CHARACTERIZATION OF *Shigella flexneri* SPECIFIC BACTERIOPHAGE INFECTIVITY UNDER ANTIBIOTIC STRESS

By

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ABSTRACT

CHARACTERIZATION OF *Shigella flexneri* SPECIFIC BACTERIOPHAGE INFECTIVITY UNDER ANTIBIOTIC STRESS

Ong Chun Hao

Bacteriophage is a bacterial virus and is known as one of the potential biocontrol agents to fight against antibiotic resistance bacteria due to its high specificity and lytic ability. However, it is a fact that bacteriophage has been co-existing with their host without causing any extinction of both parties. Besides, the relationship between bacteriophage and its host in development of antibiotic resistance is not well studied. Some studies suggested that bacteriophage may play a role in the evolution of antibiotic resistance with their viral transduction mechanism. Under adverse environment such as antibiotic stress, it was demonstrated that lytic bacteriophage may not complete the lytic cycle and exhibit a pseudolysogenic life cycle. Hence in this study, the antibiotic resistance development and genome changes in the SFN6B phage-lysogenic converted *Shigella flexneri* were investigated. Colony PCR screening for SFN6B bacteriophage infected *S. flexneri* demonstrated that the virus could remain inside the host in the event of antibiotic stress. Microbroth dilution was carried out to determine the minimal inhibitory concentrations (MICs) and to compare the antibiotic resistance level between *S.*

flexneri cultures treated with only antibiotic and cultures treated with antibiotic and SFN6B bacteriophage. The results demonstrated that SFN6B bacteriophage was able to slow down the development of trimethoprim and ampicillin resistance but had no impact on the kanamycin resistance development in the host. This suggests that SFN6B bacteriophage might be a potential option to fight against the trimethoprim and ampicillin resistance in the clinical settings. Pulsed field gel electrophoresis (PFGE) showed no significant genome changes were observed in lysogenic converted *S. flexneri* except for two additional bands that were present in the culture treated with ampicillin and SFN6B bacteriophage. The presence of these two additional bands is possibly due to incomplete restriction digestion. Further studies are needed to confirm the rationale of the two additional bands.

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DECLARATION

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

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(Ong Chun Hao)

APPROVAL SHEET

This project report entitled "**CHARACTERIZATION OF** *Shigella flexneri* **SPECIFIC BACTERIOPHAGE INFECTIVITY UNDER ANTIBIOTIC STRESS**" was prepared by ONG CHUN HAO and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **ONG CHUN HAO** (ID No: **13ADB02227**) has completed his final year project entitled "CHARACTERIZATION OF *Shigella flexneri* SPECIFIC BACTERIOPHAGE INFECTIVITY UNDER ANTIBIOTIC STRESS" under the supervision of Dr Tan Gim Cheong from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(ONG CHUN HAO)

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LIST OF ABBREVIATIONS

AMP	ampicillin
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
KAN	kanamycin
LB	Luria Bertani
MIC	minimal inhibitory concentration
OD	optical density
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pH	potential of hydrogen
S. flexneri	*Shigella flexneri*
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TMP	trimethoprim
UV	ultraviolet

%	percentage
°C	degree Celcius
µL	microliter
bp	base pair
CFU/mL	colony forming unit per milliliter
g	gram
kbp	kilobase pair
L	liter
M	molar
mm	millimeter
min	minute
rpm	revolution per minute
PFU/mL	plaque forming unit per milliliter

CHAPTER 1

INTRODUCTION

The *Shigella* spp. are Gram negative facultative anaerobic bacteria that are known for causing human gastrointestinal infection called shigellosis. There are four species of *Shigella*, namely *S. dysenteriae*, *S. flexneri*, *S.boydii*, and *S. sonnei*. Among these species, *S. flexneri* had caused the most mortality, especially in the developing countries due to lack of hygiene and incapable of affording treatment. The *S. flexneri* infection is often transmitted via fecal-oral route. It is able to survive in the acidity environment of the stomach, and then invade the colonic and rectal epithelia. This will subsequently lead to the host's inflammatory response, which destroys the epithelia, followed by the clinical symptoms of shigellosis such as watery diarrhea, severe abdominal pain, and bloody mucoid stool (Jennison and Verma, 2003). Untreated infected patients can die as *Shigella* is highly pathogenic. It can cause infection with only a small inoculum. In addition, it has high capability to survive and evade the host's defense system. Studies have shown that *Shigella* is able to survive the innate immune defense such as the acidic environment in stomach and also the antimicrobial peptides in intestinal environment. Phagocytes like macrophage are also ineffective against *Shigella* infection as the bacteria are able to escape from phagocytic vacuole and then induce macrophage apoptosis. Besides, neutrophil-mediated inflammation also promotes *Shigella* invasion by disrupting the integrity of epithelial cells (Phalipon and Sansonetti, 2007).

Current therapy of *Shigella* infection is employing antibiotics such as quinolone, trimethoprim-sulfamethoxazole, ampicillin, quinolone, and ceftriaxone (Lampiris and Maddix, 2012). However, the escalating development of antibiotic resistance by the bacteria over the years has encouraged many researchers to develop an alternative strategy to combat *Shigella* infection. One of the potential treatments is the bacteriophage therapy. Bacteriophage or phage is the virus that infects bacteria. It replicates via two types of life cycle, lytic cycle and the lysogenic cycle. In lytic cycle, it multiplies within the host cell to produce new viruses and then the new viruses are released after the host lyses. In lysogenic cycle, the virus integrates its genome into the host genome and it replicates together with the host as the host cell undergoes replication. Under certain condition such as DNA damage, prophage induction occurs where the virus genome is excised from the host genome and followed by the occurrence of lytic cycle.

One characteristic of bacteriophage is its high specificity. This is one of the advantages of phage as a potential therapeutic agent since the virus can kill the target bacteria without disturbing the other microflora in human (Haq, et al., 2012). However, the phage therapy is effective only if the virus is completely lytic (virulent bacteriophage). According to Sandeep (2006), temperate bacteriophage with a lytic lifecycle *in vitro* environment may change to adapt lysogenic cycle under certain circumstances in a body. The reason this still remains a question. Nevertheless, it is confirmed that the lysogenic

bacteriophage can cause changes in bacterial genomes through a process called transduction. There is also a possibility where the lysogenic phage cause changes in phenotype of the host. This event is known as lysogenic conversion. These changes can be beneficial or deleterious, for example the antibiotic-resistant ability of the host might increase or decrease, depending on the type of phage. Whether or not lysogenic conversion is beneficial or deleterious to the host, it is something to be examined (Thiel, 2004).

Hence in this study, *S. flexneri*-specific bacteriophage, SFN6B was hypothesized to be able to replicate via lysogenic cycle in the bacteria under antibiotic stress, which can lead to lysogenic conversion of the host bacteria. As a result, changes in minimal inhibitory concentration (MIC) of antibiotics in the lysogenic converted bacteria can occur. It is also suggested that when the host undergoes stress like antibiotic exposure, the mutational changes by the bacteriophage are accelerated.

Therefore, the objective of this study aimed:

1) To obtain SFN6B lysogenic converted *S. flexneri* under antibiotic stress.
2) To study the genome changes and evolution of SFN6B lysogenic-converted *S. flexneri*.

3) To study the changes in MIC of antibiotics in the SFN6B lysogenic converted *S. flexneri*.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteriophage

2.1.1 History of Discovery

Bacteriophage was first discovered by Twort (1915) in Great Britain and D'Herelle (1917) in France independently. Twort (1915) observed that the micrococcus bacteria colonies became glassy and transparent in the agar medium with some fluid that was commonly used for smallpox vaccination. He also found that these colonies could not be subcultured. Later, he tried to touch a pure culture of micrococcus with the glassy colonies. He noticed that the colonies of the pure culture started becoming transparent from the point of touch to the whole colonies. Then, he also filtered the glassy colonies but the filtrate retains the ability to give glassy transformation (Duckworth, 1976).

D'Herelle (1917) initially observed bacteriophage as an anomaly on bacteria colonies, which appear clear and circular. Later on, he tried to observe those transparent spots under the microscope but nothing could be seen. Thus, he deduced that the agents causing this anomaly must be very small and able to pass through the Chamberland filter that traps bacteria. During an investigation on human dysentery, he tried to isolate the agents by filtering the bloody stools. He then added

the filtrate to the broth culture and the agar plate of dysentery bacillus. In the next day, he found out that the initially turbid broth culture became perfectly clear while the agar plate had no growth at all. From this observation, he concluded that the agents causing the clear spots are virus parasitic on the bacteria (Duckworth, 1976).

