UV-A (320 to 400 nm) has the greatest effect (Murray and Jackson 1993). The penetration of sunlight depends on the water clarity and depth (Davies-Colley et al., 1994). The sampling sites A and B are believed to have higher penetration of sunlight due to the clear and fresh flowing water in shallow river, which directly exposes the phages to sunlight. The UV light may inactivate the phages present in these sites. In accordance to research work done by Chandra et al. (2011), direct sun radiation is deleterious to the phage survival. Sunlight has inactivation effect on various faecal indicator bacteria and bacteriophages (Sinton et al., 1999; Sinton et al., 2002). Phage inactivation in sunlight-exposed environment causes damage to both the capsid and nucleic acid genome (Sinton et al., 1999). The UV in sunlight can cause 5% loss in viable phage per hour for surface water due to the formation of thymine dimers (Wommack et al., 1996).

In addition, the concentration of dissolved oxygen (DO) will be reduced. As water temperature increases, the water's capacity to hold oxygen decreases (United States Environmental Protection Agency [EPA], 2012). Low level of dissolved oxygen can decrease bacterial propagation. Additionally, high temperature will also decrease infectivity of phages (Kinnunen 1978). Thus, low number of phages can be isolated from sampling sites A and B.

Unlike samples A and B, the water samples taken from sample collection sites D and E contain abundant amount of algae floating in it. The algae have symbiotic relationship with the bacteria. Photosynthetic algae convert large amount of carbon dioxide into oxygen, thereby releasing more oxygen for the growth and survival of bacteria. Bacteria respiration in turn provides carbon dioxide needed for microalgae photosynthesis (Su 2012). The concentration of dissolved oxygen increases during algal photosynthesis. The high concentration of dissolved oxygen promotes the growth of bacteria. Other than that, large amount of algae also provide an adsorption site for phage which protects the phage from inactivating factor in environment such as sunlight. As a result, higher phage count can be obtained from sample collection sites D and E.

The density of both phages and their host bacteria are important criteria for the multiplication of phages. Phage multiplication depends on host bacterial growth (Uchiyama et al., 2007). At low level of host bacteria in A and B sites, a phage has low chance of encountering a susceptible host, hence, productive infection

may not occur. A successful phage replication generally requires at least 10^4 host bacteria per mL (Goyal et al., 1987). Hence, it can be deduced that low density of host bacteria will lead to low chance of isolation of phages. Besides, the presence of *Shigella flexneri* phages in water may also used as a possible indicator of the presence of host bacterium. Therefore, it can be known that the sample collection sites C, D and E may have more bacteria than A and B sites according to the higher number of phages isolated. Drain, pond and lake of sampling site C, D and E are more prone to contamination by human activity. So, the existence of *S. flexneri* bacterium in these environmental waters may possibly caused by faecal contamination.

Bacteriophages infective to the *Shigella flexneri* can be isolated from all the water samples. The most probable reason is using the *S. flexneri* as enrichment cultures for phage isolation. When *S. flexneri* is added into the environmental sample, the phage that is infective towards the bacteria will outnumber other phages that have limited or no infectious activity. Therefore, it is easier to isolate the phages for certain bacterial strains that are being introduced in enrichment cultures (Stenholm et al., 2008).

Based on the plaque morphology obtained in plaque assay, all the phage isolates produced clear plaques, indicating all bacteria in the plaque zone are being lysed. Thus, these phages were known to be lytic where large numbers of phage progeny were released to cause lysis (rupture) of the host cell. Furthermore, the phages did

not give plaques which were uniform in size. The heterogeneity of plaque size exhibited by these phages is an indication of more than one phage type present in the water sample. This is because a slowly replicating phage or one which produces low number of infective progeny particles, will tend to form a smaller plaque compared to the rapidly replicating phage (Irving et al., 1990). Another factor that affects the plaque size is the physical size of the phage. A small phage will diffuse through the semi-solid agar more easily and quickly than the large one, therefore, larger plaque can be formed (Irving et al., 1990). This is the preliminary test to distinguish or relate the different phages on the basis on their plaque morphology.

