ISOLATION AND CHARACTERISATION OF LYTIC BACTERIOPHAGES INFECTING *Shigella* spp.

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FACULTY OF SCIENCE
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ISOLATION AND CHARACTERISATION OF LYTIC BACTERIOPHAGES INFECTING *Shigella* spp.

ABSTRACT

Shigella infected bacillary dysentery is a leading cause of morbidity and mortality worldwide. The gradual emergence of multidrug-resistant *Shigella* spp. has triggered the search for alternatives to conventional antibiotics. Bacteriophage could be one such suitable alternative for its proven long-term safety profile as well as the rapid expansion of phage therapy research. Hence the general objective of this study was to isolate and characterize different *Shigella* strains from clinical and environmental samples Forty-nine *Shigella* strains [clinical (n=39), environmental (n=10)] were isolated and identified through biochemical test, serotyping and multiplex PCR amplification. Among the strains, one was *Shigella dysenteriae,* three were *Shigella boydii*, eight were *Shigella sonnei* and 37 were *Shigella flexneri*. Antibiotic profiling of these strains was performed using ten commercially available antibiotics through disc diffusion methods where 98% of the strains were drug-resistant and 59% were multidrug resistant. Ten bacteriophages were isolated and purified against these drug-resistant *Shigella* spp. through spot plating assay. The genomic content of the isolated phages was extracted through phenol-chloroform-isoamyl alcohol (25:24:1) extraction method and digested with DNase I and RNase to validate that all phages isolated, were DNA phage in nature. The transmission electron microscopy revealed that phage TB002 and TB004 belonged to the family *Myoviridae*, TB009, TB010 and TB013 belonged to the family *Siphoviridae* while TB006 and TB014 other belonged to the family *Podoviridae*. Phage TB007, TB008 and TB011 were tailless bacteriophages and belonged to either group D or E according to Bradley's classification. The host range of the phages was determined through spot plating assay

where two of the phages TB004 and TB002 showed wider host range and lysed 49 and 48 strains out of 49 strains respectively and demonstrated the coverage on all four species of *Shigella* genus. Therefore, the TB004 phage was selected for sequencing and safety assessment while the whole genome sequencing was performed through massively parallel sequencing technology on the Illumina platform. Sequencing assembly and subsequent analysis were done through different bioinformatics tools. Genomic studies confirmed that the TB004 was a phage of T4 genus under *Myoviridae* family consisting of 169,988 bp with 35.46% G+C content having 10 tRNA and 5 repeat sequences. Two hundred and seventy three genes were encoded through GeneMarkS of which the functions of 235 genes were annotated through Swiss-Prot where 126 genes had assigned functions and 109 were hypothetical proteins. No toxic or deleterious gene products were found among this annotation. The phylogenetic analyses of five selected proteins also indicated its probability of safety as phage TB004 appeared within the same branch of some other T4 phages and their safety were approved earlier. So, phage TB004 together with other phages isolated in this study could be considered as potential and promising candidates for phage therapy and phage biology research against drug-resistant *Shigella* spp. due to their extended host range cell lysis capacity and probable safety profile. The outcomes of this study could be considered as a good possibility of using bacteriophages against *Shigella* spp. in the near future.

Keywords: *Shigella* spp., drug-resistant, bacteriophage, phage therapy, T4 phage

PEMENCILAN DAN PENCIRIAN BAKTERIOFAJ LISIS MENJANGKITI *Shigella* spp.

ABSTRAK

Disentri basilus *Shigella* adalah punca utama kemorbidan dan kematian di seluruh dunia. Kemunculan secara beransur-ansur *Shigella* spp. rintang multidrug telah mencetuskan pencarian alternatif selain antibiotik konvensional. Bakteriofaj boleh menjadi satu alternatif yang sesuai kerana profil keselamatan jangka panjang yang terbukti serta perkembangan pesat penyelidikan terapi faj. Oleh itu, objektif umum kajian ini adalah untuk memencilkan dan mencirikan pelbagai stren *Shigella* dari sampel klinikal dan alam sekitar, Sebanyak empat puluh sembilan stren *Shigella* [klinikal (n = 39), alam sekitar (n = 10)] telah pencil dan dikenalpasti melalui ujian biokimia, klasifikasi serotip dan amplifikasi multipleks PCR. Antara stren ini, satu adalah *Shigella dysenteriae*, tiga adalah *Shigella boydii*, lapan adalah *Shigella sonnei* dan 37 adalah *Shigella flexneri*. Klasifikasi rintangan antibiotik stren ini dilakukan menggunakan sepuluh antibiotik yang tersedia secara komersil melalui kaedah resapan cakera (disk diffusion assay) di mana 98% daripada stren rintang terhadap sekurang-kurangnya satu drug dan 59% adalah rintang multidrug. Sepuluh bakteriofaj telah pencil dan ditulenkan daripada *Shigella* spp. yang rintang dadah melalui kaedah ujian bintik. Kandungan genomik daripada faj terpencil diekstrak melalui kaedah pengekstrakan fenol-kloroform-isoamyl alkohol (25: 24: 1) dan dicerna dengan DNase I dan RNase di mana semua faj mempunyai sifat faj DNA. Mikroskopi elektron transmisi mendedahkan bahawa faj TB002 dan TB004 adalah daripada keluarga Myoviridae, TB009, TB010 dan TB013 adalah daripada keluarga Siphoviridae sementara TB006 dan TB014 adalah daripada keluarga Podoviridae. Faj TB007, TB008 dan TB011 adalah bakteriofaj tanpa ekor dan tergolong dalam kumpulan D atau E mengikut klasifikasi Bradley. Jangkauan hos faj ditentukan melalui kaedah ujian

bintik di mana dua daripada faj TB004 dan TB002 menunjukkan julat hos yang lebih luas, masing-masing mampu lisis sebanyak 49 dan 48 stren daripada 49 stren dan menunjukkan liputan terhadap semua empat spesies genus *Shigella*. Oleh itu, TB004 faj dipilih untuk penjujukan sekuriti dan keselamatan manakala penjujukan genom keseluruhan dilakukan melalui teknologi penjujukan secara besar-besaran pada platform Illumina. Himpunan urutan dan analisis seterusnya dilakukan melalui alat bioinformatik yang berbeza. Kajian genomik mengesahkan bahawa TB004 adalah genus gen T4 di bawah keluarga Myoviridae yang terdiri daripada 169,988 bp dengan kandungan 35.46% G + C yang mempunyai 10 tRNA dan 5 urutan berulang. Dua ratus tujuh puluh tiga gen dikodkan melalui GeneMarkS yang mana fungsi 235 gen dijelaskan melalui Swiss-Prot di mana 126 gen telah diberikan fungsi dan 109 adalah protein hipotetik. Tiada produk gen toksik atau kerosakan ditemui dalam kalangan penjelasan ini. Analisis filogenetik daripada lima protein terpilih juga menunjukkan profil keselamatannya dimana faj TB004 muncul dalam dahan yang sama dari beberapa faj T4 yang lain dan keselamatan mereka telah diluluskan. Jadi, faj TB004 bersama-sama dengan faj lain yang terpencil dalam kajian ini boleh dianggap sebagai calon yang berpotensi untuk terapi faj dan penyelidikan biologi faj terhadap *Shigella* spp. rintang drug disebabkan oleh keluasan hos dan kapasiti lisis serta profil keselamatan mereka. Hasil kajian ini boleh dipertimbangkan sebagai kemungkinan yang baik menggunakan bakteria terhadap *Shigella* spp. dalam masa terdekat.

Kata kunci: *Shigella* spp., rintang drug, bakteriofaj, terapi faj, T4 faj

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
+ve	:	Positive
-ve	:	Negative
10 X	:	Ten times
°C	:	Degree Celsius
ddH_2O	:	Double distilled water
EtOH	:	Ethanol
HCl	:	Hydrochloric acid
i.e	:	id est (in other words)
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
pH	:	Potential of hydrogen
Tm	:	Melting temperature
µg	:	Microgram
µl	:	Microliter
µM	:	Micromolar
µmol	:	Micromole
xg	:	Relative centrifugal force
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
CFU/ml	:	Colony forming unit per ml
cDNA	:	Complementary DNA
CDC	:	Centre for disease control and prevention
CS	:	Cell Suspension
CRISPR	:	Clustered Regulatory Interspaced Short Palindromic Repeats

cm	:	Centimeter
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxynucleotriphosphate
dsRNA	:	Double-stranded RNA
EDTA	:	Ethylene diamine tetra acetic acid
ETEC	:	Enterotoxigenic *Escherichia coli*
EU	:	European Union
FDA	:	Food and Drug Administration
g	:	Gram
gL^{-1}	:	Gram per liter
GRAS	:	Generally recognized as safe
h	:	Hour
KB	:	Kilo base
L	:	Liter
LB	:	Luria-Bertani
M	:	Molar
MAC	:	MacConkey agar
MDR	:	Multidrug-resistant
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	Milligram
MIC	:	Minimum inhibitory concentration
mgL^{-1}	:	Milligram per liter
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
MOI	:	Multiplicity of infection

mRNA	:	Messenger RNA
NCBI	:	National Centre for Biotechnology Information
ng	:	Nanogram
nm	:	Nanometre
NSSLRL	:	National Salmonella, Shigella & Listeria Reference Library of Ireland.
nt	:	Nucleotide
OD	:	Optical density
PBS	:	Phosphate buffer saline
PCR	:	Polymerase Chain Reaction
pmol	:	Picomole
PFGE	:	Pulsed Filed Gel Electrophoresis
PFU/ml	:	Plaque forming unit per milliliter
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
rRNA	:	Ribosomal RNA
rpm	:	Rotation per minute/ revolutions per minute
s	:	Second
SNP	:	Single nucleotide polymorphism
sp.	:	Species (singular)
Spp.	:	Species (plural)
SS	:	Salmonella-Shigella
SSR	:	Simple sequence repeat
TBE	:	Tris boric acid EDTA
TE	:	Tris EDTA
TEM	:	Transmission Electron Microscopy
Tris	:	Trisaminomethhane

Tris HCl	:	Tris hydrochloric acid
TSI	:	Triple sugar iron
U	:	Unit
UK	:	United Kingdom
USA	:	United States of America
USDA	:	United States Department of Agriculture
USSR	:	Union of Soviet Socialist Republics
V	:	Voltage
w/v	:	Weight per volume
XLD	:	Xylose lysine deoxycholate

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CHAPTER 1: INTRODUCTION

1.1 *Shigella* and Shigellosis

Shigella is one of the key pathogens responsible for the diarrhoeal disease generally known as bacillary dysentery and more specifically as Shigellosis. *Shigella* is a Gram-negative, non-motile, facultative anaerobic, rod-shaped and non-spore-forming bacteria. The bacillary dysentery caused by *Shigella* is a severe form of diarrhoea in which blood and mucus can be observed in the stool as a consequence of epithelial cell damage in the lower gut (Dodd & Jones, 1982; The et al., 2016). Worldwide, Shigellosis occurs at roughly 188 million cases in 2010 (Kotloff et al., 2018) and current estimate shows that Shigellosis causes 164,300 and 270,000 deaths in the year 2015 and 2016 respectively (12.5 % and 16.88% respectively of all diarrhoeal deaths) (Khalil, 2017; Troeger et al., 2017).

1.1.1 Classification of *Shigella* spp.

The genus *Shigella* has been divided into four species (also known as sub-groups) as per current serological classification. These subgroups/species are further subdivided into serotypes according to type-specific antigens: *S. dysenteriae* (subgroup A) has 15 serotypes; *S. flexneri* (subgroup B) has 19 serotypes and subserotypes; *S. boydii* (subgroup C) has 20 serotypes; and *S. sonnei* (subgroup D) has only one serotype. Therefore, 55 serotypes belonging to four species of *Shigella* have been identified as being responsible for bacillary dysentery and mortality worldwide (The et al., 2016).

Taxonomic position of *Shigella* spp. [source: (Castellani & Chambers, 1919)]:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobactriales

Family: *Enterobacteriaceae*

Genus: *Shigella*

Species: (1) *Shigella dysenteriae* (2) *Shigella boydii* (3) *Shigella flexneri* (4) *Shigella sonnei*

1.1.2 Symptoms of *Shigella* infection

The most common symptoms of *Shigella* infection are to develop diarrhoea, fever, and stomach cramps after being exposed to the bacteria (Bennett et al., 2014; Control & Prevention, 2010). These symptoms may arise usually within 12 to 96 h after the exposure and generally resolves in 5 to 7 days. Diarrhoea may ranges from mild to severe, it generally contains mucus and in more severe cases, the diarrhoea may appears with 25% to 50% bloody stool. The "tenesmus," commonly known as rectal spasms are common in this type of occurrence. (Keusch, 2009) (Bennett et al., 2014; Hawker et al., 2008). Different complexities include severe dehydration, rectal bleeding, invasion of the blood stream by the bacteria (bacteremia or sepsis) as well as seizures in small children may occur during Shigellosis. In addition, in few cases, the bacteria that cause Shigellosis may also cause inflammation of the lining of the rectum (proctitis) or rectal prolapse (Bennett et al., 2014; Control & Prevention, 2010; Hawker et al., 2008). Very few cases about 2% patient who are infected with *S. flexneri* later develop pains in their joints, irritation of the eyes, and painful urination which is typically diagnosed as Reiter's Syndrome and more

generally is referred to as reactive arthritis (Carter & Hudson, 2009; Hawker et al., 2008). A deadly complication called "toxic megacolon'' may arise in rare cases but more common in *S. dysenteriae* infection. This complication occurs when the colon becomes paralyze which prevents bowel movements or passing gas. The syndrome include fever, abdominal pain and swelling and disorientation. The untreated colon may rupture and cause peritonitis, a life-threatening condition may require emergency surgery. (Bennett et al., 2014; Hawker et al., 2008; Stearns)

1.1.3 Mode of transmission of *Shigella* infections

Shigella species are transmitted via fecal-oral route, and most infections are transmitted from man to man, reflecting the low infectious dose, as few as ten for *S. dysenteriae* type 1 and 180 for *S. flexneri* or *S. sonnei* colony-forming units are needed to produce symptomatic infection as opposed to *Salmonella* spp. and the various diarrhoeagenic *E. coli* pathovars, which have infectious doses of at least four orders of magnitude greater (DuPont et al., 1989; Kothary & Babu, 2001; The et al., 2016). *Shigella* is transmitted efficiently in low doses through direct or indirect human faecal contamination due to poor hygienic conditions (Weissman et al., 1975). Different food products like salads, soft cheese, vegetables and meat products are usually associated with this type of outbreak. Other modes of transmission include ingestion of contaminated food and water, contact via fomites (such as drinking devices, eating utensil and other inanimated objects) and certain modes of sexual intercourse (Morgan et al., 2006; Okame et al., 2012). Different housefly-like vectors can also play a vital role to spread the disease by physically transporting infected feces (Farag et al., 2013).

1.2 Reason of emerging drug-resistant *Shigella* spp.

Drug-resistant bacterial pathogens impose a critical challenge for clinical and pharmaceutical research due to their potentially severe impact on human health. The Infectious Disease Society of America (IDSA) is extremely concerned about the worrying growth in microbial pathogens and antibiotic-resistance in the USA and elsewhere in the world (Spellberg et al., 2008). This antibiotic resistance is caused by both bacterial and social factors, such as high mutation frequencies coupled with the exchange of genetic information by bacteria; the misuse or overuse of antibiotics by human being; and increasing population densities and global migratory movements by animals and people. (Huijbers et al., 2015; Liu et al., 2016). The acquisition of antibiotic resistance in bacteria is due to genetic exchanges via horizontal gene transfer involving three mechanisms (i.e. random transformation, transduction and conjugation). Uptake of small fragments of DNA by bacteria occurs during transformation, while transduction encompasses transfer of DNA (via bacteriophages) from one bacterium into another, and conjugation involves transfer of DNA through sexual pili involving cell-to-cell contact. The newly acquired recipients which were susceptible previously can express resistance due to the resistant genes acquired from the resistant donor (Frost et al., 2005; Oliveira et al., 2017). Moreover, the presence of R factors (plasmids) may play a major role in developing new serotypes which can foster antibacterial resistance (Tanaka et al., 1969).

1.3 Medical treatments for Shigellosis

A number of treatments of bacillary dysentery are commonly used. The World Health Organization (WHO) recommends the use of oral rehydration therapy, together with zinc supplements, for 10-14 days. The administration of zinc during Shigellosis reduces the duration and frequency of expelling loose stools (Nichter et al., 2008; UNICEF & Organization, 2006).WHO also suggests the use of effective antimicrobials against

clinically suspected Shigellosis (Christopher et al., 2009). In practice, Beta-lactams (Amoxicillin, Ampicillin, Ceftriaxone, Cefixime, and Pivmecillinam), Quinolones (Nalidixic acid, Ciprofloxacin, Norfloxacin, and Ofloxacin), Macrolides (Erythromycin and Azithromycin) and other antibiotics (Sulfonamides, Tetracycline, Furazolidone, and Cotrimoxazole) are commonly used to treat *Shigella* dysentery. Antibiotics have so far been the most common therapeutic agents against dysentery. However, the gradual emergence of drug resistant *Shigella* has caused growing concern of the long-term efficiency of antibiotics. The drug resistant characteristics of *Shigella* have been reported since 1940s, and have led to the increasing emergence of multidrug resistant strains over the past few decades. The development of new antibiotics to combat these new strains is time consuming, laborious and costly. Moreover, no effective vaccine is available to prevent Shigellosis, which is thus a serious global medical and social problem (Arias & Murray, 2009; Deris et al., 2013; Magiorakos et al., 2012; WHO, 2014). In addition the unavailability of the Food and Drug Administration (FDA) approved vaccines, has led researchers to seek alternative treatments against drug resistant bacterial pathogens (Katz et al., 2004; Wu et al., 2011). Administration of a combination of antibiotics (antibiotic cocktails) and antimicrobial peptides are promising replacements, however, these alternatives may eventually suffer a similar fate as the current treatment (Worthington & Melander, 2013). Conversely, bacteriophages have potentials to be used as an alternative to antibiotics, because phages have different modes of action and they could be rapidly 'trained' on ancestral bacterial strains via successive passages), as well as their capability to defeat bacterial resistance by evolving *in situ* mutations (Betts et al., 2013).Hence, phage therapy could be the best option for treating Shigellosis, because it has been shown to work against *Shigella* spp. since the beginning of phage therapy research. Phage treatment has also the additional advantage of causing less disruption to gut flora than antibiotic treatment (Kutter et al., 2010). Moreover, experimental anti-dysentery trials

using phages have been successfully conducted over several decades in Eastern Europe (Kutter, 2009).

1.4 Bacteriophage

Bacteriophages (phages), are viruses which specifically infect and kill their bacterial host and depend on bacterial metabolism and replication mechanism to reproduce and transmit their progeny. It is believed that the phages are the most numerous biological entities on the earth and are environmentally ubiquitous existing wherever there are suitable host. The presence of phages in the environment varying from 10^8 pfu/ml in fresh water to 10^9 pfu/ml in sediments (Ashelford et al., 2003). After the invention of the electron microscope in the 1940s aided the identification of different phage morphologies. Phages can be tailed, without tail, polyhedral, filamentous or pleomorphic, and some have the envelopes of lipoprotein or lipid (Ackermann, 2007; Bradley, 1967).

1.4.1 Classification of bacteriophages

Bradley divided phages into 6 different taxonomic groups (A to F) based on their structural morphology and nucleic acid content (dsDNA, dsRNA, ssDNA, and ssRNA). According to his classification, group A **(***Myoviridae*) phages composed of capsid and possess long tail with contractile sheath, group B **(***Siphoviridae***)** phages composed of capsid and possess non-contractile long tail and phages of group C **(***Podoviridae***)** composed of capsid and have short non-contractile tails. Both group D and E phages are tailless while group D phages represented by grand-size nucleocapsid with fibrous or spiky surface structures and group E phages incorporating one nucleocapsid. Group F phages are rod-like or filamentous where the long protein filaments are filled by ssDNA (Figure 1.1) (Bradley, 1967).

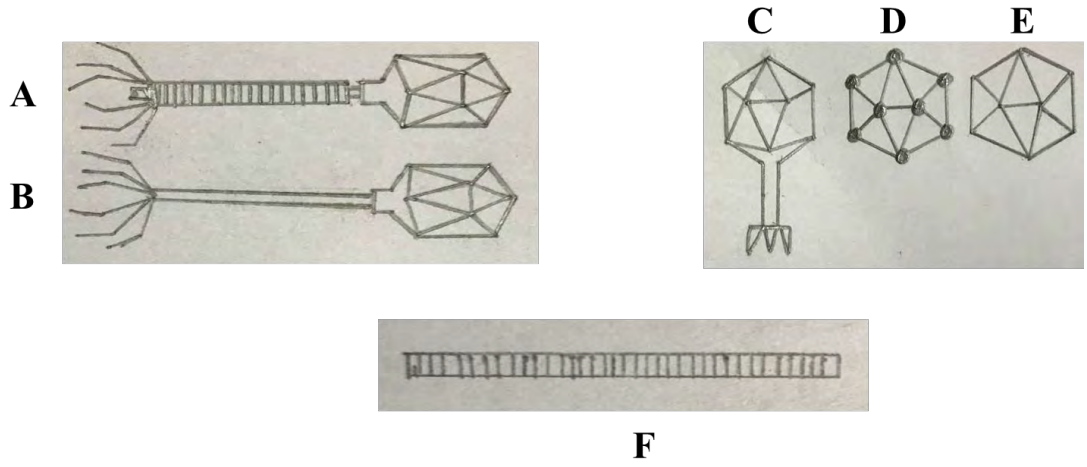

Figure 1.1: Cartoon diagram shows Bradley's bacteriophage classification scheme (Bradly, 1967; Novik et al. 2002). A: *Myoviridae* phage; B: *Siphoviridae* phage; C: *Podoviridae* phage; D and E: Tailless phage; F: Rod-like or filamentus phage.

The size of genetic material of phages (DNA or RNA) ranges from the 3.5 kb (ssRNA phage MS2) to 500 kb (dsDNA Bacillus phage G) (Rohwer, 2003). Comparing with Bradley's classification, where the phages have been divided into six morphological types, the International Committee on Taxonomy of Viruses (ICTV) has classified phages as 1 major order, 19 families, and 31 genera according to the basis of morphology, nucleic acid content, and genomic data. About 96% of isolated phages (Ackermann, 2003, 2007) belong to three large families *Myoviridae* (25%), *Siphoviridae* (61%), and *Podoviridae* (14%) under the *Caudovirales* order, which are phylogenetically related and all are tailed phages. According to the finding of Harper et al. (2011) the new model structures of those tailed phages are shown in Figure 1.2. The rest 4% of the phages are tailless with different structures (i) polyhedral (with either icosahedral or cubic symmetry) (ii) pleomorphic (asymmetric e.g. shaped like a lemon or a droplet) and (iii) filamentous with a long and thin morphology (Ackermann, 2003; Maniloff & Ackermann, 1998).

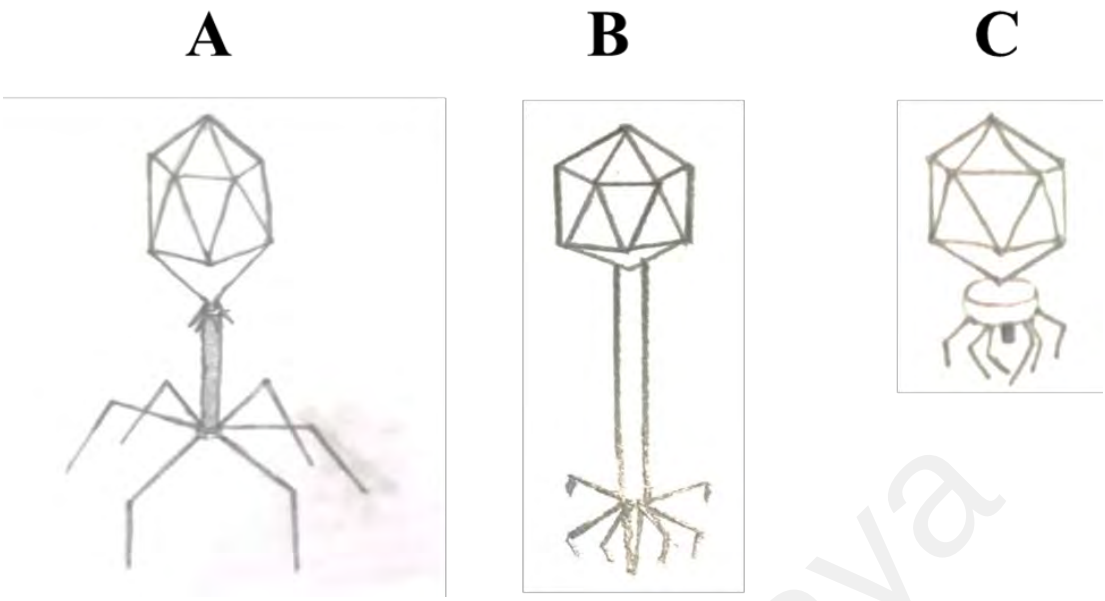

Figure 1.2: Families of the order *Caudovirales* according to Harper et al.(2011). A: *Myoviridae* phage, composed of capsid and possess long tail with contractile sheath; B: *Siphoviridae* phage, composed of capsid and possess non-contractile long tail; C: *Podoviridae* phage, composed of capsid and possess short non-contractile tail.

1.4.2 Phage biology and phage-host interaction

Bacteriophages exhibit four known life cycles inside the bacterial host: lysogenic, pseudo-lysogenic, chronic and lytic infection (Drulis-Kawa et al., 2012; Weinbauer, 2004). For phage therapy, the main interest has always been focused on lytic phages particularly the families of *Podoviridae*, *Myoviridae* and *Siphoviridae*. There are also a few reports on the applications of filamentous phages and cubic phages (Drulis-Kawa et al., 2012). For any type of life cycle, the initial step of any type of phage infection is the recognition and binding to the host receptor, which is facilitated by one type of protein named phage receptor binding protein (RBP). The host specificity of bacteriophages towards different bacterial cells depend on the different receptor binding proteins of the phages (Le et al., 2013). Three types of host receptors in *Salmonella* were identified by Shin et al. (2012): flagella, BtuB (outer membrane protein up taken by vitamin B12) and lipopolysaccharide-related O-antigen. Transmission electron microscopy analysis showed that the phages from *Podoviridae* family use O-antigen of LPS as a receptor while

phages from *Siphoviridae* family use flagella (BtuB) as a receptor. Most frequently, mutations of these receptors caused the host cells resistant to these phages (Shin et al., 2012). The recognition of phage to host cells and the subsequent binding of RBPs and host receptors stimulate the spectrum of the probable phage-bacteria interactions (Wittebole et al., 2014). After binding to the host receptor, the phages induce a pore in the bacterial cell wall and insert its DNA into the cell after binding to the host receptor, while the viral capsid remains outside of the host cell. Before the cell lysis of bacteria packing and assembly of phages are observed and finally release of phage progeny occurs (Figure 1.3). Synthesis inhibitors of different phage enzymes (lysins, holins, and murein) are then involved for the burst of the virion into the extracellular environment (Weinbauer, 2004). In molecular aspect, when a phage invades a susceptible bacterial cell, its nucleic acid enters the cell and induces production cycle of the phage. The cell is converted to a phage factory. Some of the components of the biosynthetic apparatus involved in bacterial growth and metabolisms (such as ribosomes and ATP generators) are no longer performing their normal tasks during the phage production cycle (Campbell, 2003). It is known that, while bacteria can evolve to become resistant to phages, phages can also develop new mechanisms to infect the resistant bacteria. Hosseinidoust et al (2013) demonstrated that, resistance development is linked to changes in bacterial fitness and alteration of virulence determinants that are usually maintained in the absence of the agent to which the bacteria confer resistance. The alteration of phenotypic characteristics is associated with changes in gene regulation levels (Hosseinidoust et al., 2013).

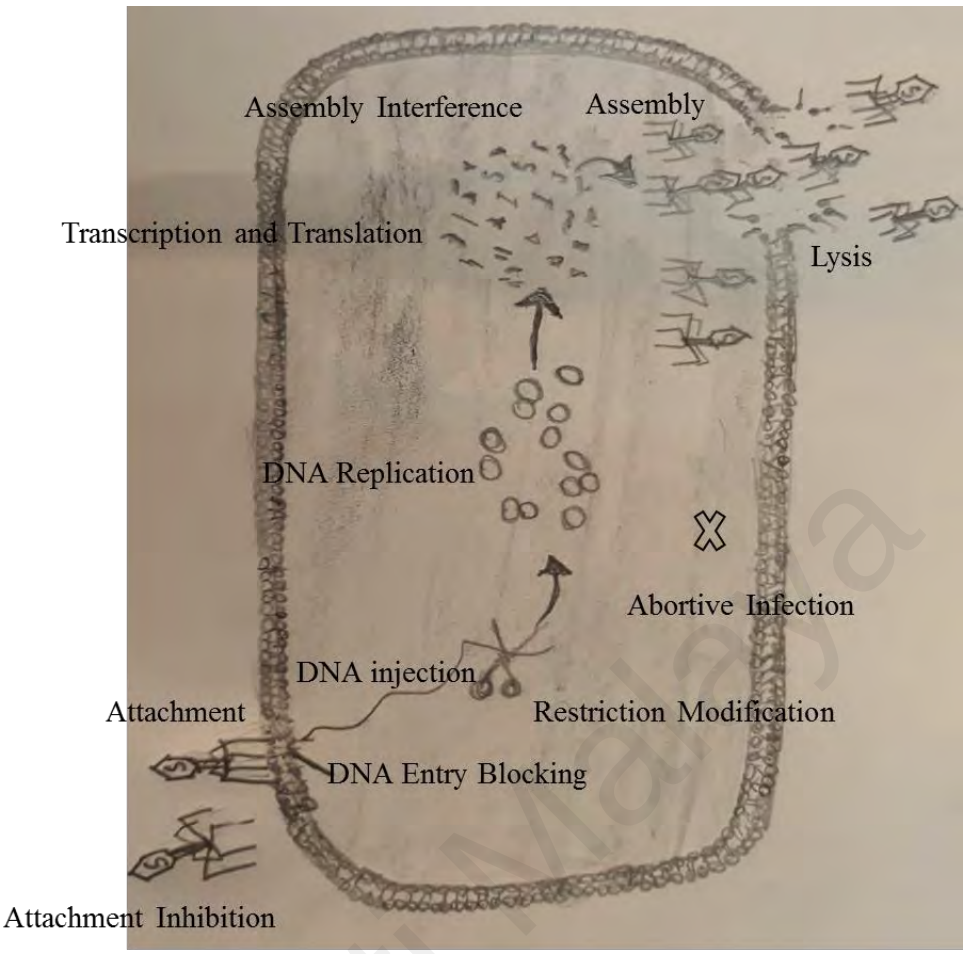

Figure 1.3: Phage-Bacteria interaction mechanism. Bacteria can modify the receptor on the cell membrane responsible for phage recognition and adsorption. Using restriction enzymes, bacteria cut and destroy the phage genetic material that entered the cell. During infection, bacteria may activate the cascade that brings to bacterial apoptosis; this altruistic mechanism takes the name of abortive infection (Seed, 2015)**.**

1.5 Bacteriophage as an alternative to antibiotic

Treating multidrug resistant *Shigella* spp. by a new antibiotic or a new combination of antibiotics tends to be more complicated than treating it with a phage or phage cocktail (Khatun et al., 2011). Generally, phages are environmentally friendly because they are not pathogenic or toxic to human. Though in both cases the clinical trial is expensive but it is usually quicker and less expensive to select, isolate and identify phages than to develop a new antibiotic, which can take also longer period (Matsuzaki et al., 2005; Weber-Dąbrowska et al., 2001). Secondary infections may happen but very rare and minimal in phage therapy compared to antibiotics.The term secondary infection during phage therapy is due to virion interaction with a bacteriophage infected bacteria, and it

can be resulted as super-infection immunity or super-infection exclusion. Alternatively, secondary infection can also be mentioned equally to super-infection or co-infection which can lead the genetic interchange among bacteriophages, phage-on-phage parasitism together with several partial decreases in phage productivity that have been termed as, mutual or partial exclusion, and the depressor effect (Abedon, 2015). With respect to antibiotics, it can cause secondary infection by attacking the normal flora of patients, in addition to the targeted pathogens. Besides these, phage resistance is less of a concern than antibiotic resistance, because phages can mutate and evolve naturally to counter phage-resistant bacteria (Ho, 2001; Matsuzaki et al., 2005). Moreover, the phage resistance development can be mitigated by using phage cocktails (combinations of multiple phages) and/or by applying phages in conjunction with antibiotics as therapy (Ho, 2001; Kutateladze & Adamia, 2010). The differences between phage therapy and antibiotic treatments are summarized in Table 1.1

Table 1.1: Comparison between phage therapy and antibiotic treatment

Feature	Phage	Antibiotic
1.Host specificity	Very specific to their host cells: usually affect primarily or exclusively the targeted bacterial species (Chernomordik, 1989).	Can target a wide range of pathogenic microbes. Can therefore be used when the exact disease-causing pathogen is unknown. However, this can lead to the emergence of new drug resistant pathogens (Sulakvelidze et al., 2001).
2.Mode of action	Bacteriophages replicate exponentially as long as the specific bacteria they are targeting are available in abundance. They replicate at the site of infection and are available where they are most needed (Smith & Huggins, 1982).	Antibiotics are metabolized and then expelled from the body, and do not necessarily concentrate at the site of infection (Sulakvelidze et al., 2001).
3.Side effects	Generally the side effects are less than the antibiotic treatment. No or very few side effects have been described (Sulakvelidze et al., 2001).	Due to their non-specificity to the host, antibiotics destroy commensal microflora. This can lead to several side effects, including allergies, intestinal disorders and secondary infections (Inal, 2003; Lehmann, 1999).
4.Time and cost for new development	The selection of new phages against drug resistant or phage resistant bacteria is a comparatively rapid process which can be carried out in days or weeks (Sulakvelidze et al., 2001). Sometimes, it also takes longer period and extra cost for safety approval and *in vivo* trial.	The development of a new antibiotic against antibiotic resistant bacteria is not only time-consuming, but can also cost millions of dollars for clinical trials, and so may not be cost-effective (Chopra et al., 1997; Silver & Bostian, 1993).
5.Dose administration	Repeated dose administration is not always essential, because the phage reproduces until the target bacterium is destroyed (Inal, 2003).	Most cases require repeated dose administration.
6.Application range	In spite of some negative effects, the range of applications of bacteriophages is broader: they can for example be applied as protective materials in food supplements, the milk industry, pharmacology, toothpastes, cleaning solutions and so on (Veiga-Crespo & Villa, 2010).	The application range of antibiotics are restricted and narrower.

As mentioned in the Table 1.1, one of the advantages of phages is that they have much lower side effects. In fact, the prolonged use of phages to medicate human infections in Eastern Europe has not shown any allergic reactions nor have animal trials in Western Europe revealed any unusual histological changes, mortality or morbidity when phages were administered orally, intravenously or intramuscularly (Biswas et al., 2002; Carlton et al., 2005; Merril et al., 1996). Indeed, intakes of the T4 phage up to 10^5 PFU (Plaque Forming Unit) have not caused any secondary effects (Bruttin & Brüssow, 2005). The intravenous injection of purified phages have not produced any side effect in either HIV-infected individuals (Fogelman et al., 2000), healthy volunteers (Ochs et al., 1993), or other infected people with immunodeficiency syndromes (Ochs et al., 1971).). Phage therapy has been successfully used to treat antibiotic resistant infections in the Southwest Regional Wound Care Centre in Texas (Clark & March, 2006) while biodegradable patches impregnated with phages have also been applied to patients with prolonged infections in Georgia (Fischetti et al., 2006).

In summary, as antibacterial agents, phages have a number of properties that make them a compelling alternative to antibiotics. Moreover, most of the concerns associated with phage therapy should be manageable through a combination of appropriate phage selection, effective formulations, clear knowledge and expertise on how to prepare and apply phages (Loc-Carrillo & Abedon, 2011)

1.6 Problem statement and research questions

Emerging resistance of *Shigella* towards antibiotics is a public health concern worldwide, especially in the developing world. New antibiotics in the development and research pipeline is scarce and has been deemed uneconomical due to the short time span of antibiotics in the market, in comparison to pharmaceuticals treating chronic diseases. Hence, there is an extreme urgency to look for an alternative treatment against infectious

disease that is both sustainable and efficacious. Phage therapy, a century old remedy for bacterial infection that has mostly been forgotten in the greater part of the world, is an interesting field to revisit and investigate. In addition, reports of the efficiency of phage therapy in eastern European countries e.g. USSR, Poland and Georgia, further instigate our interest in phage therapy as a potential therapeutic agent. Thus, in this study our aim is to address the following research questions:

1. Is phage therapy a relevant alternative treatment for bacterial infection in the current age of antibiotic resistance?
2. Does a bacteriophage with a wider host range solves the limitation of host specificity in phage therapy?

1.7 Scope of the research and objectives

Shigella spp. is an endemic in both the developing and developed world. In Malaysia, *Shigella* is the 2nd runner up bacterial agent responsible for infant diarrhoea. Lately, the uncontrolled prescription and over usage of antibiotics in the clinical environment, has led to the emergence of multidrug resistance bacteria. *Shigella*, a bacterium historically prone to acquire resistance to antibiotics has become a worrisome issue in the clinical field. Hence, to mitigate this issue, several alternatives are looked upon, one of them being, exploiting natural predators of the bacteria i.e. bacteriophages. In a recent editorial published in a highly-renowned journal 'Cell' revising bacteriophages as potential antimicrobials is currently one of the nine major research questions in microbiology (Mizrahi, 2017). So, isolation and characterization of potential bacteriophages against drug-resistant *Shigella* spp. as well as others bacterial pathogens are now burning question for the researchers. This thesis sought to investigate the efficacy of phages against drug resistant *Shigella* spp. through the study of various important aspects.

To meet the specific purpose of the thesis, the current studies have been designed with the following objectives:

(i) To isolate and identify different serotypes of *Shigella* spp. from clinical and environmental samples.

(ii) To isolate, purify and characterize the bacteriophages from sewage water samples against different serotypes of *Shigella* spp. on the basis of their lytic action.

(iii) To investigate the host specificity range of isolated bacteriophages.

(iv) To identify a wide host-range bacteriophage on the basis of lytic action and host specificity

(v) To sequence and analyse the genome of the bacteriophage with the greatest potential.

CHAPTER 2: LITERATURE REVIEW

2.1 Discovery of *Shigella* spp.

Sir William Osler (1892), the famous physician described dysentery as "one of the four great epidemic diseases of the world and it destroys more life than cholera" (Kotloff et al., 2018; Osler, 1892). Five years later, in 1897, the Japanese microbiologist Kiyoshi Shiga discovered *Shigella* during an epidemic in Japan associated with high mortality (Shiga, 1898; Trofa et al., 1999). The *Shigella* spp. discovered then was *Shigella dysenteriae* and the bacterium was termed initially as the Shiga bacillus. The *Shigella* genus was soon expanded with the discovery of *Shigella flexneri* in 1899 (Flexner, 1900), followed by *Shigella sonnei* in 1906 and *Shigella boydii* in 1921 (Barceloux, 2008; Shiga, 1936). *Shigella* spp. shows genetically resemblance with the bacterial species *Escherichia coli.* Dr. Kiyoshi Shiga reported the proximal identity between the two species of bacteria in 1898. Despite the phenotypic similarities between these two groups, variations in biochemical tests are still present. The bacterial strains can be distinguished on the basis of some key features i.e. lactose-fermentation, gas production and motility. Although these features are manifested in some *E. coli* strains termed as 'inactive' increasing the complication of discerning this two taxa (non-lactose fermenting, non-gas-producer and non-motile (Khot & Fisher, 2013).On the basis of DNA homology Brenner et al. (1973) stated that nearly all *Shigella* spp. share more than 80% nucleotide sequence similarities, and the incidence is similar in the sequence likenesses between *E. coli* and *Shigella* spp. Cilia et al. (1996) confirmed the proximal affinity while a 16S rRNA sequence study failed to differentiate *Shigella* and *E. coli* into two distinct clades, which indicates high degree of resemblance. Comparative genomic study of the whole genome, virulence genes and housekeeping genes also exhibited similar results. Beld and Reubsaet (2011) commented that *Shigella* spp. should be placed within the *E. coli* species as the degree of

similarities between the two species is remarkably higher (Beld & Reubsaet, 2011). Nevertheless the two taxa were separated because of the medical importance of Shigellosis caused by *Shigella* spp. Johnson (2000), criticized that the separation of *Shigella* spp. from the taxon *E. coli* as a 'taxonomic treachery'. Recent development of phylogenetic analysis techniques which does not require parametric changes and is not biased towards specific genes suggested that *Shigella* spp. is in fact distinct from *E. coli* (Zuo et al., 2013). Carbohydrate composition of the O-antigen, the polysaccharide constituent of the lipopolysaccharide (LPS) plays a vital role for the determination of *Shigella* serotypes (Lindberg et al., 1991).This present research would provide proper insights in the taxonomic separation of *Shigella* as a distinct species on the basis of serotyping and multiplex PCR.

2.2 Occurrence of Shigellosis

Shigellosis caused by *Shigella* is an endemic, and is one of the major causes of morbidity and mortality in all age groups in both developing and developed countries. It is particularly prevalent in children between zero and five years in developing countries (Bardhan et al., 2010; Wen et al., 2012). In recent time *S. flexneri* is the major cause of bacillary dysentery in low-income countries, particularly in Asian and sub-Saharan African countries, accounting for up to 62% of all *Shigella* infections in these areas. On the other hand, *S. sonnei* is the most common pathogen in high-income or transitional countries, especially in North America and Europe, accounting for up to 80% of all *Shigella* infections in this zone (Gu et al., 2012a). Previously, a multicenter study on Shigellosis conducted in six Asian countries (Bangladesh, China, Pakistan, Indonesia, Vietnam and Thailand) reported *S. flexneri* as the most frequent isolate of *Shigella* spp. (68%), except in Thailand (Von Seidlein et al., 2006a). In contrast, Shigellosis caused by the species *S. boydii* and *S. dysenteriae* in recent years has been reported in less than 5%

cases globally. Interestingly, *S. dysenteriae* was the main cause of dysentery more than 100 years ago, but the incidence of this pathogen is now quite rare (Bardhan et al., 2010; Gu et al., 2012b). In the late 19th and early 20th centuries, *S. dysenteriae* caused numerous outbreaks. It then disappeared for unknown reasons, although *S. dysenteriae* type 1 reappeared as an epidemic in 1968 in Central America, Asia and Africa (Gangarosa et al., 1970; Pal, 1984; Rahaman et al., 1975; Ries et al., 1994). Later, the prevalence of *S. dysenteriae* was replaced by *S. flexneri*, which in turn was gradually replaced by *S. sonnei* (Kostrzewski, 1968; Martin et al., 1983). Occurrences of *S. boydii* have meanwhile been reported on the Indian subcontinent and Latin America, but have been infrequent in other regions of the world (Fernandez-Prada et al., 2004; Niyogi, 2005; Rolfo et al., 2011). Outbreaks of *Shigella* are common, and have been reported widely. For instance, recently Michigan State of the USA experienced the largest outbreak of Shigellosis after 1988. The Michigan Department of Health and Human Services (MDHHS) reported 180 cases of *Shigella* infection (Doore et al., 2018). A serious outbreak occurred between 2014 and 2015 in California, with the causative agent being *Shigella sonnei* (Kozyreva et al., 2016). At the same time, the frequency of occurrence and severity of Shigellosis outbreaks varied greatly between different regions and countries. In Morobe province on the northern coast of Papua New Guinea, approximately 1200 cases and five deaths were reported as Shigellosis caused by the *S. flexneri* serotype 2 (Benny et al., 2014), while fifty-five cases of Shigellosis were reported in Taiwan caused by *S. flexneri* 2a, *S. sonnei* and *S. flexneri* 3b (Ko et al., 2013). In Bangladesh, a total of 10,827 isolates were identified between 2001 and 2011, with the predominant spp. detected being *S. flexneri,* followed by *S. sonnei, S. boydii* and *S. dysenteriae*, respectively (Ud-Din et al., 2013). In Sichuan province (China), about 96 students in a rural elementary school suffered from Shigellosis after drinking untreated well water; the causal organism identified in this case was *S. flexneri* 2b (He et al., 2012). In another outbreak in Parison city (Iran), 701 inmates

experienced severe diarrhoea caused by *S. flexneri* serotype 3a (Hosseini & Kaffashian, 2010). Two outbreaks were reported in Sweden in 2009, caused by *S. dysenteriae* (Löfdahl et al., 2009) and *S. sonnei,* that affected air travelers departing from Hawaii (Gaynor et al., 2009). In Austria, a foodborne outbreak of Shigellosis caused by *S. sonnei* was reported (Kuo et al., 2009) while the infection of *Shigella* spp. (Müller et al., 2009) was reported in Denmark. The above incidences show that outbreaks of shigellosis caused by *Shigella* have been occurring frequently all over the world, from developing to developed countries, with the predominant causative spp. being *S. flexneri* and *S. sonnei*.