2.1.2 Classification

The International Committee of Taxonomy of Virus (ICTV) classifies the bacteriophage based on nucleic acid and morphology. Thus far, 20 families of bacteriophage have been discovered. Most phages are tailed and have double-stranded DNA. They are classified as the *Caudovirales* order, which comprises of three families, *Myoviridae*, *Siphoviridae*, and *Podoviridae*. The other order of bacteriophage is *Ligamenvirales*, consisting of *Lipothrixviridae* and *Rudiviridae* families. They have filamentous morphology and also contain double-stranded DNA molecules as their genome. Aside from these two orders, the remaining families of bacteriophages have unassigned order. There are families of phage such as *Microviridae*, *Levivirdae*, *Coricoviridae*, and etc. with cubic symmetry and icosahedral shape; while the other families of phage such as *Plasmaviridae*, *Fuselloviridae*, *Ampullaviridae* and etc. have pleomorphic shape (Ackerman, 2009).

2.1.3 Environmental Factors that Affect Lytic or Lysogenic Development

Bacteriophage replication relies on two strategies, the lytic cycle or the lysogenic cycle. Basically, the lytic phage replicates in a horizontal manner whereas the lysogenic phage replicates vertically. Those viruses that can replicate via both lytic and lysogenic pathway are known as the temperate phage. One of the examples of well-studied temperate phage is bacteriophage lambda, which infects *Escherichia coli*. By default, bacteriophage lambda replicates via the lytic cycle, where a transcription cascade will occur to enable lytic mode of growth (Greenblatt, et al., 1998). In order for bacteriophage lambda to switch into lysogenic mode, the transcription cascade for lytic cycle needs to be turned off. The key protein that causes this switch from lytic to lysogenic development is the CII proteins. During the infection, lytic cycle is initiated when Cro proteins predominate whereas lysogenic cycle is initiated when CI proteins predominate. CII proteins are required to activate the *cI* gene expression for the production of CI proteins, but the Cro protein is able to reduce the production of CII protein. Nonetheless, when CII concentration reaches a threshold, it initiates the synthesis of other proteins including CI proteins that will cease the lytic development and begin the lysogenic cycle. (Thomas, 1971; Oppenheim, et al., 2005).

The studies on bacteriophage lambda demonstrated that lysogenic pathway is favored when the host is growing under adverse environment. Since bacteriophage relies on a host (bacteria) to grow and replicate, this is a necessary strategy for the phage to prevent its own extinction. There are several environmental conditions

that can cause the lysogenic development of bacteriophage lambda. One example is the multiplicity effect. High multiplicity of infection is a condition where the bacteriophage amount outnumbers the bacteria. In this case, a single bacterial cell might be infected by two or more phages. Thus, multiple phages can synthesize more CII proteins in a single host, which are sufficient enough to reach the CII threshold to initiate lysogenic development (Kobiler, et al., 2005).

The physiological effects also play a role in the lytic-lysogenic development of bacteriophage lambda. High nutrient status for the bacterial cells will allow the lytic cycle to predominate whereas low nutrient status favors the lysogenic growth. Bacteria growing in rich media will lead to higher expression of RNase III, which will cause degradation of CII transcript and inhibit the establishment of lysogenic pathway. Besides, the nutrient status also greatly affects the level of N protein, which is a lytic regulator protein. When bacteria grow in rich media, higher concentration of N protein can be expressed by bacteriophage lambda, thus resulting in lytic pathway. On the other hand, lower concentration of N protein is expressed by bacteriophage lambda if the host is growing in a carbon-starved media. This will increase the probability for lysogenic development (Wilson, et al., 2002).

2.2.4 Lysogenic Conversion Effects

Lysogenic cycle involves the integration of the phage genome into the bacterial genome and replicates along with the host without killing the host. This lysogenic development may sometimes cause changes of certain phenotype in the infected host. This phenomenon is known as the lysogenic conversion. The phenotypic change in the infected host is due to the gene expression of the prophage genes. Many studies have demonstrated the lysogenic conversion by temperate phage which enhances the host's virulence. A study by Waldor and Mekalanos (1996) showed that a filamentous phage infecting *Vibrio cholerae* is responsible for the host's virulence. Cholera toxin, which is a potent enterotoxin, is encoded by the filamentous phage, resulting in cholera disease.

Besides enhancing the virulence of the host, lysogenic conversion can also increase the survival ability of the host. One example of this phenomenon is displayed by the phage infecting *Bacillus anthracis*. The prophage is able to alter the host phenotype and increase its survival capabilities by inducing biofilm formation in *B. anthracis* (Schuch and Fischetti, 2009). In addition, Colomer-Lluch, Jofre and Muniesa (2011) also demonstrated that phage can induce lysogenic conversion in the antibiotic-sensitive host by encoding the antibiotic-resistant genes carried by the phage DNA. If prophage induction occurs in the infected cells, lytic pathway occurs and many copies of the antibiotic-resistant genes will be produced and released, resulting in the spread of antibiotic-resistance genes to the other hosts. As

a result, the survival of the fittest take places as there will be a positive selection for these antibiotic-resistant hosts if the antibiotic is present in the environment.

2.2 Antibiotics

2.2.1 Classes of Antibiotics

Antibiotics are well-known drugs used for the treatment of bacterial infection. The very first antibiotic discovered by Alexander Fleming in 1982 is the penicillin. Throughout the years, many antibiotics with various different mechanisms have been developed. With these many antibiotics, classification will be necessary for the convenience in drug selection during bacterial infection treatment. The antibiotics are classified based on their mechanisms. The main mechanisms are cell wall synthesis inhibition, membrane disruption, protein synthesis inhibition, folate-antagonist, and DNA gyrase inhibition. Antibiotics such as penicillin, cephalosporin, carbapenem, monobactam and glycopeptide inhibit cell wall synthesis. The lipopeptide class of antibiotic disrupts the cell membrane of bacteria (Deck and Winston, 2012a). Protein synthesis inhibition is the mechanism of tetracyclines, macrolides, clindamycin, chloramphenicol, streptogramins, oxazolidinones, aminoglycosides, and spectinomycin (Deck and Winston, 2012b). The examples of antifolate drugs are sulfonamides, trimethoprim and etc. The common DNA gyrase inhibitor is such as fluoroquinolone (Deck and Winston, 2012c).

2.2.2 Bacterial Responses to Antibiotic Stress

In the presence of antibiotics, there are two possible outcomes for the bacteria. One scenario is where the bacteria are unable to resist the bacteriostatic or bactericidal effect of the antibiotics, which resulted in inhibited growth or death of the bacteria. In another scenario, the bacteria develop resistance towards the antibiotic actions. Multiple reasons can contribute to the development of antibiotic-resistance in bacteria, such as overuse of antibiotics, incorrect antibiotic prescription, extensive agricultural use of antibiotics and etc. All these factors allow the bacteria with antibiotic-resistant genes to thrive while removing those susceptible bacteria from the bacterial population as a result of natural selection. The acquirement of antibiotic-resistant genes can attributable to horizontal gene transfer between bacteria or spontaneous mutation in the bacteria themselves (Ventola, 2015). Under antibiotic stress condition, the bacteria will have higher rate of mutation due to the actions of stress-responsive DNA polymerase IV and V, which are the low-fidelity DNA polymerases (Radman, 1999).

With the antibiotic-resistant genes, the bacteria are able to defend themselves against the actions of antibiotics via several mechanisms. The resistant mechanisms involve reducing permeability to the antibiotics, increasing efflux of antibiotics, enzymatic inactivation of antibiotics, and alteration of target sites. The decreased permeability to the antibiotics can occur by the down-regulation of the drug-binding sites such as porin. This will prevent the antibiotics from binding and entering the bacterial cells (Chambers and Sande, 1995). The efflux pumps play an active

transport role of transporting the antibiotics out of the cell. Highly resistant bacteria have high level of expression of the efflux genes, which can be usually seen in the multidrug-resistant bacteria. Enzyme inactivation is a strategy for the bacteria to modify the antibiotics via hydrolysis or transfer of a chemical group. The famous example of bacterial enzyme that can inactivate antibiotics is the β-lactamases, which inactivate antibiotics like penicillin and cephalosporin (Miller, Munita and Arias, 2014). Alteration of antibiotic target sites can take place when there is a mutation in the target site. The target sites can also be altered without mutational changes by having an addition of a chemical group. These events will prevent the access of the antibiotics to their target sites and thus no antibiotic action can take place (Blair, et al., 2014; Hawkey, 1998).

2.3 Mutagenesis in Bacteria

2.3.1 Spontaneous Mutagenesis

Mutation is an event that happens by random chances. Throughout an organism's lifespan, mutation can happen even in normal environmental condition from time to time. During DNA replication *in vitro*, the error rate for DNA polymerase was observed to be around 10^{-3} to 10^{-6} errors per base pair replicated; whereas *in vivo*, the error rate is around 10^{-9} to 10^{-11} errors per base pair replicated. When an error occurs, the "first line of defense", which is the editing function of DNA polymerase, comes into play. If the false nucleotide manages to escape the editing function, there is still "a second line of defense", namely the mismatch repair mechanism to

undo the error. Despite all these error-preventing mechanisms, there are still chances for the incorrectly added nucleotide to remain. This is especially the case if mutation had already taken place in the genes encoding DNA polymerase and DNA repair proteins (Smith, 1992).