5.2 Host Specificity Test

The 8 isolated bacteriophages were examined for their ability to lyse multiple host species. It was determined that D2 phage has broader host range compared to other phage isolates. This phage has the ability to lyse other bacterial strains which includes enterotoxigenic *Escherichia coli* (ETEC) and enteropathogenic *Escherichia coli* (EPEC). Besides, C2 and D1 phages produced a faint zone on the lawn of EPEC and ETEC respectively. Clear zone produced by D2 phage demonstrates complete lysis, whereas faint zone from C2 and D1 phages indicates minimal lysis. The clarity of plaques showed that D2 phage has a stronger lytic capability compared to the other two phages. This test illustrated that C2, D1 and D2 phages can infect more than one bacteria genus even though most of the

phages display host specificity to a particular bacterial species and strain. This phenomenon may due to the relaxed host specificity of bacteriophages, thus, allowing productive interaction with several prey genus (Jensen et al., 1998). These phages that are able to infect various bacterial host genus can easily encounter a susceptible prey and replicate in it. On the other hand, the inability of A1, B1, C1, E1 and E2 phages to lyse strains of bacteria of other genera confirms their generic specificity.

C2, D1 and D2 phages were infectious towards *Escherichia* genus because of the common characteristics shared between *Shigella* strains and members of the genus *Escherichia*. Their genetic relatedness suggested that *Shigella* belongs to the diverse species *Escherichia coli*. *Shigella* is phylogenetically similar to *E. coli*, indicating *Shigella flexneri* and *E. coli* are closely related. *Shigella* and *E. coli* are shown to be genomically indistinguishable at the species level in DNA hybridization studies. These studies have proved that *Shigella* species and members of *E. coli* are belonged to the common ancestor (Jin et al., 2002; Lan and Reeves 2002; Wei et al., 2003). Therefore, the host range of the phages shows a correlation with the degree of relatedness where those bacterial strain closely related to *Shigella flexneri* are generally susceptible to the *Shigella* phages, while the more distantly related species are resistant to these phages.

D2 phage is a potential candidate for therapeutic application due to its broad host range and strong lytic capability. These properties are desirable for the biocontrol

of pathogenic bacterial infections or foodstuff treatment without pre-analysis of sensitivity of target host cell to a given bacteriophages (Bielke et al., 2007). Hence, much attention was given for D2 phage in present study.

López-Cuevas et al. (2011) hypothesised that the differences of host range might be due to environmental origin of bacteria tested in which a loss of bacteriophage receptors may have happened as a result of antagonistic co-evolution between bacterium and bacteriophage. Apart from that, it could also be due to bacterial receptor mutation or degradation caused by restriction or modification of the resistance bacterial system which prevents the adsorption of phage to host bacteria.

The standard method of bacteriophage enrichment was used in this project to increase the number of *Shigella flexneri* that serves as the host for specific phage to multiply, hence, phages can be easily isolated from the sample. However, this method may favour bacteriophages to possess a more limited host range (Jensen et al., 1998). Jensen et al. (1998) suggested that probability to isolate broad host range bacteriophage can be increased by using two bacterial host species in isolation protocol instead of one. Cross infection between *S. flexneri* phage and *Escherichia coli* host may provide an alternate host for the multiplication of phage. So, if *E. coli* and *S. flexneri* bacteria are added together for bacteriophage enrichment, the probability of phage isolation will increase with the presence of multiple susceptible hosts. The potential to infect multiple hosts would maximise

opportunities for effective phage multiplication since the phage is more likely to encounter suitable prey.

The C2, D1 and D2 isolated phages in this study are most likely indigenous phage of *Escherichia coli*. In other words, they are possibly *E. coli* phages which able to co-infect *Shigella flexneri* bacterium due to their broad lytic spectrum. *E. coli* with shorter doubling time (20 min) is more abundant in the environmental setting compared to *S. flexneri* (doubling time, about 40 min) (Maloy et al., 1994; Lucchini et al., 2005). Phage replicates faster on the more abundant, rapidly growing host population, where new hosts can be found more easily. This applied for the concept of "killing the winner populations" (Thingstad and Lignell 1997). Thus, isolation of these phages could not conclude the presence of *S. flexneri* in the water sample since *E. coli* can be used as the host for these phages to replicate.