2.3 Global burden of Shigellosis

Shigella was ranked by the Global Burden of Disease Consortium as the 2nd major cause of diarrhoeal deaths in all age groups and the third major reason of diarrhoeal deaths in children under five years old in 2015 (Abdoli & Maspi, 2018).The worldwide deaths due to diarrhoea are estimated to have reduced between the year 2005 and 2015 by 20.8% and 34.3% among people of all ages and children younger than five years, respectively (Troeger et al., 2017). However, some diarrhoeal pathogens, including *Shigella* and ETEC are still considering as substantial causes of mortality and morbidity, especially among infants and young children in middle and low -income countries, as well as morbidity in military personnel and travelers from higher income countries (Lanata et al., 2013; Riddle et al., 2006; Shah et al., 2009; Vos et al., 2016; Wang et al., 2016). Diarrhoea was responsible for more than 1,600,000 deaths and the seventh leading cause of global death in 2016. Nearly 90% of these mortality occurred in South Asian and sub-Saharan African countries (Khalil, 2017). In 2016, *Shigella* caused about 75,000 deaths among the children under age group five and 270,000 deaths among all age groups while in 2010 *Shigella* was responsible for 28,000 deaths among the children under age five and 122,800 death among all ages (Khalil, 2017; Lozano et al., 2012). This indicate that the diarrhoeal

death due to *Shigella* has increased between the year 2010 and 2016 by 2.68 times in case of children under-five and 2.20 times in case of all age groups. The percentages of death due to Shigellosis among all types of diarrhoeal death was 8.93 in 2010 and this trend has increased gradually till 2016 (16.88%) only decreased once in 2013 (5.84%) (Table 2.1 and Figure 2.1).

Table 2.1: Year-wise comparison between all types of diarrhoeal death and number of *Shigella* death

Year	All types of diarrhoeal death	Number of *Shigella* death (age under five)	Number of *Shigella* death (adult)	Number of *Shigella* death (all age group)	% of *Shigella* death (all age group)	Reference
2010	1,445,800	28,000	94,800	122,800	8.93	(Lanata et al., 2013; Lozano et al., 2012)
2013	1,264,100	33,400	40,500	73,900	5.84	(Abubakar et al., 2015)
2015	1,31,2100	54,900	109,400	164,300	12.52	(Wang et al., 2016)
2016	1,600,000	75,000	195,000	270,000	16.88	(Khalil, 2017)

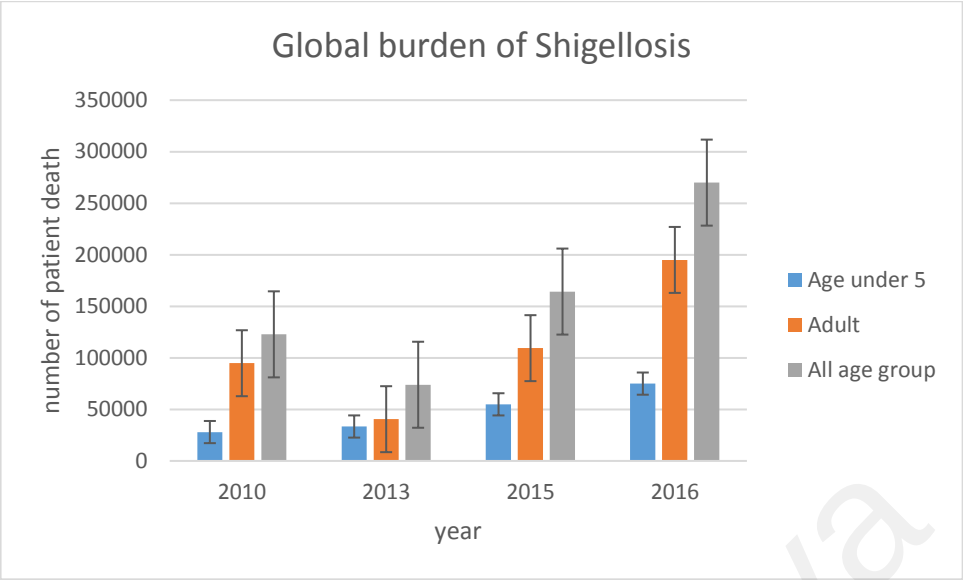

Figure 2.1: Global Burden of Shigellosis: Number of death among age group under-five, Adult, and all age group based on available data published by (Abubakar et al., 2015; Khalil, 2017; Lanata et al., 2013; Lozano et al., 2012; Wang et al., 2016)

2.4 Emergence of multidrug resistant *Shigella* spp.

The World Health Organization designated *Shigella* a priority focus for research and development of new antibiotics (WHO, 2017). From the beginning of the antibiotic era, Tetracycline, Ampicillin, Chloramphenicol, Nalidixic acid and Trimethoprim-Sulfamethoxazole were used to treat Shigellosis. As *Shigella* developed increasing resistance to these antibiotics, more recently Ciprofloxacin, Ceftriaxone and Azithromycin have served as the mainstays of Shigellosis treatments. However, many studies have reported the growing resistance of *Shigella* spp. against these antibiotics also (Table 2.2) (Klontz & Singh, 2015).

Table 2.2: First use of antibiotics for *Shigella* treatment and initial reporting of resistance

Name of drug	Beginning period	Place and initial report of resistance	References
Sulfonamide	1930s	Philippine Islands (1946) Japan (1952 - 1957) Israel (1953 - 1955) USA (1961 - 1964)	(Cheever, 1946; Haltalin & Nelson, 1965; Marberg et al., 1958; Susumu Mitsuhashi, 1969, 1971; S Mitsuhashi et al., 1960)
Ampicillin	Late 1960s to 1970s	New Zealand (1974) Bangladesh (1974) Mexico city (1976)	(Olarte et al., 1976; Rahaman et al., 1974; Smith et al., 1974)
Rimethoprim–sulfamethoxazole	1970s	Brazil (1980) Canada (1980) Korea (1981) India (1981) Finland (1975 - 1982) Bangladesh (1979 - 1983)	(Chun et al., 1981; Finlayson, 1980; Heikkilä et al., 1990; Macaden & Bhat, 1985; Taylor et al., 1980; Zaman et al., 1983)
Furazolidone	1970s	Dallas, USA (1972) India (1984)	(Bose et al., 1984; Lexomboon et al., 1972)
Nalidixic acid	1980s	Zaire (1982) India (1984) Bangladesh (1986) Burundi (1990)	(Bhardwaj & Panhotra, 1985; Munshi et al., 1987; Ries et al., 1994; Rogerie et al., 1986)
Pivmecillinam	1970s	Bangladesh (2000 - 2012)	(E. H. Klontz et al., 2014)
Fluoroquinolone	Late 1980s to 1990s	India (1984)	(Bose et al., 1984)
Azithromycin	1990s to 2000s	India (2006 - 2011) Netherlands (2012)	(Bhattacharya et al., 2014; Hassing et al., 2014)
Ceftriaxone	1990s to 2000s	Korea (2000) Vietnam (2000 - 2002) India (2006 - 2011) USA (2003 - 2012)	(Bhattacharya et al., 2014; Pai et al., 2001; Vinh et al., 2009)

There have been numerous reports of single drug resistance, cross-resistance and multidrug resistance in *Shigella* worldwide, and such cases are growing in both frequency and diversity on a daily basis (CDC, 2015; NSSRL, 2016; Von Seidlein et al., 2006b). In a study, data of approximately 1376 *Shigella* isolates were collected from the Foodborne Diseases Active Surveillance Network (FoodNet) which were tested in the US National Antimicrobial Resistance Monitoring System (NARMS) between 2000 and 2010 (Shiferaw et al., 2012). Among the tested isolates, 74% proved to be Ampicillin resistant, followed by 58% that were Streptomycin resistant, 36% Trimethoprim-Sulfamethoxazole (TMP-SMX) resistant, 32% Sulfamethoxazole-Sulfisoxazole resistant, 28% Tetracycline resistant, 2% Nalidixic acid resistant, and 0.5% ciprofloxacin resistant. Moreover, around 5% of these strains showed multiple resistance to ampicillin, streptomycin, chloramphenicol, tetracycline and Sulfamethoxazole-Sulfisoxazole (Shiferaw et al., 2012).

In 2002, *S. dysenteriae* type 1 isolates were identified in Eastern India that showed resistance to all available antibiotics, including Norfloxacin and Ciprofloxacin but with the exception of Ofloxacin (Sur et al., 2003). In the following year, similar type isolates were detected in Bangladesh that were resistant to all common antibiotics, including Ofloxacin (Naheed et al., 2004). In addition, about 200 *S. sonnei* isolates were identified in Bangladesh that demonstrated resistance to a wide range of commonly used antibiotics, including Trimethoprim-Sulfamethoxazole, Nalidixic acid, Ciprofloxacin, Mecillinam andAmpicillin, at ratios the of 89.5, 86.5, 17, 10.5, and 9.5%, respectively (Ud-Din et al., 2013).

In a study between 2001 to 2009, in Malaysia a total of 138 *Shigella* spp. strains were isolated wherein *Shigella sonnei* (50%) was the most prevalent subgroup followed by *Shigella flexneri* (49.3%), and all strains were resistant to one or more drugs among the seven tested antibiotics (Singh et al., 2011). In the annual report of the National

Salmonella, Shigella & Listeria Reference Laboratory (NSSLRL-2014, https://www.researchgate.net/publication/280804929), 93% of *Shigella* isolates were identified as multi-drug resistant. The prevalence of resistance to Azithromycin, Fluoroquinolones and Ceftriaxone do vary considerably among different regions of the world (Bhattacharya et al., 2014). More recently, a study in Iran reported high frequency of Trimethoprim/Sulfamethoxazole, Ampicillin, Cefotaxime and Nalidixic acid resistance (80, 85, 63 and 47% respectively), in 85 *Shigella* strains isolated from 211 positive stool cultures of children with gastroenteritis (Mahmoudi et al., 2017). In particular, one study demonstrated that *Shigella* exhibited far higher levels of resistance to Nalidixic acid and Ciprofloxacin in Asia-Africa than in Europe-America: 33.6% and 5.0% respectively, or 10.5 and 16.7 times higher (Gu et al., 2012b).In summary, it is extremely difficult to delimit the geographic range of drug resistant strains of *Shigella* or to control the disease through a single antibiotic, because of the dissemination of resistant pathogens through multiple vectors and the continuous emergence of new serotype

2.5. Early history of phage therapy

The idea about bacteriophage was first given by Ernest Hankin in 1896 while searching for an antibacterial agent against *Vibrio cholerae* from the water of the Ganges and the Jumna rivers in India (Hankin, 1896). He reported that an unknown substance which could pass through fine porcelain filters and was heat labile, capable of killing the pathogen *Vibrio cholera*, preventing the spread of cholera epidemics. Two years later, Gamaleya, a Russian bacteriologist, observed a similar phenomenon while working with *Bacillus subtilis* (Samsygina & Boni, 1984). However, neither of them succeeded in defining and characterizing these antimicrobial agents. It is reported that, Bacteriophage was discovered and reported independently at the beginning of the 20th century by Frederick Twort and Felix d'Herelle (Nobrega et al., 2015). Almost 20 years later, Frederick Twort hypothesized in 1915 that these agents could be viruses (Twort, 1915).

After a couple of years, in 1917, Felix d'Herelle isolated an antibacterial agent from the stools of patients who had recovered fully from dysentery. He suggested that this was a virus, and officially coined the name of "bacteriophage", derived from "bacteria" and "phagein" – meaning that phages "eat" or "devour" bacteria. He described phages as the "exogenous agents of immunity", based on their prophylactic and therapeutic functions in eliminating various types of infectious diseases (d'Herelle, 1917; Summers, 2005). In addition, d'Herelle and his co-workers isolated phages with lytic activity against pathogenic bacteria, including *Shigella* spp., and developed the idea of "phage therapy" meaning the prophylactic and/or therapeutic use of these substances (D'Herelle, 1923). Bacteriophages were then subsequently used in medicine from 1919 onwards - before the invention of the first antibiotic (penicillin). Figure 2.2 summarizes important milestones in the development of phage research. In the early stages, expectations were particularly high with regard to Shiga-phages (phages against *Shigella*), due to their success in treating dysentery patients safely. This success inspired the commercialization of therapeutic phages to treat bacterial infections in humans (Eaton & Bayne-Jones, 1934; Krueger & Scribner, 1941). However, at that time, scientists did not fully understand the mechanisms behind the treatment, and in particular how the phages killed the bacteria. As a result, the outcomes of phage treatment were inconsistent. Moreover, the introduction of antibiotics in the 1940s to treat a broader range of infections led to a reduction in phage therapy research (Matsuzaki et al., 2014). Despite the success of phage therapy in a number of Eastern European countries, it remained largely neglected in Western Europe due to the inconsistent results, the lack of a specific regulatory framework, and the complicated procedures for patenting phages (Verbeken et al., 2014).

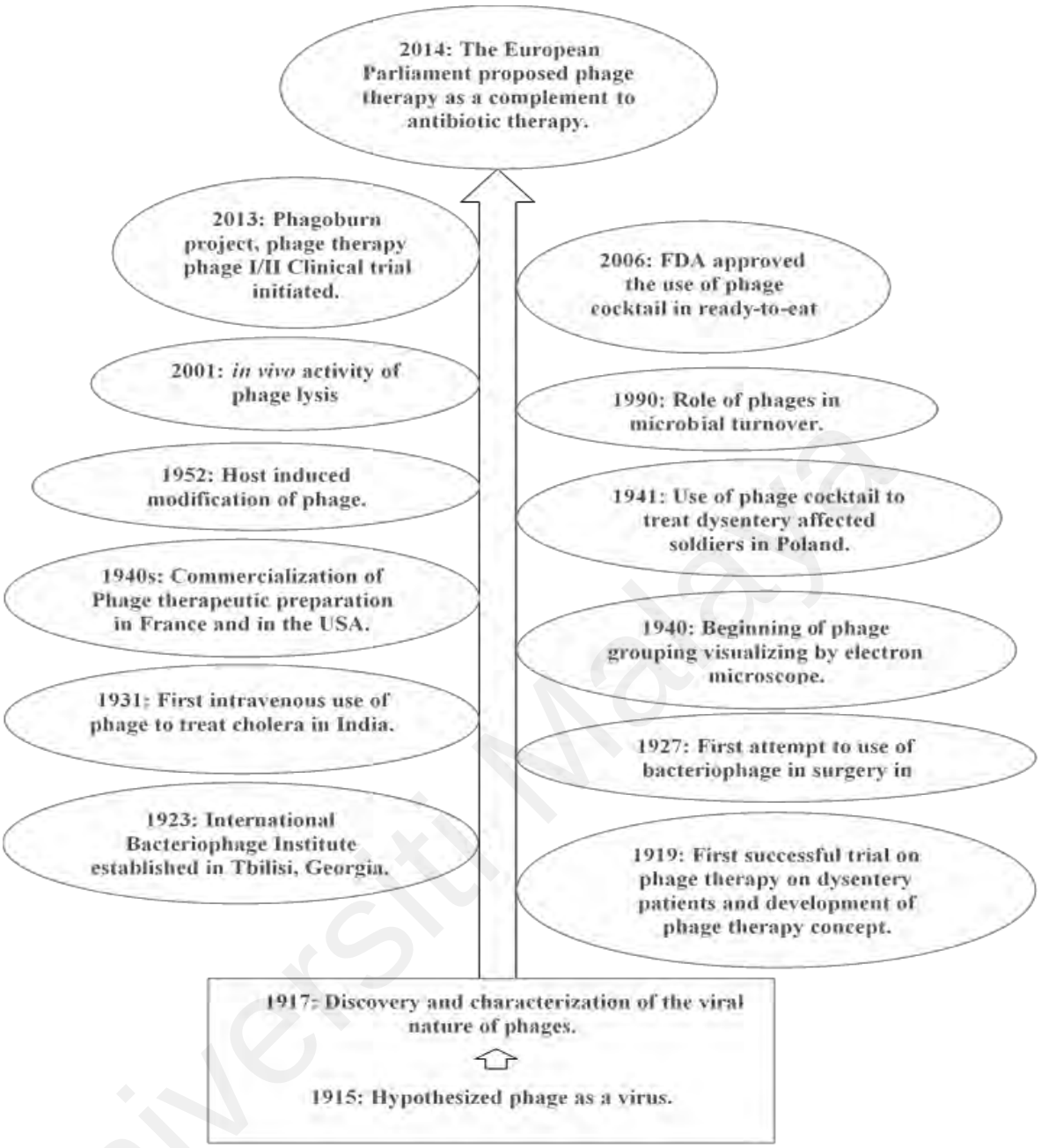

Figure 2.2: Milestones in phage therapy research (Tang et al., 2019)

2.6 Phage therapy now

The interest in phage therapy and the use of phages to control bacterial infections have been revived in recent time and the unanswered questions of phage therapy are now gradually being addressed (Fischetti, 2001; Stone, 2002; Summers, 2001). Scientists are looking back to the pre-antibiotic era with the aim of resurrecting phages as an antidote to antibiotic resistant pathogens as well as to solve other medical, agricultural, food safety

and environmental problems. Nowadays, the availability of sophisticated molecular tools, the growing understanding of phage control techniques, and the prolonged experiences of Eastern European researchers have all widened the possibility of phage therapy applications. In Eastern Europe, phages have been applied orally (tablets or liquid), topically, rectally and intravenously for almost 90 years with no serious side effects have been reported (Sulakvelidze et al., 2001). As a result of these developments, phage therapy has attracted increasing attention as a potential alternative solution in treating antibiotic-resistant bacteria. Six clinics in five different countries (the US, the UK, the Republic of Georgia, Poland and Belgium) are now offering phage therapy for treating diseases, few have shown in Table 2.3

Table 2.3: Main features and activities of phage therapy centers

Name of center	Country	Main features and activities
Center for Phage Therapy	Poland	Since 1980, specific bacteriophages have been used to treat over 1500 patients with suppurative bacterial infections, where routine antibiotic therapy has failed. (www.iitd.pan.wroc.pl).
Eliava Phage Therapy Center	Georgia	A network of eight laboratories have developed bacteriophage preparations for fighting against dangerous and antibiotic-resistant superbugs. (www.mrsaphages.com).
Novomed	Georgia	Effective treatment delivery through phage therapy in many areas of medicine, drawing on the expertise of local physicians. Treatments are available not only to local Georgians, but also to foreign patients, especially those with chronic wounds, osteomyelitis or other types of acute and chronic infections. (www.phagetherapy.com).
Phage Therapy Center	Georgia	Provides excellent treatment for patients who have bacterial infections and are difficult/non-healing, chronic, drug-resistant or have not responded to conventional antibiotic therapies. (www.phagetherapycenter.com).
Phage International Inc.	The United States	Treats patients with chronic, drug-resistant or difficult to treat infections. (www.phageinternational.com)

There are about 11 US and international biotechnology companies, as well as a number of academic investigators currently working in the field of bacteriophage technology and products. These companies and researchers are utilizing bacteriophages in the food processing industry and for the treatment of human diseases. For example, US companies

such as Intralytix and Novolytics are using bacteriophage as biotechnology tools and as platform technologies (http://www.dreamingrock.com/viridax/eviridax/cphage.htm). The FDA and the United States Department of Agriculture (USDA) have marked a milestone in phage research by approving three phage products, each comprising a "cocktail" of phages, to target and kill bacteria. ListShield™ (Intralytix, USA) is the first phage product approved by the FDA as a food additive against *Listeria monocytogenes* in ready-to-eat meat and poultry (Mead et al., 2006). The second product, EcoShield™ (Intralytix, USA), is used as a spray on red meat, before it is ground into hamburgers, in order to kill *Escherichia coli* O157:H7 (Abuladze et al., 2008; Scallan et al., 2011). The third phage product, called SalmoFresh™ (Intralytix, USA), which acts against *Salmonella enterica* and is approved as a food processing aid for the treatment of fish, shellfish, as well as fresh and processed fruits and vegetables. Lately, another phage preparation, ShigaShield™ (Intralytix, USA), is currently undergoing FDA and USDA review for the GRAS (Generally Recognized As Safe) status (GRN672). According to the report by Soffer et al. (2017) this *Shigella* phage product, ShigaShield™ is able to reduce *Shigella* levels in various foods experimentally contaminated with a *S. sonnei* strain (Soffer et al., 2017). Novolytics has the aim to lead the utilization of bacteriophage as a treatment for bacterial infections. Currently, the company's most promising products NOVO12, a phage cocktail, administered as a gel for topical treatment of MRSA (Methicillin-resistant *Staphylococcus aureus*) infections. (http://www.cobrabio.com/News/June-2013/Cobra-Biologics-and-Novolytics-Unveil-Successful-D).The European Union (EU) also shows support for phage therapy research. In 2013, a project entitled 'Phagoburn', aimed at exploring the efficacy of phage therapy in protecting patients of burn wounds against severe bacterial infection, was funded by European Commission (Matsuzaki et al., 2014). The European parliament has passed a resolution in favour of prioritizing the development of phage therapy for combating antibiotic resistance as a complement to

antibiotic therapy (Europian-council, 2014). This is an important indication of support for fostering phage therapy research and development, but a time frame is needed to see its practical impact in future.

2.7 Bacteriophages of *Shigella* spp.

Different *Shigella* phages have been isolated and their potentialities have been described by different scientists. Shahin et al. (2018) isolated a bacteriophage of T1 genus designated as vB_SsoS-ISF002 able to infect *Shigella sonnei* and *Shigella flexneri* was under *Myoviridae* family. It was a double stranded DNA phage with 50564 bp genome size , 45.53% G+C content and 76 predicted open reading frames (Shahin et al., 2018). Hamdi et al. (2017) isolated two bacteriophages SH6 and SH7 against *Shigella flexneri* belonged to family *Siphoviridae* and *Myoviridae* respectively. The genome of phage SH6 is composed of 50,552 bp of double-stranded DNA with 45.8% G+C content, in the same range as phage T1 while the genome of phage SH7 is composed of 164,870 bp of double-stranded DNA with 35.5 % G+C content in the same range of T4 genus (Hamdi et al., 2017). Jun et al. (2016) isolated *Myoviridae* bacteriophage pSs-1, showed infectivity against *Shigella flexneri* and *Shigella sonnei*. According to the genomic analysis, pSs-1 contains 164,999 bp of genome with a G+C content of 35.54% and it is considered as a member of the T4-like bacteriophage group (Jun et al., 2016).

In another studies, bacteriophage pSf-1 was isolated against *Shigella flexneri* belonged to the family *Siphoviridae*. The double-stranded DNA genome of pSf-1 is composed of 51,821 bp with a G+C content of 44.02% (Jun et al., 2013) while bacteriophage designated as SP18 having 170,605 bp long genome size and 40.4 % G+C content was isolated against *Shigella* belonged to the family *Myoviridae* (Kim et al., 2010). Jun et al. (2014) isolated another phage designated as pSb-1 against *Shigella boydii* was under *Podoviridae* family. According to the genomic analysis, pSb-1 contains 71,629 bp of

genome with a G+C content of 42.74% and it is considered as a member of the N4-like bacteriophage group (Jun et al., 2014)

There is a potential application based example of *Shigella* phages that is ShigaShield™. ShigaShield™ is a phage "cocktail" composed of 5 lytic *Shigella* phages (mixed in approximately equal concentrations) designated as SHSML-52-1, SHFML-11, SHSML-45 , SHFML-26 , and SHBML-50-1 having genome sizes of 169621 bp, 170650 bp, 108050 bp, 168993 bp and 166634 bp respectively. Among the five phages, SHSML-45 belongs to the family *Siphoviridae* and rest four phages belong to the family *Myoviridae*. It is mention worthy that, each phage genome in ShigaShield™ was fully sequenced and annotated and no toxin, virulence, repressor genes, integrases, recombinases nor any bacterial gene listed in the US Code for Federal Regulations were detected (Soffer et al., 2017).

2.8 Experimental model of phage treatment against *Shigella* infection

Many researchers have attempted to develop mammalian models for treating bacillary dysentery using mice, guinea pigs, rabbits, and macaques , of these, mice are the most suitable in terms of cost, ease of handling, and availability of gene-manipulated animals (Yang *et al*., 2014). In a study, a mouse model showed that the administration of bacteriophage cocktail 'ShigaActive™' significantly reduced the bacterial count (10- to 100 fold) compared to untreated control mice without any side effects or distortions in overall gut microbiota (Mai et al., 2015).

In another study, it is investigated that *S. flexneri* can infect the soil dwelling nematode *Caenorhabditis elegans*. This finding will help to infect *C. elegans* through *Shigella* spp. and treating that *Shigella* infection through phage treatment. This is immensely beneficial to the routine use of this new in vivo model to study phage therapy on *Shigella* pathogenesis (George *et al*., 2014).

2.9 Phage therapy for controlling *Shigella*

There is a historic relationship between *Shigella* spp. and the discovery of phages. The first application of phage against human infections was conducted by d'Herelle in 1919 to treat the symptoms of dysentery. He injected an anti-dysentery phage into a patient with severe dysentery (10 to 12 bloody stools per day). The patient made a rapid recovery, displaying no symptoms shortly after receiving the phage therapy (Summers, 1999). This pioneering experiment of d'Herrelle led to many successful applications of this therapy against dysentery, which were reported in scientific articles over the subsequent 20 years. For instance, in the US state of Maryland, *Shigella flexneri* was identified in dysentery-affected children, and phage therapy was given orally and rectally in doses ranging from 5 to 1300 ml (Davison, 1922). In one successful example, Spence and McKinley in 1924, treated Shigellosis patients through the oral administration of 10 ml phages, which substantially reduced their mortality rate and length of stay in the hospital (10% and 5.8 days, respectively) when compared to a control group in another hospital (40% and 12.8 days)(Spence & Mckinley, 1924). Another example, Querangal des Essarts (1933) treated a bacillary dysentery patient in France with a polyvalent Shiga-Flexner bacteriophage through the oral administration of 5 to 10 ml of phages with alkaline water during an outbreak on board two ships at the port of Brest in 1933. The results were remarkable, with blood and mucus rapidly ceased (2^{nd} or 3^{rd} day) and the stools reverted to normal on the 4^{th} day. The same physician also stopped an outbreak of dysentery by the prophylactic administration of bacteriophages among the newborns at a holiday camp (Querangal des Essarts, 1933). On the other hand, there have also been some failures, mainly due to the late administration of the therapy. In 1937, Vaill and Morton medicated two hundred cases of dysentery in New Jersey (USA) with bacteriophages where only 22 cases were successful (Vaill & Morton, 1937). Johnston et al. (1933) treated 70 infants aged less than 2 years old using 1 ounce of bacteriophage per hour, and found that the clinical course of

dysentery was not improved as what they expected with this therapy. These lower success rates may be due to the fact that the trials used a strain-specific bacteriophage, and only 17 out of 94 bacterial strains which is approximately 20% of bacterial strains tested *in vitro*. The British army conducted a phage therapy research in the Middle East and the experiment was divided into four small scales, of which two were reported as unsatisfactory results. The unimpressive results of third one was published in the British Medical Journal. The last experiment was administrated judiciously and among 32 enrolled cases, the control cases and phage treatment cases were 18 and 14 respectively. The outcomes of this research did not show any remarkable result but a marginally better improvement of the treated cluster than the control cluster (Boyd & Portnoy, 1944; Goodridge, 2013).Nonetheless, phage therapy has been successful in most cases. In 1938, Haler reported the phage treatment of a dysentery epidemic caused by *Shigella sonnei* in which the patients were administered with bacteriophages three times daily and the epidemic ceased after two days and no further case was observed for a year (Haler, 1938). In Poland (1941), 10 ml of local phage mixture containing sodium bicarbonate in a half cup of tea or coffee was found effective against *Shigella* infection (Kliewe & Helmreich, 1941). It has also been shown, in 1945, that the effective proportion of a phage can be diluted at a 1:10 ratio of phage-bacterium injection. In a bacterial challenge experiment, mortality can be prevented with phage treatment up to 4 days before the challenge or with maximum 3 hours delay after the challenge (Morton & Engley Jr, 1945). In 1957, the Hirszfeld Institute of Immunology and Experimental Therapy (HIIET) in Poland applied phages to treat Shigellosis and other infectious diseases caused by antibiotic resistant bacteria, which were untreatable by conventional antibiotics (Sulakvelidze et al., 2001). In 1960s, a clinical trial was conducted extensively to evaluate the efficacy of phage therapy against Shigellosis (Babalova et al., 1968). This study was performed in Tbilisi, Georgia in which 30,769 children were involved. The children, aged between 6 months

to 7 years old were divided into two groups, with one group being given tablet made of dried *Shigella* phages and the other group a placebo, orally once a week, for each child. These children were monitored for 109 days and the results showed that the occurrence of dysentery was nearly 4 fold higher in the children given placebos than those treated with phages (Babalova et al., 1968). In another investigation reported in 1984, Anpilov and Prokudin demonstrated that the phage-mediated prophylactic medication of Shigellosis produced a ten-fold reduction in the incidence of dysentery among the phage-treated patients (Anpilov & Prokudin, 1984). Miliutina and Vorotyntseva (1993) conducted an experiment on phage therapy and a combined phage-antibiotics treatment on Shigellosis and Salmonellosis in 1993. They observed that the combined phage-antibiotics treatment was more effective in some cases as compared to the antibiotics treatment alone. (Miliutina & Vorotyntseva, 1993). There are many articles reporting successful treatments of Shigellosis in 21st century. The efficacy of phages against multidrug resistant *Streptococi* and *Pseudomonas* as well as some antibiotic resistant *Enterobacteriaceae* family members, including the genera of *Shigella*, *Salmonella*, *Serratia*, *Escherichia*, *Klebsiella* and *Proteus*, have been investigated (Kumari et al., 2010). These studies have largely confirmed the viability of phage therapy as a treatment for gastrointestinal distress, particularly for *Shigella*. Zhang et al. (2013) studied the ability of *Shigella*-specific phages and phage cocktails to inhibit *Shigella* spp in chicken product. They concluded that phages with higher concentration (3×10^8 PFU/g) could lyse bacteria more effectively in comparison to phages with lower concentration (1×10^8 PFU/g), and that the *Shigella*-specific phages were able to significantly reduce or eliminate *Shigella* spp. in the edible chicken products (Zhang et al., 2013). More recently, Svab et al. (2018) isolated and characterized 12 novel *Siphoviridae* bacteriophages from confiscated food samples. All isolated phages effectively lysed *Shigella dysenteriae, S. sonnei,* multidrug-resistant (MDR) *E. coli*, different *Salmonella enterica* serovars as well

as pathogenic *E. coli* strains representing enterohaemorrhagic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and enteroinvasive (EIEC) pathotypes. In summary, from the very beginning to the present day, the success rate of phage therapy against Shigellosis has been promising. An intensive and extensive study of anti-*Shigella* phages could therefore help to identify alternative treatments for the increasing number of drug resistant bacteria, and hence reduce the pressure to find new antibiotics to combat these. In the longer term, greater use of phage therapy could help to reduce the emergence of new multidrug resistant bacterial strains.

2.10 Limitations and solutions of phage therapy

Despite all the advantages of phage therapy, it is still a long way from being the "magic bullet" for treating infections, because many parameters (e.g. optimal dose, route of administration, frequency and duration of treatment) have yet to be determined precisely through clinical trials (Wittebole et al., 2014). The eventual success of phage therapy will largely depend on the development of appropriate strategies to overcome these limitations, as well as on the creation of an adequate regulatory framework, the implementation of appropriate safety protocols, and the acceptance of phage treatment by the general public (Nobrega et al., 2015). In summary, the major limitations of phage therapy are summarized below based on the reports from a few research groups (Hermoso et al., 2007; Kutter & Gowrishankar, 2001; Matsuzaki et al., 2014; Nilsson, 2014):

I. A narrow host range as well as serotype specificity (which might reduce effectiveness and coverage).
II. A single phage is inadequate for treating illnesses caused by multiple bacteria.
III. The release of various pro-inflammatory components (endotoxins and peptidoglycans) from the bacterium lysed by phages might cause problems in the human body.

IV. There is a possibility that resistant bacteria might emerge after treating with phages, however phages can evolve and adapt to combat resistant bacteria.

V. Complicated pharmacokinetics of phage treatment and interference by anti-phage antibodies

Besides these, there are other problems associated with patenting, manufacturing, and administration which often create obstacle for development of phage therapy. The lack of a definite regulatory outline reflecting individualized therapies, or difficulties for the pharmaceutical companies to register intellectual properties for phage and phage products are some of the major problems in phage therapy (Nobrega et al., 2015; Young & Gill, 2015). However, several initiatives can be considered to minimize or overcome the limitations of phage therapy.

2.10.1 Broad host-range bacteriophage

Searching of wider host-range bacteriophages might help to minimize the problem of phage therapy. Generally, phages can infect only one or a few bacterial strains while few other phages are able to infect many species or even different genera of bacterial strains. This makes defining host range difficulties (Ross et al., 2016). Jensen et al. (1998) reported 9 broad host range phages out of 10 isolated phages and stated that broad-host-range bacteriophages are more common than that had been thought previously (Jensen et al., 1998). Ross et al. (2016) suggested to apply multiple host strains during phage isolation to produce wider host-range bacteriophages reliably. Hamdi et al. (2017) isolated two potential bacteriophages designated SH6 and SH7 using *Shigella flexneri* as host which were able to control 9 and 27 strains respectively out of 35. Sarker et al. (2018) isolated five novel bacteriophages and this set of five phages was able to control 300 *Vibrio cholera* strains. Malki et al. (2015) isolated four *Myoviridae* phages from Lake Michigan on the same bacterial host, but interestingly each phage exhibited a host-range spanning several phyla of bacteria such as *P. aeruginosa*, *E. coli*, *Arthrobacter* sp.,

Chryseobacterium sp.and *Microbacterium* sp. Until recently this type of broad host-range is rare.

2.10.2 Phage cocktail

Phage cocktails have been formulated, consisting of several phages with complementary features (different receptors) which can play a vital role to overcome the limitations of a single phage with its narrow host range (Chan & Abedon, 2012; Chan et al., 2013; Goodridge, 2010). In addition, phage cocktails containing different types of phages potentially capable of combating the same species and strains of bacteria could reduce the emergence of bacteria resistant to phage (Chan & Abedon, 2012; J. Gu et al., 2012b; Potera, 2013). A complementary approach proposed by Friman et al. (2016), where phage cocktails can also be modified by including not only various phages, but also *in vitro* evolved phages from different evolutionary time points. More recently, Bernasconi et al. (2018) investigated the promising activity of 3 commercial bacteriophage cocktails (*INTESTI*, *Septaphage*, *PYO*) against 20 *Shigella* strains via spot test. The susceptibility of *Shigella* spp. to *INTESTI*, *Septaphage* and *PYO* was 95%, 55%and 95% respectively.

2.10.3 Phage antibiotic synergy

The efficacy of phage treatment could be enhanced by utilizing the antimicrobial synergy between phages and antibiotics. A potential synergistic effect of combining phages and antibiotics on the population density of bacteria was observed by Torres-Barcelo et al. (2016). Their result demonstrated that, with the limited consequences of the evolution of bacterial virulence phages could contribute in managing the level of antibiotic resistance (Torres-Barceló et al., 2016). In another study Mai et al. (2015) reported that one phage cocktail (combination of 5 lytic *Shigella* bacteriophages and ampicillin) designated as ShigActive™ was effective on experimentally challenged mice

for safely decreasing of *Shigella* count. They did not observe any toxic side effects of phage application during the trial and this phage combination had shown much fewer impact on the usual gut microbiota than treated with a generally recommended antibiotics. Recently, Chaudhry et al. (2017) observed the combined effects of phage and antibiotics in *Pseudomonas* biofilms grown on the layers of cultured epithelial cells. The combine effect of Ciprofloxacin (1X MIC) and Ceftazidime (at 1X and 8X MIC) with phages were synergistic for killing the biofilm. The synergistic effect could hasten cell lysis and allow phages to spread more quickly (Comeau et al., 2007; Ryan et al., 2012). Thus antibiotics conjugated to phages could enable the delivery of antibiotics to specific cells and cause an increase in local drug concentrations (Yacoby & Benhar, 2008). In addition, the introduction of genes that inhibit stress responses, improve drug uptake or repress biofilm production can increase the antibiotic sensitivity of *E. coli* (Lu & Collins, 2009).

2.10.4 Phage engineering

Phage gene manipulation can be an effective tool to minimize the problem related to phage therapy. Maloy & Youderian (1996) and Maloy & Gardner (1998) studied the expression of the *ant* gene which regulate the lysis-lysogeny decision of phages. They reported that a positive *ant* can be selected for the lytic growth of the phage where the mutants cannot repress expression of *ant*. A number of foodborne pathogens from the family *Enterobacteriaceae* including *Shigella* contain prophages which encodes a major virulence factor known as Shiga-like toxin. In *S. flexneri* the O-antigen modification (serotype conversion) is a key virulence determinant, is also introduced by temperate bacteriophages (Allison & Verma, 2000). A careful screening of the phage genome for virulence genes would help to minimize the risk of phage engineering. Moreover, the host range of phages can be broadened by engineering their genomes to express endosialidase (Ackermann, 2001) and by substituting the gene encoding putative host binding proteins (Yoichi et al., 2005). In addition, the challenges of phage therapy may be overcome by

producing genetically modified phages (a recombination of phage genomes, site-directed mutagenesis, a selection of spontaneous mutants or phage display methods) (Chhibber & Kumari, 2012; Dąbrowska et al., 2014; Moradpour & Ghasemian, 2011). Mutant phages could also be used to overcome bacterial resistance as well as to prevent the human immune system against phages (Matsuzaki et al., 2014).

2.10.5 Phage-based products

Another approach for the safety use of phage therapy is to use the viral gene products (endolysins) instead of the whole virion (Fischetti, 2005; Nelson et al., 2012; Schmelcher et al., 2012). The application of gene products could eliminate or minimize the risk of phages imparting toxic properties to bacteria (Hermoso et al., 2007) and thus decrease the threat of emerging resistance (Borysowski et al., 2006; Nelson et al., 2012; Schmelcher et al., 2012).The application of phage-product was a proposed substitute to the use of whole phage due to some complexities encountered during their application such as elicitation of immunogenic response, narrow host range, (Hodyra-Stefaniak et al., 2015), sequestration of phages in the liver and spleen and the emergence of bacterial resistance (Labrie et al., 2010; Nungester & Watrous, 1934). The benefits of applying phage-based products over the organism itself are these elements are much smaller which can boost up the penetration into the infected cells and tissues. Loeffler (2001) described a successful trial of phage lysins on animal models against *Streptococcus pneumoniae* (Loeffler, 2001). Moreover, lysins of pneumococcal phage can also be synthesized on an industrial scale using *E. coli* as cell factories (Loeffler, 2001). Some other successful examples of animal trials using phage-products against *Streptococcus pyogenes* (Ferretti et al., 2001), *Bacillus anthracis* (Schuch et al., 2002) and *Enterococcus faecium* (Yoong et al., 2004) are mention worthy. But this application is limited to Gram-positive bacteria and there is no report of effective application of lysins against Gram-negative bacteria (O'Flaherty et al., 2009). As a result, further researches and studies are required to find out a way how

lysins are able to control Gram-negative bacteria. Schuch et al. (2002) described a method of genetically screened potential lysins which might be applied against Gram-negative bacteria.

2.10.6 Drug Sensitization

Though the judicious use of drugs can help to minimize the emergence of drug-resistant bacterial pathogens, the effects are very unstable, as drug-resistance quickly rises once it is used again and again (Willemsen et al., 2010). Drug sensitization with phage or phage particles can play a vital role of microbial control. A technique of exploring temperate phages for incorporating drug-sensitive genes into drug-resistant bacteria was described by Edgar et al. (2012). Briefly, the genes related to resistance against a toxic compound tellurite, was sensitized by temperate phage and became antibiotic sensitive. The antibiotic resistance of bacteria could be overcome by using phages to inject sensitizing alleles of the mutated genes (*e.g. rpsL* and *gyrA*) to restore drug efficacy. For instance, temperate phages have been used to introduce sensitizing by lysogenization where the genes *rpsL* and *gyrA* were conferred sensitivity in a dominant fashion to two antibiotics Streptomycin and Nalidixic acid respectively. This made the bacterial pathogens sensitive to antibiotics prior to host infection (Edgar et al., 2012). The transfer of the phage constructed with the sensitizing cassette would significantly enrich antibiotic-treatable pathogens on hospital surfaces (Jassim & Limoges, 2014). Yosef et al. (2015) stated other way of bacterial sensitization, where temperate phages were applied as vectors to transfer the clustered regularly interspaced short palindromic repeats (CRISPR) via CRISPR associated (Cas) system, which was specially engineered to abolish drug-resistant genes and to modify lytic phages. The application of temperate phages and the successive accumulation of modified-lytic phages, change the selective benefit towards sensitive bacterial pathogens (Yosef et al., 2015). The focusing point of the research was to counteract bacterial resistance, not to kill the bacteria. Another noticeable limitation of

bacteriophage therapy is the ability of phages to act only on extracellular bacteria and the risk of interference by anti-phage antibodies *in vivo* (Singla et al., 2016). To overcome these risks, Singla et al. (2016) used liposome as a delivery vehicle for phages. This study reinforced the growing interest in using phage therapy as a means to target multidrug resistant bacterial infections, since the liposome entrapment of phages makes them highly effective *in vitro* as well as *in vivo* by overcoming the majority of the hurdles related to the clinical use of phages.

2.10.7 Biofilm dispersal

Biofilm is a major problem either in medical or industrial settings. Besides creating problems, in case of cleaning, biofilms can be the causes of microbial risk in the food industry, as well as can deteriorate the water quality in water treatment and distribution plants (Mattila-Sandholm & Wirtanen, 1992). In medical science, the appearance of biofilms on the urinary tracts or lungs or gastrointestinal is termed as glycocalyx (Ushiyama et al., 2016). Cells of bacteria within a biofilm exhibits a higher degree of resistance towards antibiotics than an individual bacterium, and the doses of drugs required for the antibiotics to be active are generally 100 to 1000 folds more than bacteria which are free-living (Ceri et al., 1999). Besides antibiotics, some coexisting bacteriophages within a bacterial population, have shown to produce enzymes which is capable of degrading the extracellular polymeric substance (EPS) layer of biofilms. It was reported that phage K29 was able to establish the lytic action against the bacterial cells by penetrating an *E. coli* biofilm (Bayer et al., 1979). In another study, Cerca et al. (2007) reported that phage K exhibited effectiveness in eliminating a *Staphylococcus epidermidis* biofilm within a day-long challenge. The efficacy of phages to destroy a matured biofilm also exhibited noteworthy improvement when the phage in a combination with other chemical or antimicrobial agents are used. The synergistic action of antibiotics and bacteriophages aided significantly to remove a mature biofilm of

Klebsiella pneumonia (Verma et al., 2010). In an experiment, Yilmaz et al. (2013) applied the combination of phages and antibiotics on an implant model against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm and observed the increase eradication of biofilm.