2.3.2 Stress-Associated Mutagenesis and Stress-Induced Mutagenesis

MacLean, Torres-Barcelo and Moxon (2013) proposed the term stress-associated mutagenesis (SAM), which is quite different from stress-induced mutagenesis (SIM). SAM can occur in the events where DNA is damaged and then directly causing the changes in the DNA. In this case, the association between DNA damage and the DNA mutation is only happening by chance. There might or might not be an occurrence of mutation in the DNA when it is subjected to DNA damage. As for the term SIM, it involves the mutations that are induced rather than being directly caused by the stress. The DNA changes in this case are the result of error-prone DNA polymerase expression caused by the stress induction. There are numerous stress factors that can induce the increase of mutation rate, for example, DNA damage, starvation, oxidative stress, heat exposure, antibiotic exposure, and phage infection. DNA damage by DNA damaging agents will lead to the SOS response. Three polymerases, Pol II, Pol IV and Pol V take part in replicating the damaged DNA during SOS response. The Pol II is accurate as compared to Pol IV and Pol V. The two polymerases, Pol IV and Pol V belong to the γ-family of specialized polymerases. During the event of SOS response, these specialized polymerases will be recruited to replace the Pol III at the site of lesion. They are low fidelity and

error-prone, thus increasing the chance of mutation when they are replicating the DNA (Fuchs, Fujii and Wagner, 2004).

As mentioned above, exposure to antibiotics can induce mutagenesis in the bacteria. With different antibiotic mechanisms, the induction of mutagenesis can take place differently. For example, when antibiotic such as streptomycin is given at a sublethal dose, it fails to inhibit translation completely in the bacteria cells. The sublethal dosage can only cause inaccurate translation of proteins. The translated DNA replication proteins and DNA repair proteins are the factors that result in mutational changes in the bacterial genome (Al Mamun, Marians, and Humayun, 2002; Balashov and Humayun, 2002). The quinolone class antibiotics are able to inhibit DNA replication by trapping DNA gyrase and DNA topoisomerase IV enzymes. This can result in the release of DNA double-strands breaks and followed by SOS response. The involvement of low-fidelity DNA polymerase during SOS response will lead to mutational changes (Drlica and Zhao, 1997). As for the antibiotics that inhibit cell wall synthesis such as β-lactam antibiotics, they can also induce SOS response but through a very different pathway. Binding of β-lactams to the penicillin binding proteins 3 will induce the *DpiBA* two-component signal transduction system. The effector from this system, DpiA can bind to the origin of replication and then induce the SOS response, resulting in the increase in genetic variability (Miller, et al., 2004).

2.4 Evolutionary Model

Under stressful situation, mutagenesis is more likely to occur, either in a beneficial or deleterious manner. Those with deleterious mutation will result in death while those with beneficial mutation can survive. This will result in evolution, where the characteristics of the population undergo changes throughout many successive generations. There are several different evolutionary models proposed by the scientists, namely trade-off hypothesis and evolvability hypothesis (MacLean, Torres-Barcelo and Moxon, 2013). The trade-off hypothesis model describes that the evolution occurs by direct selection of the fittest in the population. According to this hypothesis, those with mutation of increased stress tolerance can survive while those without the mutation are eliminated from the population. Evidence based on the regulation of m*utS* expression in *Escherichia coli* during stationary phase supports this hypothesis. During stationary phase, overexpression of *mutS* causes decrease in the survivorship of *E. coli*; whereas those mutants with downregulated *mutS* expression have higher survivability (Saint-Ruf, Pesut, Sopta and Matic, 2007). Another evolutionary model is known as the evolvability hypothesis. In this model, natural selection favors the organisms with high evolvability rather than the fitness benefits. In other words, it is not the fittest who survives, but those with the highest ability to adapt to the changes. This hypothesis is supported by the evidence where wildtype *E. coli* with error-prone specialized DNA polymerase are selected under stressful environment. Those mutant bacteria lacking of the specialized polymerase for SOS response are replaced by the wildtype population. Nevertheless, when growing the wildtype and mutant bacteria

individually, they show indistinguishable population dynamics, which indicates that the error-prone specialized DNA polymerases do not directly provide any fitness benefit for bacterial survival (Yeiser, Pepper, Goodman, and Finkel, 2002).

2.5 Role of Bacteriophage in Bacterial Evolution

The bacterial cells have the ability of horizontal gene transfer via conjugation. It is a process where the donor bacterial cell transfers its genetic materials to the recipient bacterial cell. The F element plays an important role in this process. Donor bacteria with this F element can express F pili that form cytoplasmic bridge with the recipient bacteria, allowing the mobile genetic elements to be transferred via the bridge (Holmes and Jobling, 1996). With the introduction of bacteriophage to the bacterial population, more mechanisms of horizontal gene transfer will take place. Bacteriophage plays a role in bacterial evolution by causing changes in bacterial genome via bacteriophage-mediated transduction.

Transduction can be categorized into two types, which are generalized and specialized. Specialized transduction involves the lysogenic life cycle. When prophage induction occurs in lysogenic-converted bacteria, segments of DNA flanking the site of integration of the prophage may be excised along with the phage genome. This piece of DNA segments can be carried to another bacterial cell and integrated into the recipient's chromosome. Then, the transductants can become stable in the recipient host cell after DNA replication take place, resulting in the

replacement of the normal homologous DNA with the transducing DNA. Generalized transduction is an event that takes place in both virulent phage and temperate phage. It involves the lytic cycle and has a random occurrence. During lytic cycle, generalized transduction happens when the phage mistakenly packages the host's DNA into the virions. Unlike specialized transduction that only involves the flanking region of prophage, any genetic elements in the donor host cell can be involved in the generalized transduction process (Paul and Jiang, 2001). However in the recipient host, most of the time the transducing DNA is not stable and remains extrachromosomal. The fate of this transducing DNA in the recipient host is either being degraded or being incorporated into the recipient genome (Stenberg and Maurer, 1991). In some cases, the injected phage DNA can exhibit a pseudotemperate lifestyle. There is no integration of the phage DNA into the host genome. The phage DNA only remains as plasmid and replicates independently in the cytoplasm along with the host (Fortier and Sekulovic, 2013).

Besides transduction, another mechanism of horizontal gene transfer known as transformation can occur as well. During the event of lytic cycle, the lysed bacterial cells release their contents into the environment. The release of free DNA can be taken up by the other bacterial cells via transformation. However, the bacterial cells are required to develop competence for the uptake of free DNA from the environment. According to Charpentier, et al. (2010), they found that competence in *Legionella pneumophila* can be induced by genotoxic event such as UV radiation.

Antibiotics that cause genotoxicity can also cause competence development, which will allow transformation to take place.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strain

The bacteria used in this project is a clinical isolate *Shigella flexneri*, obtained from Hospital University Science Malayasia (HUSM)

3.2 Chemical, Reagents and Equipment

The lists of chemicals and reagents used in this project are listed in Table 3.1.

Table 3.1 List of chemicals and reagents.

Chemicals and reagents	**Manufacturers**
1 kbp DNA ladder	Thermo Scientific
95% ethanol	Chemical Industries (Malaya) Sdn. Bhd.
96 wells plate	Hamburg Chemicals
Agarose powder	PhileKorea
6X loading dye	Thermo Scientific
Ampicillin	Sigma-Aldrich
Acetic acid	Hamburg Chemicals

Table 3.1 List of chemicals and reagents (continued).

Chemicals and reagents	**Manufacturers**
BSA	Thermo Scientific
Deoxyribose nucleoside triphosphate (dNTPs)	Nano Helix
Ethylenediaminetetraacetic acid (EDTA)	Hamburg Chemicals
Gel Red	Biotium
Glycerol	Fisher Scientific
Hydrochloric acid	Fisher Scientific
Kanamycin	Sigma-Aldrich
Luria Bertani agar	Conda
Luria Bertani broth	Conda
Magnesium Chloride	1st BASE
Proteinase K	Nacalai Tesque Inc.
Restriction enzyme *Xba*I	BOIRON
Sodium hydroxide	QRec
Tango Buffer	Thermo Scientific
Taq buffer	1st BASE
Taq polymerase	1st BASE
Trimethoprim	Sigma-Aldrich
Tris	Bio Basic Inc.

The list of equipment and apparatus used in this project are listed in Table 3.2.

Table 3.2 List of equipment and apparatus.