5.3 Molecular Characterisation

5.3.1 Phage DNA Extraction

This method was done to verify extraction yield and absence of bacterial genomic DNA. The gel image shows that the phage DNA was successfully extracted using phenol chloroform method and it was free of genomic contamination. It can be observed that the genome size of all the bacteriophages were above 3,000 bp. The banding pattern was clear showing high concentration of phage DNA extracted.

By visualising the band pattern, it was shown that the concentration and purity of the extracted DNA are high and suitable to proceed for restriction digestion analysis. This also demonstrates that polyethylene glycol (PEG) 6000 used for the purification and concentration of phage particles was useful and effective in obtaining highly purified phage preparations. This method is independent of phage concentration where phages can be concentrated even with low titer lysates. It is also fast which provides a 100-fold phage concentration after low speed centrifugation with little loss of infectivity (Boulanger 2009). As a result, both PEG 6000 and phenol chloroform method have proved to produce high yields and purity DNA, hence, these methods are reliable to be used for future research work or molecular study.

5.3.2 Restriction Digestion of Phage DNA

Differences between phages were confirmed by comparison between the restriction endonuclease cleavage patterns of the phage DNA. Based on the digestion pattern by *Eco*RI, all the phages have similar restriction patterns especially B1, D1, D2, E1 and E2 phages with an extra band at 2,000 bp. This indicated that they are possibly the same bacteriophage which belonged to the same strain. However, these phages presented different host ranges. This demonstrates that similarity in genotypic characteristics does not imply similarity in other phenotypic characteristics of the phages. Likewise, similar host range does not imply that phages are genetically related. This observation is supported

by Stenholm et al. (2008) that showed no indication of a relation between lytic potential and the genome sizes or the morphological characteristics of the phages tested. The most probable factor is the differences in phage receptor properties between host strains. Surprisingly, no band can be seen in Lane 8 loaded with A1 phage. This phenomenon might due to the contamination of DNase that causes degradation of phage DNA.

The D2 phage digestion pattern by *Bam*HI was not very clear, probably due to overlapped bands. The inability to digest phage DNA by *Not*I and *Xba*I enzymes could be due to the absence of target sites for the restriction enzyme tested (Krylov et al., 2006). Moreover, the *Not*I and *Xba*I enzymes are rare-cutters which recognize rare cutting sites that are not commonly found in genome (Table 5.1).

Table 5.1: Restriction endonucleases with rare-cutting frequency in genomes (Adapted from Michel 2003).

Recognition particularity	**Enzyme**	**Recognition sequence**
8-nucleotide recognition sequence	*Not*I	GC/GGCCGC
Overlap on TAG, a rare stop codon on prokaryotes	*Xba*I	T/CTAGA

The digested DNA with 600 -1000 bp was preferred for cloning and sequencing in future. It was shown that the *Sac*I, *SaI*I and *Bam*HI enzymes able to produce the desired size for insert. 600 – 1000 bp was chosen as the range for insert because the manufacturer information of the PCR cloning kit stated that short

DNA fragments (<1 kb) are cloned with a higher efficiency compared to long one (Thermo Scientific 2012). The efficiency of cloning depends on insert size. Larger inserts are amenable to cloning in high-copy number vectors, however, at a lower efficiency (Invitrogen Corporation 2013). In addition, inserts with the same size as vector will compete equally with other vector molecules for ligation. So, if the inserts are smaller than the vector molecules, they will outcompete other vectors that exist (Nybo 2011). 600 bp was decided as the lower limit because insert size that is too small will reduce specificity and accuracy of sequence identification. Short fragments contain less sequence information, hence, it is hard to be identified and compared. A few more sequence added may contain the key information that define and represent the feature of a particular phage.

5.4 Limitations and Further Study

The major problem encountered was the high contamination risk obtained from environmental sample. There is wide variety of bacteria present in the environmental sample despite the desired phage and host bacteria. This unrelated bacteria or contaminant can affect the accuracy and reliability of result. The growth of this contaminants cause difficulty in the inspection of plaque formation. This poses problem for the isolation and selection of plaque. Centrifugation and filtration through 0.45 µM cellulose acetate syringe filter were not sufficient to eliminate the bacterial debris and particulates completely. Instead, chloroform can

be added to eliminate all viable bacterial cells so that the phage obtained is more purified (Jensen et al., 1998).