2.11 Future prospect of phage therapy

Anti-microbial resistance (AMR) is regarded as one of the greatest challenges for human being and has been indicated as an ultimate threat to our civilization. It is predicted that ten million people may face death yearly and the economic burden may hit $100 trillion by 2050 (Neill, 2016). This crisis has focused interest greatly on bacteriophages and most recent review published in the high impact journals namely the Lancet (Watts, 2017), Journal of American Medical Association (JAMA) (Lyon, 2017) and Science (Guglielmi, 2017) addressing different aspect of phage therapy indicates the growing interest of phage in current antimicrobial research. The application of phage therapy in wider clinical practice was noted in the USA and Belgium when individual patients were successfully treated through intravenous phage application (Duplessis et al., 2017; Jennes et al., 2017; Schooley et al., 2017). Gorski et al (2017) recently discussed in detail and reported the anti-inflammatory and immunomodulating effects of phages including some non-communicable diseases. As things now stand, most of the drawbacks of phage therapy have been addressed to a lesser or greater degree, and phages are now capable of being successfully incorporated into the era of multi-drug resistant treatment. As further steps, next-generation sequencing could be employed to determine genomic DNA sequences from multiple phage products, which could reduce further the risks of phage therapy by eliminating harmful genes and gene products (Matsuzaki et al., 2014). Recently the whole genome of all five lytic bacteriophages of the cocktail ShigaShield™ have been sequenced and analyzed, and no undesirable genes have been found, including those listed in the US Code for Federal Regulations (40 CFR Ch1) (Soffer et al., 2017).

In addition, the multi-route administration of phages (intramuscular, intravenous, intraperitoneal, subcutaneous, intranasal and oral) would broaden the use of phage therapy as a potential agent in the future. Moreover, the prophylactic use of phages and the development of vaccines using phages or phage products would open up a new dimension for the prevention of antibiotic resistant pathogens (Chanishvili, 2012; Morello et al., 2011). Furthermore, the active participation of dysentery patients and a large-scale trial of phage therapy against multidrug resistant Shigellosis and other dysenteries would enhance the acceptance of phage therapy as a common treatment. Finally, it is essential to build up public awareness of phage therapy as well as expand the availability of phages and phage therapy centers in order to expand and exploit this potentially fruitful innovation.

Some recent researches on bacteriophages against *Shigella* spp. published in different renowned journals are given in table 2.4.

Table 2.4: List of some recent potential researches on bacteriophages infecting *Shigella* spp.

Research title	**Name of Journal**	**Name of Scientists and published year**
Isolation, characterization and genomic analysis of a novel lytic bacteriophage vB_SsoS-ISF002 infecting *Shigella sonnei* and *Shigella flexneri*	Journal of Medical Microbiology	(Shahin et al., 2018)
Characterization of two polyvalent phages infecting *Enterobacteriaceae*	Scientific Report	(Hamdi et al., 2017)
Bacteriophage preparation lytic for *Shigella* significantly reduces *Shigella sonnei* contamination in various foods	PLoS ONE	(Soffer et al., 2017)
Characterization and Genomic Study of the Novel Bacteriophage HY01 Infecting Both *Escherichia coli* O157:H7 and *Shigella flexneri*: Potential as a Biocontrol Agent in Food	PLoS ONE	(Lee et al., 2016)

Table 2.4, continued

Research title	Name of Journal	Name of Scientists and published year
Bacteriophage application to control the contaminated water with *Shigella*	Scientific Report	(Jun et al., 2016)
Isolation and characterization of bacteriophages against equine pathogens-novel phages revealed as phage therapy candidate	Journal of Equine Veterinary Science	(Anand et al., 2016)
Characterization of new *Myoviridae* bacteriophage WZ1 against multi-drug resistant (MDR) *Shigella dysenteriae*	Journal of Basic Microbiology	(Jamal et al., 2015)
Bacteriophage administration significantly reduces *Shigella* colonization and shedding by *Shigella*-challenged mice without deleterious side effects and distortions in the gut microbiota	Bacteriophage	(Mai et al., 2015)
Bacteriophages for managing *Shigella* in various clinical and non-clinical settings	Bacteriophage	(Goodridge, 2013)
Isolation and genomic characterization of SfI, a serotype-converting bacteriophage of *Shigella flexneri*	BMC Microbiology	(Sun et al., 2013)

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection for *Shigella* isolation

A total of 176 stool specimens (from local clinics and hospitals of northern part in Bangladesh) and 48 water samples (from 3 locations of central Kuala Lumpur Malaysia) were collected from September 2015 to February 2018. All specimens and samples were cultured within 2h of collection to detect pathogen using standard bacteriological procedure.

3.2 Biochemical test

Biochemical test of *Shigella* spp. was performed according to standard protocol (Bopp, 1999; WHO, 1987, 1995). Briefly the collected samples were diluted serially from 10^{-2} to 10^{-8} with autoclaved distilled water and 100 µl of each dilution was evenly plated onto Salmonella-Shigella (SS) agar. MacConkey agar (MAC) was used as medium of low selectivity and Xylose lysine deoxycholate (XLD) agar was used as a more selective agar medium. Suspected colonies of *Shigella* strains were picked and sub-cultured onto a non-selective LB agar media (APPENDIX A) and incubated overnight at 37 °C. Then single colonies were selected for subsequent triple sugar iron (TSI) screening test, oxidase test and catalase test. In case of TSI screen test a single colony of bacterial strain was picked using a sterile inoculating loop and the TSI slant was inoculated by stabbing the bottom as well as subsequent streaking on the top. After incubation for 18 to 24 h the *Shigella* characteristically produced an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H_2S. In case of oxidase test 1 – 2 drops of 1% NNNN- tetramethyl-p-phenylenediamine dihydrochloride was placed on a filter paper and let it to soak. Then a single colony was picked using a sterile loop and smeared onto the filter paper already soaked with the solution. Changing of colour was examined within 10 – 30 seconds. For the catalase test, one to three drops of the 3% hydrogen peroxide solution was placed onto

a glass slide and a single colony of bacterial strain was picked and smeared into the solution. The production of bubbles were observed within a few seconds.

For long-term storage 50% (v/v) glycerol stock of *Shigella* spp. was prepared and stored at −80 °C.

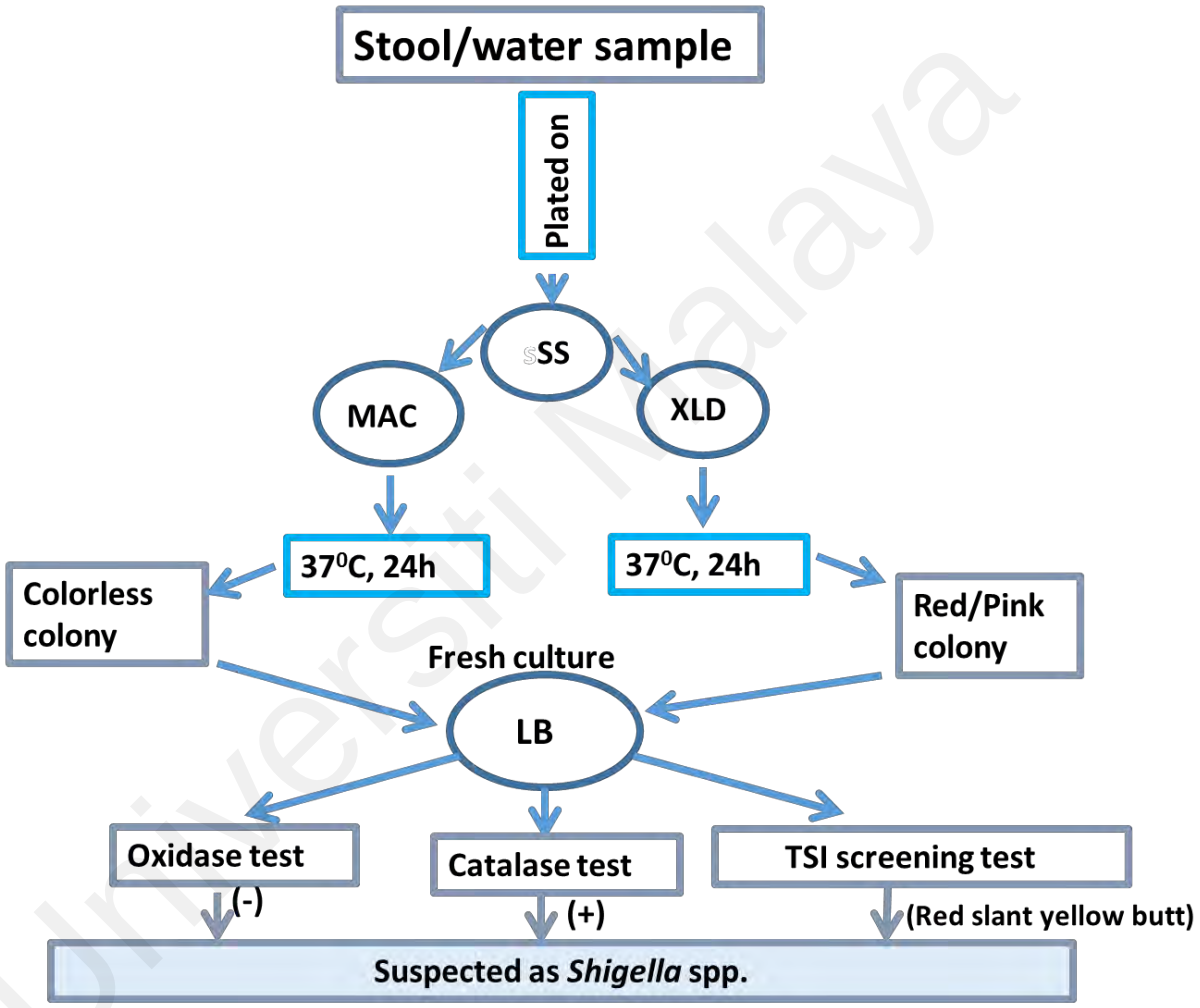

Figure 3.1: Schematic diagram of biochemical test. Preliminary identification of *Shigella* strains through selective media, screening media as well as oxidase test and catalase test.

3.3 Serotyping

Serotyping of the *Shigella* strains was done through a commercially available antisera kit (Denka Seiken Co., Ltd., Tokyo, Japan) specific for all type and group-factor antigens. Bacterial strains were sub-cultured on LB agar plates and after 18 h of incubation,

serological reactions were performed by the slide agglutination test in accordance with the manufacturer's instructions. Polyvalent sera was used for species identification and monovalent sera was used for serotypes identification.

3.4 Molecular characterization

For further serotypes confirmation, molecular identifications of different serotypes of *Shigella flexneri* were done through single and nonaplex PCR amplifications using specific primer for different serotypes.

3.4.1 Preparation of DNA templates

Boiling method was used for bacterial DNA extraction. Concisely, a single colony from an overnight culture (at 37 °C) of LB agar plate was suspended into 30 µl of distilled water. The suspension was then boiled for 10 min at 100 °C. The mixture was instantly transferred into ice and cooled it for 5 min. The sample was centrifuged for 10 min at 13,000 x*g* and the supernatant, containing DNA, was used as the template for PCR amplification (Sun et al., 2011).

3.4.2 PCR primers

Nine set of primers were used in this study listed in Table 3.1. O-antigen flippase gene, *wzx* amplification primer was used from Li et al. (2009), the rest 8 set of primers for *S. flexneri* serotype-specific genes for O-antigen modification *gtrI*, *gtrIC*, *gtrII*, *oac*, *gtrIV*, *gtrV*, and *gtrX* were from Sun et al. (2011) These primers were produced by IDT (USA) and dissolved in ddH_2O to obtain a 100 mM stock solution.

Table 3.1: List of primers

Gene name	Accession No.	Orientation	Sequence(5'-3')	Amplicon size (bp)	Primer (bp)
gtrI	AF139596	F R	CTGTTAGGTGATGATGGCTTAG ATTGAACGCCTCCTTGCTATGC	1122	22+22=44
gtrII	AF021347	F R	ATTTATTGTTATTGGGGGTGGTTG ATTTGTTCTTTATTTGCTGGTT	1272	24+22=46
oac	AF547987	F R	CTGTTCGGCTTTGAAAGTGCTG CGTAGGCGTACATAGCAAGCAAAGA	604	22+25=47
gtrIV	AF288197	F R	ATGTTCCTCCTTCTTCCTTT TCCTGATGCTACCTTATCCA	378	20+20=40
gtrV	U82619	F R	AATACGATTCTCCTGGTGCTAAAC TAGGGCATTGCTTGTATCTTTCAT	905	24+24=48
gtrX	L05001	F R	AATGCTGGATGGGATAATCACCTT GAGACGGCTTCTCCATGTTTTGCT	425	24+24=48
Wzx$_{1-5}$	AE005674	F R	CACTTGTTGGGTATGCTGG CCGGCAAACAGATTAGAAA	782	19+19=38
gtrIC	FJ905303	F R	AGGGAATGGCATTAGGGATCGG GCTGCAAGTGGTTTTTGTTGGA	518	22+22=44
Wzx$_6$	EU294165	F R	TTAAGAGCGATCATTTC CCATCCAAGCGGACATT	739	17+17=34

3.4.3 PCR amplification and detection

Singleplex and Nonaplex PCR were performed using KOD FX Neo kit (Tayobo, Japan). The reaction mixture for each PCR was consisted of 2X PCR buffer for KOD FX Neo, KOD FX Neo enzyme, 2 mM dNTPs, 0.2 µM concentrations of each primer, and 1.5 µl of template DNA in a final reaction volume of 20 µl. PCR amplification was performed using a standard thermal cycle for multiplex PCR optimized by Sun et al. (2011). Briefly, denaturing step 95 °C for 15 min, followed 30 cycles of 94 °C for 30 s, annealing step 55 °C for 90 s, and extending step 72 °C for 60 s, with a final extension of 72 °C for 10 min in a thermocycler (G-Strom, UK). A small volume of PCR product (5

µl) was mixed with loading dye, for electrophoresis in 1.5% agarose gel premixed with red safe dye.

3.5 Antibiotic profiling of isolated *Shigella* serovars

The antibiotic profiling of the isolated *Shigella* serotypes was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Cockerill & Patel, 2015). The conventional disc diffusion method was used against ten antibiotics of six different groups (Table 3.2) namely Ampicillin, Tetracycline, Chloramphenicol, Streptomycin, Kanamycin, Amoxicillin, Nalidixic Acid, Ciprofloxacin, Ceftriaxone, and Cefepime. Minimum inhibitory concentration of each antibiotic was used in this study. Mid log phase bacterial strains (100 µl) were swabbed uniformly on LB agar petridishes and the antibiotic discs were placed cautiously in a specific distance and kept them for overnight incubation at 37 °C.

Table 3.2: Specification of the drugs used for antibiotic profiling

Name of Antibiotics	Short Name	Concentration (μg)	Group
Amoxicillin	AML	5	Penicillins
Ampicillin	AMP	10	Penicillins
Tetracycline	TE	30	Tetracyclines
Streptomycin	S	25	Aminoglycosides
Kanamycin	K	30	Aminoglycosides
Ciprofloxacin	CIP	5	Fluoroquinolones (2^{nd} generation)
Cefipime	FEP	20	Cephalosporins (4^{th} generation)
Ceftriaxone	CRO	30	Cephalosporins (3^{rd} generation)
Nalidixic Acid	NA	30	Fluoroquinolones (1^{st} generation)
Chloramphenicol	C	30	Chloramphenicols

3.6 Extraction and profiling of plasmid DNA of three novel strains of *Shigella flexneri* 1c

3.6.1 Extraction of plasmid DNA

Plasmids of three novel strains *Shigella flexneri* 1c were extracted using the Plasmid Miniprep System Kit (Promega). Briefly, a single well-isolated colony of *Shigella flexneri* 1c from a fresh Luria-Bertani (LB) agar plate was inoculated to 10 ml of LB broth medium. The inoculum was incubated overnight (18 h) at 37 °C in a shaking incubator. An A600 reading of 2–4 ensured that cells had reached the proper growth density for harvesting and plasmid DNA isolation. 1.5ml of bacterial culture was centrifuged in a

tabletop centrifuge at 10,000 x *g* for 5 min. The supernatant was discarded and blotted by inverting the tube on filter paper to remove excess media. Harvesting of bacterial cells was done twice in the same tube to get high copy number of plasmids. 250µl of Cell Resuspension Solution was added in the harvested tube and the bacterial cell pellets were completely resuspended by vortexing or pipetting. Then 250µl of Cell Lysis Solution was mixed by inverting the tube 4 times (no vortexing) and the mixture was incubated until the clear cell suspension was observed (approximately 1–5 min). In next step the suspension was again incubated for 5 min at room temperature after adding 10µl Alkaline Protease Solution and mixed by inverting the tube 4 times. Then 350 µl of Neutralization Solution was mixed immediately with the cell suspension by inverting the tube 4 times. The cell suspension was centrifuged at around 14,000 x *g* for 10 min at room temperature. After centrifugation, the top cleared lysate (approximately 850µl) was transferred into the preset spin column and the supernatant was again centrifuged at 14000 x g for 1 minute. The spin column was removed from and the flowthrough was discarded from the collection tube. 750 µl of Column Wash Solution previously diluted with 95% ethanol was added after reinserting the spin column into the collection tube and the tube was centrifuged at 14000 xg for 1 minute . This step was repeated once again where 250 µl of Column Wash Solution was used. After washing properly, the solution was centrifuged at maximum speed in a microcentrifuge for 2 min at room temperature. Finally, the spin column was transferred to a new sterile 1.5ml microcentrifuge tube and the plasmid DNA was eluted by adding 100µl of nuclease-free water to the spin solumn by centrifuging at maximum speed for 1 minute at room temperature. After eluting the plasmid DNA, the assembly was removed from the 1.5ml microcentrifuge tube and the spin column was discarded. The plasmid DNA was stored at – 20 °C for further use.

3.6.2 Profiling of plasmid DNA

The concentration and purity of extracted plasmid DNA were determined by measuring the absorbance at 260nm (A_{260nm}) and 280 nm (A_{280nm}) using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples with an A_{260nm} /A_{280nm} ratio between 1.8 - 2.0 were used for plasmid profiling. The plasmid DNA was observed via electrophoresis on a 0.6% (w/v) agarose gel premixed with red-safe dye and running for 60 min with 70 volt in 1X TBE buffer (APPENDIX B). 1 kb marker and reference plasmids PGMET (3kb) and HTB (4.8 kb) were used as marker for comparison.

3.7 Collection of water samples for bacteriophage isolation

For bacteriophage isolation, water samples were collected from Sewage Treatment Plant, Pantai Sewage Works, Jalan Pantai Dalam, Indah Water Konsortium, Kuala Lumpur, Malaysia. The samples were collected in 50 ml screw cap plastic tubes and stored at 4 ºC until use.

3.7.1 Preparation of host bacterial Culture

Three serotypes of *Shigella flexneri* i. e. 2a, 1c and 4a were used as host to isolate the bacteriophages. A single colony was selected and inoculated into a 50 ml screw cap plastic tube containing 5 ml LB broth media. The inoculum was incubated overnight with a gentle shaking of 150-180 rpm at 37 ºC.

3.7.2 Isolation of bacteriophage

The bacteriophages were isolated from sewage water samples following a previously described methods with some optimizations (Ghasemi et al., 2014). Briefly 4.5 ml of sewage water and 0.5 ml of 10X- LB broth (Tryptone 10g, Yeast extract 5g, NaCl 10g, dH_2O 100ml) were enriched with 1 ml of mid-log phase *Shigella* strains. The enriched sewage water was then incubated in a shaking incubator at 180 rpm and at 37 °C for 24 h. The supernatant was filtered through a 0.2 µm pore-size cellulo-acetate syringe filter

(Sartorius, Gottingen, Germany) after removing debris and bacterial cells by centrifuging the suspension for 10 min at 6000 xg. The crude phage lysate was stored at 4 ºC until use and the presence of lytic bacteriophages were observed through spot plating assay. In brief, 100 µl of bacterial culture was spread evenly into a lawn on LB agar media and 5 – 10 µl of phage lysate was spotted onto the bacterial lawn The plates were incubated overnight at 37 ºC to observe the clear plaque zones..

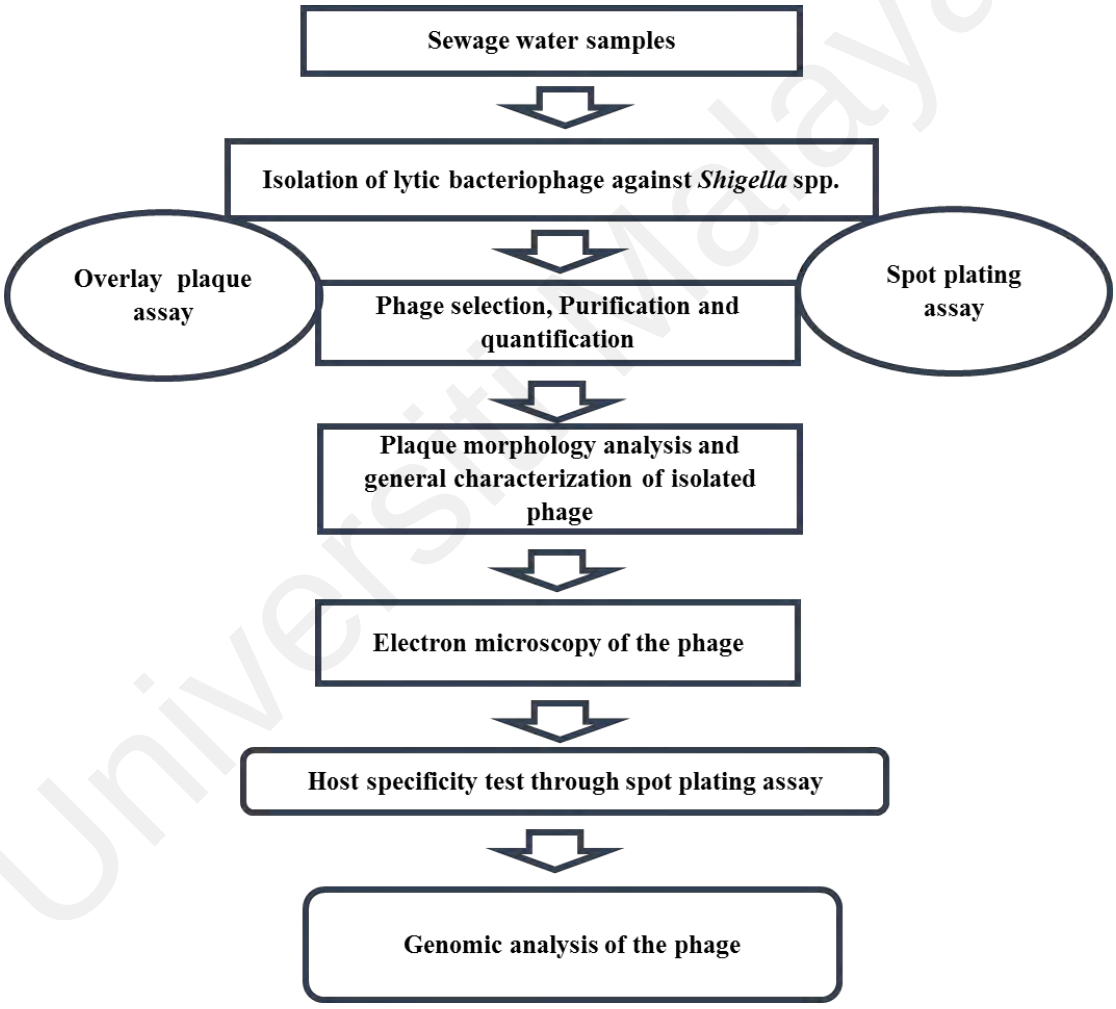

Figure 3.2: Schematic diagram of bacteriophage isolation and characterization. Isolation and purification of bacteriophages through spot plating assay and overlay plaque assay respectively. Characterization of the phages through transmission electron microscopy, host specificity test and genomic analysis.

3.7.3 Purification of bacteriophages

Crude lysate may contain different types of bacteriophages. For the purification of individual phages, agar over lay assay was applied (Adams, 1959). Briefly, crude lysate of bacteriophage was serially diluted in LB broth media with the dilution ranges from 10^{-2} to 10^{-8}. 100 µl of phage lysate dilution was added to 100 µl of a bacterial culture which was followed by an addition of 3 ml of the top agar to the tube and immediate pouring onto the bottom agar layer. The plaques morphology was observed after overnight incubation at 37 °C. Single plaque was selected from each type of morphology for subsequent purification. Then 5 ml LB broth media was inoculated with 0.2 ml host bacterium and a single distinct plaque was picked by a sterile pipette tip and the mixture was incubated for 24 h at 37°C with gentle shaking at 150-180 rpm. The supernatant was filtered through 0.2-µm cellulose acetate syringe filters after centrifuging for 10 min at 10 000 × g. The crude lysate was again serially diluted with the dilution ranges from 10^{-2} to 10^{-8} and agar overlay assay was applied to calculate the phage titre. For getting homogeneous plaque morphology this step was repeated at least 3 times.

3.7.4 Preparation of purified high titre phage lysates

The modification of ammonium acetate purification method (Fortier and Moineau, 2007) was performed to obtain purified high titre phage lysates. In brief, 10 to 15 plaques of same bacteriophage and 600 µl of mid log phase host bacterial culture were added to 15 ml LB broth media and kept it for incubation at 37 °C in 180 rpm for 24 h. The co-culture was centrifuged for 10 min in 10000 xg and 10 ml of top aquas solution was filtered by 0.2 um cellulo-acetate syringe filter to remove host bacteria. Then 10 ml of crude phage lysate was centrifuged for 1 h at 4 °C in 32000 xg (Rotor Beckman JA20). A fraction of supernatant approximately 9 ml was gently discarded and 5 ml of ammonium acetate (0.1 M, pH 7.5, previously filtered through 0.2 um syringe filter and stored at 4 °C) was added to the remaining lysate which was then centrifuged for another

one hour as described above (at 4 °C and in 32000 xg). After centrifugation 5 ml of supernatant was discarded from the sample and another 5 ml of fresh ammonium acetate was added and the centrifugation step was performed once again. Then the supernatant was discarded and 500 µl to 1 ml of concentrated and washed phage lysate was kept in an eppendorf tube. This phage lysate was filtered again through 0.2µm syringe filter to avoid any type of impurity and to ensure the removal of host bacteria before use it for TEM or DNA extraction. The purified phage lysate generally contained a titre around 10^{10}- 10^{12} plaque forming units per ml (PFU/ml). For short-term storage, phage suspension in LB broth medium was directly kept at 4 °C and for long-term storage, 15% (v/v) glycerol stock of phage lysate was prepared and stored at −20 °C. and−80 °C respectively.

3.7.5 Phage morphology examination by transmission electron microscopy (TEM)

For TEM, traditional negative staining with 1% uranyl-acetate was applied with few modifications. Briefly One drop of high titre phage lysate (10^{10} - 10^{12} pfu/ml) was dropped onto a layer of parafilm. A carbon-coated copper grid (200 mesh) was picked up carefully using a negative-action style tweezers at the very top of the grid and shiny surface was placed atop the suspension. The grid was left to suspend atop the suspension for 3 min. The grid was picked up, additional fluid was dried off by a filter paper and transferred onto 1% Uranyl-acetate staining solution for 3 min. Excess liquid was dried off using wedged filter paper gently. This copper grid was kept inside the desiccator 24-48 h for drying before viewing in TEM-Zeiss Leo Libra-150. For the classification of isolated bacteriophages the guideline of the International Committee on Taxonomy of Viruses was followed (Krupovic et al., 2016).

3.8 General characterization of bacteriophages

3.8.1 One step growth curve

The one-step growth curve experiment of all isolated bacteriophages was performed according to (Ellis & Delbruck, 1939) with some modification. Briefly the host suspension (~ 10^8 cfu/ml) and the phage lysate (~10^6 pfu/ml) were kept in the 37 °C water bath and maintained this temperature throughout the experiment. For achieving 0.01 multiplicity of infection (MOI = 0.01) 0.1 ml of the phage lysate and 0.9 ml of host culture were added and mixed properly in an absorption tube. After 10 min of incubation at 37 ºC, the mixture was serially diluted to 10^{-6} and 0.1 ml of this dilution was inoculated into 3 ml LB soft agar media with 0.1 ml mid-log phase host bacterial. The mixture was immediately poured onto a pre-warmed LB solid agar media. This step was continued in every 10 min interval until the 100 min. The experiment was performed in duplicate to verify the data. The titre of phages was calculated as pfu/ml with the formula described before and converted into log pfu/ml for graphical presentation.

3.8.2 Thermal tolerance of bacteriophages

Thermal tolerance of different bacteriophages were determined with some modification of the method previously described by Capra et al. (2006) and Jamal et al. (2015). Briefly different temperatures ingredients -20 ºC, 25 ºC, 37 ºC, 50 ºC, 70 ºC and 90 ºC were used to incubate bacteriophages. After one hour of incubation at 37 ºC the phage titre was determined using agar overlay assay. This assay was performed in triplicates for each temperature ingredient.

3.8.3 pH stability of bacteriophages

The pH Stability of different bacteriophages was determined with some modification of the method previously described by Capra et al. (2006) and Jamal et al. (2015). In brief a pH gradient was established ranging from 1 to 11 (pH 1, 3, 5, 7, 9, 11) using

hydrochloric acid (HCl) and sodium hydroxide solution (NaOH). Different phage lysates were incubated at these specific pH solution and the phage titre was determined using agar overlay assay after overnight incubation at 37 ºC. This experiment was performed in triplicates for each pH gradient.

3.9 Extraction of phage DNA and digestion with DNase I and RNase

Purified and highly concentrated phage lysate (10^{10}- 10^{12} pfu/ml) was used for DNA extraction. Traditional phenol-chloroform-isoamyl alcohol extraction method was used for phage DNA extraction (Sambrook et al., 1989). Briefly 500 µl of the purified phage lysate was added with 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) in an eppendorf tube and mixed properly, the mixture was then centrifuged at 14,000 x*g* for 10 min to separate the phages. The top aqueous phage solution was transferred to a new sterile 1.5 ml eppendorf tube and 500 µl of isopropanol (100% cold) and 50 µl of 3 M sodium acetate were added into the solution. The mixture was left to incubate at room temperature for 20 min to allow precipitation. Then the supernatant was discarded after centrifuging the mixture for 15 min at 14000 xg and at 4 ºC temperature. The DNA pellet of phage existing at bottom of the tube was washed with 70% ethanol twice and air dried. Finally, the DNA pellet was eluted with 50 µl distilled water and stored at -20 ºC. The extracted phage DNA was analyzed through electrophoresis on 0.7% (w/v) agarose gel in 1X Tris-Borate-EDTA (TBE) buffer. To determine the type of nucleic acid the phage DNA was digested with DNase I and RNase following the method described by Klieve and Gilbert (2005) (Klieve & Gilbert, 2005)

3.10 Determination of host range

To determine the host range of 10 isolated bacteriophages against 49 *Shigella* strains, spot plating technique was applied (Hamdi et al., 2017). Here 5 µl of the phage lysate with a titre of 10^6 -10^8 PFU/ml was used. The plates were checked after 4–6 h of

incubation and again 18 h of incubation to observe bacterial lysis. Spot testing is an efficient and the rapid technique to determine the host range of a large population of bacteria

3.11 Determination of adsorption rate of phage TB004

Adsorption rate of phage was determined according to Lau et al. (2012). In briefly the log phase host bacteria were inoculated and infected the phage at 37 ºC with an MOI of 0.01. Then, 300 µl of co-culture was removed every 2 min for the first 10 min and continued to every 5 min for the next 15 min. The samples taken in all time intervals were centrifuged immediately for 3 min at 5000 × *g* and at 4 ºC to remove adsorbed phage and the bacteria. Then the titre of the phage was calculated through agar overlay assay described previously. The adsorption rate of the phage was estimated as per following formula:

Adsorption rate = [(initial titre of phage – titre of unadsorbed phage)/initial titre of phage] ×100%.

3.12 Determination of multiplicity of infection of phage TB004

The phage was combined with log phase host bacteria at different MOIs ranging from 0.01 to 100 to estimate the optimal multiplicity of infection (MOI). The co-cultures were incubated with an agitation of 180 rpm at 37 ºC. Hundred µl of co-cultures were taken in every 20 min until 2 h to determine the phage titre through the overlay plaque assay. The number of viable bacteria was estimated through the spread plating method (Kudva et al., 1999; Z. Lu et al., 2003; Pasharawipas et al., 2011)

3.13 Genomic study of TB004 bacteriophage

3.13.1 Genome sequencing and assembly

Whole-genome sequencing of the Phage TB004 was performed with a MPS (massively parallel sequencing) technology on the Illumina platform. For the library construction, a-tailed which ligated to paired-end adaptors, a PCR amplified with a 500 bp insert and a mate-pair library with an insert size of 5 kb were used. The low quality reads the Illumina PCR adapter reads from mate pair library and the paired-end were filtered by compiling pipeline of quality control. The paired-end reads were assembled into 16 scaffolds with minimum contig length of 1000 bp using the SPAdes 3.12.0 (Bankevich et al., 2012) version and the QC of the resulting assembly was obtained using QUAST (Gurevich et al., 2013). To verify the top five longest contigs were subjected to megaBLAST against the NCBI nucleotide database. To verify if the two longest contigs (NODE 1 and NODE 2) are part of a single genome, the 16 scaffolds were used to map against the reference genome of *Shigella* phage Shfl2 (HM0350250) and *Shigella* phage SHFML-11 (NC_030953) using LASTZ version 1.02.00 (http://www.bx.psu.edu/miller_lab/dist/README.lastz-1.02.00/README.lastz-1.02.00a.html).

3.13.2 Annotation of genome components

The constitution of sequenced genome was detected through coding gene prediction, repeat gene prediction and non-coding RNA prediction. The coding genes were predicted by GeneMarkS (Besemer et al., 2001). Interspread repeat was predicted using RepeatMasker (Saha et al., 2008) and tandem repeat is analyzed using TRF (Tandem repeat finder) (Benson, 1999).The prediction about the presence of transfer RNA (tRNA) genes were done by the tRNAscan-SE (Lowe & Eddy, 1997), the presence of ribosome RNA (rRNA) genes were predicted by the rRNAmmer (Lagesen et al., 2007) and the

presence of small nuclear RNAs (snRNA) were predicted by the BLAST against the Rfam (Gardner et al., 2008; Nawrocki et al., 2009) database.

3.13.3 Genome map annotation

The two scaffolds NODE 1 and NODE 2 were annotated using the RAST pipeline (Besemer et al., 2001) and were aligned using LASTZ (Chiaromonte et al., 2001; Harris, 2007)

3.13.4 Gene function annotation

Two databases namely GO and Swiss-Prot were used for Gene function prediction of TB004 phage genome. A whole genome Blast (Altschul et al., 1990) search (E-value less than 1e-5, minimal alignment length percentage larger than 40%) was performed against these databases.

3.13.4.1 Gene ontology (GO) annotation

The GO is a database which is created by The Gene Ontology Consortium in 1988 (Ashburner et al., 2000). GO is the established standard for the functional annotation of gene products which covers three domains namely (i) Cellular component: the parts of a cell or its extracellular environment (ii) Molecular function: the elemental activities of a gene product at the molecular level and (iii) Biological process: operations or sets of molecular events with a defined beginning and end.

3.13.4.2 Swiss-Prot annotation

Swiss-Prot is a database (Bairoch & Apweiler, 2000) containing high-quality, comprehensive and freely available resources of protein sequence as well as functional information. It combines the computed features, experimental results and scientific conclusions together. For the alignment of the amino acid sequences against the Swiss-Prot database the BLAST was applied. The amino acid sequences from the best hit

together with highest alignment of length percentage and the identity of matching were assigned to annotate the of predicted gene.

3.13.5 Comparative genomics analysis

3.13.5.1 Whole genome phylogeny

The phylogenetic analysis of whole genome of TB004 was done through Megablast using NCBI database of complete bacteriophages from microbial nucleotide sequences section. The phylogenetic tree was constructed with most likely 34 bacteriophages using neighbor joining method.

3.13.5.2 Evolutionary relationship of five selected proteins

Whole genome sequence of 33 *Enterobacteria Myoviridae* phages (List of phages shown in Appendix O) were downloaded from NCBI. All genomes were re-annotated using RAST(Overbeek et al., 2014) to find out five selected genes encoded the proteins namely major capsid protein, portal vertex protein, terminase large subunit protein, DNA polymerase protein and thyamidylate synthase protein . Selected genes were individually extracted from each genome based on name search. The genes of the selected proteins were translated into amino acid sequences and aligned using MUSCLE 3.8.425 (R. C. Edgar, 2004). The resulting alignment file was manually curated. Maximum likelihood tree with 1000 boostrapping was constructed using PHYML (Guindon et al., 2009) with LG substitution model. All trees were rooted using appropriate gene sequence as out-group.

3.14 Statistical treatment

Simple statistics such as mean and standard error of mean were calculated for interpretation and graphical presentation of data.

Means

Data on individual treatment were added together and divided by the total number of observations to obtain the mean value as follows.

$$\bar{x} = \frac{\sum xi}{n}$$

Where

$\bar{x}$ = mean of the x variable

n= no of observations

i= 1,2,3.....n

Σ= Summation

Standard error of means (SE)

If instead of considering one replication, several replications were considered, the standard deviation of the different replication also vary. This variation was measured by the standard error, which was calculated as follows.

$$SE = \frac{Sd}{\sqrt{n}}$$

Where,

SE= standard error of mean

Sd= standard deviation

n= total no. of individuals.

CHAPTER 4: RESULTS

The present investigation was carried out to isolate and characterize bacteriophages against different drug-resistant *Shigella* strains. To achieve the objectives of this study, firstly, 49 *Shigella* strains (APPENDIX C) were isolated and characterized through biochemical tests, serotyping and molecular detection via nonaplex PCR amplification. The bacterial strains were subsequently tested for resistance towards antibiotics. Secondly, ten bacteriophages were isolated and characterized based on plaque morphology, pH stability, thermal tolerance, one step growth curve, phage morphology in TEM and lytic activity against different strains of *Shigella*. Thirdly, the bacteriophage with the widest host range was selected among the characterized phages and subjected to subsequent experiments i.e. MOI, adsorption rate and whole genome sequencing. Finally the analysis of the whole genome i.e. genome assembly, gene component prediction, gene function annotation and phylogenetic relationship were determined. The results of this study are described in this chapter

4.1 Biochemical characterization of *Shigella* spp.

After incubation for about 18-24 h at 37 ^{o} C, the colonies of *Shigella* on MacConkey (MAC) agar appeared as convex, colorless colonies around 2 to 3 mm in diameter. *Shigella* colonies on Xylose lysine deoxycholate (XLD) agar were transparent pink or red smooth colonies around 1 to 2 mm in diameter, while producing red slant and yellow butt in Triple Sugar Iron (TSI) agar screening media (APPENDIX D). *Shigella* strains were positive for catalase test and negative for oxidase test (produce bubbles and no change in colour, respectively). Among 176 clinical and 48 environmental samples 39 clinical strains (Table 4.1) and 10 environmental strains (Table 4.2) were suspected as *Shigella* respectively.

Table 4.1: Biochemical test of different isolated *Shigella* strains (clinical samples)

Lab code	MAC	XLD	TSI	Oxidase test	Catalase test	Comments
SS1001	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SS1002	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SB1003	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SD1007	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1008	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1011	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1013	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1014	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1015	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1016	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1017	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1018	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1019	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1020	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1021	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.

Table 4.1, continued

Lab code	MAC	XLD	TSI	Oxidase test	Catalase test	Comments
SF1023	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1024	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1025	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1027	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1028	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1029	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1031	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1032	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1033	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1034	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1035	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SB1036	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SS1051	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1052	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1053	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1054	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.

Table 4.1, continued

Lab code	MAC	XLD	TSI	Oxidase test	Catalase test	Comments
SF1055	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SS1056	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1057	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1058	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1059	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SS1060	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1061	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SS1062	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.

Table 4.2: Biochemical test of different isolated *Shigella* strains (environmental samples)

Lab code	MAC	XLD	TSI	Oxidase test	Catalase test	Comments
SF1041	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1042	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1043	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1044	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1045	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SB1046	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1047	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1048	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1049	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.
SF1050	Colourless colony	Pinkish Colony	Yellow butt and red slant	-	+	Suspected as *Shigella* spp.

4.2 Serological characteristics of *Shigella* spp. with polyvalent sera

In the case of serotyping with polyvalent sera *S. dysenteriae* showed agglutination with Poly-A, *S. flexneri* showed agglutination with Poly-B, *S. boydii* showed agglutination with Poly-C2, and *S. sonnei* showed agglutination with Poly-D (Table 4.3). Among 49 strains *Shigella flexneri* (n=37) was predominant followed by *Shigella sonnei* (n=8), *Shigella boydii* (n=3) and *Shigella dysenteriae* (n=1).