Equipment and apparatus	**Brand/Model**
Autoclave machine	HIRAYAMA
Cellulose acetate syringe filter (0.45 µM)	Pall corporation
Gel electrophoresis set	Major Science
Gel imaging systems	Bio-Rad
Microcentrifuge tubes	Thermo Scientific
Incubator	Memmert
Laminar flow cabinet	ESCO
Media bottle	KIMAX
Microcentrifuge	Thermo
Micropipette	WATSON NEXTY
Microwave	SANYO
PCR machine	Biometra
PCR tubes	AXYGEN
Petri dishes	Greiner bio-one
pH meter	Sartorius
Portable Bunsen burner	CAMPINGAZ®
Pulsed-field gel electrophoresis	Bio-Rad

Table 3.2 List of equipment and apparatus (continued).

Equipment and apparatus	Brand/Model
Refrigerator and freezer	Haier
Shaking incubator	N-BIOTEK
Spectrophotometer	Bio-Rad
Spectrophotometer cuvettes	Bio-Rad
Syringe	TERUMO
Water bath	Memmert
Weighing balance	KERN

3.3 Preparation of Culture Media, Reagents, and Solutions

3.3.1 Preparation of LB Agar

Luria Bertani agar was prepared by weighing 40 g of LB agar powder and then dissolved in 1 L of distilled water. The mixture was mixed well and autoclaved at 121 °C for 15 min. Then, the molten LB agar was poured into sterile petri dish and left to solidify. The prepared agar plates were stored at room temperature.

3.3.2 Preparation of LB Broth

Luria Bertani broth was prepared by weighing 25 g of LB broth powder and then dissolved in 1 L of distilled water. The mixture was mixed well and transferred into universal bottles (5 mL each). The universal bottles containing LB broth were sent

for autoclaved at 121 °C for 15 min. The prepared LB broths were stored at room temperature.

3.3.3 Ethanol (70%)

Five hundred milliliters of 70% ethanol was prepared by topping up 368 mL of 95% ethanol to 500 mL with distilled water.

3.3.4 Preparation of Antibiotics

Trimethoprim was prepared in concentrations of 100 µg/mL and 10 µg/mL. The 100 µg/mL trimethoprim was prepared by dissolving 10 µL of 10 mg/mL trimethoprim into 1.0 mL of sterile distilled water. The 10 µg/mL trimethoprim was prepared by dissolving 1 µL of 10 mg/mL trimethoprim into 1.0 mL of sterile distilled water.

Ampicillin was prepared in concentrations of 1 mg/mL and 100 µg/mL. Ten microliters of 100 mg/mL ampicillin was added to 1.0 mL of sterile distilled water to make 1 mg/mL ampicillin. One microliter of 100 mg/mL ampicillin was added to 1.0 mL of sterile distilled water to make 100 µg/mL.

Kanamycin was prepared in the concentrations of 10 mg/mL and 100 µg/mL respectively. Two hundred and fifty microliters of 50 mg/mL kanamycin was added to 1.0 mL of sterile distilled water to make 10 mg/mL kanamycin. Ten microliters of 10 mg/mL kanamycin was added to 1.0 mL of distilled water to make 100 µg/mL.

The prepared antibiotics were stored in 4^{o}C fridge.

3.3.5 Preparation of Tris-Acetate-EDTA Buffer

Tris-Acetate-EDTA (TAE) buffer was prepared in 10X concentration as stock solutions. Five hundred milliliters of 10x TAE buffer was prepared. Firstly, 24.2 g of Tris Base was dissolved in distilled water. Then, ten millimeter of 0.5 M EDTA (pH adjusted to 8.0 with NaOH) was added into the mixture. Next, 5.7 mL of acetic acid was added into the mixture. The solution was topped up to 500 mL to make 10X TAE buffer. The working solution of TAE buffer (1X) was prepared by mixing 50 mL of 10X TAE buffer with 450 mL of distilled water.

3.3.6 Glycerol

To prepare 100 mL of 80% glycerol, 80 mL of glycerol was mixed with 20 mL of sterile distilled water.

3.3.7 Agarose Gel (1%)

One gram of agarose powder was dissolved in 100 mL of 1X TAE buffer to make 1% agarose gel. The mixture was heated in microwave to completely dissolve the agarose powder. The molten agarose was poured into the gel cast and left to solidify.

3.3.8 Tris-EDTA Buffer

Ten milliliters of 1 M Tris was mixed with 2.0 mL of 0.5M EDTA. The pH was adjusted to 8.0 and the mixture was topped up to 1000 mL with sterile distilled water to make TE buffer (10 mM Tris:1 mM EDTA, pH 8.0).

3.3.9 Cell Suspension Buffer

One hundred milliliters of 1 M Tris and 200 mL of 0.5 M EDTA were mixed and adjusted to pH 8.0. Then, the mixture was topped up to 1000 mL using sterile distilled water.

3.3.10 Cell Lysis Buffer

Fifty milliliters of 1 M Tris and 100 mL of 0.5 M EDTA were mixed and adjusted to pH 8.0. Then, Tris and EDTA were mixed with 100 mL of 10% Sarcosyl. The mixture was then topped up to 1000 mL using sterile distilled water.

3.3.11 Restriction Enzyme Master Mix

For each plug slice, 200 µL of master mix was prepared. It consists of 173 µL of sterile distilled water, 20 µL of 10X restriction buffer, 2 µL of bovine serum albumin (10 mg/mL), and 5 µL of the *Xba*I restriction enzyme (10 U/µL).

3.4 Research Methodology Overview

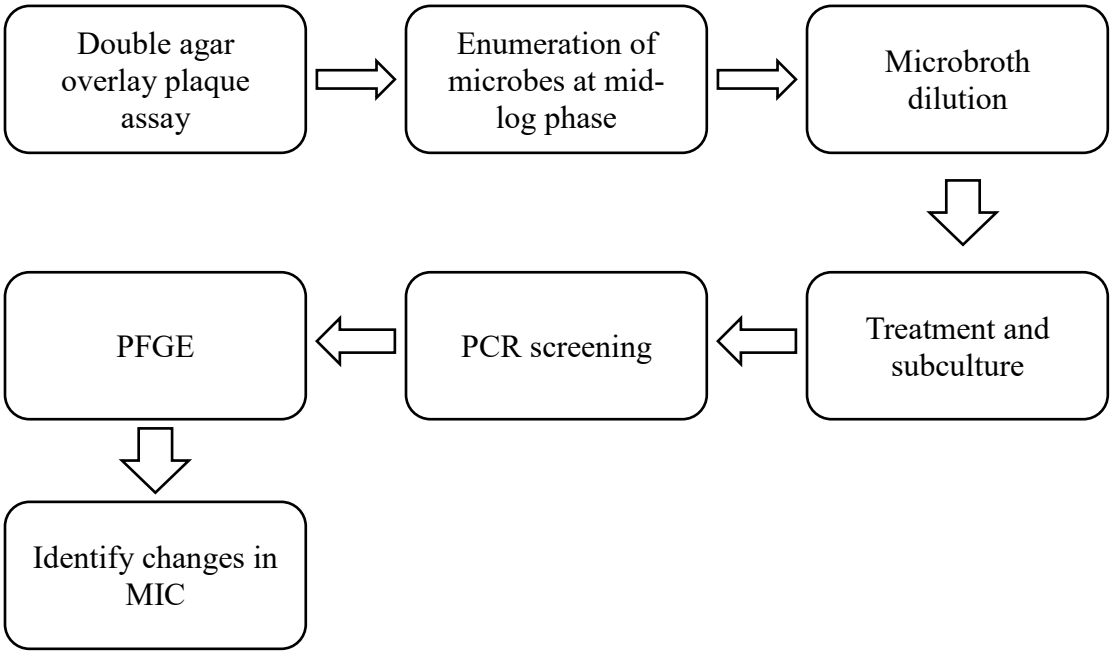

Figure 3.1: **Project methodology outline.**

3.5 Double Agar Overlay Plaque Assay

The double agar overlay plaque assay was adapted from Kropinski, et al. (2009). Two microliters of SFN6B phage was added into mid-log phase (0.4-0.6 OD_{600}) *S. flexneri* culture and incubated for 3 hours at 37 °C with shaking (200 rpm). After

incubation, the supernatant was filtered through 0.45 µM cellulose acetate syringe filter to collect the filtrate containing phage.

Into 11 tubes, 2 mL of molten LB agar (1.5%) were mixed to 2 mL of LB broth to make the overlay top agar (0.75%). Then, the tubes were kept in 50 °C water bath to maintain its liquid state.