As shown in the plaque assay, the phage titre for the sample collection sites C, D and E was too high. The plaques formed were found to be close to each other and they were nearly joined together. When the number of plaques is too numerous, there is high possibility to pick up the neighbouring plaques. The chances of contamination by other phages will therefore increase. To overcome this problem, more serial dilution should be carried out to dilute the phage to achieve low plaque count, preferably 30 – 300 pfu (plaque forming units) (Panec and Katz 2003). In addition, phages can also be plated repeatedly to assure similar phage plaque parameters are obtained.

There were only 6 bacterial strains used for the host specificity test in this study. This limited number of bacterial strains was insufficient to determine the host range of phages. For successful therapeutic application of bacterial infection, a group of broad host range phages are needed in order to cover a possible broad spectrum of potential pathogenic host strains involved in an outbreak of disease. Hence, more bacterial strains from *Shigella* species or even other genera, particularly antibiotic resistant strains, should be included in this test to help in the selection of phage candidate for phage therapy.

Apart from that, use of only one restriction enzyme, *Eco*RI, in this study was inadequate to distinguish and relate the isolated phages. In future, more restriction enzymes should be used in restriction endonuclease analysis to determine the genetic variability of the isolated phages and to improve the reliability of the result. Molecular analysis such as cloning and sequencing can also be used to identify the phage of interest. Molecular analysis normally produces more rapid, sensitive and reproducible result.

In addition, the banding pattern obtained for restriction analysis was not clear and the bands were extremely thin because of low concentration of DNA used for the analysis. The faint bands cause difficulty in the estimation of size. Perhaps more DNA can be used in future to obtain a clear and strong band. Moreover, the phages isolated in this study were found to be above 3000 bp. However, the actual genome size of the phage was not known due to the small size marker used. In future, larger size marker should be used to estimate the size of the phage.

Detailed characterisation of phage properties is a prerequisite for evaluating the potential of phages as biocontrol agent. The newly isolated phages need to be examined in detail especially when they are intended to be used for therapeutic application. Therefore, more characterisation tests should be carried out in future to analyse the isolated phages. These include visualisation of phage morphology using transmission electron microscope. Based on morphological characteristics, the phages can then be classified to families following the guidelines of the

International Committee on Taxonomy of Viruses. Besides, one-step phage growth curve can be performed to determine the lytic ability, adsorption time, latent period, exponential growth and burst size of the phages. This method is useful to study the properties of phages which are going to be used as controller of a pathogenic host. The heat and pH stability of phages can also be tested to study the adaptability of the phages in adverse environmental conditions. In addition, sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) profile could be carried out to analyse the protein composition of the phages. This protein profile provides additional information to differentiate the isolated phages.

The D2 phage isolated in the present study may be an ideal therapeutic phage candidate due to its broad host specificity and strong infectious ability. However, more identification and characterisation tests need to be done to examine the ability of this newly isolated phage in overcoming outbreaks and spread of shigellosis. Same as drug, its form and mode of action have to be known in detail. One of the effective methods is genomic analysis that provides assessment for the safety of phage by examining the presence or absence of undesirable genes such as antibiotic resistance and virulence factor. Other than that, the therapeutic effectiveness of the phage can be tested *in vivo* because the bacteriocidal effectiveness is different *in vitro* and *in vivo*.

CHAPTER 6

CONCLUSION

In conclusion, all of the bacteriophage isolates collected from Kampar area were able to infect *Shigella flexneri*. All the 8 isolated phages (A1, B1, C1, C2, D1, D2, E1 and E2) are virulent phages which produced round, clear and medium sized (1.0-3.0 mm in diameter) plaques. Sample collection sites C, D and E have higher plaque count compared to A and E due to the environmental factor. It has shown that sampling sites C, D and E provide favourable condition for the growth of phage. Based on the number of phages isolated, higher number of host bacteria population was present in C, D and E than A and B.