Table 4.3: Serotyping of different strains of *Shigella* spp. with polyvalent sera

Name of strains	*S. dysenteriae*		*S. flexneri*	*S. boydii*				*S. sonnei*	Comment
	Poly-A	Poly-A1	Poly-B	Poly-C	Poly-C1	Poly-C2	Poly-C3	Poly-D	
SS1001	-	-	-	-	-	-	-	+	*Shigella sonnei*
SS1002	-	-	-	-	-	-	-	+	*Shigella sonnei*
SB1003	-	-	-	-	-	+	-	-	*Shigella boydii*
SD1007	+	-	-	-	-	-	-	-	*Shigella dysenteriae*
SF1008	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1011	-	-	+	-	-	-	-		*Shigella flexneri*
SF1013	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1014	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1015	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1016	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1017	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1018	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1019	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1020	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1021	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1023	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1024	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1025	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1027	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1028	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1029	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1031	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1032	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1033	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1034	-	-	+	-	-	-	-	-	*Shigella flexneri*

Table 4.3, continued

Name of strains	*S. dysenteriae*		*S. flexneri*	*S. boydii*				*S. sonnei*	Comment
	Poly-A	Poly-A1	Poly-B	Poly-C	Poly-C1	Poly-C2	Poly-C3	Poly-D	
SF1035	-	-	+	-	-	-	-	-	*Shigella flexneri*
SB1036	-	-	-	-	-	+	-	-	*Shigella boydii*
SF1041	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1042	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1043	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1044	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1045	-	-	+	-	-	-	-	-	*Shigella flexneri*
SB1046	-	-	-	-	-	+	-	-	*Shigella boydii*
SF1047	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1048	-	-	-	-	-	-	-	+	*Shigella sonnei*
SF1049	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1050	-	-	-	-	-	-	-	+	*Shigella sonnei*
SS1051	-	-	-	-	-	-	-	+	*Shigella sonnei*
SF1052	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1053	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1054	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1055	-	-	+	-	-	-	-	-	*Shigella flexneri*
SS1056	-	-	-	-	-	-	-	+	*Shigella sonnei*
SF1057	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1058	-	-	+	-	-	-	-	-	*Shigella flexneri*
SF1059	-	-	+	-	-	-	-	-	*Shigella flexneri*
SS1060	-	-	-	-	-	-	-	+	*Shigella sonnei*
SF1061	-	-	+	-	-	-	-	-	*Shigella flexneri*
SS1062	-	-	-	-	-	-	-	+	*Shigella sonnei*

4.3 Serological characteristics of *Shigella flexneri* with monovalent sera

Seven different serotypes of *Shigella flexneri* were confirmed by serotyping with monovalent sera (Table 4.4). *Shigella flexneri* 2a (n=25) was more prevalent followed by *Shigella flexneri* 6 (n=4), *Shigella flexneri* 1 atypical (n = 3), *Shigella flexneri* 1b (n=2), *Shigella flexneri* 3a (n=1), *Shigella flexneri* 4 atypical (n=1) and *Shigella flexneri* Y (n=1)

Table 4.4: Serotyping of different strains with monovalent sera specific for *Shigella flexneri*

Name of Strain	Type						Group			Comment
	I	II	III	IV	V	VI	3(4)	6	7(8)	
SF1008	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1011	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1013	-	-	-	-	-	-	+	-	-	*Shigella flexneri* Y
SF1014	+	-	-	-	-	-	+	+	-	*Shigella flexneri* 1b
SF1015	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1016	+	-	-	-	-	-	-	-	-	***Shigella flexneri* 1 atypical**
SF1017	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1018	-	-	-	-	-	+	-	-	-	*Shigella flexneri* 6
SF1019	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1020	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1021	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1023	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1024	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1025	-	-	+	-	-	-	-	+	+	*Shigella flexneri* 3a
SF1027	-	-	-	-	-	+	-	-	-	*Shigella flexneri* 6
SF1028	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1029	-	-	-	-	-	+	-	-	-	*Shigella flexneri* 6
SF1031	-	-	-	-	-	+	-	-	-	*Shigella flexneri* 6

Table 4.4, continued

Name of Strain	Type						Group			Comment
	I	II	III	IV	V	VI	3(4)	6	7(8)	
SF1032	-	-	-	+	-	-	-	-	-	***Shigella flexneri* 4 atypical**
SF1033	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1034	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1035	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1041	+	-	-	-	-	-	-	+	-	*Shigella flexneri* 1b
SF1042	+	-	-	-	-	-	-	-	-	***Shigella flexneri* 1atypical**
SF1043	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1044	+	-	-	-	-	-	-	-	-	***Shigella flexneri* 1atypical**
SF1045	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1047	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1049	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1052	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1053	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1054	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1055	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1057	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1058	-	+	-	-	-	-	+	-	-	*Shigella flexneri 2a*
SF1059	-	+	-	-	-	-	+	-	-	*Shigella flexneri* 2a
SF1061	-	+	-	-	-	-	+	-	-	*Shigella flexneri 2a*

4.4 Serotyping with monovalent sera specific for *Shigella sonnei*:

Serotypes of *Shigella sonnei* were confirmed by serotyping with monovalent sera specific of *Shigella sonnei* (Table 4.5).

Table 4.5: Serotyping with monovalent sera specific for *Shigella sonnei*

Name of the strains	Phage -I	Phage-II	Comment
SS1001	-	+	*Shigella sonnei* phage-II
SS1002	-	+	*Shigella sonnei* phage-II
SS1048	-	+	*Shigella sonnei* phage-II
SS1050	-	+	*Shigella sonnei* phage-II
SS1051	-	+	*Shigella sonnei* phage-II
SS1056	-	+	*Shigella sonnei* phage-II
SS1060	-	+	*Shigella sonnei* phage-II
SS1062	-	+	*Shigella sonnei* phage-II

4.5 Molecular characterization

All the serotypes of *Shigella flexneri* were further confirmed by singleplex and nonaplex PCR amplification. In addition the three *Shigella flexneri* 1 (atypical/untypical) were detected as *Shigella flexneri* 1c and the *Shigella flexneri* 4 atypical was detected as *Shigella flexneri* 4a (Figure 4.1 and 4.2). The expected gene expression in multiplex PCR for the strains were as follows: *Shigella flexneri* 1b : *wzx*1-5, *gtrI*, and *oac* (782 bp, 1122 bp and 604bp); *Shigella flexneri* 1c : *wzx*1-5, *gtrI*, and *gtrIC* (782 bp, 1122 bp and 518bp); *Shigella flexneri* 2a : *wzx*1-5 and *gtrII* (782bp and1272 bp); *Shigella flexneri* 3a : *wzx*1-5, *oac*, and *gtrX* (782 bp, 604 bp and 425bp); *Shigella flexneri* 4a :*wzx*1-5 and *gtrIV* (782 bp and 378 bp); *Shigella flexneri* Y : *wzx*1-5 (782 bp); *Shigella flexneri* 6 : *wzx6* (739 bp)

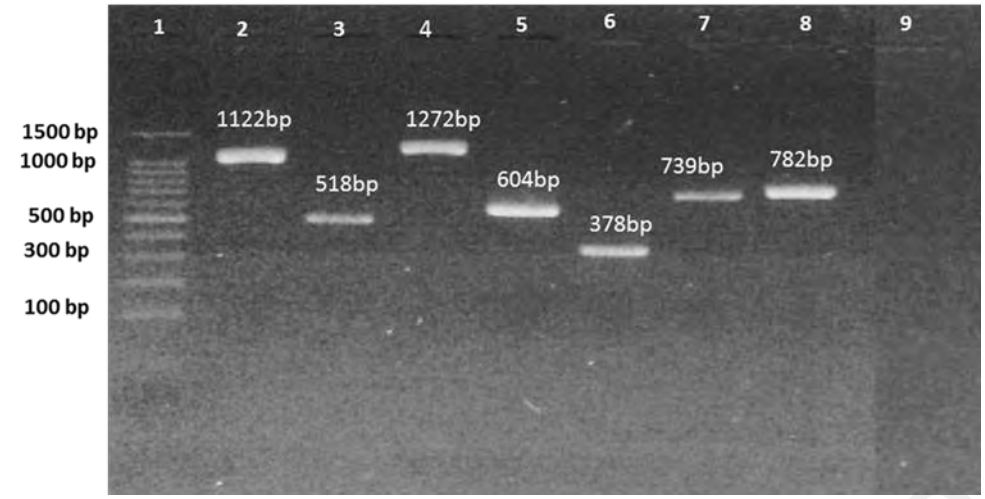

Figure 4.1: Singleplex PCR, representatives of all seven serotypes are showing specific bands.1: 100bp marker; 2: *S. flexneri* 1b (SF1014); 3: *S. flexneri* 1c (SF1016); 4: *S. flexneri* 2a (SF1017); 5: *S. flexneri* 3a (SF1025); 6: *S. flexneri* 4a (SF1032); 7: *S. flexneri* 6 (SF1018); 8: *S. flexneri* Y (SF1013); 9: negative control.

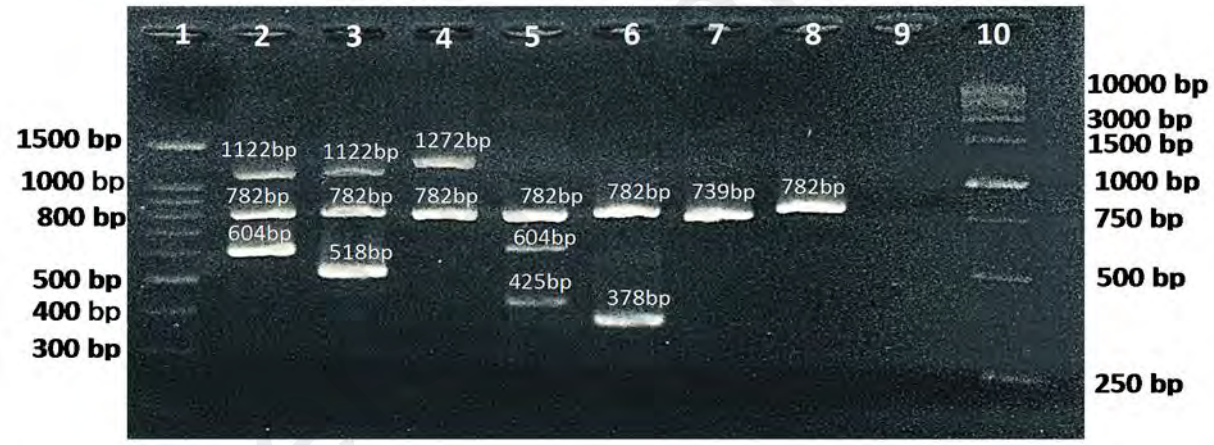

Figure 4.2: Nonaplex PCR, representatives of all seven serotypes are showing their respective multiple gene expression. 1: 100bp marker; 2: *S. flexneri* 1b (SF1014); 3: *S. flexneri* 1c (SF1016); 4: *S. flexneri* 2a (SF1017); 5: *S. flexneri* 3a (SF1025); 6: *S. flexneri* 4a (SF1032); 7: *S. flexneri* 6 (SF1018); 8: *S. flexneri* Y (SF1013); 9: negative control; 10: 1kb marker.

4.6 Antibiotic profiling of 49 serotypes of *Shigella* spp.

Antibiotic profiling of all 49 *Shigella* strains demonstrated that 98% (48) strains were drug resistant and 59% (29) were multi-drug resistant (Table 4.6 and Figure 4.3). Highest number of resistance was found against Nalidixic Acid (79%) followed by Tetracycline (57%), Streptomycin (55%), Ampicillin (49%) Amoxicillin (40%), Kanamycin (26%), Chloramphenicol (20%) and Ciprofloxacin (16 %), Ceftriaxone (2%) and Cefepime (2%) (Figure 4.4). Among all the strains, three serotypes of *Shigella flexneri* 2a (SF1033,

SF1045 and SF1058) showed most resistance range against seven antibiotics out of ten while nine *Shigella flexneri* 2a strains (SF1023, SF1028, SF1034, SF1035, SF1043, SF1047, SF1049, SF1055 and SF1057) and one *Shigella flexneri* 1C (SF1044) strains showed resistance against six antibiotics (APPENDIX E showing the resistant capacity of the strain SF1056). Five strains showed resistance against five antibiotics and the strains were one *Shigella flexneri* Y (SF1013), two *Shigella flexneri* 2a (SF1008 and SF1021) and two *Shigella boydii* (SB1036 andSB1046). Four *Shigella flexneri* 2a (SF1020, SF1019, SF1024 and SF1059)) one *Shigella flexneri* 4a (SF1032) and one *Shigella boydii* (SB1003 showed resistance against four antibiotics while one *Shigella flexneri* 1c (1016), one *Shigella flexneri* 2a (SF1061) two *Shigella flexneri* 6 (SF1027 and SF1029) and one *Shigella sonnei* (SS1060) showed resistance against three antibiotics. One *Shigella flexneri* 3a (SF1025), one *Shigella flexneri* 2a (SF1015) one *Shigella flexneri* 1b (SF1041), one *Shigella flexneri* 1c (SF1042) and one *Shigella sonnei* (SS1051) showed resistance against two antibiotics. Only one strain *Shigella flexneri* 2a (SF1011) did not show resistance against any antibiotics and all the rest of the 14 *Shigella* strains showed resistance against only one antibiotic out of ten (Table 4.6).

Table 4.6: Antibiotic profiling of 49 *Shigella* strains

Lab code	Name of the Strains	Name of antibiotics										Comment
		AMP (10µg)	AML (10µg)	TE (30µg)	S (25µg)	K (30µg)	CIP (5µg)	FEP (30µg)	NA (30µg)	CRO (30µg)	C (30µg)	
SS1001	*Shigella sonnei*	S	S	S	S	S	I	S	R	S	S	Drug resistant
SS1002	*Shigella sonnei*	S	S	S	S	S	I	S	R	S	S	Drug resistant
SB1003	*Shigella boydii*	R	S	R	R	I	S	S	R	S	S	Multidrug resistant
SD1007	*Shigella dysenteriae*	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1008	*Shigella flexneri* 2a	R	R	R	R	I	I	S	R	S	S	Multidrug resistant
SF1011	*Shigella flexneri* 2a	S	S	I	I	I	I	S	I	S	S	Intermediate
SF1013	*Shigella flexneri* Y	S	S	R	R	R	R	S	R	S	S	Multidrug resistant
SF1014	*Shigella flexneri* 1b	S	S	S	R	I	S	S	I	S	S	Drug resistant
SF1015	*Shigella flexneri* 2a	I	I	I	I	S	S	S	R	S	R	Drug resistant
SF1016	*Shigella flexneri* 1c	R	R	I	R	R	S	S	I	S	I	Multidrug resistant
SF1017	*Shigella flexneri* 2a	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1018	*Shigella flexneri* 6	S	S	R	I	I	S	S	S	S	S	Drug resistant

Table 4.6, continued

Lab code	Name of the Strains	Name of antibiotics										Comment
		AMP (10µg)	AML (10µg)	TE (30µg)	S (25µg)	K (30µg)	CIP (5µg)	FEP (30µg)	NA (30µg)	CRO (30µg)	C (30µg)	
SF1019	*Shigella flexneri* 2a	S	S	R	R	S	R	S	R	S	S	Multidrug resistant
SF1020	*Shigella flexneri* 2a	R	R	R	I	S	I	S	R	S	S	Multidrug resistant
SF1021	*Shigella flexneri* 2a	R	R	R	R	I	I	S	R	S	R	Multidrug resistant
SF1023	*Shigella flexneri* 2a	R	R	R	R	S	R	S	R	S	S	Multidrug resistant
SF1024	*Shigella flexneri* 2a	S	S	R	R	I	R	S	R	S	S	Multidrug resistant
SF1025	*Shigella flexneri* 3a	S	S	S	R	I	S	S	R	S	I	Drug Resistant
SF1027	*Shigella flexneri* 6	S	S	R	R	R	S	S	S	S	S	Multidrug resistant
SF1028	*Shigella flexneri* 2a	R	R	R	R	I	R	S	R	S	S	Multidrug resistant
SF1029	*Shigella flexneri* 6	S	S	R	R	R	S	S	S	S	S	Multidrug resistant
SF1031	*Shigella flexneri* 6	S	S	R	S	S	I	I	S	I	S	Drug resistant
SF1032	*Shigella flexneri* 4a	R	R	S	R	R	S	S	I	S	S	Multidrug resistant
SF1033	*Shigella flexneri* 2a	R	R	R	R	I	R	S	R	S	R	Multidrug resistant
SF1034	*Shigella flexneri* 2a	R	R	R	R	R	S	S	R	S	I	Multidrug resistant

Table 4.6, continued

Lab code	Name of the Strains	Name of antibiotics										Comment
		AMP (10µg)	AML (10µg)	TE (30µg)	S (25µg)	K (30µg)	CIP (5µg)	FEP (30µg)	NA (30µg)	CRO (30µg)	C (30µg)	
SF1035	*Shigella flexneri* 2a	R	R	R	R	R	S	S	R	S	I	Multidrug resistant
SB1036	*Shigella boydii*	R	R	R	R	S	S	S	R	S	S	Multidrug resistant
SF1041	*Shigella flexneri* 1b	S	S	S	R	I	S	S	R	S	S	Drug resistant
SF1042	*Shigella flexneri* 1c	R	S	R	S	S	S	S	S	S	S	Drug resistant
SF1043	*Shigella flexneri* 2a	R	R	R	R	S	S	S	R	S	R	Multidrug resistant
SF1044	*Shigella flexneri* 1c	R	R	R	R	S	S	S	R	S	R	Multidrug resistant
SF1045	*Shigella flexneri* 2a	R	R	R	R	R	S	S	R	S	R	Multidrug resistant
SB1046	*Shigella boydii*	R	R	R	R	S	S	S	R	S	S	Multidrug resistant
SF1047	*Shigella flexneri* 2a	R	R	R	R	S	S	S	R	S	R	Multidrug resistant
SF1048	*Shigella sonnei*	S	S	S	S	S	I	S	R	S	S	Drug resistant
SF1049	*Shigella flexneri* 2a	R	R	R	R	S	S	S	R	S	R	Multidrug resistant
SF1050	*Shigella sonnei*	S	S	S	S	S	I	S	R	S	S	Drug resistant
SS1051	*Shigella sonnei*	S	S	S	S	R	S	S	R	S	S	Drug resistant

Table 4.6, continued

Lab code	Name of the Strains	Name of antibiotics										Comment
		AMP (10µg)	**AML (10µg)**	**TE (30µg)**	**S (25µg)**	**K (30µg)**	**CIP (5µg)**	**FEP (30µg)**	**NA (30µg)**	**CRO (30µg)**	**C (30µg)**	
SF1052	*Shigella flexneri* 2a	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1053	*Shigella flexneri* 2a	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1054	*Shigella flexneri* 2a	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1055	*Shigella flexneri* 2a	R	S	R	S	R	S	R	S	R	R	Multidrug resistant
SS1056	*Shigella sonnei*	S	S	S	S	S	S	S	R	S	S	Drug resistant
SF1057	*Shigella flexneri* 2a	S	R	S	R	R	R	S	R	S	R	Multidrug resistant
SF1058	*Shigella flexneri* 2a	R	R	R	R	R	R	S	R	S	S	Multidrug resistant
SF1059	*Shigella flexneri* 2a	R	S	R	S	R	S	S	R	S	S	Multidrug resistant
SS1060	*Shigella sonnei*	R	R	S	S	S	S	S	R	S	S	Multidrug resistant
SF1061	*Shigella flexneri* 2a	R	S	R	S	S	S	S	R	S	S	Multidrug resistant
SS1062	*Shigella sonnei*	S	S	S	S	S	S	S	R	S	S	Drug resistant

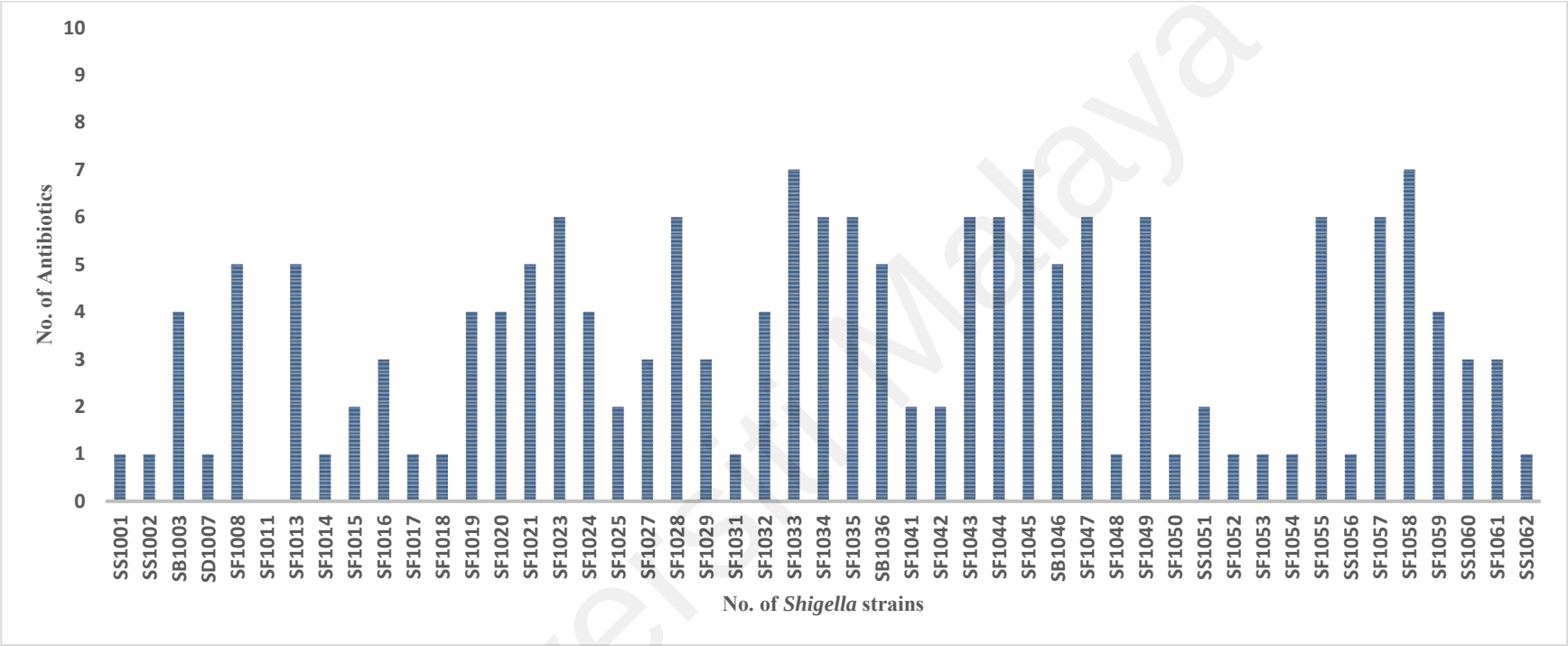

Figure 4.3: Antibiotic profiling of 49 *Shigella* strains. The strains showing resistance to more than two antibiotics are regarded as multidrug resistant.

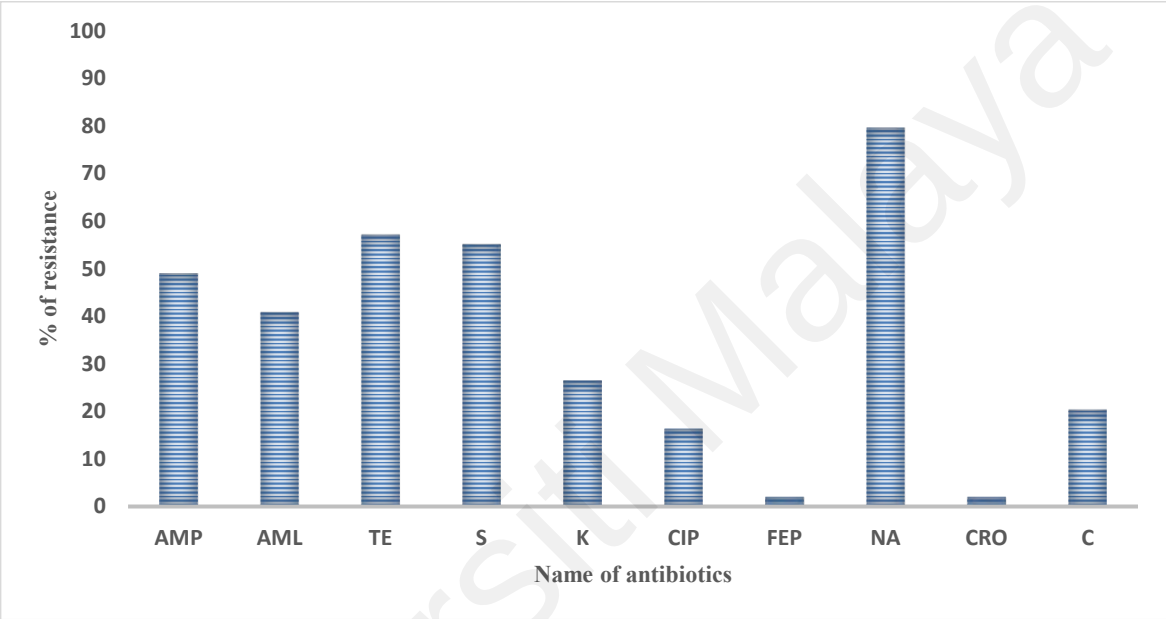

Figure 4.4: Percentage of resistance pattern against 10 antibiotics. AMP: Ampicillin; AML: Amoxicillin; TE: Tetracycline; S: Streptomycin; K: Kanamycin; CIP: Ciprofloxacin; FEP: Cefepime; NA: Nalidixic Acid; CRO: Ceftriaxone; C: Chloramphenicol.

4.7 Plasmid profiling of three novel serotypes *Shigella flexneri* 1c

Four types of plasmids of different sizes were observed through agarose gel electrophoresis in all three strains of *Shigella flexneri* 1c: SF 1016, SF 1042 and SF1044. Three strains showed similarity in their plasmids. The size of plasmids were approximately 12kb, 3kb, 2.5kb and 1.4kb respectively (Figure 4.5).

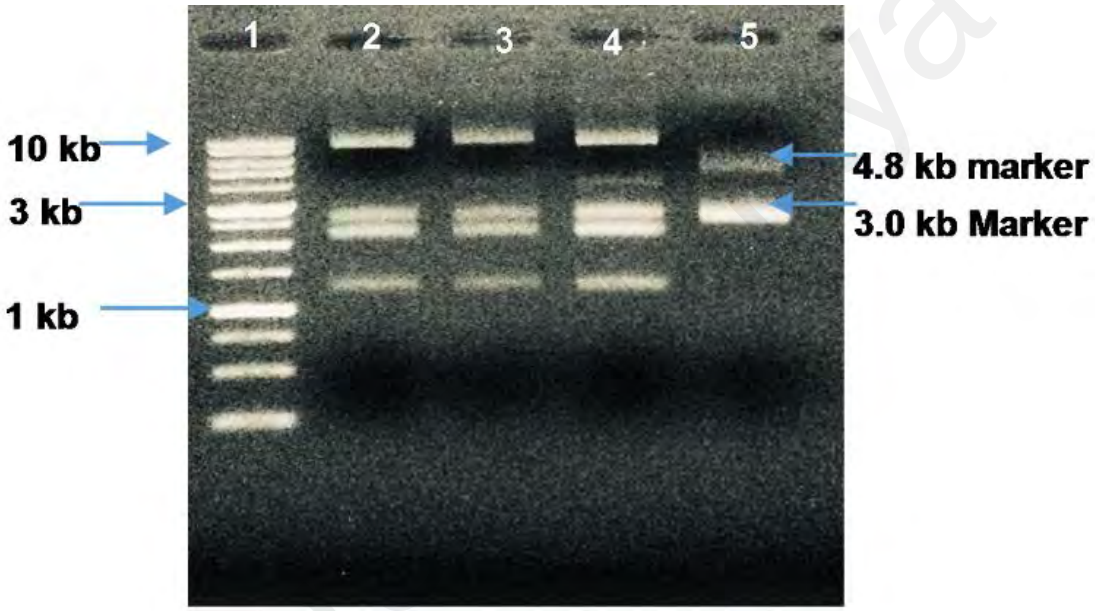

Figure 4.5: Plasmid profiling of three *S. flexneri* 1c strains, 1: 1kb marker; 2: SF1042; 3: SF 1044; 4: SF1016; 5: reference plasmids PGMET (3kb) and HTB (4.8 kb) in same lane.

4.8 Isolation, purification, and naming of bacteriophage

Ten bacteriophages were isolated and purified based on their lytic activities and designated as SF2aV1TB002, SF1cV2TB004, SF2aV2TB006, SF1cV3TB007, SF4aV1TB008, SF4aV1TB009, SF4aV2TB010, SF2aV5TB011, SF2aV7TB013 and SF4aV4TB014. Three bacterial strains *Shigella flexneri* 2a, *Shigella flexneri* 1c and *Shigella flexneri* 4a were selected randomly as hosts in this isolation and purification process. Short name of these phages l (TB002, TB004, TB006, TB007, TB008, TB009,

TB010, TB011, TB013 and TB014) have been used throughout the thesis for easy communication. The host name of the phages are given in the Table 4.7 below-

Table 4.7: List of isolated bacteriophage and their host bacteria

Name of bacteriophage	Name of host bacteria
SF2aV1TB002	*Shigella flexneri* 2a
SF1cV2TB004	*Shigella flexneri* 1c
SF2aV2TB006	*Shigella flexneri* 2a
SF1cV3TB007	*Shigella flexneri* 1c
SF4aV1TB008	*Shigella flexneri* 4a
SF4aV1TB009	*Shigella flexneri* 4a
SF4aV2TB010	*Shigella flexneri* 4a
SF2aV5TB011	*Shigella flexneri* 2a
SF2aV7TB013	*Shigella flexneri* 2a
SF4aV4TB014	*Shigella flexneri* 4a

4.9 Plaque morphology, size and characters

On the basis of plaque size, the phages were categorised into three classes i. e small (0.92-1.23 mm), medium (2.61-3.63 mm) and large (5.34-8.31 mm) (Table 4.8).Clear plaques were observed for all bacteriophages while TB013 showed clear plaques with halos (bull eye shaped) **(** Figure 4.6 and 4.7)

Table 4.8: Plaque size and morphology of 10 isolated bacteriophages

Category	Name of the phages	Plaque size and characteristics
Small	SF2aV1TB002	0.92 ± 0.107 mm (Small clear plaques)
	SF1cV2TB004	1.23 ± 0.070 mm (Small clear plaques)
Medium	SF2aV2TB006	3.26 ± 0.076 mm (Medium clear plaques)
	SF4aV1TB008	3.17 ± 0.131 mm (Medium clear plaques)
	SF4aV1TB009	3.28 ± 0.172 mm (Medium clear plaques)
	SF4aV2TB010	3.15 ± 0.141 (Medium clear plaques) mm
	SF2aV5TB011	2.61 ± 0.141 (Medium clear plaques) mm
	SF2aV7TB013	3.63 ± 0.098 mm (Medium plaques with halos)
Large	SF1cV3TB007	8.31 ± 0.156 mm (Large clear plaques)
	SF4aV4TB014	5.34 ± 0.193 mm (Large clear plaques)

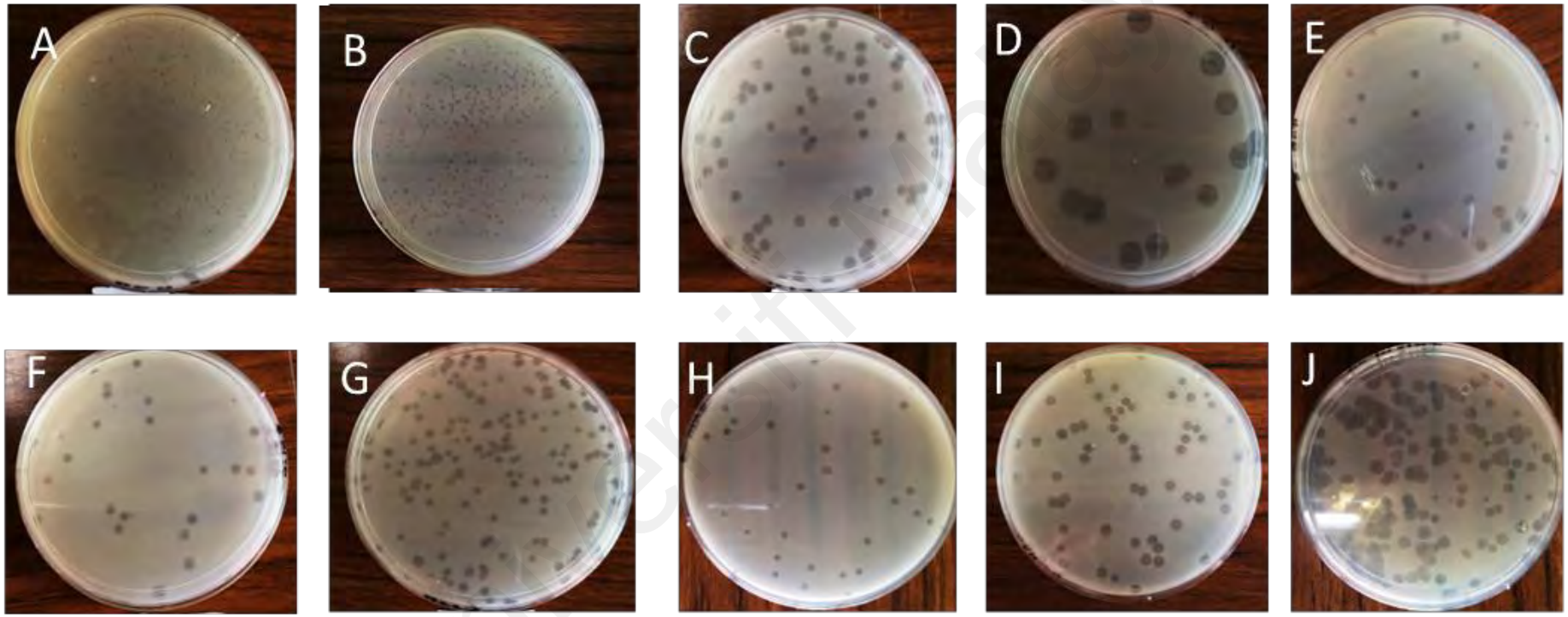

Figure 4.6: Plaque morphology of bacteriophages; A: TB002; B: TB004; C: TB006; D: TB007; E: TB008; F: TB009; G: TB010; H: TB011; I: TB013; J: TB014.

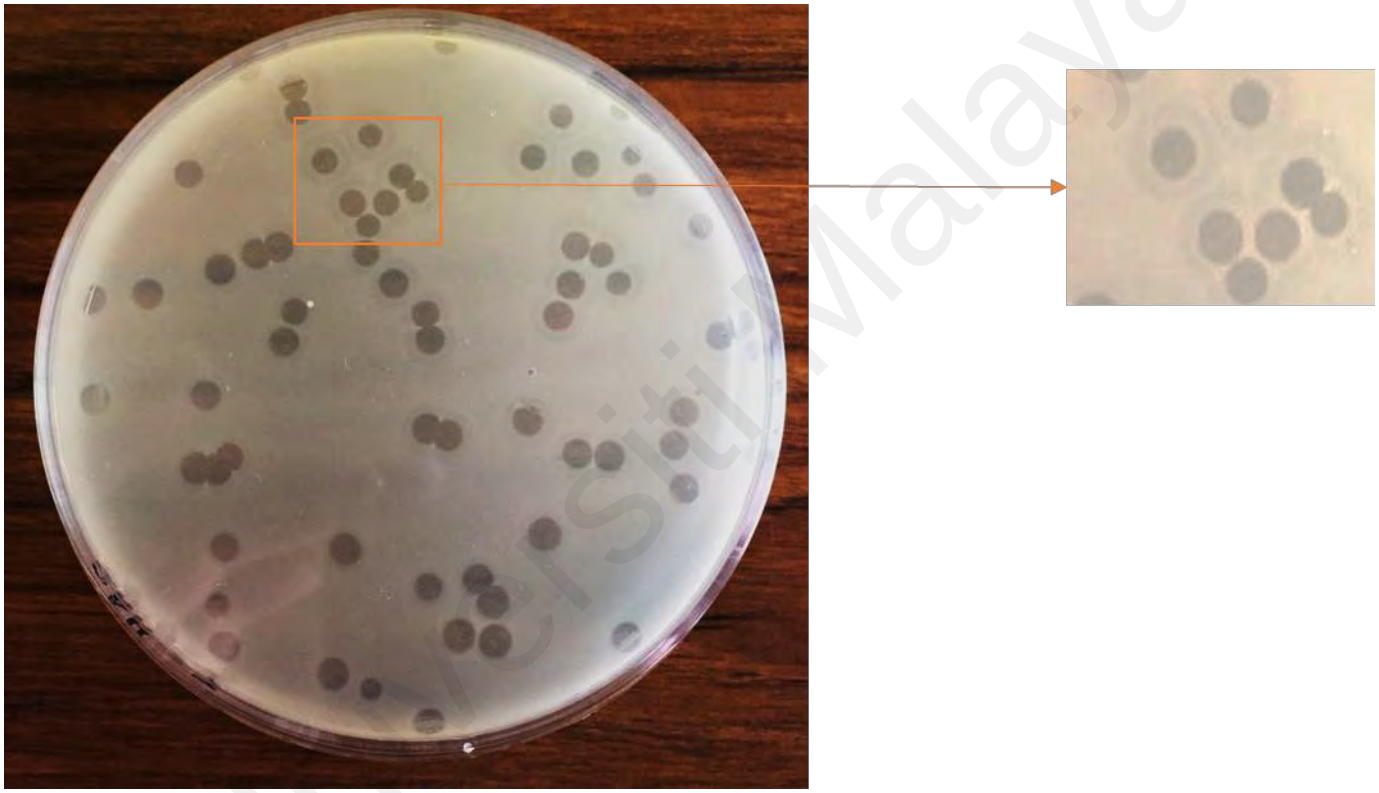

Figure 4.7: Plaque morphology of bacteriophage TB013 showing the halos morphology of plaques

4.10 Transmission electron microscopy and phage morphology

The morphologies of different bacteriophages obtained from TEM (Figure 4.8) were classified according to the guideline of the International Committee on Taxonomy of Viruses (Krupovic et al., 2016) where TB002, TB004, TB006, TB009, TB011, TB013 and TB014 were under *Caudovirales* order because all these seven phages were tailed phage. Among these tailed phages TB002 and TB004 belonged to the family *Myoviridae* as the phages composed of capsid and possess long tail with contractile sheath while phage TB009, TB010 and TB013 belonged to the family *Siphoviridae* as the phages composed of capsid and possess non-contractile long tail. TB006 and TB014 belonged to the family *Podoviridae* as the phages composed of capsid and have short non-contractile tail. TB007 was tailless bacteriophage with hexagonal head, TB008 and TB011 were tailless bacteriophages with round head. These three tailless phages belong to either group D or E (*Corticoviridae, Tectiviridae* or *Microviridae*) according Bradley's classification (Bradley, 1967). Further studies are required to confirm the family of these three tailless bacteriophages.

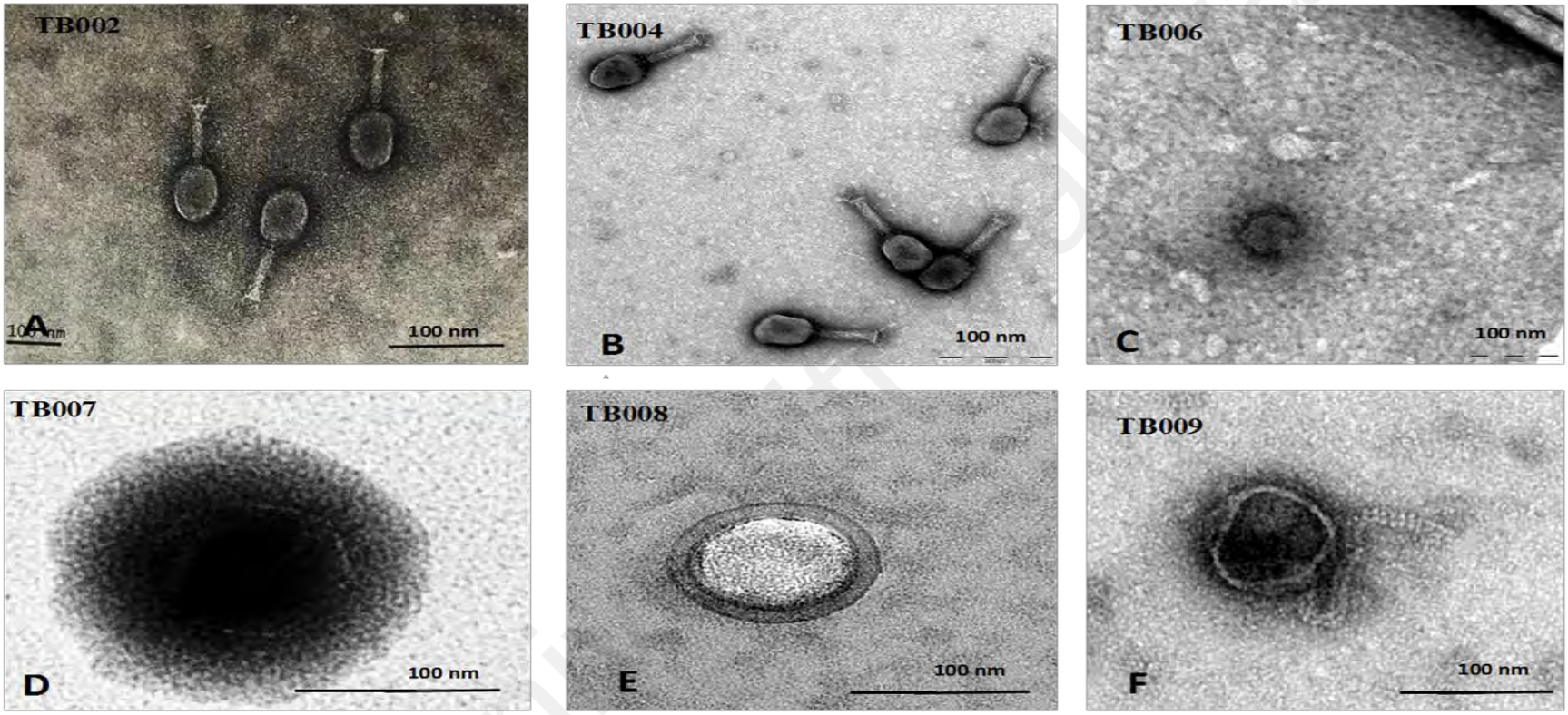

Figure 4.8: Phage morphology under Transmission Electron Microscope (TEM). A: TB002 (magnification × 100000); B: TB004 (magnification × 80000); C: TB006 (magnification × 94500); D: TB007 (magnification × 150000); E: TB008(magnification × 150000); F: TB009 (magnification × 150000); G: TB010 (magnification × 94500); H: TB011(magnification × 100000); I: TB013(magnification × 100000); J: TB014 (magnification × 100000)

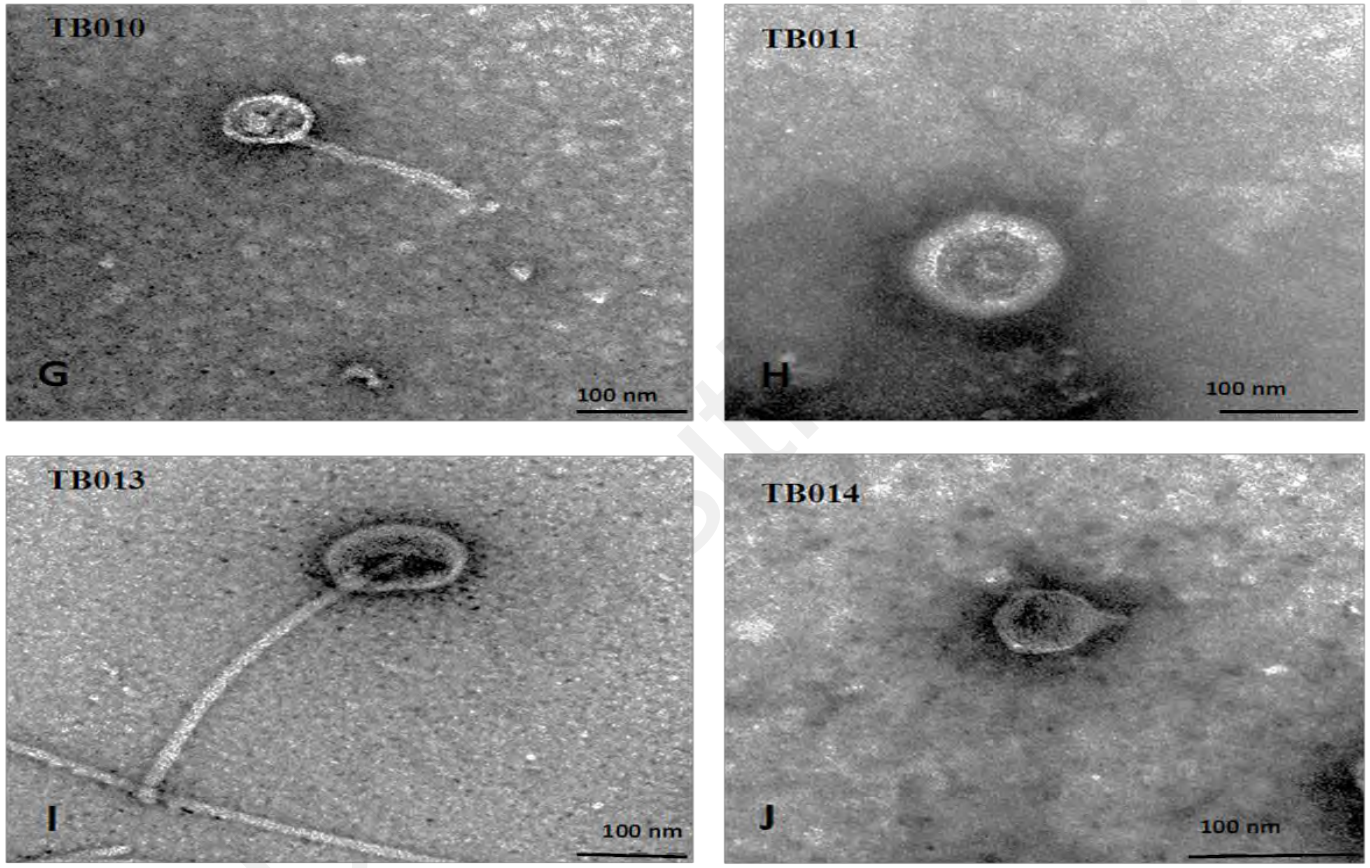

Figure 4.8, continued

4.11 Genetic nature of the bacteriophages

Extracted nucleic acids of all 10 bacteriophages isolated in this study were treated with DNase I and RNase and found that the nucleic acids of 10 phages were DNA in nature as they were digested with DNase I but not digested with RNase (Figure 4.9)

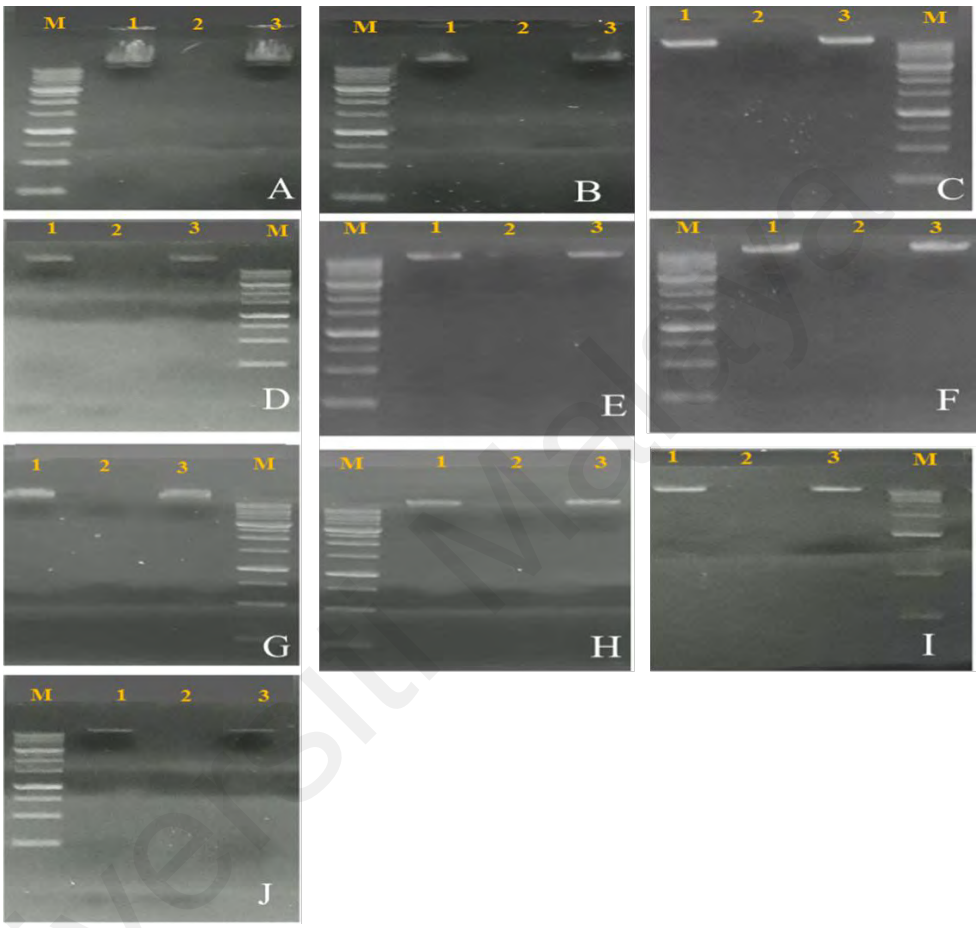

Figure 4. 9: Digestion of phage nucleic acids with DNase I and RNase. A: TB002; B: TB004; C: TB006; D: TB007; E: TB008; F: TB009; G: TB010; H: TB011; I: TB013; J: TB014; Lanes. M: 1 kb marker; 1: digested with RNase; 2: digested with DNase I; 3: undigested phage DNA.