One milliliters of LB broth was added to another eleven tubes. Then, serial dilution of 1:1000 of the phage filtrate was performed by transferring 1 µL of the phage filtrate to the first tube, followed by 1 µL from first tube to second tube and so on. The dilutions are 10^{-3}, 10^{-6}, 10^{-9}, 10^{-12}, 10^{-15}, 10^{-18}, 10^{-21}, 10^{-24}, 10^{-27} and 10^{-30}. The last tube was not added with any phage to act as negative control. Next, 100 µL of *S. flexneri* culture was added into each eleven tubes.

Working with one tube at a time, the diluted phage was added with the prepared overlay agar and then immediately poured onto LB agar plates. The agar plates were swirled gently to spread the mixture evenly over the surface of bottom agar. The plates were left for 15 min at room temperature to allow the overlay agar to solidify. Then, the plates were incubated at 37 °C overnight. On the next day, the plaques on each plate were counted and the PFU/mL was calculated.

3.6 Enumeration of *Shigella flexneri* at mid-log phase

Shigella flexneri was grown in 5.0 mL LB broth until mid-log phase (0.4 - 0.6 OD_{600}). Serial dilution of the mid-log phase culture was made (10^{-2}, 10^{-4}, and 10^{-6}). One hundred microliters of diluted bacteria culture was used to spread plate on LB agar plates. The agar plates were incubated at 37 °C overnight. On the next day, number of colonies for each plate was counted and the CFU/mL was calculated.

3.7 Antibiotic Susceptibility Testing with Microbroth Dilution

The minimal inhibitory concentration (MIC) of each antibiotic was tested using the microbroth dilution method. Colonies of *S. flexneri* were inoculated into LB broth and incubated at 37 °C with shaking (200 rpm). The turbidity of the culture was checked periodically until it reached absorbance of 0.08 to 0.13 OD_{625}, which is approximately equal to 1 x 10^8 CFU/mL. While waiting for the incubation, antibiotic two-fold serial dilution was performed. Trimethoprim was diluted from 10 µg/mL using LB broth as diluent. Ampicillin and kanamycin were both diluted from 100 µg/mL using LB broth as diluent. Fifty microliters of diluted antibiotic was added into the each well respectively. An 1:100 dilution of bacterial culture was first performed by transferring 50 µL of the culture to 5 mL LB broth. Then, 50 µL of the diluted cultures were added into each well containing antibiotic. The positive control well was added with 50 µL LB broth and 50 µL of 1:100 diluted culture. The negative control well was added with 100 µL LB broth. After that, the 96 well plate was incubated at 37°C overnight. On the next day, MIC was

determined by identifying the lowest concentration of antibiotic that could inhibit visible growth (Wiegand, Hilpert and Hancock, 2008). Then, 100 µL from the wells of MIC and sub-MICs were spread plate onto LB agar plate to observe colony growth. The concentration that can give colony growth was determined as the sub-MIC to be used in the subsequent treatment.

3.8 Treatment and Subculture

A series of subculture was performed for 8 different sets of *S. flexneri* cultures that were given different treatment. The eight sets are listed below:

A = *S. flexneri* + SFN6B + trimethoprim (Final concentration = 0.08 µg/mL)

B = *S. flexneri* + SFN6B + ampicillin (Final concentration = 6.25 µg/mL)

C = *S. flexneri* + SFN6B + kanamycin (Final concentration = 12.5 µg/mL)

D = *S. flexneri* + trimethoprim (Final concentration = 0.08 µg/mL)

E = *S. flexneri* + ampicillin (Final concentration = 6.25 µg/mL)

F = *S. flexneri* + kanamycin (Final concentration = 12.5 µg/mL)

G = *S. flexneri* + SFN6B

H = *S. flexneri*

The subculture was begun from passage 0 by inoculating few colonies of *S. flexneri* into 5 mL broth and incubated at 37 °C with shaking (200 rpm) until it reached absorbance of 0.08 to 0.13 OD_{625}. Then, 1:200 dilution was performed by transferring 25 µL to 8 universal bottles respectively, each containing 5 mL broth. The eight universal bottles were labeled accordingly based on their respective treatments. Bacterial glycerol stock was made using the passage 0 culture.

The antibiotic treatment was added to the respective cultures. After incubation at 37 ºC with shaking (200 rpm) until 0.4 to 0.6 OD_{600}, bacteriophage treatment was given.

On the next day, the passage 1 cultures were diluted to absorbance of 0.08 to 1.3 OD_{625}. Then, 25 µL of the diluted cultures were transferred to passage 2 broths (2A to 2H) respectively. The same treatment was given to the passage 2 cultures.

For every fifth passage (5, 10, 15, 20…), the bacterial cultures were plated. On the next day after incubation, the colonies were used to perform PCR screening for SFN6B.

Bacterial glycerol stocks were also made for every fifth passage. One millimeter of each bacterial culture was transferred into three sterile microcentrifuge tubes. After centrifugation of the tubes, 600 µL of the supernatant was discarded and the remaining 400 µL was used to suspend the cell pellets. Next, 100 µL of 80 % glycerol was added to each bacterial suspension to make bacterial glycerol stocks with final concentration of 16%. The bacterial glycerol stocks were stored in -80 °C.

3.9 Colony PCR Screening

Twenty microliters of PCR master mix was aliquoted into PCR tubes. Colony was picked using a pipette tip and placed into the PCR tube with master mix. Then, PCR reaction was performed using the following parameters in Table 3.3.

Table 3.3: PCR reaction parameters

Steps	**Temperature (°C)**	**Time (s)**	**Cycle**
Initial denaturation	95	300	1
Cyclic denaturation	95	30	30
Cyclic annealing	58	30	
Cyclic extension	72	30	
Final extension	72	300	1

After PCR, the PCR products were used to run gel electrophoresis. Five microliters of PCR products were mixed with 1 µL of 6X loading dye and then loaded into the well. After running gel electrophoresis, the positive result was compared to positive control (around 500 bp DNA band).

3.10 Pulsed Field Gel Electrophoresis

The protocol of PFGE in this project was adapted from the standard operating procedures from Centers for Disease Control and Prevention (2013).

3.10.1 Making Agarose Plugs

One percent SeaKem Gold Agarose was prepared in TE buffer. Two milliliters of cell suspension buffer was transferred to each microcentrifuge tube. Colonies from agar plates were then transferred to the tubes containing cell suspension buffer. The concentration of cell suspensions was adjusted to the absorbance of 0.8 to 1.0 OD_{610} by either diluting more with cell suspension buffer or concentrating it with more colonies.

3.10.2 Casting Agarose Plugs

Microcentrifuge tubes were labeled and 200 µL of the adjusted cell suspension was transferred into the tubes accordingly. Ten microliters of Proteinase K was added into each tube and mixed well. Then, 200 µL of melted SKG agarose was added to

the cell suspension and mixed gently. The mixture was then dispensed into the plug mold and allowed to solidify.

3.10.3 Lysis of Cells in Agarose Plugs

Cell Lysis Buffer was prepared. The master mix of Cell Lysis Buffer/Proteinase K was prepared by mixing 25 µL of proteinase K with every 5 mL of Cell Lysis Buffer. Then, 5 mL of the prepared master mix was added into each empty labeled tube. The agarose plug was then removed from the mold and placed into the master mix. The tubes were incubated in water bath at 55 °C for 1.5 hours with constant and vigorous shaking.

3.10.4 Washing of Agarose Plugs

After incubation, the master mix was discarded while remaining the agarose plug inside the tubes. Ten milliliters of pre-heated sterile distilled water was added into the tube and the tube was incubated in 55 °C water bath with shaking for 10 min. This washing step was repeated one more time with the pre-heated sterile distilled water, followed by another four more times of washing using pre-heated TE buffer. After discarding the last wash, 10 mL of fresh sterile TE buffer was added to the tube. The plugs were stored at 4°C until the restriction digestion steps.

3.10.5 Restriction Digestion of DNA in Agarose Plugs

Two hundred microliters of 1X restriction buffer was added to each labeled microcentrifuge tube. The agarose plug was cut to about 2 mm wide slice and transferred to the microcentrifuge tube containing restriction buffer. Then, the plug slice was incubated in 37 °C water bath for 5 min. After incubation, the restriction buffer was removed from the plug slice. Restriction enzyme master mix was prepared and 200 µL was added to each tube. The tube was then incubated in 37 °C water bath for 2 hours.

3.10.6 Casting of Agarose Gel

The 1% SKG agarose was prepared in 0.5X TBE buffer by mixing 1.5 g of SKG agarose powder in 150 mL of 0.5X TBE buffer. After microwave heating and subsequent cooling, the agarose was poured into the gel form. The comb was removed after the agarose solidified. Then, 0.5X TBE running buffer was added into the electrophoresis chamber.