In addition, C2, D1 and D2 bacteriophages possessed broad spectrum of infectious activity while other bacteriophages have narrow host range. D2 phage was able to lyse enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) while C2 and D1 phages have lysogenic effect against EPEC and ETEC respectively. Thus, these bacteriophages were able to infect bacterial strains of other genus. Conversely, the inability of A1, B1, C1, E1 and E2 phages to lyse strains of bacteria of other genera confirms their generic specificity.

Based on the digestion pattern by *Eco*RI, B1, D1, D2, E1 and E2 phages were found to have similar restriction patterns. This indicated that they are most

probably belonged to the similar phage lineages. However, these bacteriophages have different host specificity. This demonstrates that similarity in genotypic characteristics does not reflect similar phenotypic characteristics among the phages. Moreover, *Sac*I, *Sal*I and *Bam*HI enzymes were able to digest phage DNA into desirable size of DNA fragments (600 – 1000 bp) that are required for cloning and sequencing in future.

D2 phage isolated in this study was suggested to be a potential candidate for therapeutic application due to its broad host range and strong lytic capability. Nevertheless, more identification and characterisation tests should be done to confirm this hypothesis.

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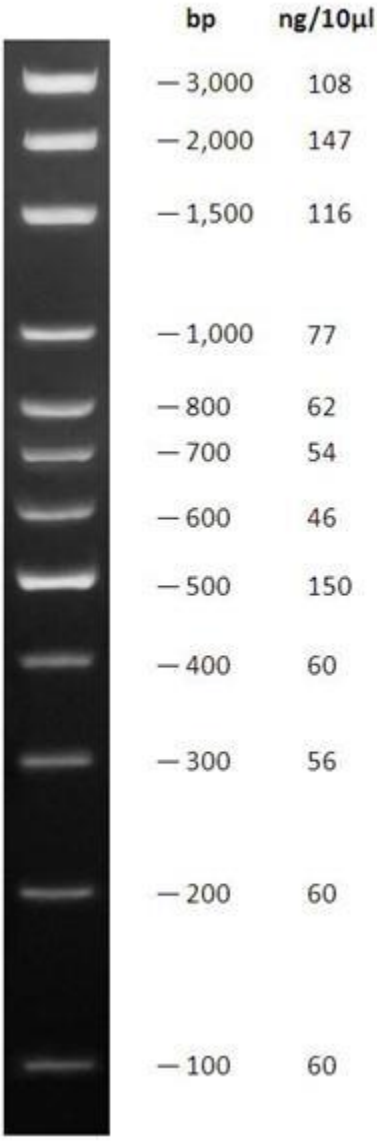
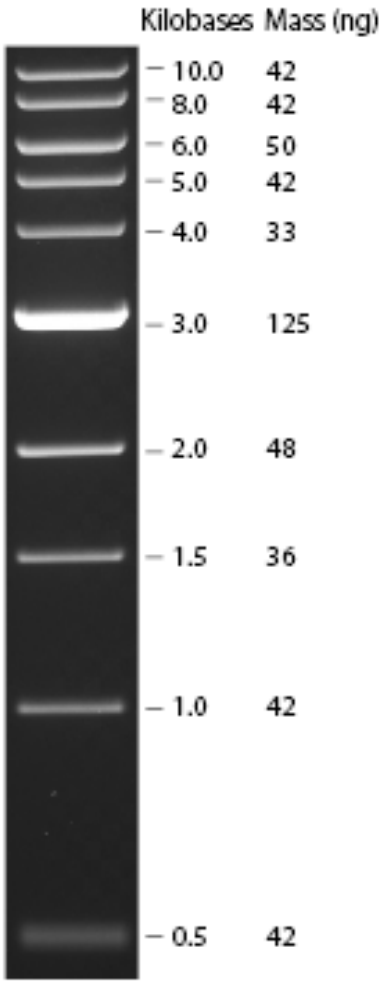
APPENDIX A



100 bp DNA ladder by SOLIS BIODYNE used in this study. This figure is obtained from https://www.sbd.ee/EN/products/dna_ladders/100_bp_dna_ladder.

APPENDIX B



1 kbp DNA ladder by NEW ENGLAND Bio Labs Inc. used in this study. This figure is obtained from

http://66.155.211.155/nebecomm/products_intl/productN3232.asp.