4.12 One step growth curve

All phages showed gradual increase of growth until the 100 min while TB011 showed 10% decrease at the 100th minute (Table 4.9 A and APPENDIX G). There were some variations in their latent period and burst size too. TB002 and TB014 showed the longest latent period and it was 40 min while TB007 and TB013 showed a latent period of 30 min

and the latent period for TB004 and TB010 was 20 min. The rest of the phages TB006, TB008, TB009 and TB011 showed short latent period and it was 10 min only (Figure 4.10 and 4.11). Within the latency period the highest burst size was estimated for the phage TB002 and the value was 634 virions/cell, followed by TB004 (486.5 virions/cell), TB007 (221 virions/cell), TB013 (161.5 virions/cell), TB014 (158.25 virions/cell), TB008 (157.75 virions/cell), TB009 (109 virions/cell), TB010 (102 virions/cell), TB011 (52.6 virions/cell) and TB006 (51.9 virions/cell).

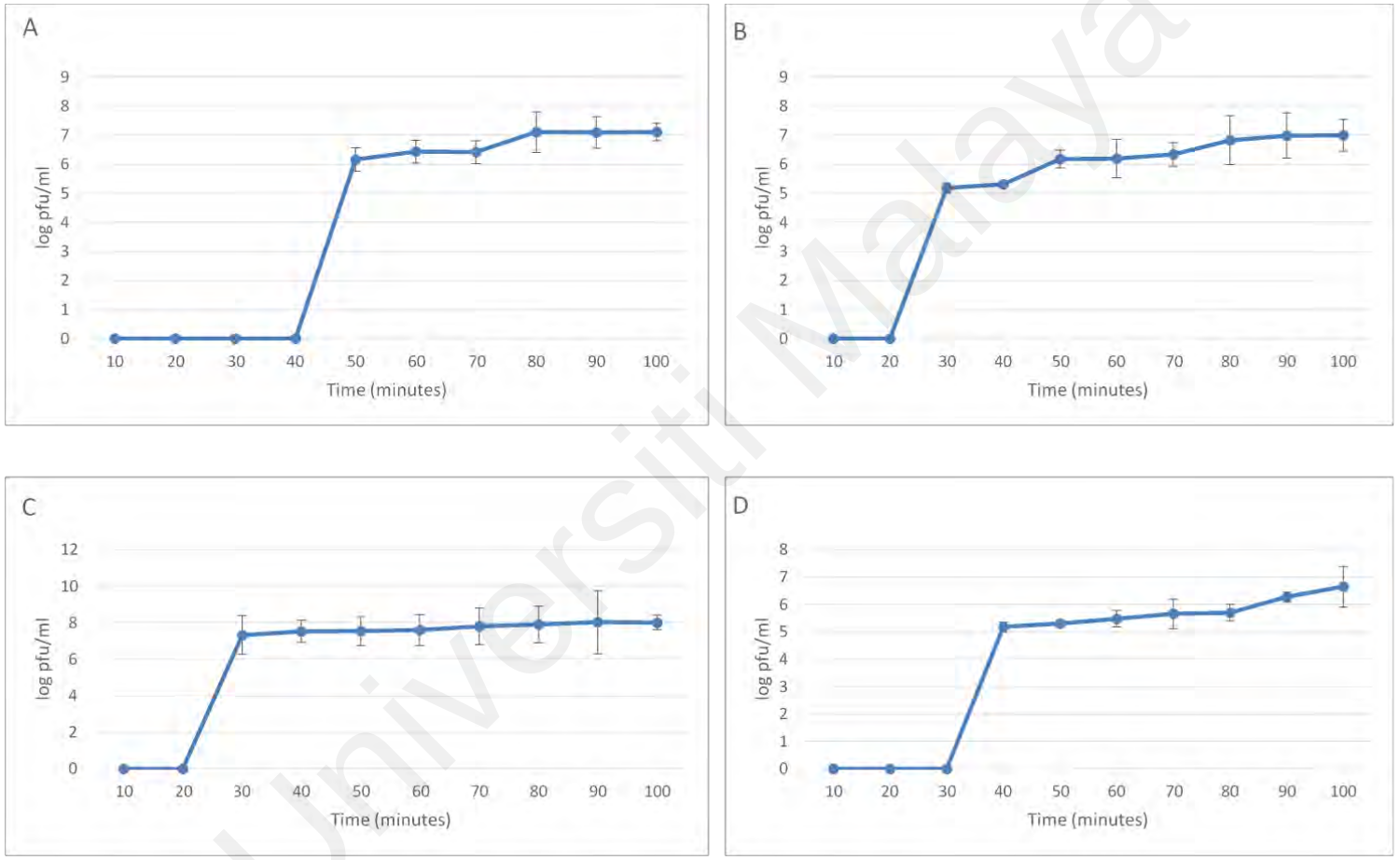

Figure 4.10: One step growth curve of 10 bacteriophages. A: TB002; B: TB004; C: TB006; D: TB007; E: TB008; F: TB009; G: TB010; H: TB011; I: TB013; J: TB014.

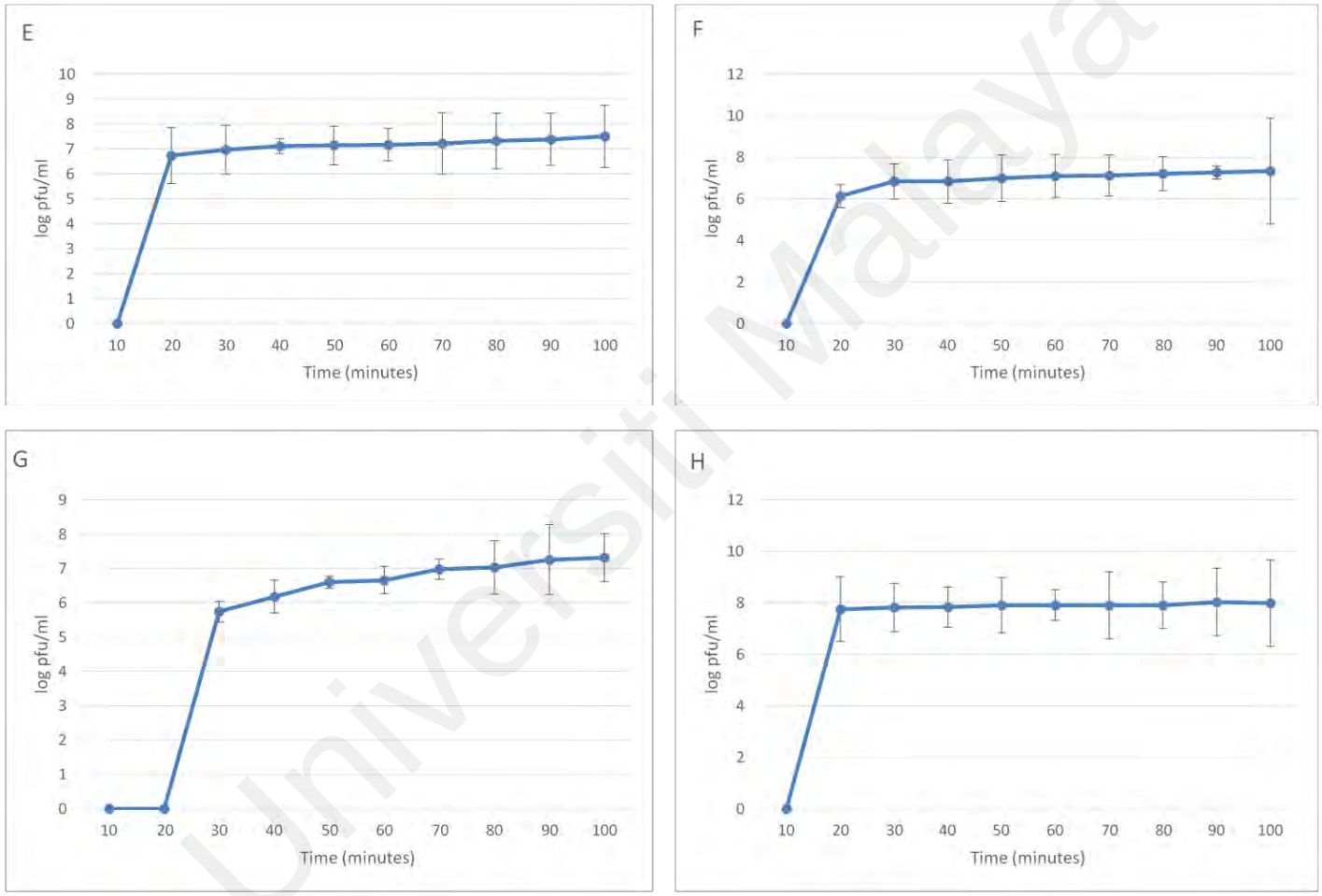

Figure 4.10, continued

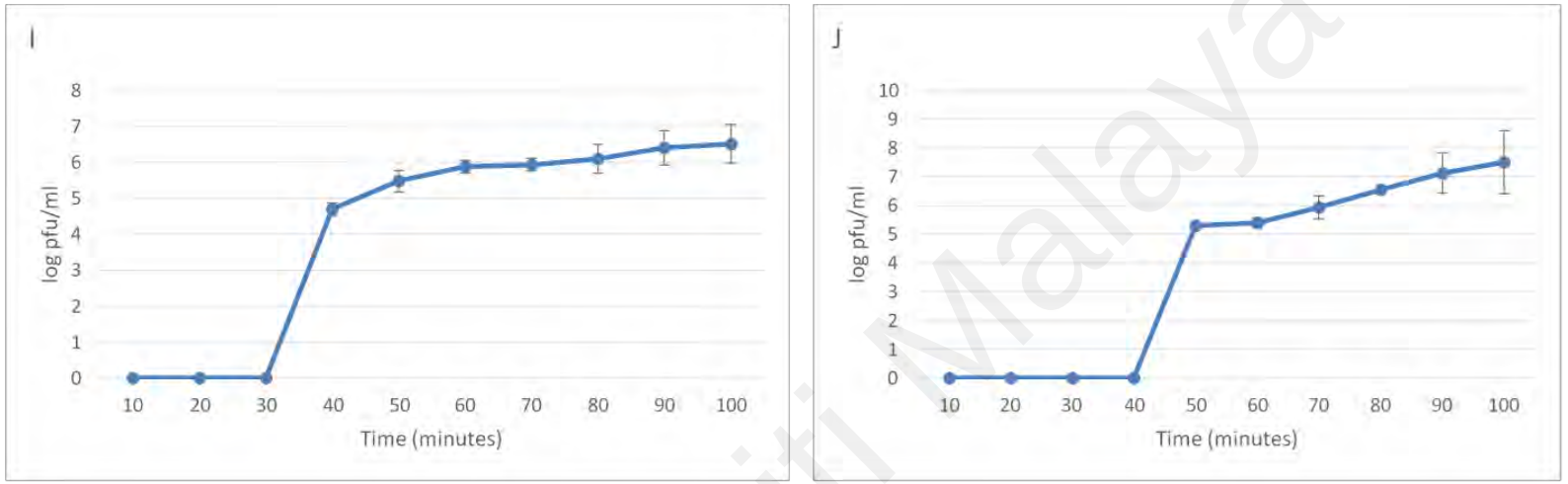

Figure 4.10, continued

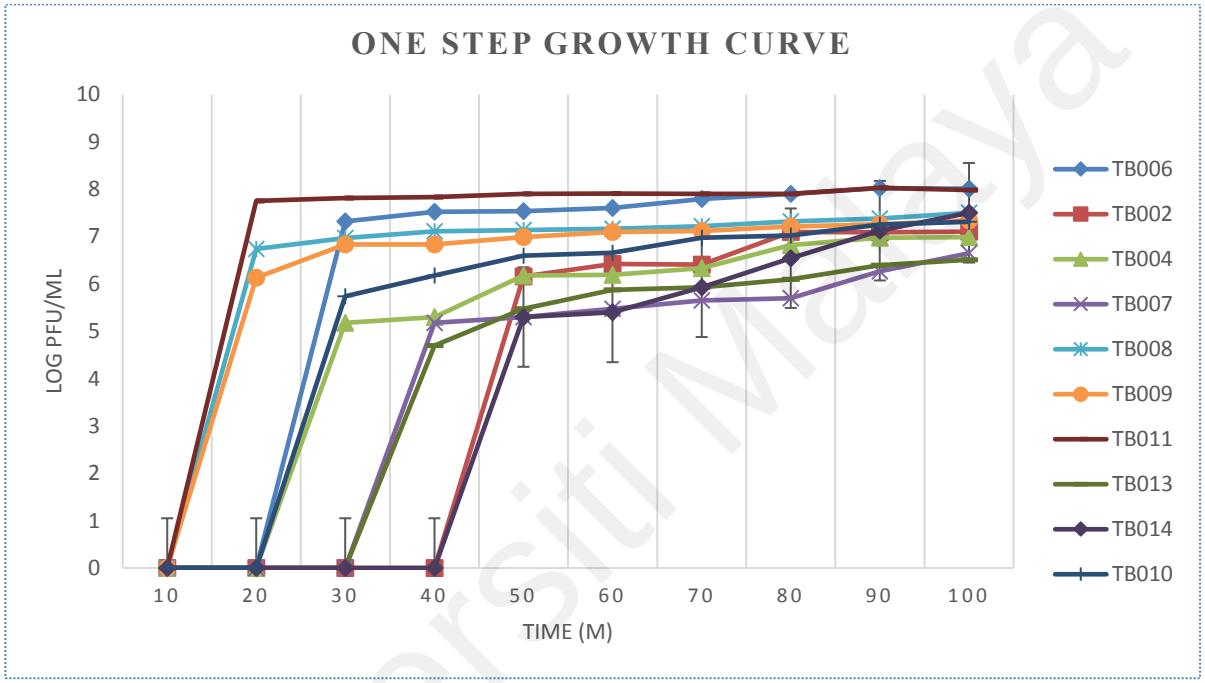

Figure 4.11: Comparison of one step growth curve of 10 bacteriophages. TB002 and TB014 showed the highest latent period (40 min) while TB007 and TB013 showed the latent period of 30 min and TB004 and TB010 showed the latent period of 20 min. The rest of the phages showed shortest latent period (10 min).

4.13 Determination of optimum temperature tolerance

All the 10 phages showed normal growth after 1 h incubation at minus20 °C, 25 °C, 37 °C and 50 °C while TB014 and TB013 showed more than 50% survival at 70 °C also (Table 4.9 B and APPENDIX H). Other phages failed to grow at 70 °C (Figure 4.12 and 4.13).

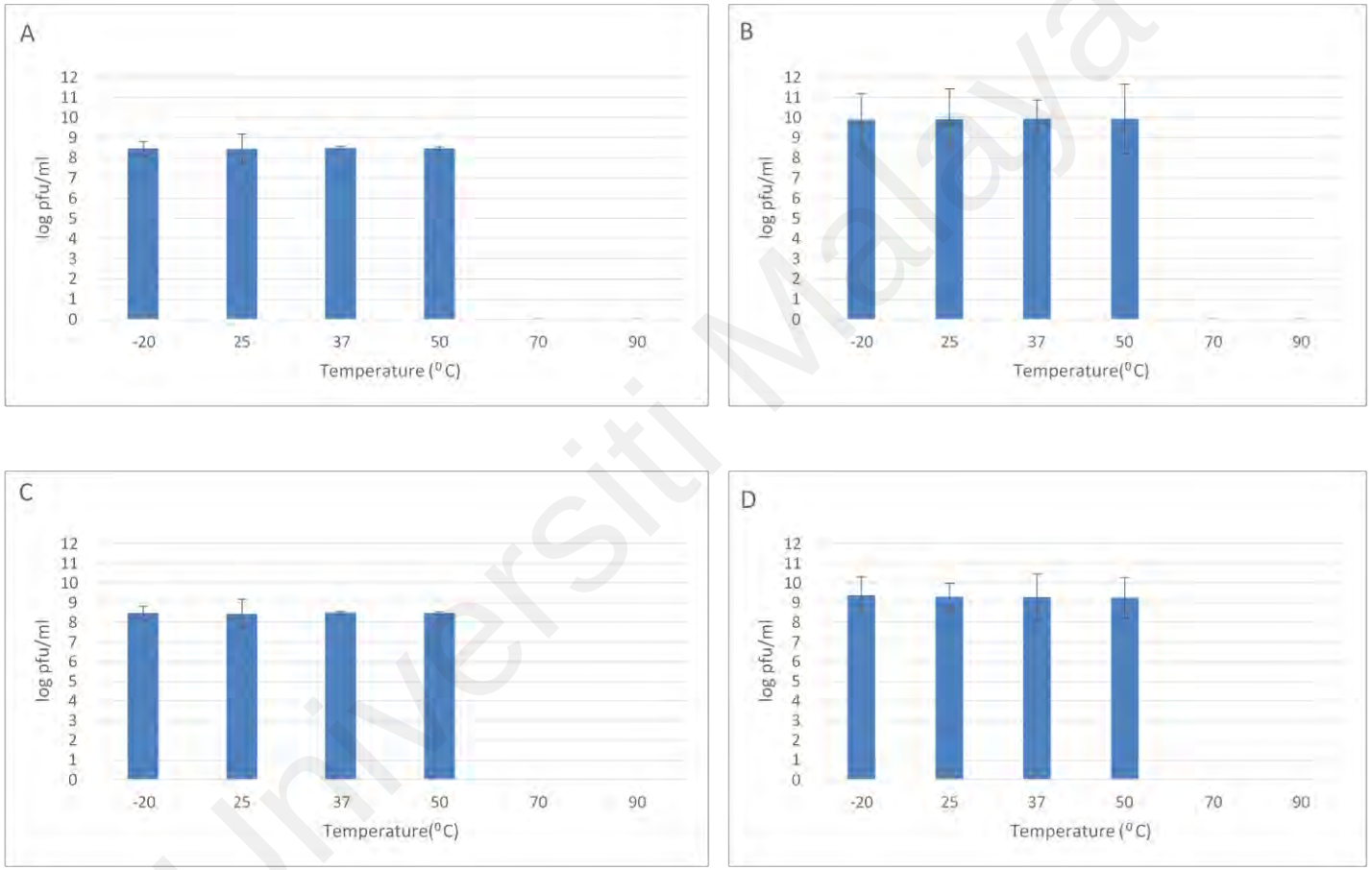

Figure 4.12: Thermal stability of 10 bacteriophages. A: TB002; B: TB004; C: TB006; D: TB007; E: TB008; F: TB009; G: TB010; H: TB011; I: TB013; J: TB014.

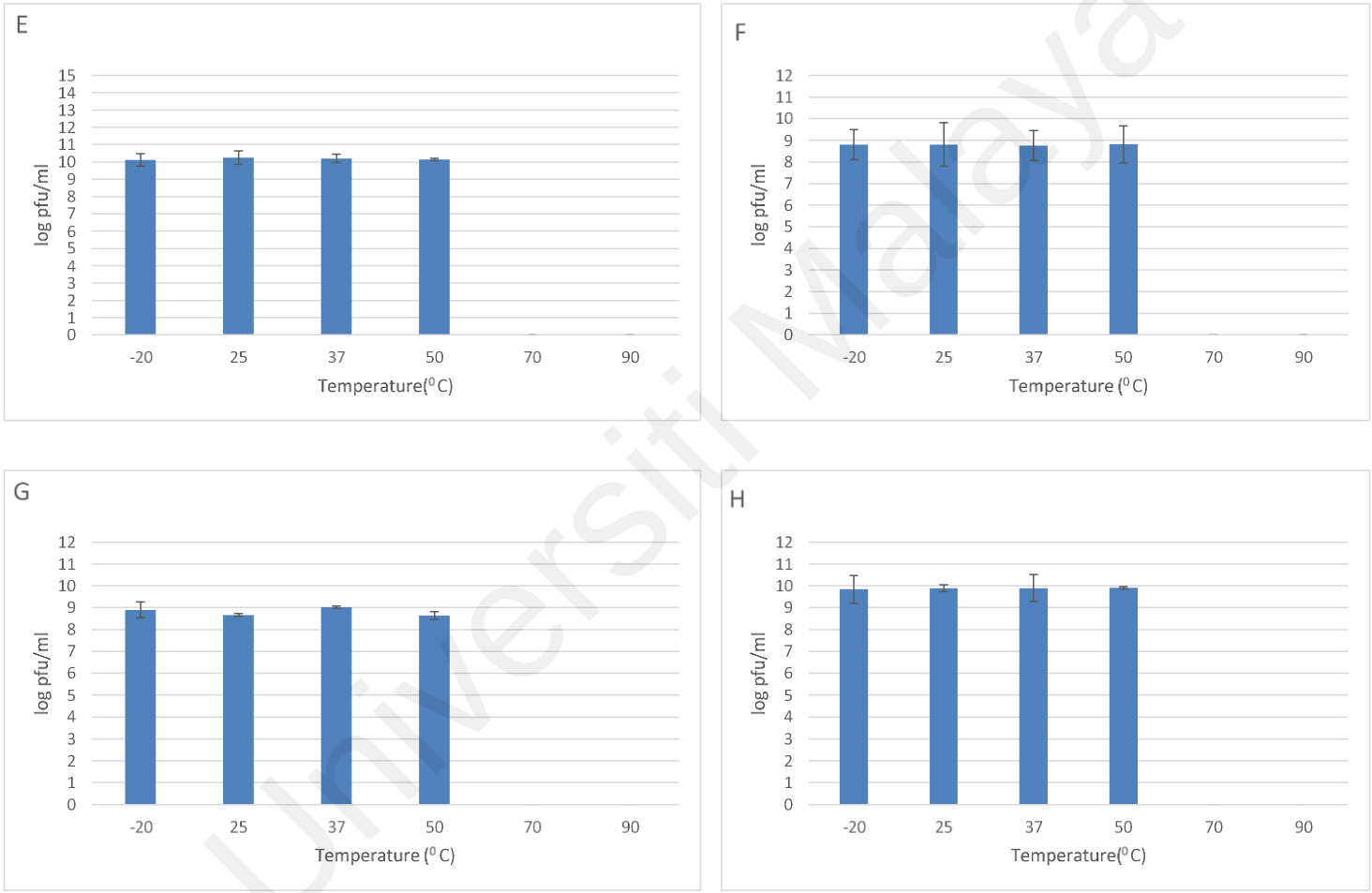

Figure 4.12, continued

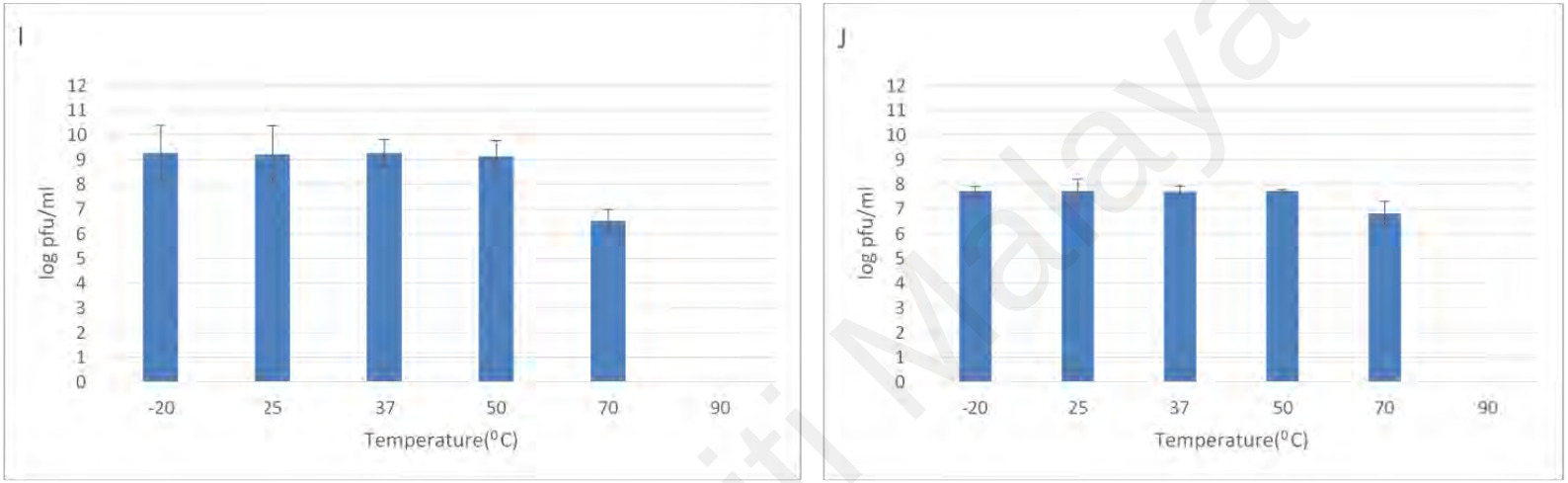

Figure 4.12, continued

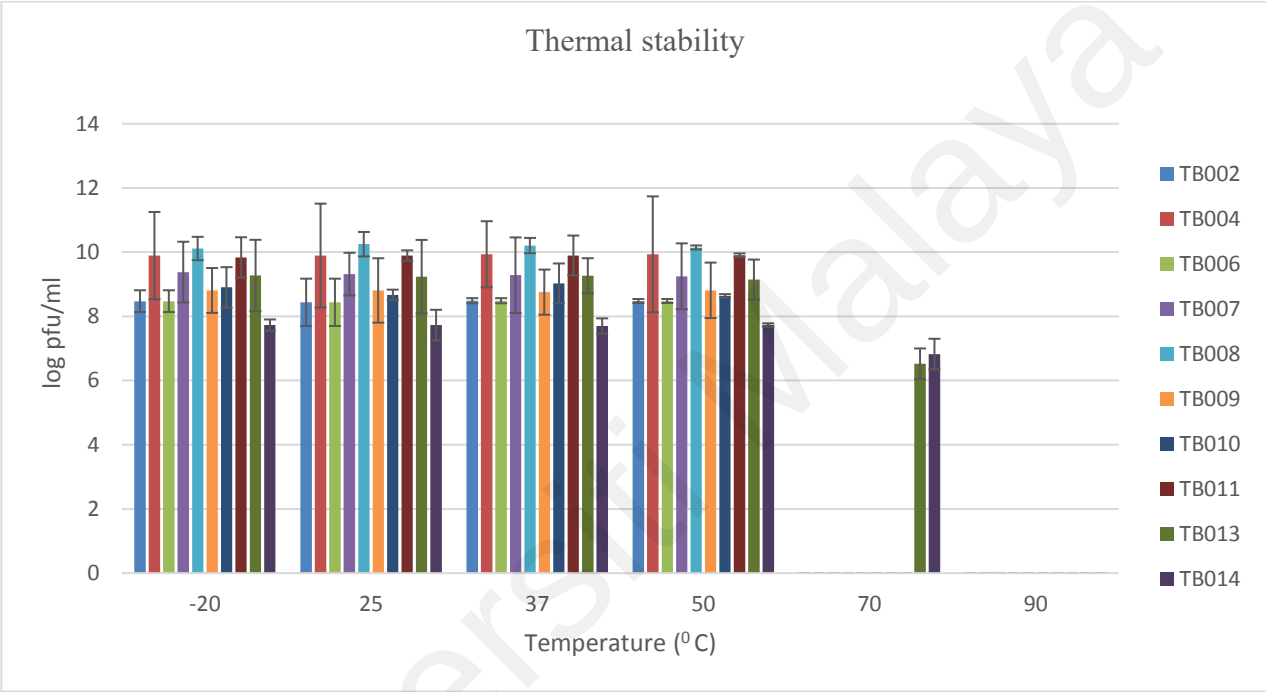

Fig 4.13: Comparison of thermal stability of 10 bacteriophages. All the phages showed normal growth after incubation at -20 °C, 25 °C, 37 °C and 50 °C temp while TB014 and TB013 showed minimum survival at 70 °C temp and others failed to survive at 70 °C temp.

4.14 Determination of optimum pH stability

All ten phages showed no growth at pH 1 and pH 3 in LB liquid media. Phages TB007 and TB014 showed no growth at pH 5 also (Table 4.9 C and APPENDIX I). But all phages showed normal growth in pH 7 and pH 9 and the growth decreased at pH 11 except TB013 which showed normal growth (Figure 4.14 and 4.15).

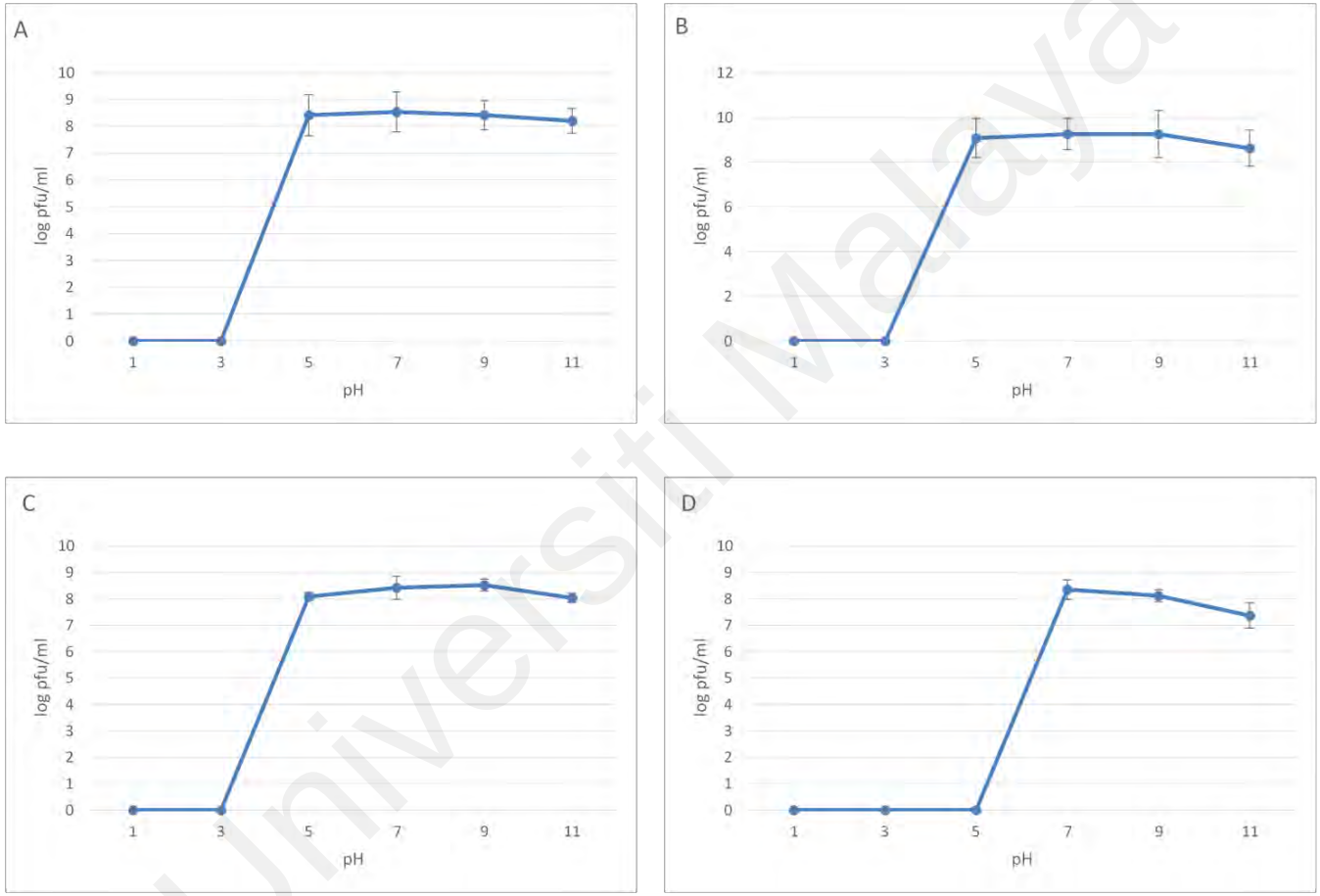

Figure 4.14: pH stability of 10 bacteriophages. A: TB002; B: TB004; C: TB006; D: TB007; E: TB008; F: TB009; G : TB010; H: TB011; I :TB013; J: TB014.

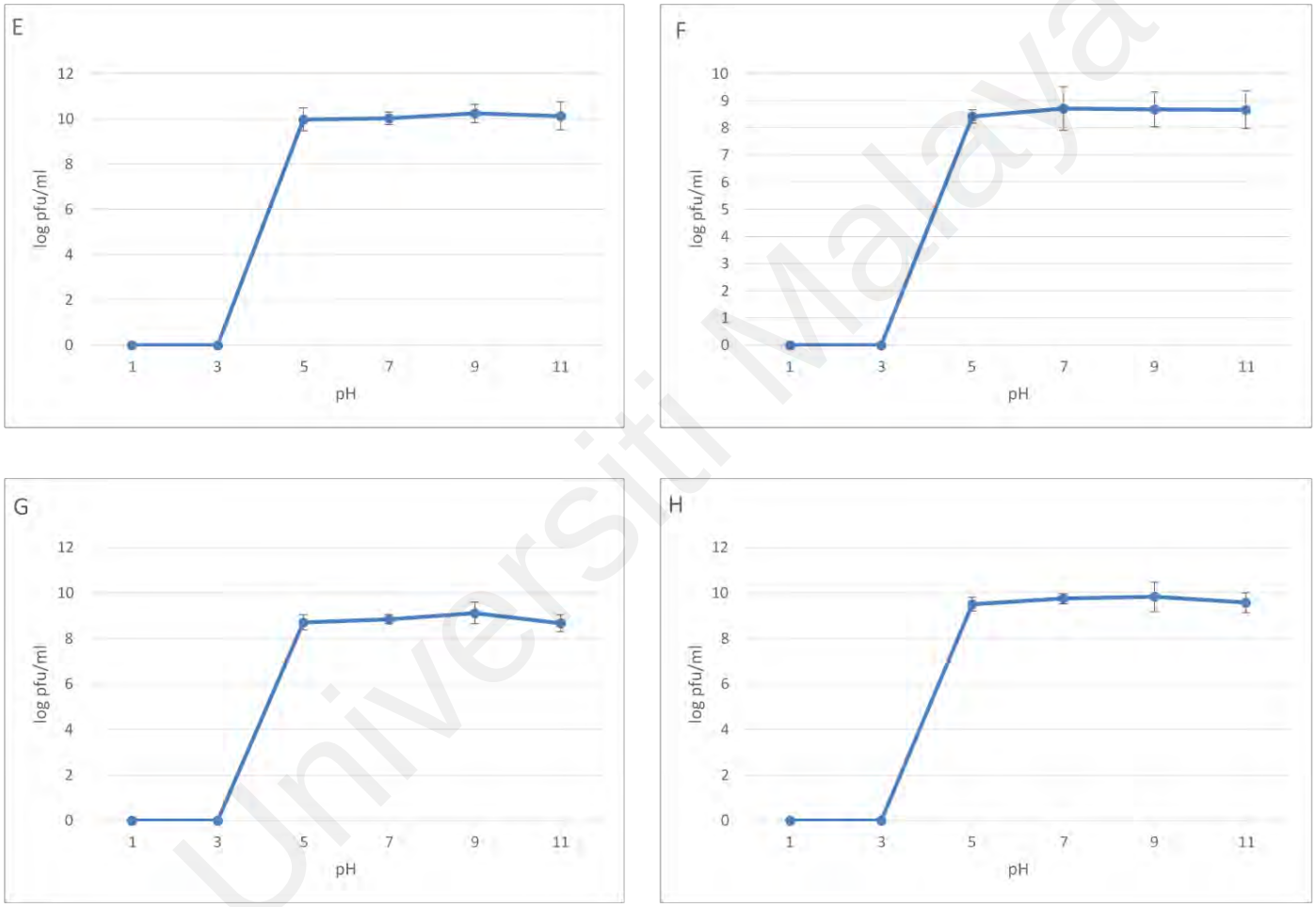

Figure 4.14, continued

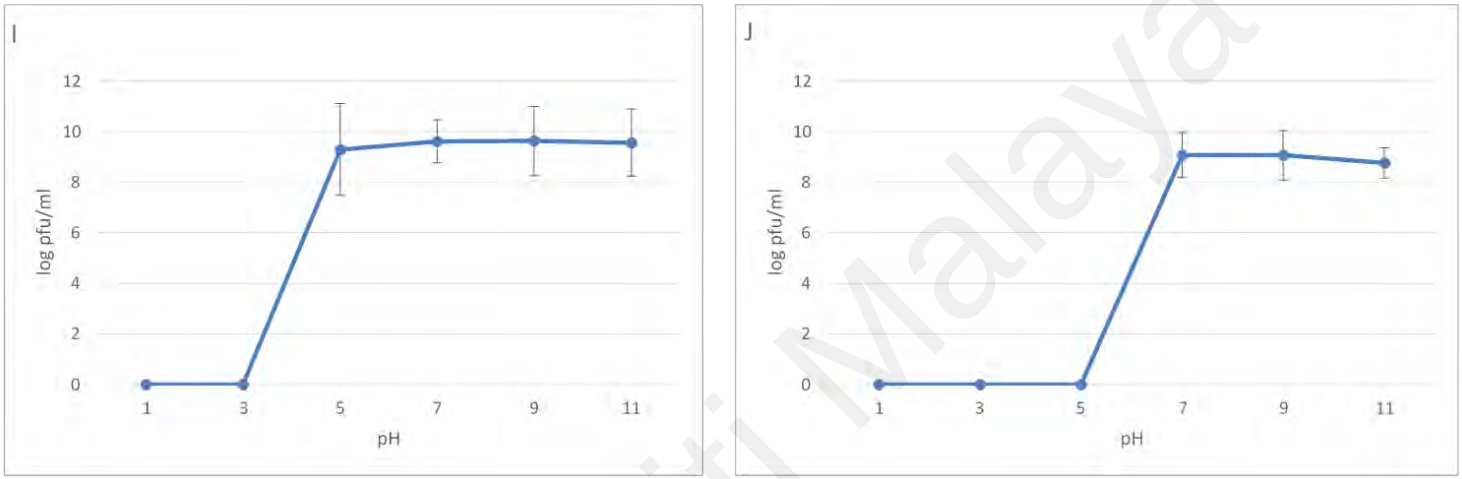

Figure 4.14, continued

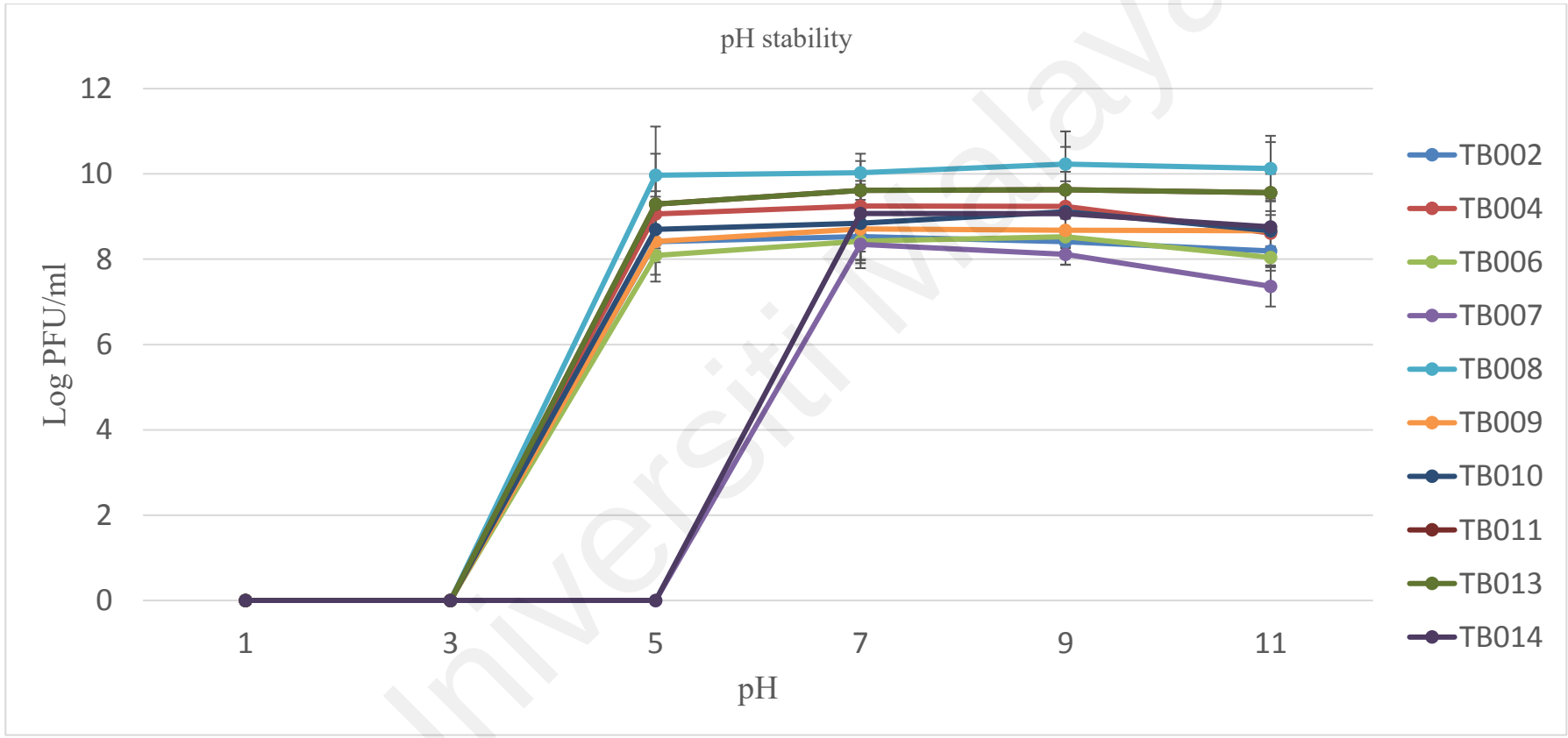

Fig 4.15: Comparison of pH stability of 10 bacteriophages**.** All the phages failed to survive at pH 1 and pH 3 while the phage TB007 and TB014 failed to survive at pH 5 also. But all the phages showed normal growth between pH 7 to pH 11

Table 4.9: Mean (Average value of 3 replications, raw data refers to Appendix G) log pfu/ml of 10 bacteriophages (A) one step growth curve (B) Thermal stability (C) pH stability

A

Time (min)	Titre of different phages (log pfu/ml)									
	TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014
10	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	6.736397	6.130334	0	7.752816	0	0
30	0	5.176091	7.321184	0	6.966142	6.832509	5.740363	7.813247	0	0
40	0	5.30103	7.523746	5.176091	7.113943	6.832509	6.176091	7.836324	4.69897	0
50	6.161368	6.176091	7.539076	5.30103	7.136721	6.991226	6.596597	7.900913	5.477121	5.30103
60	6.423246	6.190332	7.607455	5.477121	7.165838	7.098644	6.658011	7.907411	5.875061	5.39794
70	6.40654	6.332438	7.79379	5.653213	7.217484	7.115611	6.973128	7.90309	5.929419	5.929419
80	7.100371	6.819544	7.904174	5.69897	7.318063	7.213518	7.025306	7.905256	6.09691	6.544068
90	7.088136	6.977724	8.024486	6.267172	7.383815	7.257679	7.251638	8.030195	6.39794	7.123852
100	7.103804	6.989005	8.009663	6.64836	7.501744	7.342423	7.313867	7.984077	6.511883	7.503109

B

Tem(^{0}C)	Titre of different phages (log pfu/ml)									
	TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014
-20	8.472269	9.891352	8.472269	9.377792	10.11394	8.80618	8.90309	9.834633	9.272615	7.726999
25	8.436693	9.892466	8.436693	9.31597	10.24715	8.80618	8.669007	9.893947	9.236369	7.726999
37	8.486667	9.933993	8.486667	9.280275	10.20412	8.753328	9.028029	9.897627	9.264818	7.69897
50	8.477121	9.931458	8.477121	9.247973	10.14613	8.81068	8.636822	9.906694	9.147161	7.726999
70	0	0	0	0	0	0	0	0	6.522879	6.823909
90	0	0	0	0	0	0	0	0	0	0

C

pH	Titre of different phages (log pfu/ml)									
	TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014
1	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
5	8.409369	9.061955	8.09108	0	9.970037	8.414973	8.69897	9.50515	9.292994	0
7	8.531479	9.247155	8.420506	8.348954	10.02803	8.710399	8.845098	9.750765	9.615248	9.071882
9	8.409369	9.24221	8.5272	8.113943	10.23045	8.678215	9.113943	9.826075	9.62941	9.069421
11	8.194977	8.6163	8.041393	7.367977	10.12494	8.665894	8.669007	9.572097	9.564271	8.760925

4.15 Host range determination

Host range was determined for all ten phages against 49 *Shigella* strains through spot plating assay. TB004 and TB002 showed wider host range and were capable of lysing 49 and 48 strains respectively out of 49. These two phages were able to infect of all four species of *Shigella*. TB006, TB007, TB008, TB009, TB010, TB011, TB013 and TB014 lysed 32, 34, 33, 33, 35, 31, 30 and 32 strains respectively out of 49 where TB007 and TB010 infected two species of *Shigella* namely *Shigella flexneri* and *Shigella sonnei* and the rest of the phages infected the strains from *Shigella flexneri* only (Table 4.10 and APPENDIX F).