3.10.7 Loading Plug Slices into Wells

The restriction enzyme master mix was removed from the restricted plug slice and then the tube was added with 200 µL of 0.5X TBE buffer. The tubes were incubated at room temperature for 5 min. After that, the plug slices were removed from the

tubes and carefully loaded into the wells. The loaded wells were then filled with 1% SKG agarose and allowed to solidify.

3.10.8 Pulsed Field Gel Electrophoresis

The electrophoresis conditions were set as follow:

- Initial switch time = 5 s
- Final switch time = 35 s
- Start Ratio = 1.0
- Voltage = 6 V (for CHEF DR-III) or 200 V (for CHEF DR-II)
- Included Angle = 120°
- Run time = 18 to 19 hours

3.10.9 Staining

After electrophoresis, the gel was stained with ethidium bromide for 30 min. The gel image was observed later.

3.10.10 Software Analysis of PFGE Results

BioNumerics software was used for the analysis of PFGE results.

CHAPTER 4

RESULTS

4.1 Double Agar Overlay Plaque Assay

The agar plates added with 10^{-3}, 10^{-6} and 10^{-9} dilution of phage were almost clear of bacterial growth with only a few colonies remained, which could be the phage-resistant bacteria. This can indicate presence of bacteria that are resistant to bacteriophage infections. Plaques formation can be observed from the phage dilution of 10^{-12} onwards. The plaque has a round circular shape, consisting of a small clear center surrounded by a thicker halo ring as shown in Figure 4.1. The semi-transparent halo that surrounds the clear plaque center shows decreasing lytic efficiency of SFN6B towards the plaque periphery. The number of plaques are too numerous to be counted for 10^{-12} and 10^{-15} dilution of phage. It was only countable from 10^{-18} dilution of phage onwards. Therefore, the virus titer was estimated based on the number of plaques of 10^{-18} phage dilution. The calculated virus titer are as shown in Table 4.1 was 4.5×10^{19} pfu/mL.

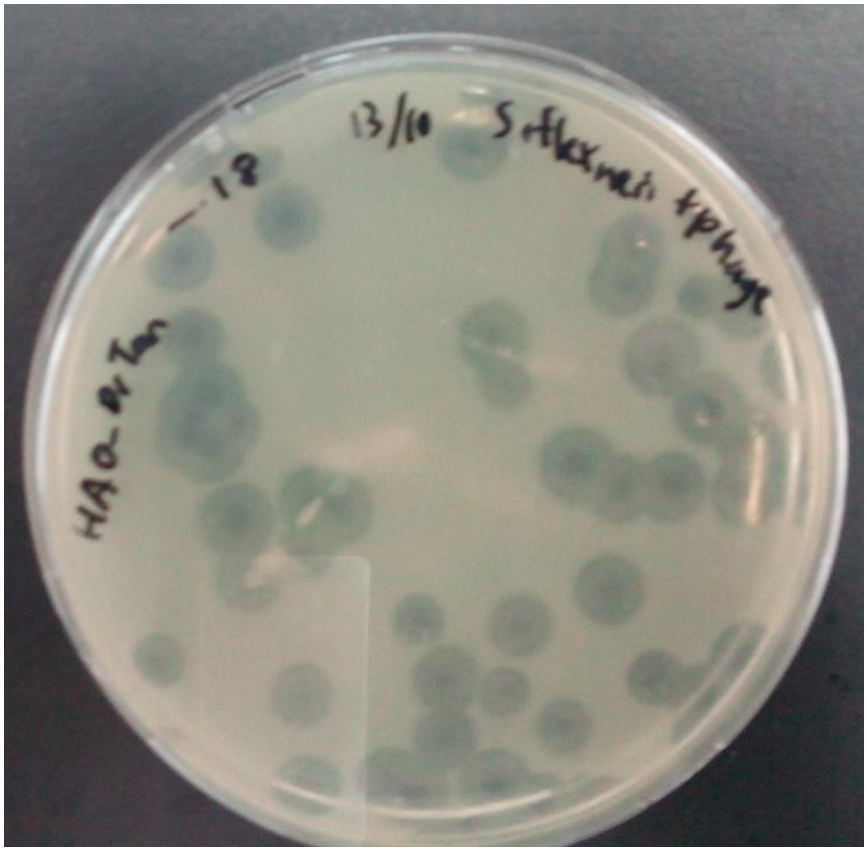

Figure 4.1: The morphology of plaque formed by SFN6B bacteriophage.

Table 4.1 Number of plaques and virus titer count.

Dilution	Number of plaques	Virus titer (PFU/mL)
10^{-3}	-	-
10^{-6}	-	-
10^{-9}	-	-
10^{-12}	Too numerous	-
10^{-15}	Too numerous	-
10^{-18}	45	4.5×10^{19}

4.2 Enumeration of *Shigella flexneri* at mid-log phase

Formation of bacterial colonies can be observed after incubation of the diluted mid-log phase *S. flexneri*. The number of colonies was too numerous to be counted for 10^{-2} and 10^{-4} dilution of the mid-log phase *S. flexneri* cultures. Countable number of colonies was observed at the dilution of 10^{-6} of mid-log phase *S. flexneri* culture (Figure 4.2). Thus, the colony-forming unit was calculated with 10^{-6} dilution and estimated to be 3.5 x 10^{8} cfu/mL (Table 4.2).

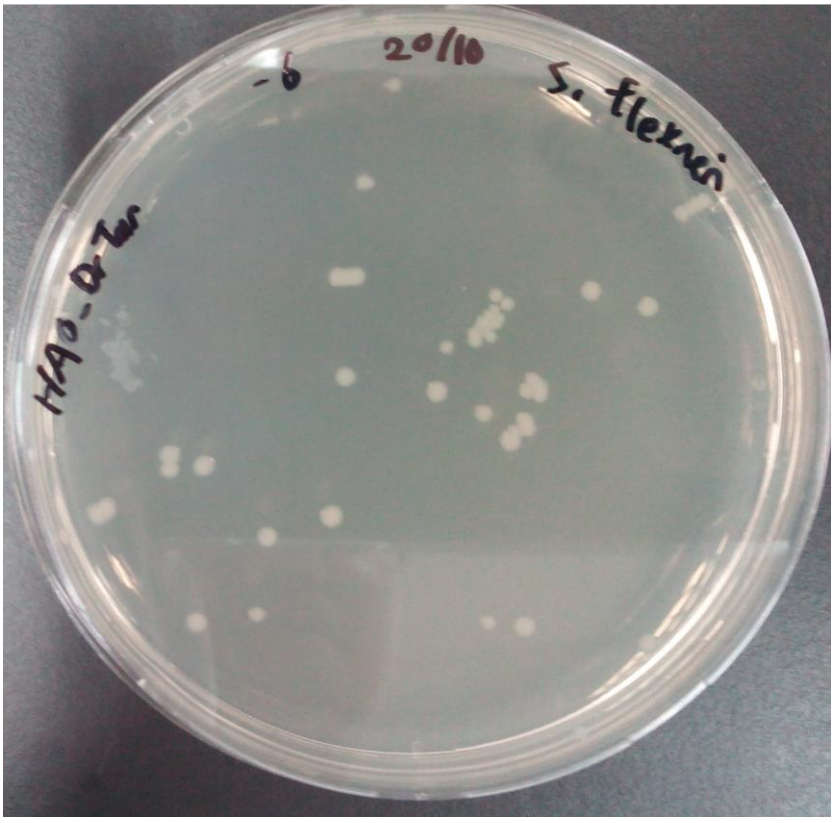

Figure 4.2: The number of colonies formed by the mid-log phase *S. flexneri* at the dilution of 10^{-6}.

Table 4.2 Number of colonies and colony-forming unit calculation

Dilution	Number of colonies	CFU/mL
10^{-2}	Too numerous	-
10^{-4}	Too numerous	-
10^{-6}	35	3.5×10^{8}

Since the virus titer was estimated to be 4.5×10^{19} pfu/mL whereas the mid-log phase bacteria was estimated to be 3.5×10^{8} cfu/mL, the 5 mL mid-log phase culture in every passage was treated with at least 1 µL of SFN6B bacteriophage. It is because multiplicity of infection (MOI) of at least 5 is needed to allow the SFN6B bacteriophage to infect almost every bacterial cell in the culture.

4.3 Initial Minimal Inhibitory Concentration of Antibiotics

Minimal inhibitory concentration is the minimal concentration of antibiotic that inhibits visible growth of bacteria; whereas sub-MIC is the concentration of antibiotic below the MIC value. The passage zero *S. flexneri* culture was tested with MIC assay in order to determine the sub-MIC value of each antibiotic to be given for the subsequent passages. Treatment using sub-MIC of antibiotic did not inhibit bacterial growth but only slow down the growth rate of bacteria throughout the passages. The MICs and sub-MICs of the three antibiotics used, trimethoprim (TMP), ampicillin (AMP) and kanamycin (KAN) are shown in Table 4.3. Based on the result, the passage zero *S. flexneri* was susceptible to trimethoprim (MIC = 0.15625 µg/mL). It also showed intermediate susceptibility to ampicillin (MIC = 12.5 µg/mL) and kanamycin (MIC = 25 µg/mL).