Table 4.10: Host range of 10 bacteriophages against 49 *Shigella* strains.

Lab code	Host	Name of Phage										No. of potential phages against single strain
		TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014	
SS1001	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2
SS1002	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2
SB1003	*Shigella boydii*	+	+	-	-	-	-	-	-	-	-	2
SD1007	*Shigella dysenteriae*	+	+	-	-	-	-	-	-	-	-	2
SF1008	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1011	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1013	*Shigella flexneri Y*	+	+	+	+	+	+	+	+	+	+	10
SF1014	*Shigella flexneri 1b*	+	+	+	+	+	+	+	+	+	+	10
SF1015	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1016	*Shigella flexneri 1c*	+	+	+	+	+	+	+	+	-	-	8
SF1017	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1018	*Shigella flexneri 6*	+	+	-	-	-	-	-	-	-	-	2

Table 4.10, continued

Lab code	Host	Name of Phage										No. of potential phages against single strain
		TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014	
SF1019	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1020	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1021	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1023	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1024	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1025	*Shigella flexneri 3a*	+	+	+	-	+	+	+	+	+	+	9
SF1027	*Shigella flexneri 6*	+	+	-	-	-	-	-	-	-	-	2
SF1028	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1029	*Shigella flexneri 6*	+	+	-	-	-	-	-	-	-	-	2
SF1031	*Shigella flexneri 6*	+	+	-	-	-	-	-	-	-	-	2
SF1032	*Shigella flexneri 4a*	+	+	+	+	+	+	+	+	+	+	10
SF1033	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1034	*Shigella flexneri 2a*	+	+	-	+	+	+	+	+	+	+	9

Table 4.10, continued

Lab code	Host	Name of Phage										No. of potential phages against single strain
		TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014	
SF1035	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SB1036	*Shigella boydii*	-	+	-	-	-	-	-	-	-	-	1
SF1041	*Shigella flexneri 1b*	+	+	+	+	+	+	+	+	+	+	10
SF1042	*Shigella flexneri 1c*	+	+	+	+	+	+	+	-	-	+	8
SF1043	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SF1044	*Shigella flexneri 1c*	+	+	+	+	+	+	+	-	-	+	8
SF1045	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SB1046	*Shigella boydii*	+	+	-	-	-	-	-	-	-	-	8
SF1047	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SS1048	*Shigella sonnei*	+	+	-	+	-	-	+	-	-	-	4
SF1049	*Shigella flexneri 2a*	+	+	+	+	+	+	+	+	+	+	10
SS1050	*Shigella sonnei*	+	+	-	+	-	-	+	-	-	-	4
SS1051	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2

Table 4.10, continued

Lab code	Host	Name of Phage										No. of potential phages against single strain
		TB002	TB004	TB006	TB007	TB008	TB009	TB010	TB011	TB013	TB014	
SF1052	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SF1053	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SF1054	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SF1055	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SS1056	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2
SF1057	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SF1058	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SF1059	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SS1060	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2
SF1061	*Shigella flexneri* 2a	+	+	+	+	+	+	+	+	+	+	10
SS1062	*Shigella sonnei*	+	+	-	-	-	-	-	-	-	-	2

4.16 Adsorption rate

On the basis of wider host range and cell lysis capacity phage TB004 was selected for further biological test and sequencing. The adsorption rate of phage TB004 by the host bacteria was observed and the rate of adsorption was 95.51 % within the 2 min of infection at 37 ºC (Figure 4.16**).** The adsorption rate constant describes the likelihood of a single phage adsorbing to a single bacterium within some unit volume over some unit of time.The adsorption rate gradually increased to 99.36% at 10 min and at 25 min it increased to 99.94%.

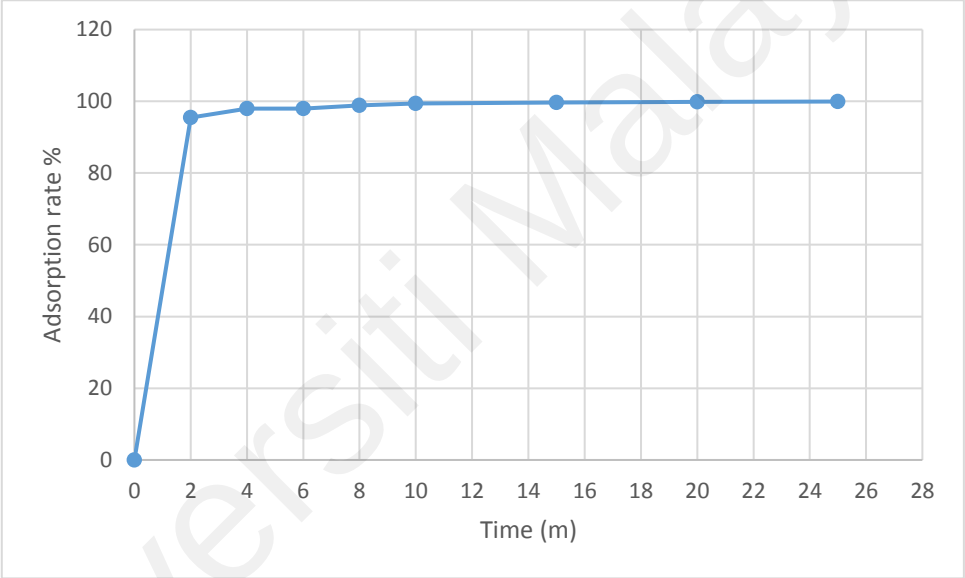

Figure 4.16: Adsorption ability of phage TB004. The adsorption rate reached 95.51 % within the 2 min of infection and it gradually increased to 99.36% after 10 min and to 99.94% after 25 min.

4.17 Multiplicity of infection

To determine the multiplicity of infection the phage lysate was added at different MOIs (0.01, 0.1, 1, 10 and 100) to log phase host bacteria. Significant reduction of host bacterial cells and increase of bacteriophage cells were observed. The infection of host bacteria by TB004 at all tested MOIs led to a decrease in bacteria titre to a similar level as expected (from ~10.20 to ~8 log cfu/ml) during the experiment (Figure 4.17 A). Actually, the efficiency of reduction was dependent on the MOI. At an MOI of 10 the decrease in

bacteria titre was higher at 40 min of post-infection but at the end of the experiment at 120 min the decrease in bacteria titre was higher at MOI 0.1. On the other hand the phage titre increased from 6.5 to 10.5 log pfu/ml at the same MOIs as recorded throughout the experiment (Figure 4.17 B). At MOIs of 0.01 and 0.1 the growth of phage was higher at the end of the experiment and it was 3.77 pfu/ml and 2.80 pfu/ml respectively.

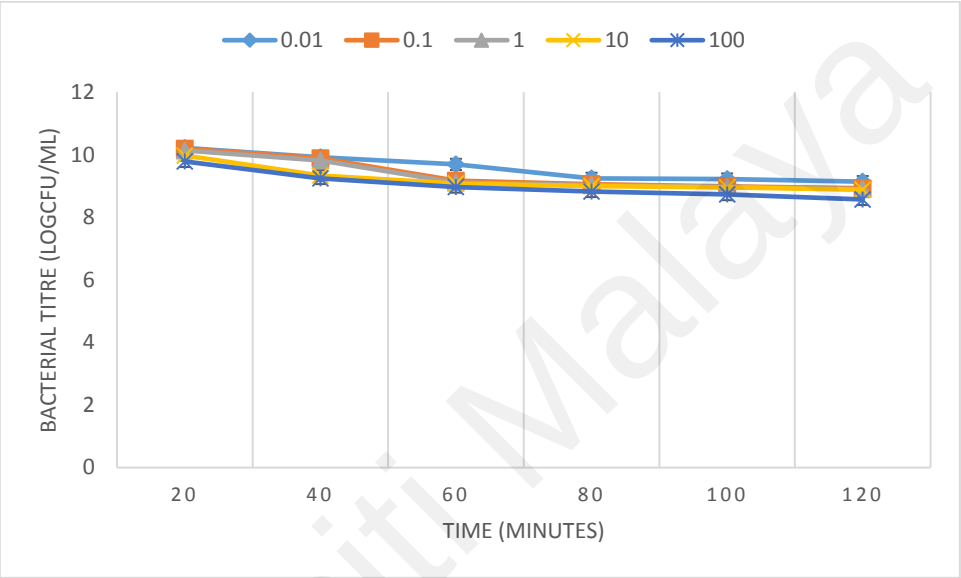

Figure 4.17: Populations of (A) *Shigella flexneri* [log cfu/ml] and (B) Phage TB004 [log pfu/ml] at different MOIs. Values represent the mean of triplicate evaluation.

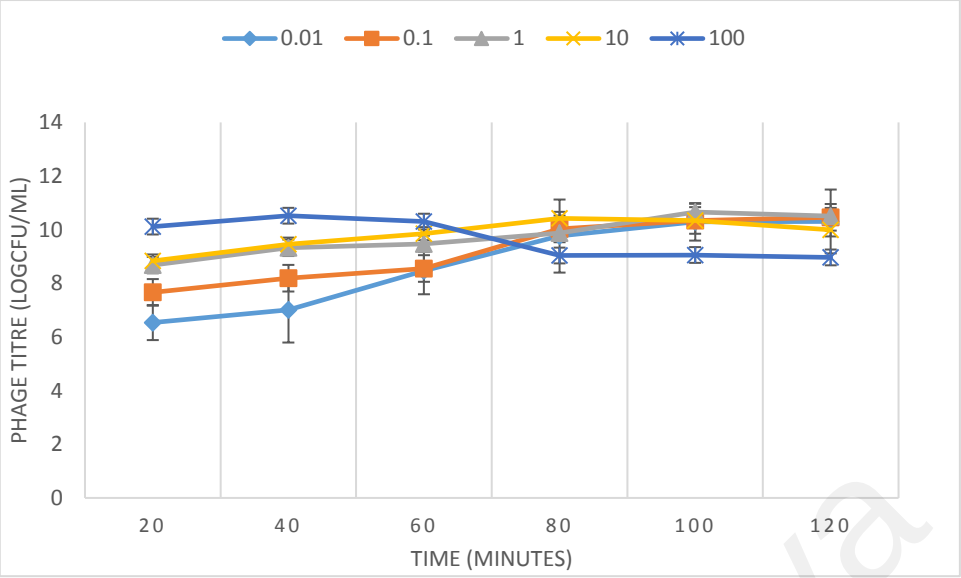

Figure 4.17, continued

4.18 Sequence and assembly of TB004 phage genome

As described earlier phage TB004 was selected for sequencing on basis of its wider host range and cell lysis capacity. Furthermore, this phage was able to infect of all four species of *Shigella* genus. Among the 16 scaffolds (>= 1000 bp), the two longest scaffolds (126081 bp and 43907 bp, node 1 and node 2 respectively) had the coverage value of 3708 and 3745 respectively (APPENDIX J). The third longest scaffold (42079 bp) only had the coverage value of 14, and the rest were all shorter than 6 kbp with the coverage less than 10. Based on the sequence coverage, it was therefore hypothesized that the two longest contigs are the part of a single genome. These two longest contigs had pairwise similarity >95% to genomes of *Shigella* phage Shfl2 (HM0350250), *Escherichia* phage slur07 (LN881732) and other phages (mainly *Shigella* and *Enterobacteria* phages). Based on the sequence coverage results and mapping against closely related phage genomes [*Shigella* phage Shfl2 (HM0350250), *Escherichia* phage slur07 (LN881732)], it was therefore concluded that scaffold NODE 1 and NODE 2 are the part of a single genome. The genome of this phage is composed of 169,988 bp (Appendix K) of double-stranded DNA with G+C content of 35.46%.

4.19 Gene component annotation

Two hundred and seventy three genes were predicted by GeneMarkS for TB004 bacteriophage. The size of smaller gene (ORF-248) was 104 bp and the size of longest gene (ORF-128) was 3873 bp. The coding region of this phage genome was 160,554 bp which was about 94.45% of the whole genome. The highest number of genes lies between 200 to 300 bp and the number of genes in this category was 64 (Figure 4.18). Besides these, 5 repeat sequences (APPENDIX N) were predicted through RepeatMasker and analyzed through TRF (Tandem repeat finder). All the repeat sequences were in minisatellite category and the length of the longest and shortest repeats were 60 bp and 24 bp respectively. The prediction of tRNA, rRNA and small nuclear RNAs were done through tRNAscan-SE, rRNAmmer and Rfam respectively while 10 tRNA (APPENDIX M) were predicted and no rRNA or small nuclear RNAs were found. Total length of tRNA was 788 bp while the longest and shortest tRNA were 90 bp and 73 bp respectively.

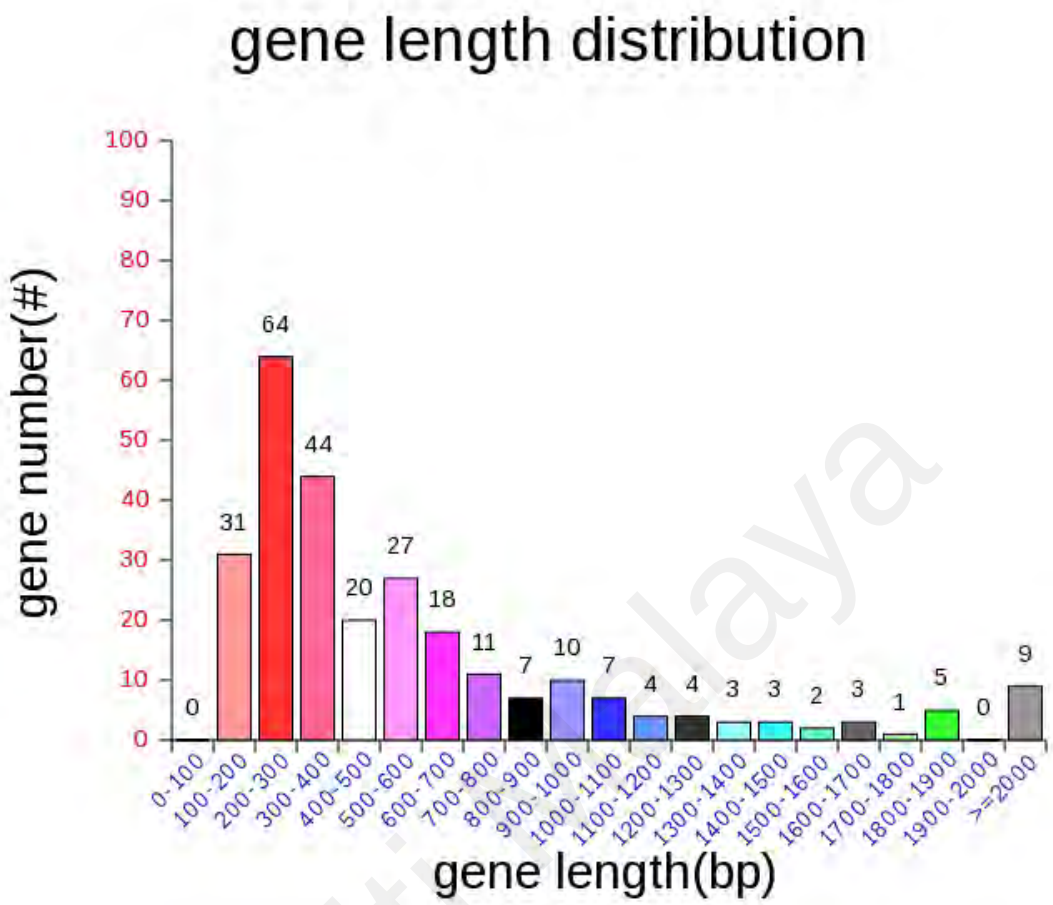

Fig 4.18: Distribution of genes based on their length

4.20 Gene function annotation

Function of genes of TB004 phage genome were predicted through GO (Gene ontology) annotation and Swiss-Prot annotation.

4.20.1 GO (Gene ontology) annotation

Functions of 93 genes out of 273 were predicted through GO annotation and it was observed that different single genes might be responsible for multiple functions. The functional annotation of gene products generally covers three major domains namely (i) biological process (ii) cellular component and (iii) molecular function. This general GO annotation was not specific for phage genome. Hence, the annotated result was further curated based on phage specific genes e.g. early genes, middle genes and late genes and a total of 57 specific genes was found with GO identity (Table 11). In this case, 31 genes

were categorized as early genes associated with DNA replication, 7 were categorized as middle genes associated with metabolisms and 19 were categorized as late genes associated with structure formation. For early genes, 4 genes were found with GO identity associated with endonuclease activity followed by 3 genes for each group were associated with polymerase, kinase and helicase activity while 2 genes for each group were responsible for exonuclease, ligase, topoisomerase, anaerobic ribonucleoside-triphosphate reductase and ribonucleoside-diphosphate reductase activity. Single gene for each function was found with GO identity for DNA binding, ribonuclease, methylase, endolysis, sliding-clamp-loader, dihydroflotate reductase and glutaredoxin. For middle genes, single gene for each function was found for thymidylate synthase, deoxycytidylate deaminase, NAD-protein ADP-ribosyltransferase, autonomous glycyl radical cofactor, protein rIIB , internal protein II, protein inh activity. For genes, 6 genes were found with GO identity associated with baseplate, followed by 4 genes were associated with head, 3 genes in each group for capsid and tail, 2 genes for terminal and one gene for neck formation (Figure 4.19).

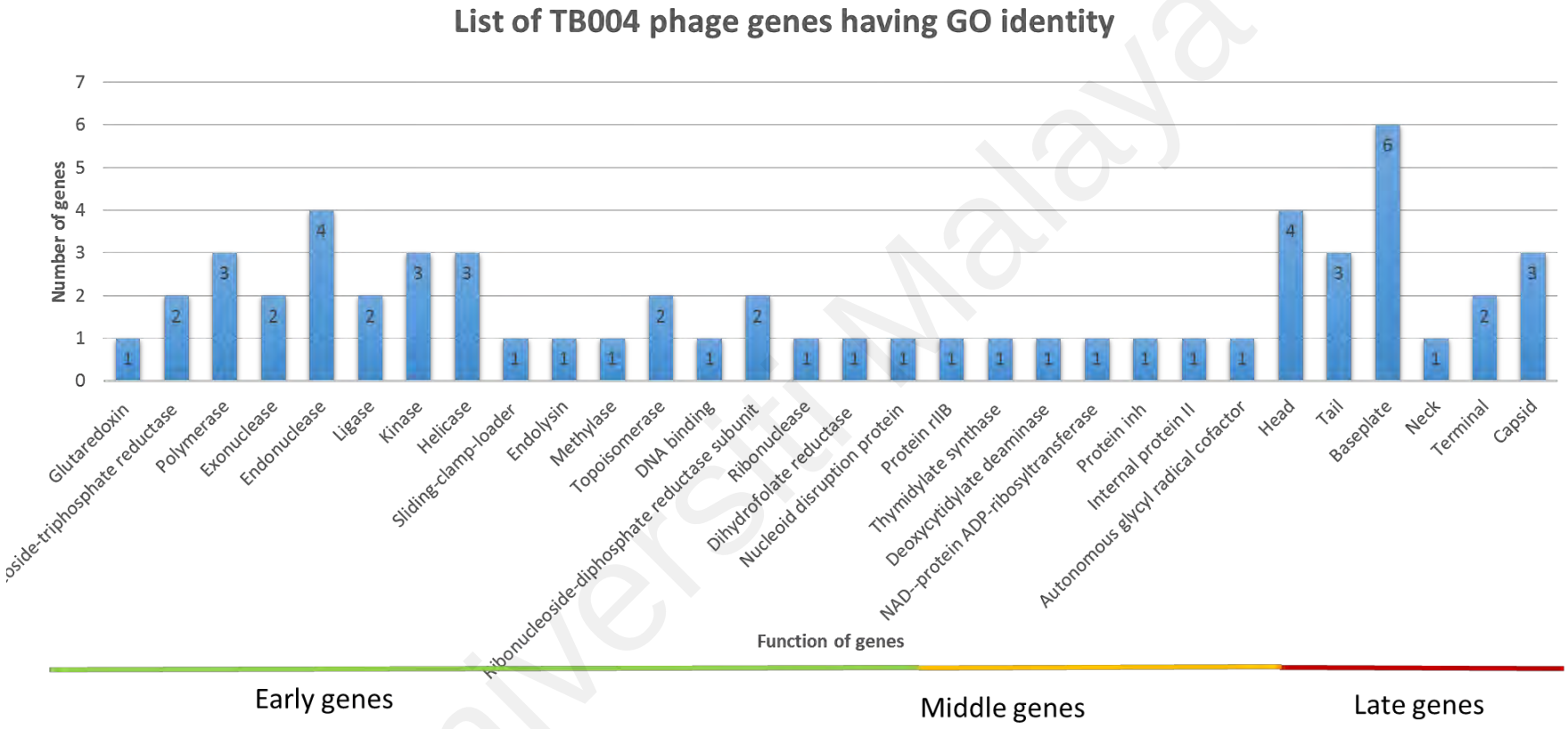

Figure 4.19: Distribution of gene functions, gene numbers and their GO identity of phage TB004

Table 4.11: Features of TB004 phage genome with GO identity

Gene ID	Gene name	GO id	Annotated function	Category of phage genes
ORF22	*NRDC*	GO:0005515; GO:0009055;GO:0015035;	Glutaredoxin	Early gene
ORF28	*nrdD*	GO:0008152	Anaerobic ribonucleoside-triphosphate reductase	early
ORF30	*NRDG*	GO:0003824	Anaerobic ribonucleoside-triphosphate reductase-activating protein	early
ORF42	*gp55*	GO:0003700; GO:0016987;GO:0005634; GO:0003677; GO:0006355; GO:0006352	RNA polymerase sigma factor	early
ORF47	*mobB*	GO:0003677	Probable mobile endonuclease	early
ORF49	*gp47*	GO:0016787	Exonuclease subunit 1	early
ORF52	*gp46*	GO:C20:R200016021; GO:0030674; GO:0005524; GO:0005577; GO:0005102; GO:0000166; GO:0008608; GO:0006281GO:0005876; GO:0007049;GO:0051301; GO:0004518;GO:000367;GO:0007165;GO:0042729;GO:0051258; GO:0016887; GO:0008270; GO:0030168; GO:0007059	Exonuclease subunit 2	early
ORF55	*gp45*	GO:0006260	DNA polymerase clamp	early
ORF56	*gp44*	GO:0006310; GO:0016301; GO:0005681; GO:0009378;GO:0006281; GO:0003724; GO:0007067;	Sliding-clamp-loader	early
ORF59	*gp43*	GO:0055114; GO:0004345;GO:0000166;	DNA-directed DNA polymerase	early
ORF67	*gp41*	GO:0005524; GO:0003678; GO:0006260	ATP-dependent helicase	early

Table 4.11, continued

Gene ID	Gene name	GO id	Annotated function	Category of phage genes
ORF76	*DAM*	GO:0005524;GO:0005634; GO:0003676;GO:0009007;	DNA adenine methylase	early
ORF90	*dda*	GO:0016851;GO:0016787; GO:0006310; GO:0098519; GO:0043531;GO:0008134; GO:0015979;GO:0008026; GO:0003677;GO:0003676; GO:000412GO:001688;GO	ATP-dependent DNA helicase	early
ORF101	*Gp39*	GO:0005524; GO:0003918; GO:0003677; GO:0006265	DNA topoisomerase large subunit	early
ORF111	*ndd*	GO:0009058;GO:0005794; GO:0016021;GO:0001733	Nucleoid disruption protein	early
ORF114	*Gp52*	GO:0005524;GO:0003918; GO:0003677: GO:0006265	DNA topoisomerase	early
ORF130	*rnh*	GO:0003824; GO:000367;GO:0008409	Ribonuclease H	early
ORF134	*Gp32*	GO:0003697;GO:0006950; GO:0009415	Single-stranded DNA-binding protein	early
ORF140	*frd*	GO:0006545;GO:0009165; GO:0004146;GO:0055114	Dihydrofolate reductase	early
ORF143	*ITEVIR*	GO:0003677	Intron-associated endonuclease 1	early
ORF146	*ITEVI*	GO:0003677	Intron-associated endonuclease 1	early
ORF147	*NRDA*	GO:000626;GO:005511;GO:0005971;GO:000474;GO:0005524	Ribonucleoside-diphosphate reductase subunit alpha	early
ORF148	*NRDB*	GO:005511;GO:0009186	Ribonucleoside-diphosphate reductase subunit beta	early
ORF149	*denA*	GO:001507;GO:0015986;GO:0000276	Endonuclease II	early
ORF156	*pseT*	GO:0003993;GO:0019001; GO:0031683;GO:0005524; GO:0016301;GO:0004871; GO:0007186;GO:0016740; GO:0003924	Polynucleotide kinase	early
ORF177	*Gp30*	GO:0005524;GO:0006281; GO:0006310;GO:0003910	DNA ligase	early

Table 4.11, continued

Gene ID	Gene name	GO id	Annotated function	Category of phage genes
ORF195	*uvsW*	GO:0016787;GO:0006139; GO:0003676;GO:0004386; GO:0005524;GO:0003677; GO:0016818;GO:0008026	ATP-dependent DNA helicase	early
ORF231	*gp1*	GO:0004631; GO:0006695; GO:0005737	Deoxynucleotide monophosphate kinase	early
ORF251	*E*	GO:0003796; GO:0009253; GO:0016998	Endolysin	early
ORF264	*vs*	GO:0019089	Valyl--tRNA ligase modifier	early
ORF270	*TK*	GO:0000160; GO:0005524; GO:0004673; GO:0004797; GO:0000155; GO:0016020	Thymidine kinase	early
ORF104	*rIIB*	GO:001698;GO:0005975;GO:0006352;GO:0016773;GO:0006310;GO:0003700;GO:0005524GO:0000150;GO:0003677;GO:0006355	Protein rIIB	middle
ORF142	*TD*	GO:0006231;GO:0004799	Thymidylate synthase	middle
ORF163	*CD*	GO:0008270;GO:0016814;	Deoxycytidylate	middle
ORF179	*alt*	GO:0009405;GO:0005576	NAD--protein ADP-	middle
ORF197	*inh*	GO:0098519; GO:0031412	Protein inh	middle
ORF253	*ipi2*	GO:0015948;GO:0008168	Internal protein II	middle
ORF258	*grcA*	GO:0003824; GO:0008152	Autonomous glycyl	middle
ORF66	*gp40*	GO:0005737; GO:0042325	Head formation protein	late
ORF166	*Gp31*	GO:0006457;GO:0005737	Capsid assembly protein	late
ORF183	*gp54*	GO:0005198	Baseplate tail-tube junction protein	late
ORF185	*g29*	GO:0004826;GO:0000166; GO:0005737;GO:0005525 GO:0005524;GO:0006432	Tape measure protein	late
ORF188	*gp51*	GO:0008270;GO:0006351; GO:0003676	Baseplate hub assembly protein	late
ORF198	*hoc*	GO:0016021;GO:0007155	highly immunogenic outer capsid protein	late

Table 4.11, continued

Gene ID	Gene name	GO id	Annotated function	Category of phage genes
ORF204	*gp22*	GO:0015078;GO:0006457; GO:0016491;GO:0007165; GO:0005096;GO:0045263; GO:0005634;GO:0004674; GO:0015986;GO:0006352; GO:0055114; GO:0006468;	Capsid assembly scaffolding protein	late
ORF206	*gp68*	GO:0003714;GO:0006351	Prohead assembly protein	late
ORF207	*gp67*	GO:0005681;GO:0005634; GO:0003677; GO:0032040; GO:0006270; GO:0006351GO:0000398; GO:0003887; GO:0008270; GO:0019013	Prehead core component	late
ORF209	*gp19*	GO:0005198	Tail tube protein	late
ORF216	*wac*	GO:0005198; GO:0005102; GO:0030168; GO:0019028; GO:0019031;GO:0007165; GO:0030674; GO:0051258; GO:0007155;GO:0005577	Fibritin wac	late
ORF219	*gp10*	GO:0019058	Baseplate wedge protein	late
ORF220	*gp9*	GO:0019058	Baseplate protein	late
ORF222	*gp7*	GO:0005515; GO:0006355	Baseplate wedge protein	late
ORF226	*gp5*	GO:0016998; GO:0009253; GO:0003796	Baseplate central spike complex protein	late
ORF228	*gp50*	GO:0009036; GO:0003677; GO:0009307	Head completion protein	late
ORF229	*gp2*	GO:0005524;GO:0005634; GO:0042157; GO:0005576; GO:0006351; GO:0006355; GO:0003677	Terminal DNA protecting protein	late
ORF230	*gp3*	GO:0005198	Tail tube terminator protein	late
ORF232	*gp57*	GO:0003677	Tail fiber assembly helper protein	late

4.20.2 Swiss-Prot annotation

The functions of 235 gene products were predicted out of 273 through Swiss-Prot annotation (APPENDIX L). Among the predicted proteins, 126 have their specific functions and 109 are uncharacterized hypothetical protein. Of all annotated gene products, 223 showed closest hit with *Enterobacteria* T4 phage proteins, followed by *Enterobacteria* T2 phage proteins (n= 3), *Enterobacteria* K3 phage proteins (n=3), *Enterobacteria* T6 phage proteins (n=2), *Enterobacteria* LZ3 phage protein (n= 1), *Enterobacteria* LZ5 phage protein (n=1), *Enterobacteria* RB18 phage protein (n=1) and *Enterobacteria* AR1 phage protein (n=1). One hundred seventy five of them showed > 95% identity, 29 of them showed >90% identity, 16 of them showed >80 % identity and rest of them showed <80% identity with the closest hit phage proteins. Different gene products associated with DNA and RNA manipulation, packaging, structural protein as well as lysis protein were predicted. In case of DNA and RNA metabolism, RNA polymerase (*gp55* encoded by ORF-42, identity 100%) RNA polymerase binding protein (*rpbA* encoded by ORF-54, identity 99.2%), DNA polymerase clamp (*gp45* encoded by ORF-55, 97.8% identity) DNA polymerase (*gp43* encoded by ORF-59, 99.3% identity), RNA polymerase associated protein (*gp33* encoded by ORF-132, 100% identity), DNA primase (*gp58-61* encoded by ORF-73, 98.2% identity), exonuclease subunit 1(*gp47* encoded by ORF-49, 98.8% identity), exonuclease subunit 2 (*gp46* encoded by ORF-52, 99.1% identity), endonuclease IV (*denB* encoded by ORF-106, identity 97.8%), recombination endonuclease (*gp49* encoded by ORF-27, 99.4% identity),DNA ligase (*gp30* encoded by ORF-177, 98.8% identity), RNA ligase (*Y10A* encoded by ORF-101, 98.2% identity), DNA topoisomerase (*gp52* encoded by ORF-114, 99.3% identity) and DNA binding protein (*gp32* encoded by ORF-135, 97.7% identity) were predicted. With respect to lysis protein, holin (*T*, encoded by ORF-124 99.1% identity), endolysin (*E* encoded by ORF-251, 97% identity) were predicted. Protein involved in structure and

assembly, head formation protein (*gp40* encoded by ORF-66, 100% identity), long tail fibre proximal subunit (*gp34* encoded by ORF-129, 96.4% identity), long tail fibre protein (*gp35* encoded by ORF-128, 97.8% identity), tail fibre protein (*gp36* encoded by ORF-127, 98.2% identity), baseplate wedge protein (*gp25* encoded by ORF-190, 99.2% identity), baseplate hub assembly protein (*gp26* encoded by ORF-189, 97.6% identity), baseplate central spike complex protein (*gp27* encoded by ORF-187, 99.5% identity), baseplate tail-tube junction protein (*gp48* encoded by ORF-184, 98.6% identity), capsid vertex protein (*gp24* encoded by ORF-202, 99.5% identity), major capsid protein (*gp23* encoded by ORF-203, 98.1% identity), capsid assembly scaffolding protein (*gp22* encoded by ORF-204, 98.5% identity), prehead core protein (*gp67* encoded by ORF-207, 90% identity), tail tube protein (*gp19* encoded by ORF-209, 98.8% identity), tail sheath protein (*gp18* encoded by ORF-210, 98.2% identity), neck protein (*gp14* encoded by ORF-214 encoded by 99.6% identity), baseplate protein (*gp9*, ORF-220 encoded by 99.7% identity) baseplate wedge protein(*gp10* encoded by ORF-219, 99% identity), head completion protein (*gp50* encoded by ORF-228, 98% identity) were predicted. In case of packaging protein, terminase large subunit (*gp17* encoded by ORF-211, 98.9% identity), terminase small subunit (*gp16* encoded by ORF-212, 100% identity), prohead assembly protein (*gp6* encoded by ORF-206, 100% identity), portal protein (*gp20* encoded by ORF-208, 99.6% identity) prohead protease (*gp21* encoded by ORF-205, 99.5% identity) were predicted.

4.21. Construction of genetic map

From the genetic map it was observed that all the essential genes and core genes together with additional genes related to T4 series phages were aligned in the map of TB004 bacteriophage genome. The core genes for DNA replication, repair, and recombination *gp43* encoded by ORF-59, *gp44* encoded by ORF-56, *gp45* encoded by ORF-55, *gp62* encoded by ORF-42, *gp41* encoded by ORF-67, *gp59* encoded by ORF-133 were aligned accordingly. The core genes for gene expression *gp33* encoded by ORF-132, *gp55* encoded by ORF-42, *regA* encoded by ORF-58 while packaging genes *gp16* encoded by ORF-121, *gp17* encoded by ORF-211, *gp20* encoded by ORF-208 were present in the genetic map. Structural genes of bacteriophage *gp3* encoded by ORF-230, *gp5* encoded by ORF-226, *gp6* encoded by ORF-223, *gp8* encoded by ORF-221, *gp13* encoded by ORF-215 , *gp14* encoded by ORF-214, *gp15* encoded by ORF-213 , *gp18* encoded by ORF-210, *gp19* encoded by ORF-209, *gp21* encoded by ORF-205, *gp22* encoded by ORF-204, *gp23* encoded by ORF-203, *gp24* encoded by ORF-202 ,*gp25* encoded by ORF-190, *gp26* encoded by ORF-189, *gp34* encoded by ORF-129, *gp35* encoded by ORF-128, *gp36* encoded by ORF-127, *gp37* encoded by ORF-126, *gp53* encoded by ORF-227 together with other genes for auxiliary metabolism were also aligned in the genetic map related to T4 series bacteriophage. Function and alignment of the genes of phage TB004 have shown in Figure no. 4.20

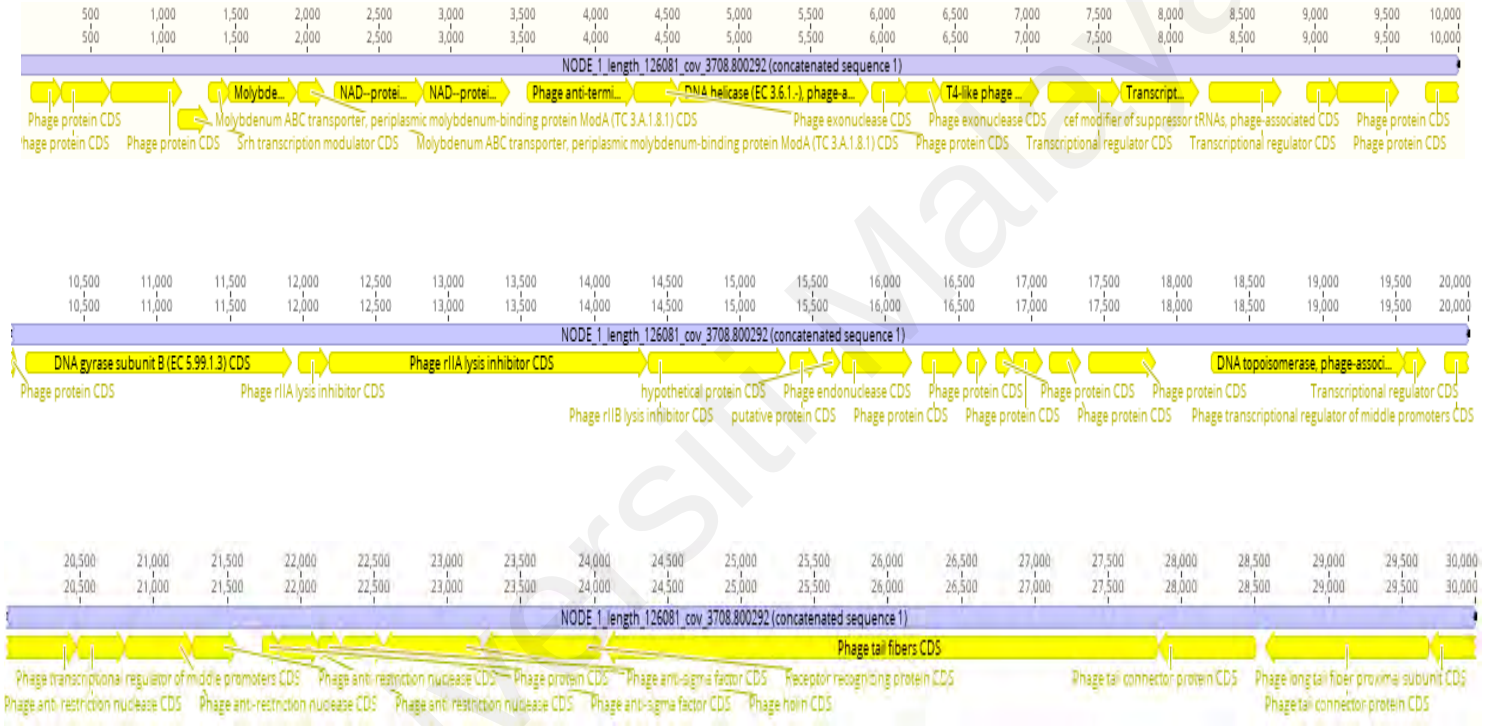

Figure 4.20: Linear map of the TB004 bacteriophage genome. 270 CDS (genes) and transcription direction are indicated as arrows. Nucleotides number and gene names are indicated above and below the arrows, respectively.

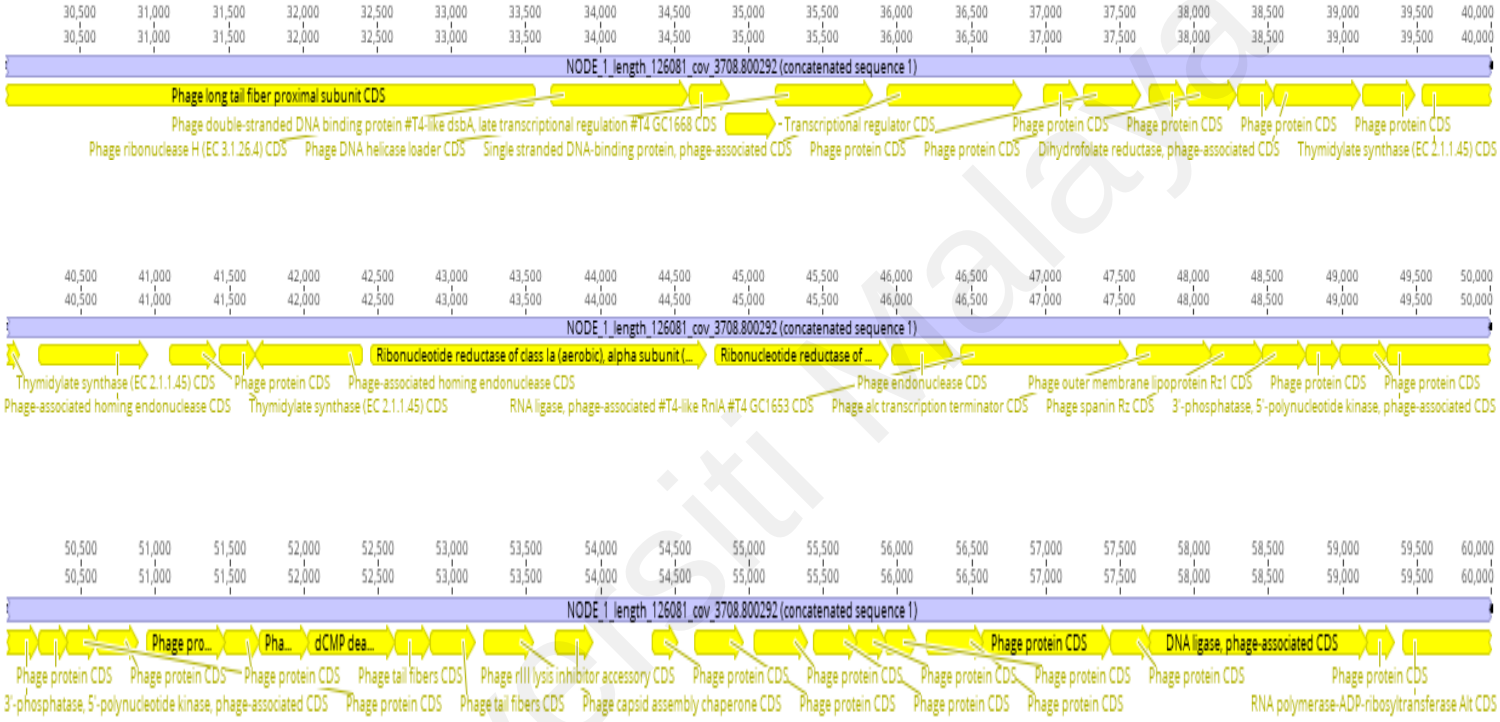

Figure 4.20, continued

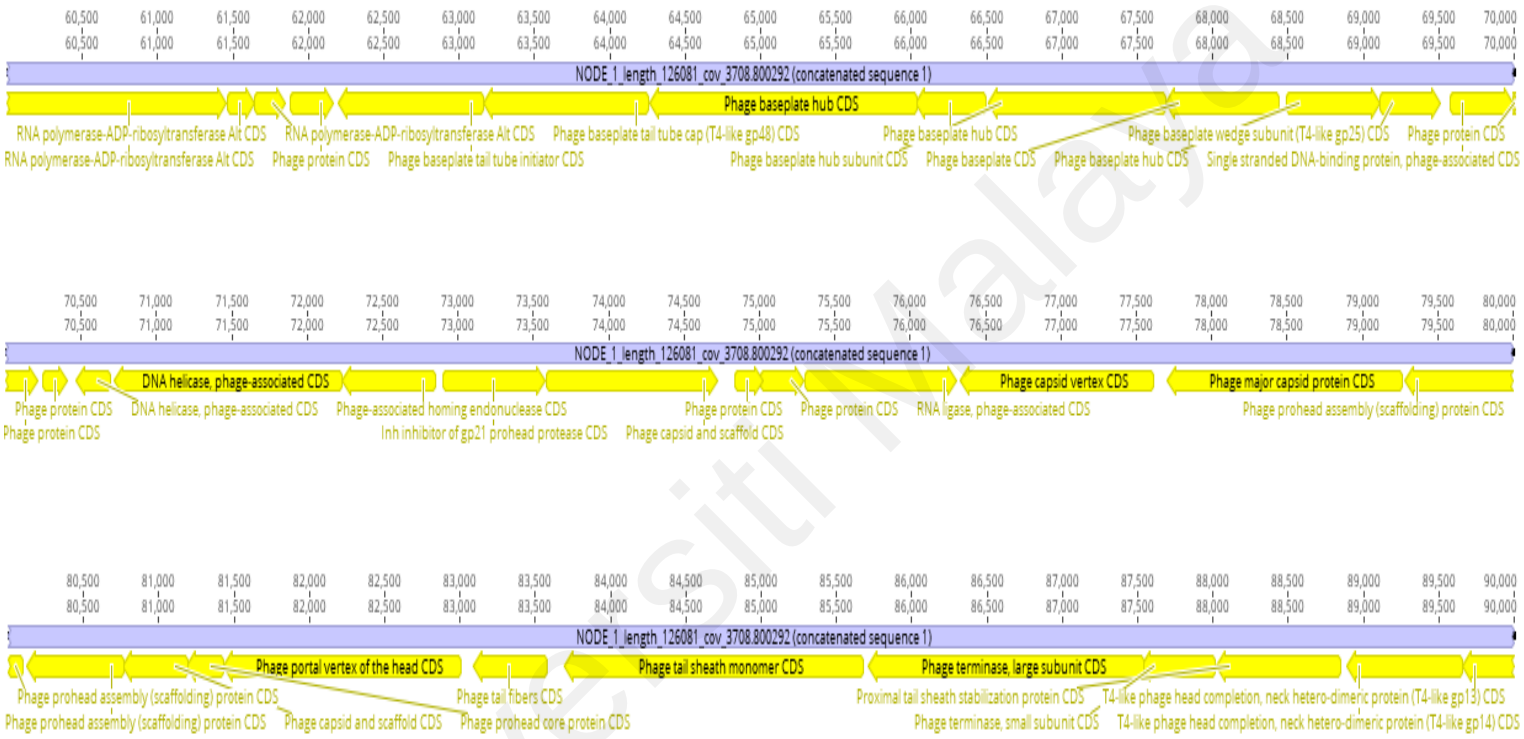

Figure 4.20, continued

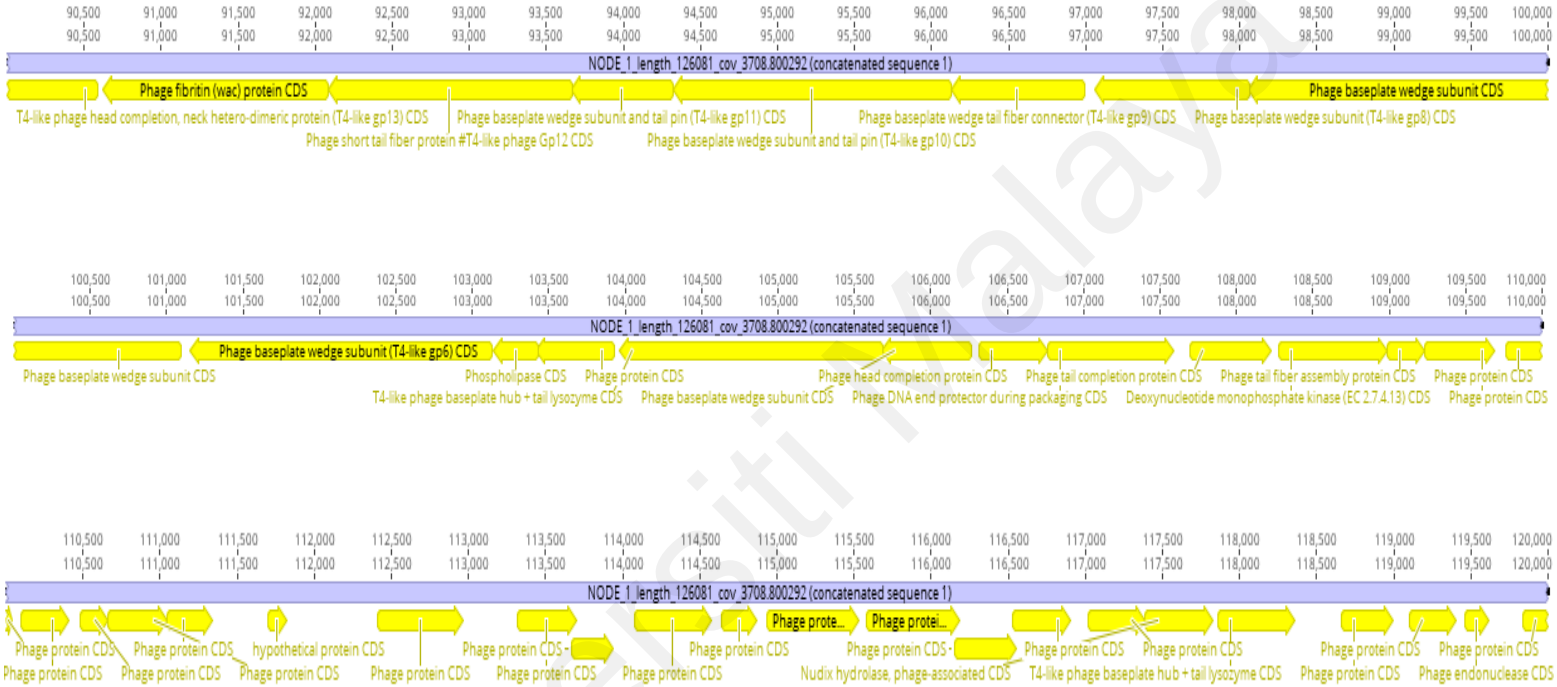

Figure 4.20, continued

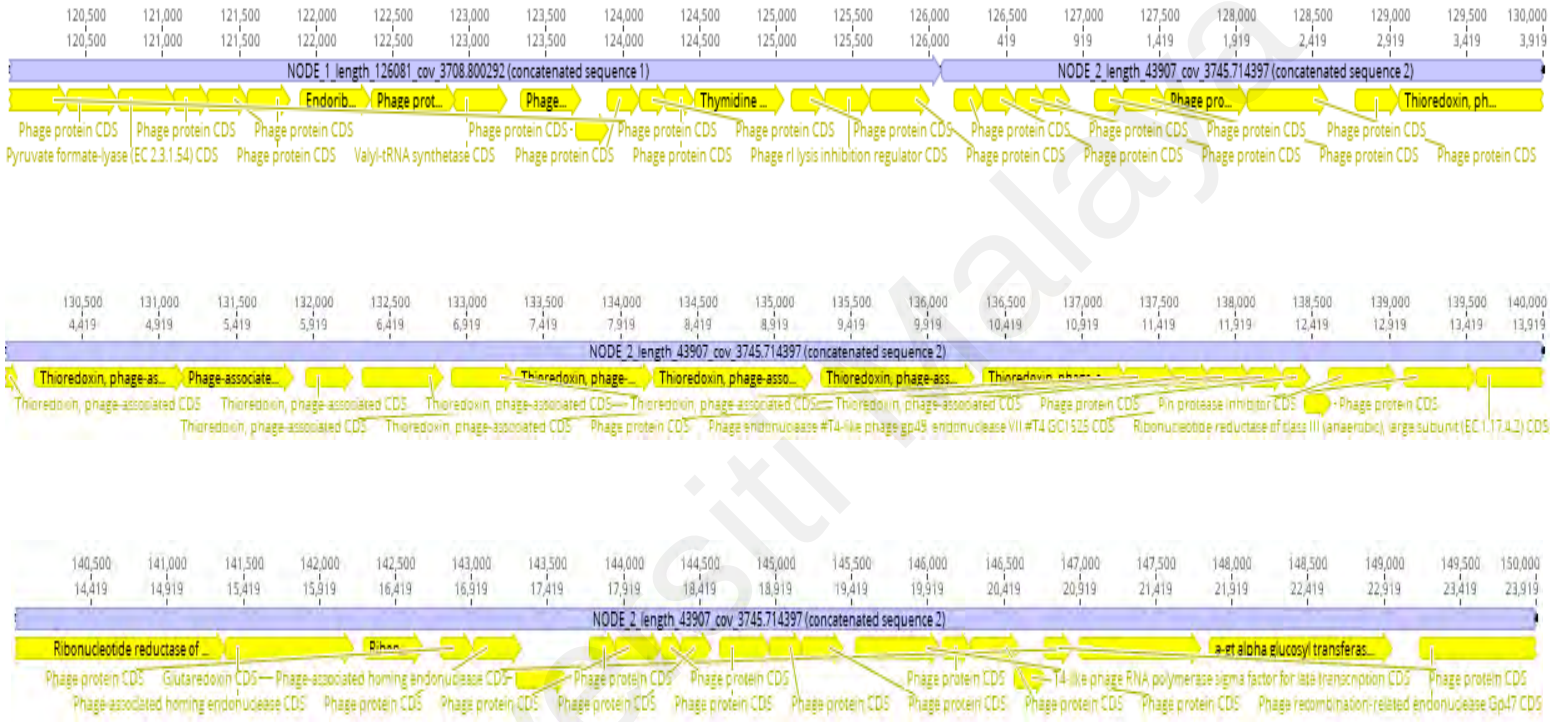

Figure 4.20, continued

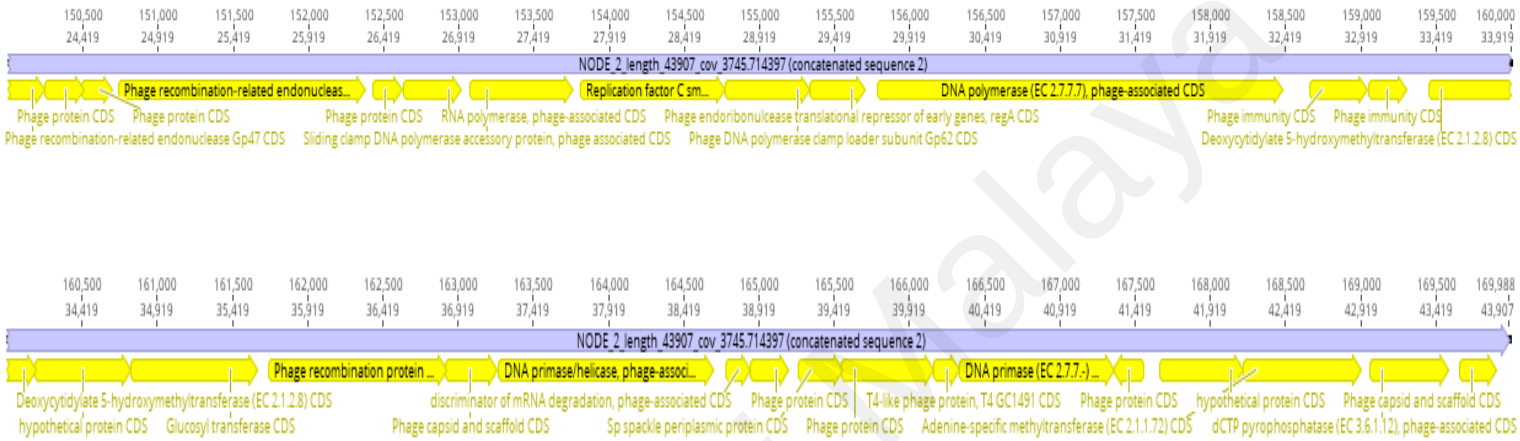

Figure 4.20, continued

4.22 Comparative Genomics Study

4.22.1 Whole genome phylogeny

The phylogenetic tree of whole genome of TB004 was constructed using the neighbor -joining method through Megablast from the NCBI database of complete bacteriophages from microbial nucleotide sequences section (Figure 4.21). Thirty four closest hit complete genome of bacteriophages were selected to construct the maximum likelihood tree. The TB004 phage showed highest query cover (91%) with *Shigella* phage Shfl2 (accession no: NC_015457) where the identity was 97 and showed highest identity (98%) with *Escherichia* phage ime09 (accession no: NC_019503) where the query cover was 88%. It also showed 97 similarity and 88% query coverage with the phage phiD1 (accession no: NC_027353) and phage RB3 (accession no: NC_025419). It was observed that these 4 phages together with another 30 top hit bacteriophages belonged to the family *Myovirdae* which validated the transmission electron microscopy result as TB004 exhibited *Myoviridae* phage like morphology in TEM. In spite of the similarities, the phage TB004 clustered in a separate sub branch of the tree which meant that it is a novel strain of T4 series bacteriophage.

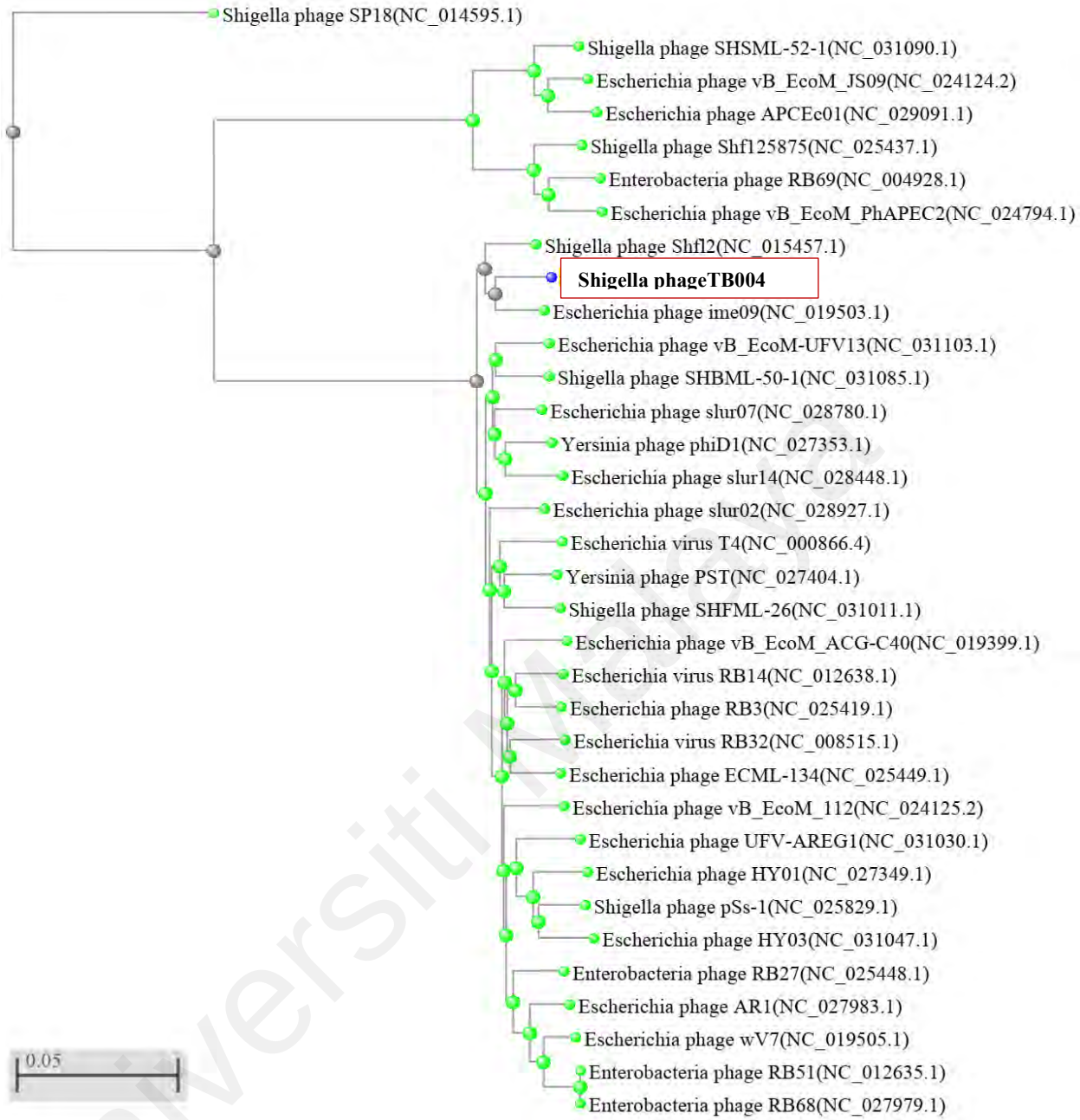

Fig 4.21: The phylogenetic relationship of phage TB004 with the complete genome of 34 closest hit bacteriophages. The maximum likelihood tree was constructed through Megablast using neighbor-joining method. The Gen-bank accession number is also provided after each phage's name.

4.22.2 Evolutionary relationship of 5 selected proteins:

The evolutionary relationship of 5 selected proteins namely major capsid protein, portal vertex protein, terminase large subunit protein, DNA polymerase protein and thyamidylate synthase protein were evaluated. These proteins are the key functional proteins of tailed bacteriophages. The large terminase sub-unit is regarded as the most universally preserved gene sequence in bacteriophages. So this protein is used to construct phylogeny and to describe evolutionary associations among the phages (Casjens et. al, 2005). The major capsid protein is the primary unit for assembling head capsid which shows high similarity among the tailed bacteriophages from the same family of *Podoviridae, Siphoviridae*, or.*Myoviridae* (Gao et. al, 2012). Over one fourth of known dsDNA phage carry the DNA polymerase gene which is known as one of the most widely distributed phage genes because of its essential role in DNA replication (Nasco et. al, 2018). The portal protein is known as a commonly used marker for constructing phylogenetic trees of T4-like bacteriophages and other *Myoviridae* phages (Sullivan et. al, 2011) Furthermore, sufficient information of these proteins are available in databases to perform an effective phylogenetic analysis. Hence, these five protein were selected for further phylogenetic studies.

The phylogenetic tree of major capsid protein (structural protein) (Figure 4.22A) exhibited the TB004 phage in the same branch of *Shigella* phage SHML-11, *Shigella* phage SHMBL-50-1, *Shigella* phage SHML-26 and *Shigella* phage pSs-1 while the phylogenetic tree of both portal vertex protein (Figure 4.22B) and terminase large subunit protein (packaging protein) (Figure 4.22C) exhibited the TB004 phage in same branch of *Shigella* phage SHML-11, *Shigella* phage SHMBL-50-1,*Shigella* phage SHML-26 and *Shigella* phage pSs-1 together with the *Shigella* phage SH7 and *Shigella* Phage shfl2 . The phylogenetic tree of DNA polymerase protein (Figure 4.22D) exhibited the TB004 phage in same branch of *Shigella* phage pSs-1, *Shigella* phage SH7, *Shigella* phage

SHML-11, *Shigella* phage SHMBL-50-1 and *Shigella* phage Shfl2 which were common in the previous three phylogenetic trees. In case of the phylogenetic tree (Figure 4.22E) of thymidylate synthase protein of TB004 phage, it was appeared in the same branch of *Shigella* phage Shfl2 while the *Shigella* phage pSs-1, *Shigella* phage SHML-11, *Shigella* phage SH7, *Shigella* phage SHML-26 and *Shigella* phage SHMBL-50-1 were appeared at the sub branch of the same branch. So the phylogenetic trees of each of these five proteins indicate that the phage TB004 is in the same cluster of phage Shfl2, SH7, SHML-11, SHML-26, SHMBL-50-1 and pSs-1. All these phages belonge to T4 genus under *Tevenvirinae* subfamily and *Myoviridae* and family.

A

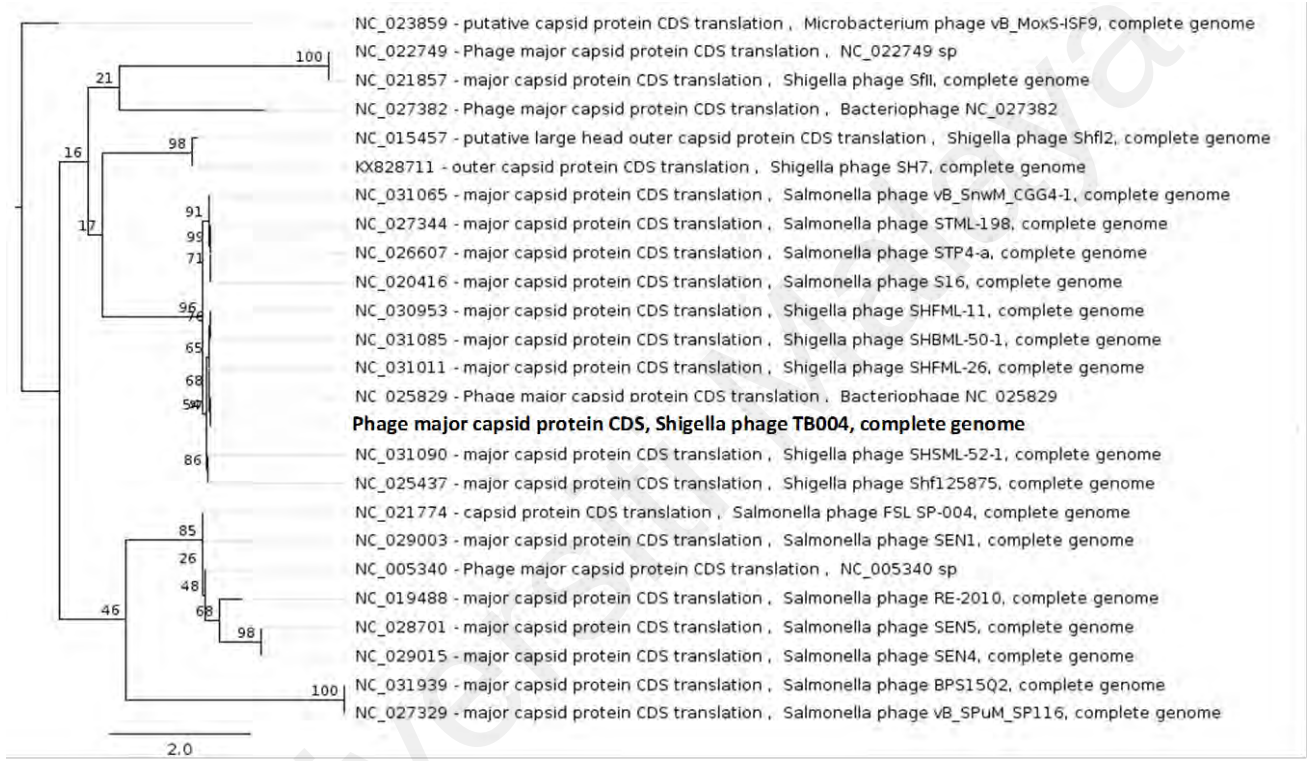

Figure: 4.22: Phylogenetic trees of of TB004 phage protein. A: Capsid protein; B: Portal vertex protein; C: Terminase large subunit protein; D: DNA polymerase protein; E: Thymidylate synthase protein. Maximum likelihood tree with 1000 boostrapping was constructed using PHYML with LG substitution model. All trees were rooted using appropriate gene sequence as out-group.

B

Figure: 4.22, continued

C

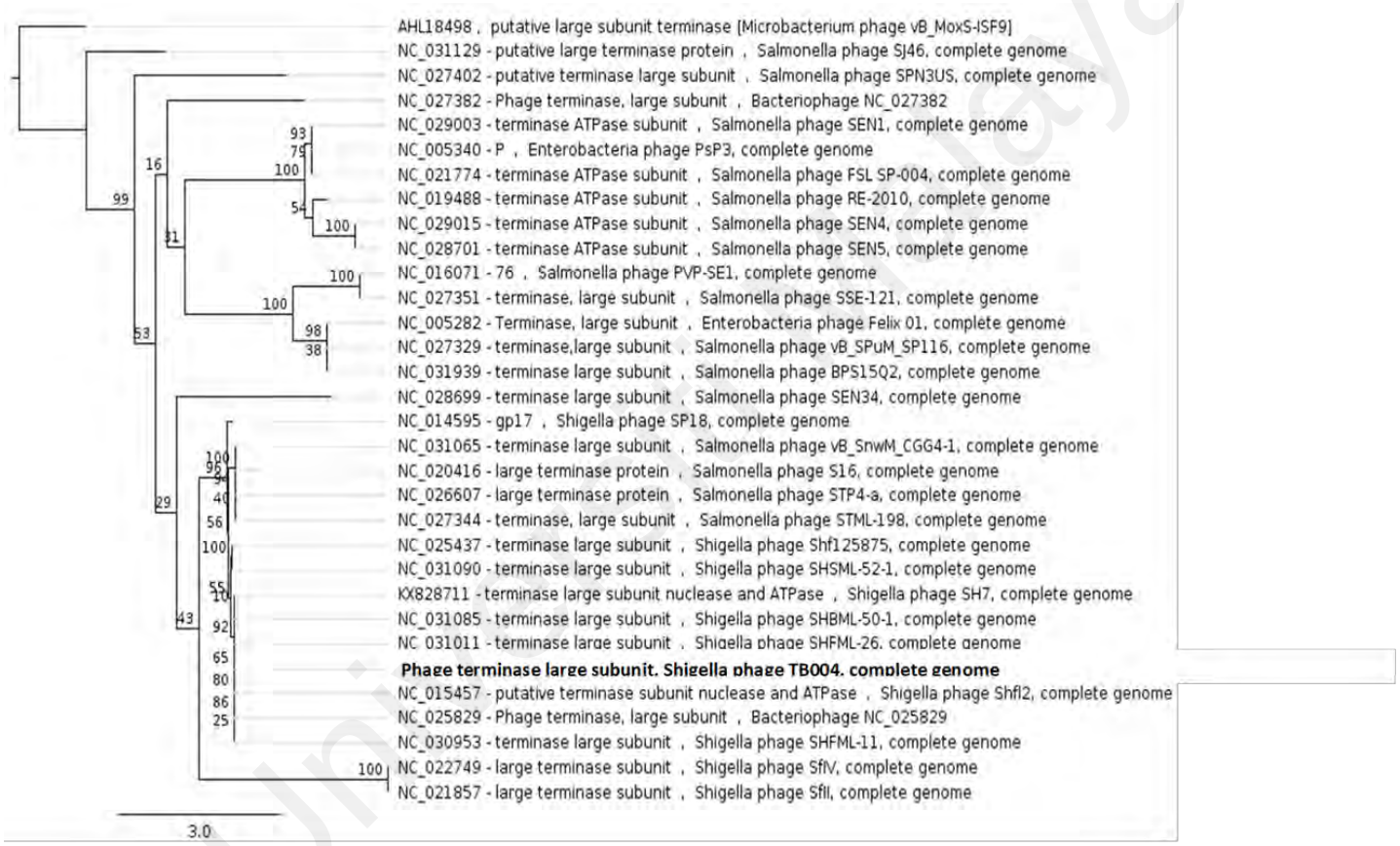

Figure: 4.22, continued

D

Figure: 4.22, continued

E

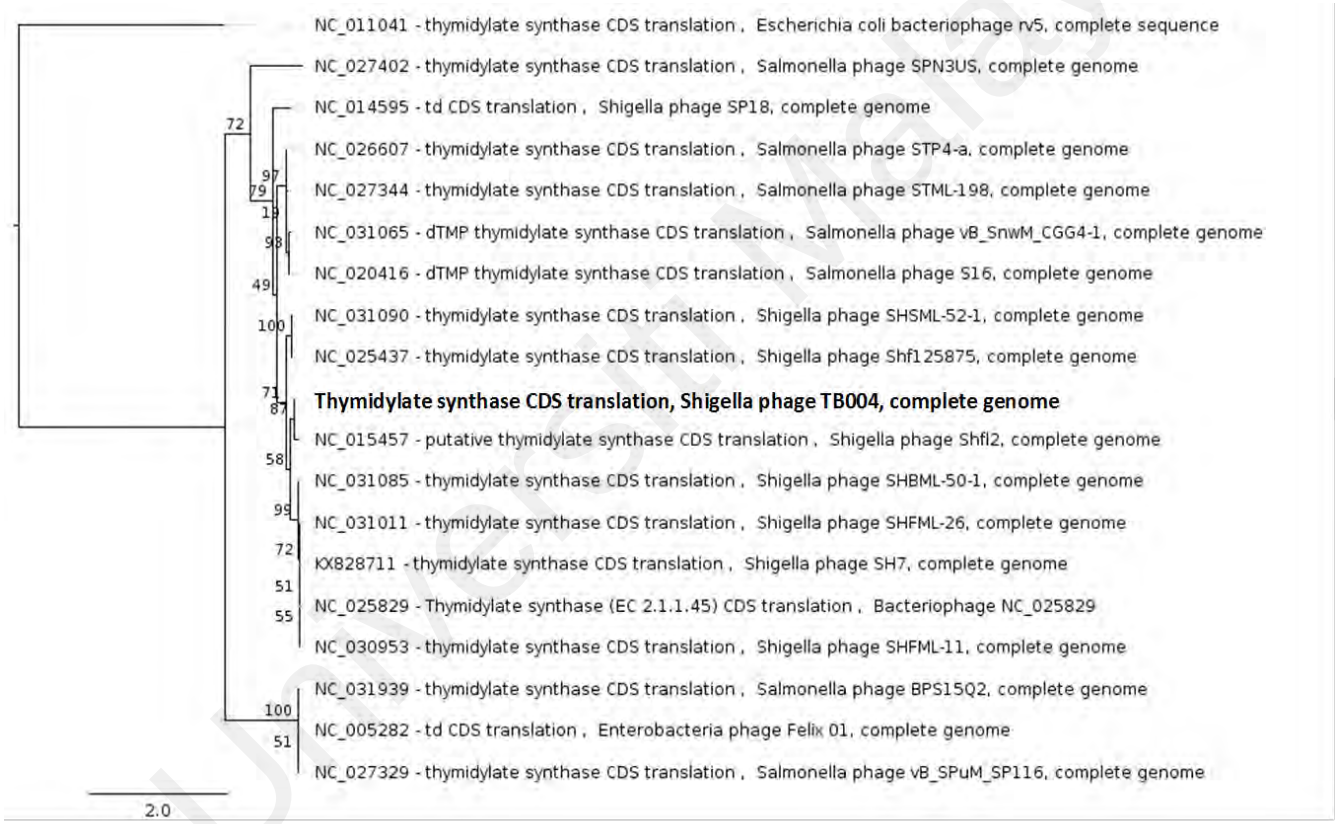

Figure: 4.22, continued

CHAPTER 5: DISCUSSION

The main objective of this current study was to isolate a bacteriophage against drug/multidrug resistant *Shigella* spp., and it was hoped that it could be used as a potential antimicrobial agent in future prophylactic and therapeutic treatment of Shigellosis. It has been stated in the literature review section, that phage therapies for *Shigella* spp. and other pathogenic bacteria have been studied and applied for more than hundred years, but phage therapy as an antibacterial treatment in general has not received much attention due to lack of clinical knowledge and public awareness of phages. The potential of phage therapy has been acknowledged and revisited by many scientists over the last few decades, and there has been a rejuvenation of research into phage therapy. In spite of some limitations, phage-based bio-control and bacteriophage therapy are very promising approaches to combat the challenge of pathogenic bacterial infections, particularly when the search for new antibiotics is stagnating.

Shigella, a dysentery causing enteric pathogen comprising of four species and 55 serotypes, is predominant in low to middle income countries especially in South Asia and sub-Saharan Africa (Gu et al., 2012a; The et al., 2016). The occurrence of shigellosis is also very frequent in many developed countries, for instance, in Malaysia (Singh et al., 2011) and in the USA (Gupta et al., 2004) it is the third most common diarrhoeal pathogen. Diarrhoea was the seventh leading cause of global death in 2016 (Khalil, 2017) while *Shigella* was ranked as the second leading cause of diarrhoeal deaths (Abdoli & Maspi, 2018). Gradual emergence of multidrug resistant *Shigella* requires urgent attention, and it is imperative that focus should be directed towards the development of efficient drugs. However, the development of new drugs is time consuming, laborious and may cost millions of dollars (Chopra et al., 1997). Thus, the emergence of multidrug-resistant pathogenic bacteria is underlying a great challenge for drug development. By

giving emphasis in this current drug-resistant phenomenon, WHO has convened a global consultation for better understanding of the challenge and the status of determining *Shigella* and ETEC burden as well as the strategic policy of vaccine development against these pathogens (Hosangadi et al., 2017). In this situation, scientists are thinking about an alternative or complementary treatment to antibiotic. Hence, phage therapy could be a suitable alternative or supplement as it had a lot of proven success in the past and is still used confidently in Eastern-European countries. At the beginning of antibiotic era 1940s when antibiotic appeared as a magic bullet, the USA, USSR and other part of the world lost interest on phage therapy research except for a few Eastern-European countries (Matsuzaki et al., 2014; Verbeken et al., 2014). Now scientists from a lot of countries are re-focusing on phage therapy research and trying to master the art of phage therapy from the Eastern Europe. Recently the highly-renowned journal 'Cell' has published an editorial entitled "Big Questions in Microbiology" giving emphasis on bacteriophages as potential antimicrobials where bacteriophage was mentioned as one of the nine major research questions. According to the editorial, bacteriophages are the most prevalent bacterial predators in nature (Mizrahi, 2017). In addition, isolation and identification of wider host range bacteriophages in recent years has created a new opportunity to solve the problem of host specificity. Furthermore, advancement of molecular detecting techniques and bioinformatics analysis of the whole genome i.e. genomics, proteomics and metabolomics can ensure the safety prophylactic and therapeutic application of bacteriophages.

The emergence of multi-drug resistant *Shigella* spp. and the necessity to develop a treatment against it made *Shigella* spp. an excellent candidate for preliminary work on phage therapy. Forty-nine *Shigella* strains from all four species covering ten serovars were characterized from both clinical and environmental samples. Among 49 strains, 48 were drug resistant while 29 were multidrug resistant. Hence, ten bacteriophages of

different families with variable host ranges were isolated based on their lytic action and a wider host range bacteriophage TB004 was selected for subsequent experiments as well as whole genome sequencing for genomic studies

5.1 Isolation and characterization of *Shigella* spp.

As Shigella genus has 4 species including 55 serotypes, and one of the major objective of this study was to characterize wider host range bacteriophages. Therefore, to materialize the objective, 49 *Shigella* strains were detected in this study through biochemical test and serotyping of which 37 were *Shigella flexneri*, eight were *Shigella sonnei*, three were *Shigella boydii* and one was *Shigella dysenteriae*. Among the 49 strains, 39 were isolated from clinical samples and 10 strains were isolated from environmental samples. *Shigella flexneri* of seven different serotypes were observed (serotype 1b =2, 1c=3 2a=25, 3a=1, 4a=1, 6=4, y=1) and were confirmed further through multiplex PCR (nonaplex PCR) amplification. For quick identification of different serotypes of *Shigella flexneri,* multiplex PCR (Nonaplex) assay was performed successfully in this study. It is worth to mention that, Sun et al. (2011) developed multiplex PCR (octaplex, using 8 sets of primer) for the quick identification of different serotypes of *Shigella flexneri* but failed to optimize and develop nonaplex (using 9 sets of primer) for the identification of the serotype *Shigella flexneri* 6 together with other serotypes. In this study, the primer (wzx6) for the detection of *Shigella flexneri* 6 was added and optimized successfully. Henceforth, nine pairs of primers now could be used in multiplex PCR for quick identification of different serotypes of *S. flexneri*. Currently, the outbreaks of Shigellosis is very frequent from developing to developed countries where different serotypes of *S. flexneri* and *S. sonnei* are predominating. Recently in the USA, 180 cases of *Shigella* infection in Michigan State (Doore et al., 2018) and a serious outbreak of Shigellosis due to *S. sonnei* in California State (Kozyreva et al., 2016) have been reported. Besides these, 1200 cases of Shigellosis in Papua New Guinea caused by

the *S. flexneri* serotype 2 (Benny et al., 2014), fifty-five cases in Taiwan caused by *S. flexneri* 2a, *S. sonnei* and *S. flexneri* 3b (Ko et al., 2013), 96 cases in China caused by *S. flexneri* 2b (He et al., 2012), 701 cases in Iran caused by *S. flexneri* serotype 3a (Hosseini & Kaffashian, 2010) have been reported widely. These reports and our findings have demonstrated that the samples of Shigellosis are dominated by *S. flexneri* and *S. sonnei*. In the present study, out of 49 isolated *Shigella* strains, 37 were *Shigella flexneri* and 8 were *S. sonnei* which represented 91.84% of the total isolation. Further explanation has been mentioned in the section 5.3.

5.2 Antibiotic profiling of isolated *Shigella* spp.

The outcome of antibiotic profiling of all the *Shigella* isolated in this study demonstrated that 98% strains were resistant to at least one antibiotic whereas 59% were multi-drug resistance. The highest number of resistant was observed against Nalidixic Acid (79%) followed by Tetracycline (57%), Streptomycin (55%), Ampicillin (49%), Amoxicillin (40%), Kanamycin (26%), Chloramphenicol (20%) and Ciprofloxacin (16 %), Ceftriaxone (2%) and Cefepime (2%) in this study. Recently, high frequency of Trimethoprim/Sulfamethoxazole (80%), Ampicillin (85%), Cefotaxime (63%) and Nalidixic acid (47%) resistant *Shigella* spp. has been reported in Iran (Mahmoudi et al., 2017). Furthermore, three subsequent annual reports published by the National Salmonella, Shigella & Listeria Reference Laboratory, Ireland (NSSLRL) (http://www.saolta.ie/sites/default/files/publications/NSSLRL%20Annual%20Report%202016.pdf) in 2014, 2015 and 2016 revealed that the percentages of multidrug resistant *Shigella* were 93%, 91% and 82.5 % respectively. The organization reported 14 Ciprofloxacin resistant and 8 Azithhromycin resistant *Shigella* strains in 2015 while 17 Ciprofloxacin resistant and 6 Azithhromycin resistant strains were reported in 2016 (Rabaa et al., 2016) . Recently, Puzari et al. (2017) noticed that *Shigella* have developed resistance against Fluoroquinolones, Cephalosporins and Azithromycin, but earlier they

were susceptible to Ampicillin, Chloramphenicol, Cotrimoxazole and Nalidixic acid (Puzari et al., 2017). In current study, out of 49 *Shigella* strains 29 strains demonstrated multidrug-resistant where three strains showed the highest resistance against seven antibiotics out of ten followed by nine strains against six antibiotics; five strains against five antibiotic; six strains against four antibiotics and five strains against three antibiotics. This scenario of emerging multidrug resistant is alarming which indicates the urgency of an alternative or complementary treatment for multidrug resistant *Shigella.*

5.3 First report of *Shigella flexneri* 1c isolate in Malaysia

Three serotypes of *Shigella flexneri* 1c (also designated as 7a serotype) also known as novel serotype, as it is unable to agglutinate with antibodies specific for the recognized serotypes (two from waste water sample, Malaysia and one from clinical sample, Bangladesh) were identified in this study. The amplification of *gtrIc* (518 bp) gene in singleplex PCR as well as amplification of *wzx1-5* (782bp), *gtrI* (1122 bp) and *gtrIc* (518bp) genes in nonaplex PCR confirmed that, the three suspected serotypes are absolutely *Shigella flexneri* 1c. Wehler and Carlin (1988) first reported two atypical *Shigella flexneri* in Bangladesh that failed to agglutinate with antibodies specific for the conventionally recognized serotypes. The variation in chemical composition of the 'O' antigen revealed that these strains belonged to a new serotype, which was designated as serotype 1c due to the similarity with the serotype 1a and 1b in case of 'O' antigen sharing. Besides Bangladesh, *S. flexneri* 1c was isolated and identified in six other countries namely Vietnam, Egypt, Pakistan, China, Canada and UK (Parajuli et al., 2017). Parajuli et al. (2017) reported that *S. flexneri* 1c is a predominant serotype responsible for more recent *Shigella* outbreaks in the developing countries. Singh et al. (2011) isolated 138 *Shigella* strains from clinical samples where *S. sonnei* was the major species, followed by *S. flexneri* and *S. boydii* and reported *Shigella* as the third most common pathogen in Malaysia. No *S. flexneri* 1c was reported in their study and to the best of our

knowledge, the two *S. flexneri* 1c isolated in this study would be the first reporting in Malaysia. Plasmids of four similar sizes were isolated from all the three *S. flexneri* 1c (approximately 12kb, 3kb, 2.5kb and 1.4 kb). Stagg et al. (2008) observed five different plasmid profiles (P1-P5) of which type P5 was very close to our plasmid profiling. Even though, these three *S. flexneri* 1c serotypes demonstrated similarity in plasmid profiling, they showed variation in antibiotic sensitivity. Among these three strains, SF1044 showed highest resistance against six antibiotics out of ten namely Ampicillin, Amoxicillin, Tetracycline, Streptomycin, Nalidixic acid and Chloramphenicol while SF1016 showed resistance against Ampicillin, Amoxicillin, Streptomycin and Kanamycin followed by SF1042 showed resistance against Ampicillin and Tetracycline. The emergence of novel and drug-resistant bacterial serotypes due to antigenic variation is a great challenge for vaccine development. Since the 1980s, several new *S. flexneri* serotypes especially 1c has been emerged and spread gradually. Currently, this serotype has become the most prevalent serotype in some countries. The current communication is the first report of the isolation of *S. flexneri* serotype 1c in Malaysia and indicates the expansion of this serotype in this country.