Table 4.3 Minimal inhibitory concentrations (MIC) and sub-MIC of trimethoprim, ampicillin and kanamycin in passage zero *S. flexneri*.

Antibiotics	**MIC (µg/mL)**	**Sub-MIC (µg/mL)**	**Reference range (µg/mL)**	
			S	R
Trimethoprim (TMP)	0.16	0.08	≤8	≥16
Ampicillin (AMP)	12.5	6.25	≤8	≥32
Kanamycin (KAN)	25	12.5	≤16	≥64

"S" represents susceptible; "R" represents resistance.

(Drugs.com, 2015; Drugs.com, 2016; Drugs.com, 2017)

4.4 Colony PCR Screening

In every 5 passage, PCR was carried out using SFN6B-specific primer to screen for the presence of SFN6B bacteriophage in the colonies to obtain lysogenic-converted *S. flexneri*. Based on Table 4.4, SFN6B bacteriophage remained in the colonies of *S. flexneri* culture that was treated with trimethoprim throughout the passages. As for the *S. flexneri* culture treated with ampicillin and SFN6B bacteriophage, the virus seems to be lost in passage 15 and 20, but was able to infect the bacteria again during passage 25 and 30. The colonies of *S. flexneri* culture treated with kanamycin and SFN6B bacteriophage also lost the virus at passage 20 and later the virus was detected again in passage 25 onwards. As for the *S. flexneri* culture

treated with only SFN6B bacteriophage, the result is rather inconsistent. The SFN6B bacteriophage was not detected in the colonies initially in passage 5, but was detected in passage 10. There was no detection again in passage 15 and 20, but was detected later in the colonies of passage 25 and 30.

Table 4.4: Colony PCR screening result from passage 5 to 30.

Passage\Treatment	TMP+Phage	AMP+Phage	KAN+Phage	Phage
5	+	+	+	-
10	+	+	+	+
15	+	-	+	-
20	+	-	-	-
25	+	+	+	+
30	+	+	+	+

"+" represents positive detection; "-" represent negative detection; "TMP" represent trimethoprim; "AMP" represents ampicillin; "KAN" represents "kanamycin"

4.5 Changes in Minimal Inhibitory Concentration of Antibiotics

The minimal inhibitory concentrations (MIC) of trimethoprim, ampicillin and kanamycin were determined for the *S. flexneri* cultures in passage 10, 20 and 30. The assay was repeated for two times and the results of MIC were consistent. The MIC values for each treated culture from passage 10 to 30 were displayed in scatter

plots as shown in Figure 4.3 (Trimethoprim), Figure 4.4 (Ampicillin) and Figure 4.5 (Kanamycin).

Based on Figure 4.3, the MIC of trimethoprim in untreated culture shows consistent value (0.16 µg/mL) throughout the passages, which is also the same value as the initial MIC in passage zero culture shown in Table 4.3. Both *S. flexneri* cultures that were treated with only trimethoprim and cultures that were treated with SFN6B bacteriophage and trimethoprim show increase in MIC as compared to the untreated culture. By comparing both of these two cultures, the trimethoprim-only treatment gave rise to higher and faster increase in MIC. In passage 5, the *S. flexneri* culture that was treated with SFN6B bacteriophage and trimethoprim only had slight increase in MIC. Later in passage 10, its MIC was further increased.

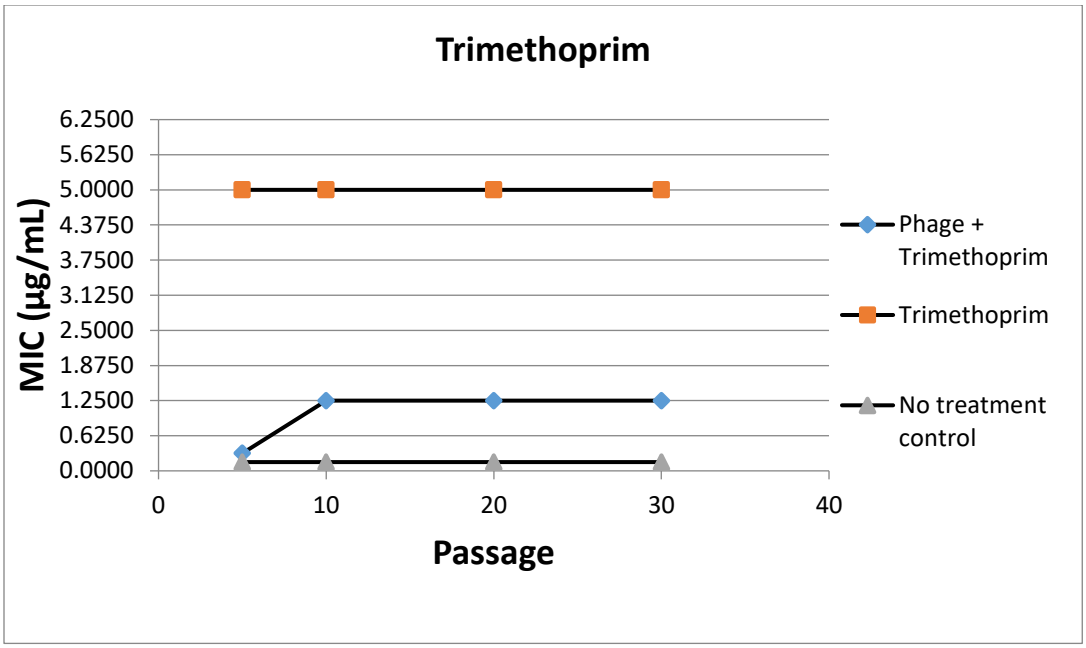

Figure 4.3: Changes in MIC of trimethoprim in trimethoprim-treated and combined phage-trimethoprim treated *S. flexneri* culture throughout 30 passages.

Based on Figure 4.4, the MIC of ampicillin in untreated culture shows consistent value (12.5 µg/mL) throughout the passages, which is also the same value as the initial MIC in passage zero culture shown in Table 4.3. Both *S. flexneri* cultures that were treated with only ampicillin and cultures that were treated with SFN6B bacteriophage and ampicillin show increase in MIC as compared to the untreated culture. Both these cultures had the same MIC values initially in passage 10. Later in passage 20 and 30, only the culture treated with only ampicillin had further increase in MIC from 25 µg/mL to 50 µg/mL; whereas the culture treated with

SFN6B bacteriophage and ampicillin remained as 25 µg/mL. By comparing these two cultures, the ampicillin-only treatment gave higher increase in MIC.

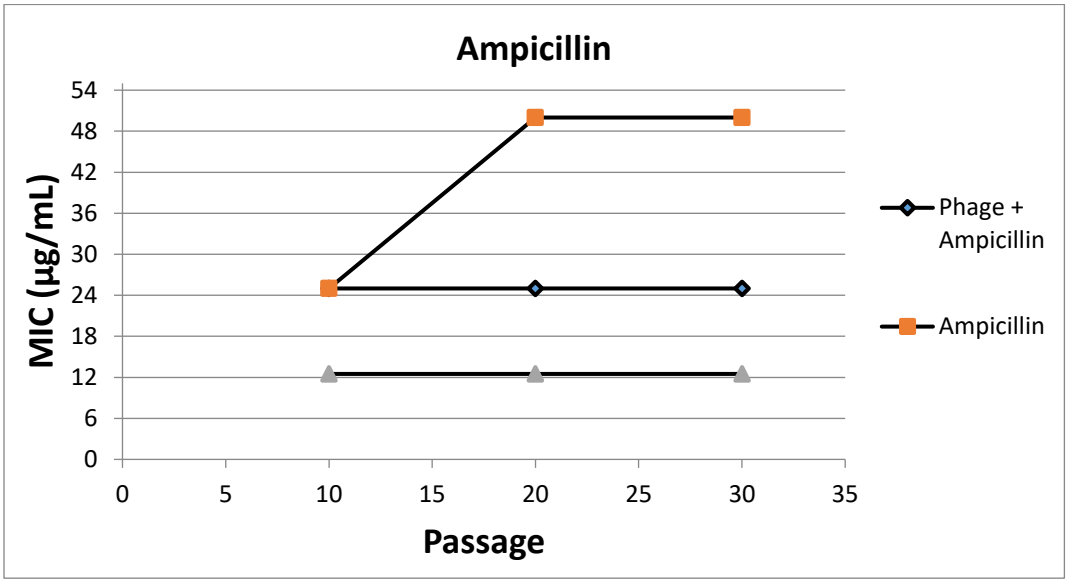

Figure 4.4: Changes in MIC of ampicillin in ampicillin-treated *S. flexneri* culture and combined phage-ampicillin treated *S. flexneri* culture throughout 30 passages.