5.4 A diversity of bacteriophages isolated against *Shigella* spp.

Ten *Shigella* phages designated as TB002, TB004, TB006, TB007, TB008, TB009, TB010, TB011, TB013 and TB014 were isolated from sewage water samples against *Shigella* spp. These phages exhibited diversities in their plaque size, morphology, pH stability, thermal stability, one step growth curve, burst size and host ranges. It was revealed from plaque morphology analysis that all the phages produced very clear plaques where the plaque size of TB002, TB004 were small (0.92-1.23 mm) and plaque size of TB007 and TB014 were large (5.34-8.31mm) while the plaque size of rest of the phages were medium (2.61-3.63 mm). Interestingly, the plaque morphology of TB013 was different from the others which demonstrated bull-eye morphology (Table 4.7). Jurczak-

Kurek et al. (2016) reported plaques with different morphologies while performing large scale phage diversity analysis. Out of 83 phages, 73 showed clear plaques and 10 showed turbid plaques while 35 of them showed clear plaques with halo/bull's eye morphology. The plaque size of their reported *Myoviridae* phages ranging from 0.5-2 mm, *Siphoviridae* phages ranging from 1-6 mm and *Podoviridae* phages ranging from 2-7 mm. The range of plaque sizes was similar to our findings showed in Table 4.8.

According to the guideline of the International Committee on Taxonomy of Viruses (Fauquet et al., 2005; Krupovic et al., 2016) and Bradley's classification (Bradley, 1967), phage TB002 and TB004 belong to the family *Myoviridae,* phage TB009, TB010 and TB013 belong to the family *Siphoviridae* while TB006 and TB014 belong to the family *Podoviridae*. Phage TB007, TB008 and TB011 were tailless and belong to either group D or E (*Corticoviridae, Tectiviridae* or *Microviridae*) according to Bradley's classification as they were DNA phage in nature (Bradley, 1967). Further investigation and study are required to confirm the family of these three tailless bacteriophages. Whole genome sequencing with *de novo* assembly and phylogenetic analysis would help to determine their family and genus.

Shahin et al. (2018) isolated a *Myoviridae* bacteriophage designated as vB_SsoS-ISF002 able to infect *Shigella sonnei* and *Shigella flexneri* was under *Myoviridae* family (Shahin et al., 2018). Svab et al. (2018) isolated two T5-like bacteriophages of *Siphoviridae* family which can infect both *Shigella dysenteriae* and *Shigella sonnei* (Sváb et al., 2018). Hamdi et al. (2017) isolated two bacteriophages SH6 and SH7 against *Shigella flexneri* belonged to *Siphoviridae* and *Myoviridae* family respectively (Hamdi et al., 2017) while Jun et al. (2016) isolated *Myoviridae* bacteriophage pSs-1, showed infectivity against *Shigella flexneri* and *Shigella sonnei* (Jun et al., 2016). In another study, Jamal et al. (2015) isolated *Myoviridae* bacteriophage WZ1 against multidrug resistant *Shigella dysenteriae*. . Bacteriophage pSf-1 was isolated against *Shigella flexneri*

belonged to the family *Siphoviridae* (Jun et al., 2013) while bacteriophage designated as SP18 was isolated against *Shigella* belonged to the family *Myoviridae* (Kim et al., 2010). Jun et al. (2014) isolated phage pSb-1 against *Shigella boydii* under *Podoviridae* family (Jun et al., 2014) while Faruque et al. (2003) isolated *Podoviridae* bacteriophage SF-9 against *Shigella dysenteriae* (Faruque et al., 2003). Most of the reported bacteriophages against *Shigella* spp. are tailed bacteriophage. Thus, isolation of tailless bacteriophages against *Shigella* is a rare occurrence. Interestingly, we have isolated three tailless bacteriophages together with seven tailed bacteriophages in our study which will aid the future researchers to study phage diversity against *Shigella.* Phages belonging to different families and having variable host ranges are the key components of a phage cocktail and phage-based product, therefore, the diversity of phage observed in this study could help to produce an efficient phage cocktail or phage-based product in future.

5.5 Biological properties of the bacteriophages

One step growth curve was constructed using the respective host strain of the phages. It was observed that, the latent period of the phages ranges from 10 min to 40 min and within these latency periods, the burst size ranges from 51.9 to 634 virions/cell. The highest burst size was observed in the phage TB002 (634 virions/cell) followed by TB004 (486.5 virions/cell). In case of pH stability, all phages were unable to grow when the pH was 3 and below while phage TB007 and TB014 failed to grow from pH 5 and below. All the phages showed normal growth from pH 7 to pH 9 and a little reduction of growth was observed in pH 11. In case of thermal stability, all the phages showed normal growth at the temperature -20 ºC to 50 ºC and only TB011 and TB014 showed minimum growth at the temperature up to 70 ºC. Similar study by Hamdi et al. (2016) reported that phage SH6 and SH7 showed 16 min and 23 min latent period and were stable at pH 5-11 and pH 3-11 respectively. In another study, Jamal et al. (2015) reported that phage WZ1 was stable up to 65 ºC and the pH stability was 7 to 11 having 24 min latent period with the

burst size of 430 virions /cell. In a very recent study, Shahin et al. (2018) observed that phage vB_SsoS-IS002 showed highest growth at the temperature -20 ºC to 40 ºC and pH 7 to 9 while its latent period was 15 min with a burst size of 76 virions /cell. Jun et al. (2013) reported phage pSf-1 having a short latent period of 10 min with a burst size of 86.86 pfu /ml and was stable in pH 5 to 7 as well as was active at the temperature from 4 ºC to 50 ºC. For the therapeutic and prophylactic application of bacteriophages, the microbiological properties are very important. The phages with high burst size and stable in various temperature and pH can be used for biosanitation purposes or to treat infectious diseases (Hamdi et al. 2016). The phage having small latency period, high burst size and stable in wider range of pH and temperature means that the phage have the capacity to lyse large number of bacteria in a shorter period of time in variable environments. Comparing the microbiological properties of the isolated bacteriophage with different bacteriophages reported by Hamdi et al. (2016); Jamal et al. (2015); Jun et al. (2013) and Shahin et al. (2018), it is observed that TB002 and TB004 are very potential to be used in further research.

5.6 Selection of bacteriophage based on the breadth of host range

Phage TB004 and TB002 showed wider host range and lysed 49 and 48 *Shigella* strains respectively out of 49 (Figure 5.1). Surprisingly, these two phages were able to infect and lyse all four species of *Shigella* genus, could be considered as a significant finding in comparison to the findings of other researchers. Other phages TB006, TB007, TB008, TB009, TB010, TB011, TB013 and TB014 also showed wide host range and were able to infect 32, 34, 33, 33, 35, 31, 30 and 32 strains respectively out of 49 strains where TB007 and TB010 infected two species of *Shigella* namely *S. flexneri* and *S. sonnei* and the rest of the phages infected the strains *S. flexneri* only. Hamdi et al. (2016) reported the SH7 and SH6 had wider host range infecting 27 and 9 strains out of 35 strains respectively. In their study they used three species of *Shigella* namely *S. flexneri*, *S.*

sonnei and *S. dysenteriae* but the phages were able to infect *S. flexneri* only (Hamdi et al., 2016). Phage pSf-1 infected seven strains out of nine *Shigella* strains used in the experiment and had the coverage of three species of *Shigella* except *S. dysenteriae* (Jun et al., 2013). In another study, phage chee24 and chee130_1 were tested against all four species of *Shigella* but phage chee24 infected *S. sonnei* and *S. dysenteriae* and phage chee130_1 infected *S. sonnei* only (Sváb et al., 2018). By comparing the reported *Shigella* phages and their host range (Table 5.1) it is suggested that our isolated TB004 and TB002 showed wider host range and their coverage of all four species of *Shigella* genus are significant findings.

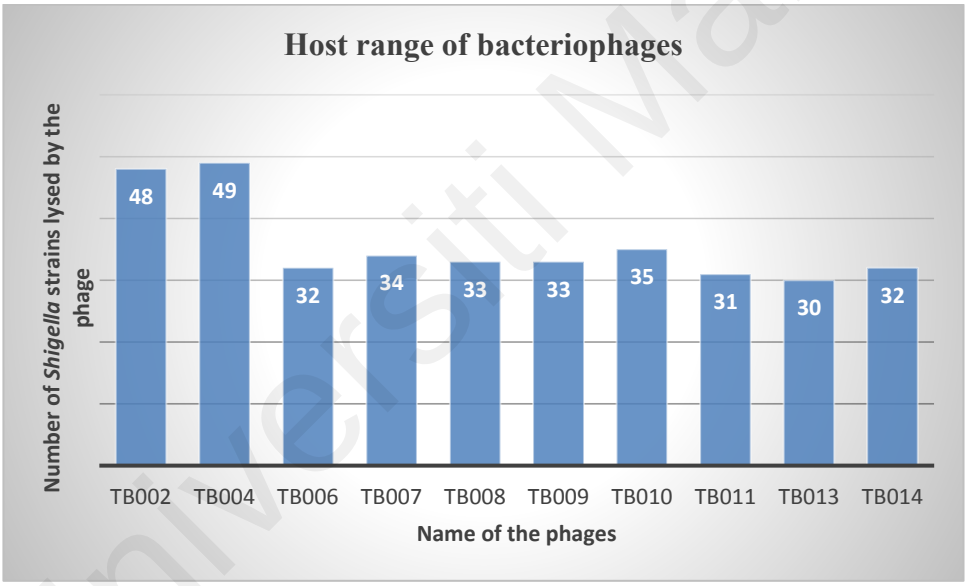

Figure 5.1 : Host range of bacteriophages showing the number of strains infected by the phages out of 49 strains

Table: 5.1 Host range comparison of different phages isolated against *Shigella* spp.

Name of phage	Host range	Species of *Shigella* used (number)	Species of *Shigella* infected	Number of *Shigella* strains infected by the phage	Reference
Phage chee24	11 out of 29	*S. dysenteriae* (1), *S. Sonnei* 2), *S. boydii* (1), *S. flexneri* (1)	*S. dysenteriae, S. Sonnei,*	3	(Sváb et al., 2018)
Phage chee130_1	6 out of 29	*S. dysenteriae* (1), *S. Sonnei* 2), *S. boydii* (1), *S. flexneri* (1)	*S. Sonnei*	1	(Sváb et al., 2018)
vB_SsoS-ISF002	9 out of 19	*S. dysenteriae* (1), *S. Sonnei* (7), *S. boydii* (1), *S. flexneri* (2)	*S. Sonnei, S. flexneri*	9	(Shahin et al., 2018)
SH6	9 out of 35	*S. dysenteriae* (1), *S. Sonnei* (1), *S. flexneri* (1)	*S. flexneri*	1	(Hamdi et al., 2016)
SH7	27 out of 35	*S. dysenteriae* (1), *S. Sonnei* (1), *S. flexneri* (1)	*S. flexneri*	1	(Hamdi et al., 2016)
pSs-1	7 out of 11	*S. Sonnei* (4), *S. boydii* (2), *S. flexneri* (3)	*S. Sonnei, , S. flexneri*	7	(Jun et al., 2016)
WZ1	5 out of 34	*S. dysenteriae* (7)	*S. dysenteriae*	3	(Jamal et al., 2015)
pSb-1	2 out of 18	*S. Sonnei* (4), *S. boydii* (2), *S. flexneri* (3)	*S. boydii*	2	(Jun et al., 2014)
pSf-1	7 out of 18	*S. Sonnei* (4), *S. boydii* (2), *S. flexneri* (3)	*, S. Sonnei, S. boydii, S. flexneri*	7	(Jun et al., 2013)

Table 5.1, continued

Name of phage	Host range	Species of *Shigella* used (number)	Species of *Shigella* infected	Number of *Shigella* strains infected by the phage	Reference
SP18	3 out of 21	*S. Sonnei* (3), *S. boydii* (1), *S. flexneri* (1)	*S. Sonnei*	3	(Kim et al., 2010)
TB004	49 out of 49	*S. dysenteriae* (1), *S. Sonnei* (8), *S. boydii* (3), *S. flexneri* (37)	*S. dysenteriae, S. Sonnei, S. boydii, S. flexneri*	49	This study

Bacteriophage with a broad host range and large burst size is very rare. TB004 is an exceptional bacteriophage that has the ability to extend its host range to all the species (or subgroups) of *Shigella* spp. Short latent period, large burst size, and fast adsorption rate indicates that TB004 has a favourable propagation rate where new host cell exist which strengthening the justification of selecting the phage TB004 for subsequent genome sequencing.

5.7 Adsorption rate and MOI of TB004

The adsorption rate of TB004 phage was observed 95.51 % within the first 2 min of infection at 37 ºC and the rate of adsorption increasingly reached to 99.94% at 10 min where the initial phage concentration was 2×10^6 pfu/ml. In case of MOI, significant reduction of host bacterial cells and increase of bacteriophage cells were observed. The infection rate of bacterial host by the phage TB004 in each tested MOIs (0.01 to 100) showed a reduction of bacteria titre ranging ~10.20 to ~8.00 log cfu/ml and the titre of phage amplified from ~ 6.50 to ~10.50 log pfu/ml at the same MOIs as recorded throughout of the experiment. Lau et al. (2012) observed 98.9 % adsorption within the 2 min of infection and the phage was able to decrease the bacterial titre from ~8.5 to ~4.5 log cfu/ml while the titre of phage increased from ~ 6.5 to ~11.1 log pfu/ml. It is reported that, in the *in vivo* therapeutic application approach, the efficient phage treatment regime would be the rapid elimination of host cells in a short period of time using high MOI ratios (O'Flynn et al., 2004). In present study, phage TB004 was adsorbed by the host nearly 100% within a short period of time and the titre of bacteria was reduced while the titre of phages increased gradually over time. The ratio of phage to host bacteria during co-infection is very important for efficient lysis of the host cells and it only occur at the right ratio of phage and host bacteria. In this study the optimum MOI range for TB004 was determined from 1 to100 and at these MOIs, effective elimination of bacterial cells were observed. The relatively high phage titre in comparison to the lower numbers of host

cells, the chances of phage to bind to the targeted bacteria would be increased but lysis of cells without phage multiplication would also be observed (Goode et al., 2003; Huff et al., 2006). Adsorption of higher percentages of phages by the host bacteria doesn't mean that multiplication of phages will be higher. It depends on the lytic activity of the phages.

5.8 Genomic study of TB004

The genomic study of TB004 was performed through whole genome sequencing and assembly, gene component prediction, gene function annotation and phylogenetic analysis.

5.8.1 Whole genome assembly and gene component prediction of TB004

The whole genome sequencing revealed that TB004 was a phage of double stranded DNA containing 169,998 bp nucleotides with the 35.46 % of G+C content..Two hundred seventy three genes were predicted for TB004 bacteriophage while 5 repeat sequences and 10 tRNA were also annotated. The specificities of the tRNAs were Gln (UUG), Leu (UAA), Gly (UCC), Pro (UGG), Ser (UGA), Thr (UGU), Met (CAU), Tyr (GUA), Asn (GUU) and Arg (UCU). The number of tRNA in TB004 was more than the average number of tRNA in T4-like phage group. The number of tRNAs varies considerably among the genomes of phages of the T4 virus genus (Nolan et al., 2006). Though the accurate function of tRNA in phage genome is still unclear, it is assumed that it may influence to short latent period and large burst size since tRNA in phage is known to contribute its reproduction within the host, and enable the enhancement of propagation and the decrease of latent period (Bailly-Bechet et al., 2007). A few similar type of *Myoviridae* bacteriophages has been reported for example, phage pSs-1 contains 164, 999 bp of genome with a 35.54% G+C content, 266 ORFs and 10 tRNAs (Jun et al., 2016) while SH7 is composed of 164,870 bp with a G+C content of 35.5% having 265 putative ORFs and 11 tRNAs (Hamdi et al., 2017). Kim et al. (2010) reported that phage SP18 composed of 170,605 bp with a G+C content 40.4 % of having 286 ORFs and 3 tRNAs

(Kim et al., 2010). Comparatively smaller size *Myoviridae* phage also reported against *Shigella* spp. for instance, phage WZ1 (Jamal et al., 2015) and SflIV (Jakhetia et al., 2013) are composed of 38000 bp and 39758 bp respectively.

5.8.2 Gene function annotation

5.8.2.1 GO (Gene ontology) annotation

In case of gene ontology, almost 40,000 of terms grouped into three categories i.e. biological process, cellular component and molecular function. So far widespread bacterial virus biology has not been comprehensively described in this ontology. Hulo et al. (2017) listed 68 terms for bacterial viruses with GO identity which provides a bit more clarification for GO annotated phage genes. Is case of TB004 phage, the functions of 57 genes were annotated with GO identity out of 273 genes. It is common that one gene can share multiple GO id classes of 68 listed terms among biological, cellular and molecular functions. In general, around 50% of the genes cluster into families of three or more members (Toussaint et al., 2007). The function of the GO annotated genes hasbeen described elaborately in the result section (4.20.1). In this study, 57 genes were curated manually based on early genes, middle genes and late genes having GO identity. Among the 57 genes 31 were categorized as early genes, 7 were categorized as middle genes and 19 were categorized as late genes. It is worth mentioning that, from the Swiss-Prot analysis only specific/ single function of genes could be annotated but Go annotation revealed the probable multiple functions of the genes. In this study, the highest 24 GO identity was found for the early gene *dda* (ORF-90) followed by the early gene *gp46* (n=20,ORF-52), early gene *gp22* (n=12,ORF-204), early gene *gp44* (n=11,ORF-56), middle gene *rIIB* (n=10,ORF-104), late gene *gp67* (n=10,ORF-207), late gene *wac* (n=10,ORF-216), early gene *pseT* (n=9,ORF-156), early gene *gp43* (n=8,ORF-59), early gene *uvsW* (n=8,ORF-195), early gene *dam* (n=7,ORF-76), late gene *gp2* (n=7,ORF-229) early gene *gp55* (n=6,ORF-42),early gene *tk* (n=6,ORF-270),late gene *gp29* (n=6,ORF-185), early gene *nrda* (n=5,ORF-147), early gene *nrdc* (n=4,ORF-22), early gene *gp39*

(n=4,ORF-101),early gene *ndd* (n=4,ORF-111), early gene *gp52* (n=4,ORF-114),early gene *frd* (n=4,ORF-140), early gene *gp30* (n=4,ORF-177), early gene *gp41* (n=3,ORF-67), early gene *rnh* (n=3,ORF-130), early gene *gp32* (n=3,ORF-134), early gene *denA* (n=3,ORF-149), early gene *gp1* (n=3,ORF-231), early gene *e* (n=3,ORF-251), middle gene *cd* (n=3,ORF-163),late gene *gp51* (n=3,ORF-185), late gene *gp5* (n=3,ORF-226), late gene *gp50* (n=3,ORF-228) and rest of the 11 genes had double GO identity and 14 of the genes had only one GO identity. The specificity of knowledge that Gene Ontology (GO) annotations currently can represent is still restricted by the legacy format of the GO annotation file. Thus, as a step forwards, the GO consortium has introduced a new field into the annotation format, annotation extensions, which can be used to capture valuable contextual detail. This provides experimentally verified links between gene products and other physiological information that is crucial for accurate analysis of pathway and network data (Huntley & Lovering, 2017). Annotation of bacteriophage genome through Gene Ontology (GO) is still not frequent as the tools and database for GO of bacteriophages are in development stage. Hence, the annotated findings of Gene Ontology of phage TB004 would help future researchers as a reference to compare their findings.

5.8.2.2 Swiss-Prot annotation

For TB004 phage, 235 genes were annotated out of 273 genes of which 126 had their assigned function and 109 of them were uncharacterized hypothetical protein. Of all annotated gene products, 223 showed closest hit with *Enterobacteria* T4 phage proteins followed by T2 phage (n=3), K3 phage (n=3), T6 phage (n=2), LZ3 phage (n= 1), LZ5 (n=1), RB18 (n=1) and AR1 (n= 1) while 25 ORFs (ORF-22, ORF-42, ORF-43, ORF-44, ORF-66, ORF-100, ORF-109, ORF-123, ORF-131, ORF-132, ORF-166, ORF-167, ORF-168, ORF-181, ORF-193, ORF-206, ORF-224, ORF-227, ORF-230, ORF-233**,** ORF-236, ORF-263, ORF-271, ORF-273) and 1 ORF(ORF-58) showed 100% identity with T4 phage and RB18 phage respectively. Miller et al. (2003) predicted 62 essential ORFs related to T4 phage including phage structure and assembly, transcription

regulation and metabolism. Petrov (2010) also enlisted 62 essential genes with 38 core genes. From the gene function annotation and genetic map alignment, it was revealed that all the 62 essential genes and 38 core genes were predicted for TB004 phage (Table 5.2).

Table: 5.2 List of essential and core genes of TB004 phage, which showed similarity of the essential and core genes of T4 phage (Miller et al., 2003; Petrov et al., 2010).

Category of important genes	**List of essential genes of T4 phage**	**List of core genes of T4 phage**
Structural	*gp2; gp3; gp4; gp5; gp6; gp8; g13; gp 14; gp15, gp18; gp19; 22; gp23; gp25; gp26; gp34;g35; gp36; g37; g49; gp53*	*gp3, gp4, gp5, gp6, gp8, gp13 , gp14 gp15, gp18, gp19, gp22, gp23, gp24 ,gp25, gp26, gp34, gp35, gp36, gp37, gp53*
Packaging	*g16, gp17, gp20, gp21*	*gp16, gp17, gp20, gp21,*
DNA replication, repair and metabolism	*gp43; gp45; gp44 and gp62; gp41, gp61, g59; gp32; gp46 & gp47; uvsW; uvsX, uvsY; gp30; rnh; gp 39+60 &52; dda; gp49*	*gp43,gp45, gp44, gp62, gp41, gp59, gp32, gp46, gp47, uvsW*
Gene expression	*gp33; gp55; regA*	*gp33, gp55, reggA*
Auxiliary metabolism	*nrdA &nrdB; nrdC; nrdG; nrdH; gp56; cd; frd; td; tk; 1; denA; dexA*	*nrdA, nrdB*

Though 204 of annotated gene products showed >90% identity with T4 phage, significant differences were observed with a few genes. Gene *Gp37* responsible for long tail fiber protein encoded by ORF-126 showed only 62.9 % similarity with K3 phage (one type of T4 series phage). Moreover, *gp56* responsible for pyrophosphatase protein encoded by ORF-56 showed 73.3% similarity, *gp38* responsible for receptor recognition protein encoded by ORF-125 showed 76% similarity, *comCA* responsible for *alpha* protein encoded by ORF-98 showed 76.6% similarity, and *mrh* responsible for modulating protein encoded by ORF-81 showed 76.9% similarity with T4 phage. These low percentages of similarity in few genes indicate that TB004 is a novel strain of T4

genus. Hamdi et al. (2016) isolated T4 phage designated as SH7 and observed homologous of all these essential ORFs related to T4 phage, with high identities including ten ORFs with 100% identity to phage T4. They also reported few differences for instance, *gp37* and *gp12* shared only 37% and 64% identity with T4 phage. In case of phage structure comparison reported earlier by Karam and Drake (1994) and Miller et al. (2003), all proteins were present in TB004 phage (Karam & Drake, 1994; Miller et al., 2003) except internal protein IP III . The proposed diagram of TB004 phage have shown in Figure 5.2

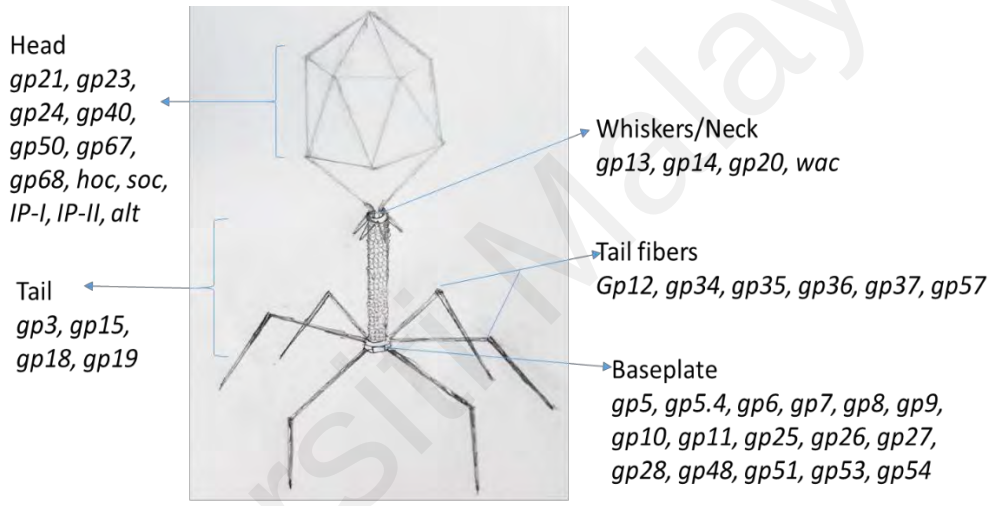

Figure 5.2: Proposed diagram of TB004 phage based on different genes responsible for structure formation (i.e head, tail, neck, tail fibers and baseplate)

5.8.3 Comparative genomic study

The phylogenetic analysis of whole genome and five selected core protein i.e capsid protein, portal vertex protein, terminase large subunit protein, DNA polymerase protein and thyamidylate synthase protein confirmed that TB004 bacteriophage belonged to T4 genus under the family *Myoviridae*. The morphology of this phage under transmission electron microscope also indicated that this phage belonged to the family *Myoviridae*. A detailed comparison with the ten closest hit bacteriophage sequence revealed that the TB004 phage belonged to T4 genus of subfamily *Tevenvirinae* and family *Myoviridae* as all the ten phages belonged to T4 genus under the subfamily *Tevenvirinae* and family

Myoviridae as well (Table 5.3). The phylogenetic trees of each of these five proteins revealed that the phage TB004 was in the same branch of phages Shfl2 (accession no: NC_015457), SH7 (accession no:KX828711), SHFML-11(accession no: NC_030953), SHFML-26 (accession no:NC_031011), SHMBL-50-1 (accession no: NC_031085) and phage pSs-1(accession no: NC_025829). All these phages also belonged to T4 genus under the subfamily and family *Tevenvirinae* and *Myoviridae* respectively (Table 5.4).

Table 5.3: Comparison of TB004 bacteriophage with top 10 closest hit bacteriophages

Phage Name	**Shfl2**	**Slur07**	**ime09**	**vB_Ecom-UFV13**	**PhiD1**	**Slur14**	**RB14**	**PST**	**SHBML-50-1**	**T4**	**TB004**
Host	*Shigella flexneri*	*E. coli*	*E. coli*	*E. coli*	*Yersinia*	*E. coli*	*E. coli*	*Yersinia*	*Shigella flexneri*	*E. coli*	*Shigella flexneri*
Accession No.	NC_015457	NC_028780	NC_019503	NC_031103	NC_027353	NC_028448	NC_126380	NC_027404	NC_031085	NC_000866	This study
Genome length	165919	167124	166499	165771	167063	167467	165429	167785	166634	168903	169988
G-C content	35.6	35.4	35.7	34.8	35.5	35.4	35.3	35.3	35.4	35.3	35.46
No. of genes	265	275	277	269	277	282	274	280	276	288	273
No. of Protein	265	266	268	269	277	272	274	271	265	278	273
No. of tRNA	0	9	9	10	0	0	10	9	11	8	10
Identity %	97	96	98	96	97	96	96	96	96	96	This study
Query level	91	89	88	89	88	89	89	89	88	88	This study
E value	0	0	0	0	0	0	0	0	0	0	This study
Family	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*	*Myoviridae*
Subfamily	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*	*Tevenvirinae*
Genus	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4

Table 5.4: Details of the phages those were appeared in the same branch with TB004 phage during 5 selected proteins phylogenetic tree construction

Name of the phage	Accession no.	Genome size	G-C content	Gene	Host	Family	Sub family	Genus
Shfl2	NC_015457	165919	35.6	265	*Shigella flexneri*	*Myoviridae*	*Tevenvirinae*	T4
SH7	KX828711	164870	35.5	265	*Shigella flexneri*	*Myoviridae*	*Tevenvirinae*	T4
SHMFL-11	NC_030953	170650	35.2	277	*Shigella sonnei*	*Myoviridae*	*Tevenvirinae*	T4
SHMFL-26	NC_031011	168993	35.4	277	*Shigella sonnei*	*Myoviridae*	*Tevenvirinae*	T4
SHMBL-50-1	NC_031085	166634	35.4	276	*Shigella sonnei*	*Myoviridae*	*Tevenvirinae*	T4
pSs-1	NC_025829	164999	35.5	266	*Shigella sonnei*	*Myoviridae*	*Tevenvirinae*	T4
TB004	This study	169988	35.46	273	*Shigella flexneri*	*Myoviridae*	*Tevenvirinae*	T4

5.9 Safety assessment of TB004 phage in comparison to the phages used in phage therapy

It is very important to ensure the safety of any phage before implementing it in phage therapy or phage based product. Intralytix, a world renowned company of phage-based products developer has received the approval form FDA/USDA of three phage based products namely EcoShield™, SalmoFresh™, and ShigaShield™. Among these three phage based products, ShigaShield™ is a product designed to control waterborne or foodborne *Shigella* infection. The active ingredient within ShigaShield™ are naturally occurring lytic bacteriophages that specifically and selectively kill *Shigella* species

(http://www.intralytix.com/index.php?page=news&id=79). ShigaShield™ is composed of five bacteriophages namely SHSML-52-1, SHFML-11, SHFML-26, SHBML-50-1 and SHSML-45. Among these five phages SHSML-45 is a T5 phage of *Siphioviridae* family, SHSML-52-1 is a Rb69 phage of *Myoviridae* family and the rest three phages are T4 phage of *Myoviridae* family (Soffer et al., 2017). Though the genome size of SHSML-52-1 phage (169,621bp) was very close to TB004 (169988 bp) phage, but it showed only 80% identity in case of whole genome phylogeny. The genome size of SHBML-50-1 (166,634 bp) and SHFML-26 (168993 bp) were lesser while the genome size of SHFML-11 (170,650 bp) was higher in comparison to TB004 (169988 bp) phage genome, but showed closer identity in whole genome phylogeny which were 96%, 95% and 96% respectively. The number of encoded genes by SHFML-11 (n=277), SHFML-26 (n=277), SHBML-50-1 (n=276) were slightly higher than TB004 (n=273) phage while the encoded genes by SHSML-52-1 (n=271) was slightly less than the TB004 (n=273) phage. The G+C content of three phages SHFML-11, SHFML-26 and SHBML-50-1 were closer to TB004 phage and the percentages are 35.2, 35.4 and 35.4 respectively. The percentage of G+C content of SHSML-52-1 is relatively higher than the TB004 and the value was 37.6%. The number of tRNA sequences in the phage SHFML-11, SHFML-26, SHBML-50-1 and SHSML-52-1 were 8, 8, 11 and 2 respectively while the number tRNA in TB004 was 10. Interestingly, the protein phylogenetic analysis of the five selected proteins (major capsid protein, portal vertex protein, terminase large subunit protein, DNA polymerase protein and thymidylate synthase protein) revealed that TB004 was appeared in the same branch with SHFML-11, SHFML-26 and SHBML-50-1 phages which were the active ingredient of ShigaShild^{TM} having safety profile. In addition, recently, oral administration of T4 phage has been shown to be safe. In a study on hospitalized bacterial diarrhoea affected children in Bangladesh, the T4-like coliphages or Russian coliphage product or placebo were given orally over 4 days. The study showed a safe gut transit in

children without any adverse implications (Sarker et al., 2016). In another study, *Escherichia coli* phage T4 was given to 15 healthy volunteers in their drinking water where one with a lower dose of 10^3 PFU/ml and another with a higher dose of 10^5 PFU/ml. Though the phage did not cause a decrease in total fecal *E. coli* counts, no adverse events related to phage application were reported (Bruttin & Brüssow, 2005). Later, a mouse model showed the administration of bacteriophage cocktail 'ShigaActive™' significantly reduced the bacterial count (10- to 100-fold) compared to untreated control mice without any side effects or distortions in overall gut microbiota (Mai et al., 2015). ShigaActive™ is also a combination of five phages SHSML-52-1, SHFML-11, SHFML-26, SHBML-50-1 and SHSML-45 mentioned earlier. Moreover, among the 273 genes of TB004, the function of 93 genes were predicted through gene ontology (GO) and function of 235 genes were predicted through Swiss-Prot where no deleterious or toxic gene products were found. So it can be assumed that the possibility of having deleterious gene in TB004 phage is low and it might be used as a potential candidate for phage therapy and phage based product.

Bacteriophages have the considerable untapped potential as a counterpart to antibiotics, not only due to the range of inherent variation in their action mechanisms, but also due to the almost infinite diversity of phages and their ability to develop *in situ* to minimize the challenge of bacterial resistance. The FDA has approved bacteriophages as GRAS and allowed the application of phages as food additives in 2006, which was a significant boost to phage therapy research. It is a matter of hope that, the European Parliament passed a resolution in favour of prioritizing the development of phage therapy as a complement to antibiotic therapy in order to combat antibiotic resistance (Europian-council, 2014). This is an important milestone fostering phage therapy research and development. Recently, a novel phage therapy has successfully treated patients with multidrug-resistant *Acinetobacter baumannii* infections. The treatment was jointly conducted by the

University of California San Diego, School of Medicine, the U.S. Navy Medical Research Center and the Texas A&M University (LaFee & Buschman, 2017). Besides these, other examples of safety oral administration of bacteriophages (Bruttin & Brüssow, 2005; Mai et al., 2015; Sarker et al., 2016) have triggered the new hope for phage therapy research. In this study, the gene profile of TB004 phage has been assessed by the gene function annotation and comparing the gene profile with other similar types of T4 phages belonging to the family *Myoviridae*. The analysis carried out in this study suggested that phage TB004 has the great potential and the probability of having deleterious gene is very low. Therefore, it can be used in further phage therapy research.

CHAPTER 6: CONCLUSION

The death due to Shigellosis has increased gradually over the years. In 2010, the death due to Shigellosis was 122,800 and in 2016 it increased to 270,000 which was more than double from 2010 (Khalil, 2017; Lozano et al., 2012). The discovery of new antibiotics is not sufficient to combat these multidrug resistant *Shigella* pathogens. Hence, phage therapy and phage-based product could be the suitable alternatives or complementary treatments against the drug-resistant bacterial pathogens. There is probably a role for phage therapy in future medicine, but more research is needed in order to solve the problems and the limitations of the method need to be accepted. By giving emphasis on current situation, this study was carried out based on five rationale objectives.

The major objective of this study was to isolate, purify and characterize the bacteriophages against different serotypes of *Shigella* spp. from sewage water samples on the basis of their lytic action. Based on this objective different types of bacteriophages were isolated against *Shigella* spp. on the basis of lytic action. Their plaque morphologies were examined through plaque overlay assay and structures were observed through transmission electron microscopy. Among these phages seven are tailed bacteriophage under *Caudoviralies* order where two of them belonge to the family *Myoviridae*, two of them belonge to the family *Podoviridae*, and three of them belonge to the family *Siphoviriade*. The rest of three phages are tailless bacteriophage, one with hexagonal head, and another two with round heads, might be the member lies between Bradley's D or E group of *Corticoviridae, Tectiviridae* or *Microviridae*. Hence the isolation of both tailed and tailless bacteriophages, belonging different families will facilitate the future research to control wider range of enteric pathogens through individual uses and developing phage cocktails.

The third objective of this study was to determine the host specificity range of isolated bacteriophages. Based on the second objective, host range of 10 bacteriophages against 49 *Shigella* strains were determined through spot plating assay. All the bacteriophages showed a satisfactory host range based on cell lysis capacity while two them TB004 and TB002 showed an extended host range. The fourth objective of this study was to identify a unique wide host-range bacteriophage on the basis of lytic action and host specificity. Based host specificity, phage TB004 was selected as it demonstrated wider host range (49 out of 49). Other subsequent experimental results such as one step growth curve, burst size, pH stability, thermal stability, adsorption rate as well as multiplicity of infection strengthens the justification of TB004 phage for further genomic study.

The final objective of this study was to sequence and analyse the genome of the most potential bacteriophage. Based on the final objective, whole genome sequencing, assembly and genomics study was performed to determine the genus of the phage as well as to assess the safety profile of the phage for therapeutic use. The genomic study confirmed that TB004 was a double stranded DNA phage of T4 genus under *Myoviridae* family consists of 169,988 bp nucleotides. The phage encoded 273 genes and among the 273 genes 109 genes produced hypothetical protein and 126 genes were assigned for producing functional protein while no toxic or deleterious genes were found. In case of phylogenetic trees of five selected proteins, the TB004 phage appeared in the same branch with the three phages SHFML-11, SHFML-26 and SHBML-50-1 which were the active ingredient of ShigaShild^{TM} with an established safety profile.

Now it is time to consider an alternative or a complementary treatment for multidrug resistant *Shigella* spp. The isolated wider host range bacteriophages especially TB004 of T4 genus could be considered as potential and promising candidates for phage therapy and phage biology research against drug resistant *Shigella* spp. due to their wider host range, cell lysis capacity and safety profile. Nevertheless, the therapeutic application of

phages still requires extensive studies, judiciously performed clinical trials, and importantly well-defined regulatory guidelines to overcome the limitations of phage application. It is a matter of hope that, most of the drawbacks of phage therapy have been addressed to a lesser or greater degree, and phages are now capable of being successfully incorporated into the era of multi-drug resistant treatment. Functional genomic study could reduce the further risks of phage therapy by eliminating harmful genes and gene products. In addition, the multi-route administration of phages (intramuscular, intravenous, intraperitoneal, subcutaneous, intranasal and oral) would broaden the use of phage therapy as a potential antimicrobial agent in the future. Moreover, the prophylactic use of phages and the development of vaccines using phages or phage products would open up a new dimension for the prevention of antibiotic resistant pathogens. Furthermore, the active participation of dysentery patients and a large-scale trial of phage therapy against multidrug resistant *Shigella* and other dysenteries would enhance the acceptance of phage therapy as a common treatment. Finally, it is essential to build up public awareness of phage therapy as well as expand the availability of phages and phage therapy centers in order to expand and exploit this potential innovation.

Currently, phage therapy is encouraged in many parts of the world because decision-makers consider growing multidrug resistance as a serious health problem. This awareness should further encourage researchers to study the biological properties of phages, which will eventually increase their safety and efficacy.

FUTURE EXPERIMENTS

In order to develop a successful phage based therapeutic, it is essential to understand the complex biology of phages as well as the unique relationship between phages and their host. This thesis has isolated and characterized phages against drug resistant *Shigella* and investigated biological and applied aspects that will be important in the development of phage therapeutics. In order to ease presentation the thesis has been broken into two broad section: isolation and characterization of *Shigella* spp. and isolation and characterization of bacteriophage against *Shigella* spp.

The first identification of two *Shigella flexneri* 1c in Malaysia revealed that this serotype is being extending to new countries gradually. Though they showed similarity in plasmid profiling but they exhibited variation in antibiotic resistance. Since its emergence in the late 1980s, the *S. flexneri* serotype 1c remains poorly understood, particularly with regard to its origin and genetic evolution. In future, the whole genome of these two strains SF1042 and SF1044 could be sequenced and comparative genomic study with other *Shigella flexneri* 1c could be performed to understand the origin of new pathogenic serotypes and the molecular basis of serotype conversion in *S. flexneri* and to observe the variation and evolution in genetic level due to their migration in new countries.

In this study, biochemical test, serotyping, and multiplex PCR amplification assay were performed to identify different species and serotypes of *Shigella*. Though these identification techniques are very useful, a quick identification technique is still required to identify *Shigella* spp. form a large number of clinical samples. Development of aptasensor could be considered in future for quick identification of *Shigella* serotypes. In case of aptasensor, the aptamer detects the molecular target against which it was previously *in vitro* selected. The aptamer-target reaction is self-determining of both the kind of transducer employed and the type of detection system. In addition aptasensors can

be easily multiplexed to detect a variety of aptamer-target reactions simultaneously (Moreno, 2015).

Forty-nine strains of *Shigella* covering all four species and 10 serotypes out of 55 serotypes were identified and the strains were used against ten bacteriophages to estimate their host specificity. The host range of TB004 phage, which was able to lyse 49 strains out of 49 has created further interest to justify its efficacy against the rest 45 serotypes of *Shigella* to use the phage TB004 phage as a unique and potential antimicrobial agent against drug-resistant *Shigella* spp. Hence, the rest 45 serotypes of *Shigella* spp. from different geographical locations could be collected through collaborative research for the justification cell lysis efficiency of TB004 phage as well as other phages against them.

Host specificity is a major limitation in phage therapy research but broad-host-range bacteriophages are more common than that had been thought previously (Jensen et al., 1998). Malki et al. (2015) isolated four *Myoviridae* phages using same bacterial host, but interestingly each phage exhibited a host-range spanning several phyla of bacteria such as *P. aeruginosa*, *E. coli*, *Arthrobacter* sp., *Chryseobacterium* sp. and *Microbacterium* sp. Hence, the host specificity range of the ten isolated phages could be performed on other enteric pathogens specially *Salmonella, E.coli* and *Vibrio* to select one single phage for wide therapeutic application.

Functional genomic, proteomic and systematic safety studies are essential for the well characterisation and safety assessment of bacteriophages before implementing them in any phage cocktail and phage-based product like ShigaActive^{TM} and ShigaShield^{TM} respectively. Therefore, five top performing phages could be selected for functional genomic study through whole genome sequencing and proteomic study through liquid chromatography/tandem mass spectrometry (LC-MS/MS) to ensure their safety profile to use them in phage therapy and phage based products.

Finally, it is necessary to evaluate the efficacy of bacteriophage on an animal model before applying to human. Animal studies are important for evaluating the efficacy of phage treatment due to the difficulties in performing randomized controlled trials with human subjects. Several animal models specially, mice models have been used to investigate the efficacy of bacteriophages. To evaluate the efficacy of phage therapy as an antimicrobial treatment the mouse model will be the best selection for in vitro study.

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