Based on Figure 4.5, the MIC of kanamycin in untreated culture shows consistent value (25 µg/mL) throughout the passages, which is also the same value as the initial MIC in passage zero culture shown in Table 4.3. Both *S. flexneri* cultures that were treated with only kanamycin and cultures that were treated with SFN6B bacteriophage and kanamycin show increase in MIC as compared to the untreated culture. However, unlike the previous two antibiotics, kanamycin-only treatment

did not result in higher MIC value than the cultures with SFN6B bacteriophage and kanamycin treatment.

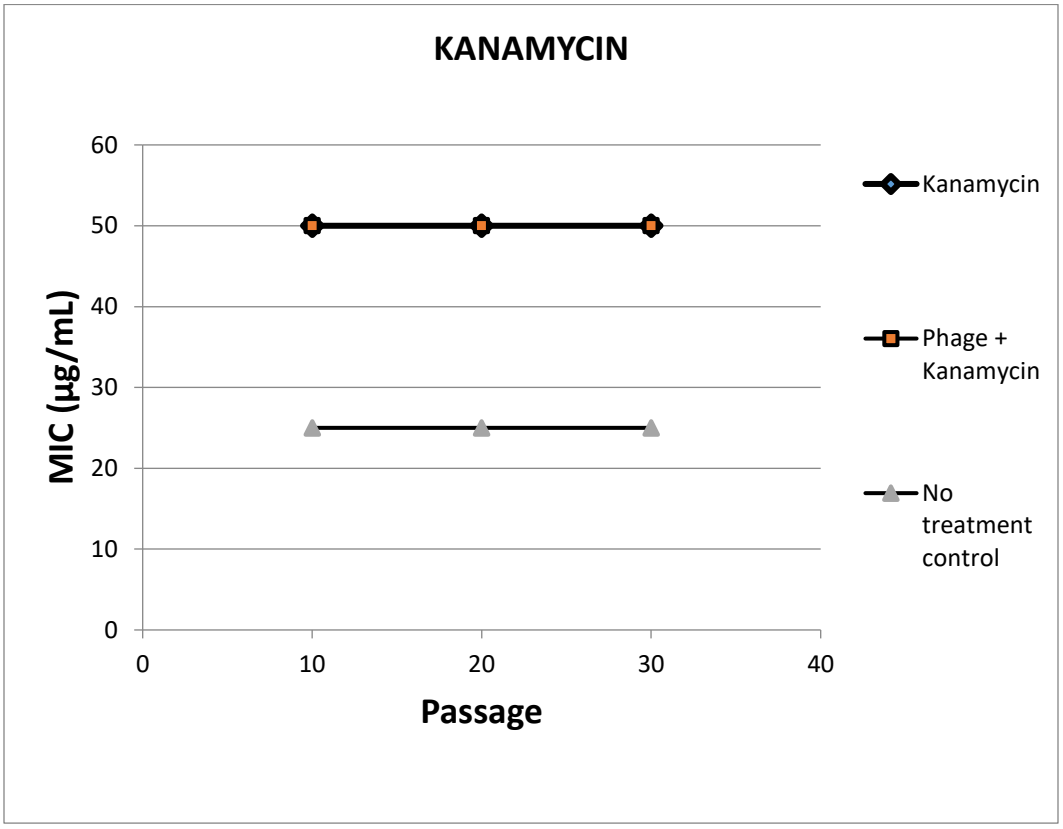

Figure 4.5: Changes in MIC of kanamycin for kanamycin-treated *S. flexneri* culture and combined phage-kanamycin treated *S. flexneri* culture throughout 30 passages.

4.5 Pulsed Field Gel Electrophoresis Analysis

Due to time constraint, pulsed-field gel electrophoresis (PFGE) was not carried out for every *S. flexneri* culture in every passage. Passage 30 cultures with various

different treatments were used to carry out PFGE (Figure 4.6). Overall there is no significant changes when comparing with the initial *S. flexneri* culture (Passage zero). Some slight difference can be observed in three lanes, which is the culture that was treated with trimethoprim and SFN6B bacteriophage, ampicillin and SFN6B bacteriophage, and kanamycin and SFN6B bacteriophage respectively (second to fourth lane in Figure 4.6). For the cultures treated with trimethoprim and SFN6B bacteriophage and the cultures treated with kanamycin and SFN6B bacteriophage, there was one additional band, estimated to be about 55 kbp in size. As for the culture treated with ampicillin and SFN6B bacteriophage, there were two additional bands observed in that lane, which are estimated to be 325 kbp and 285 kbp in size respectively.

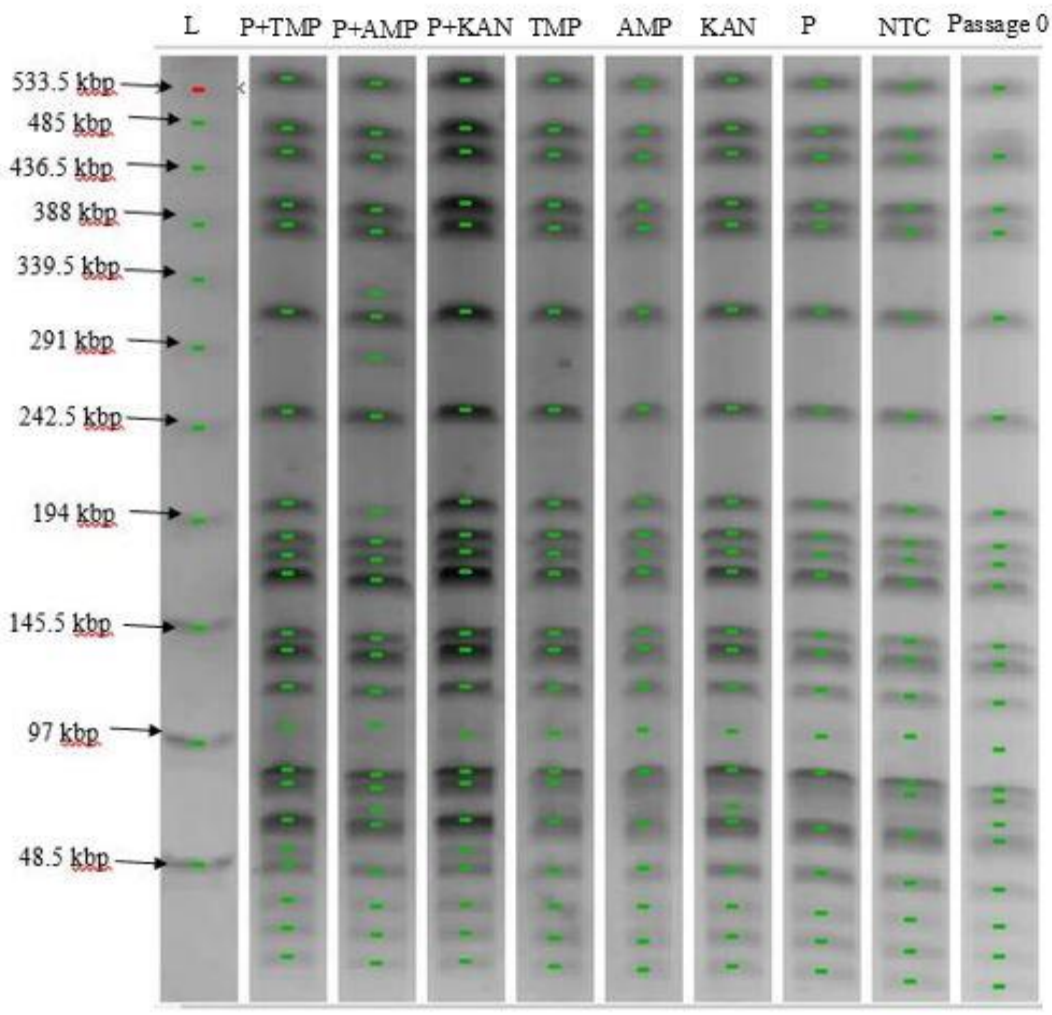

Figure 4.6: Result analysis of Pulsed-Field Gel Electrophoresis photo for passage 30 cultures. "L" represents Ladder; "P" represents SFN6B phage treatment; "TMP" represents trimethoprim treatment; "AMP" represents ampicillin treatment; "KAN" represents kanamycin treatment; "NTC" represents no treatment control.

PFGE was also carried out for the cultures that were treated with SFN6B bacteriophage and ampicillin from passage 5 to passage 30 (Figure 4.7). The same additional bands (325 and 285 kbp) was observed for passage 15, 20 and 30. The additional bands were not observed in passage 5 and 10.

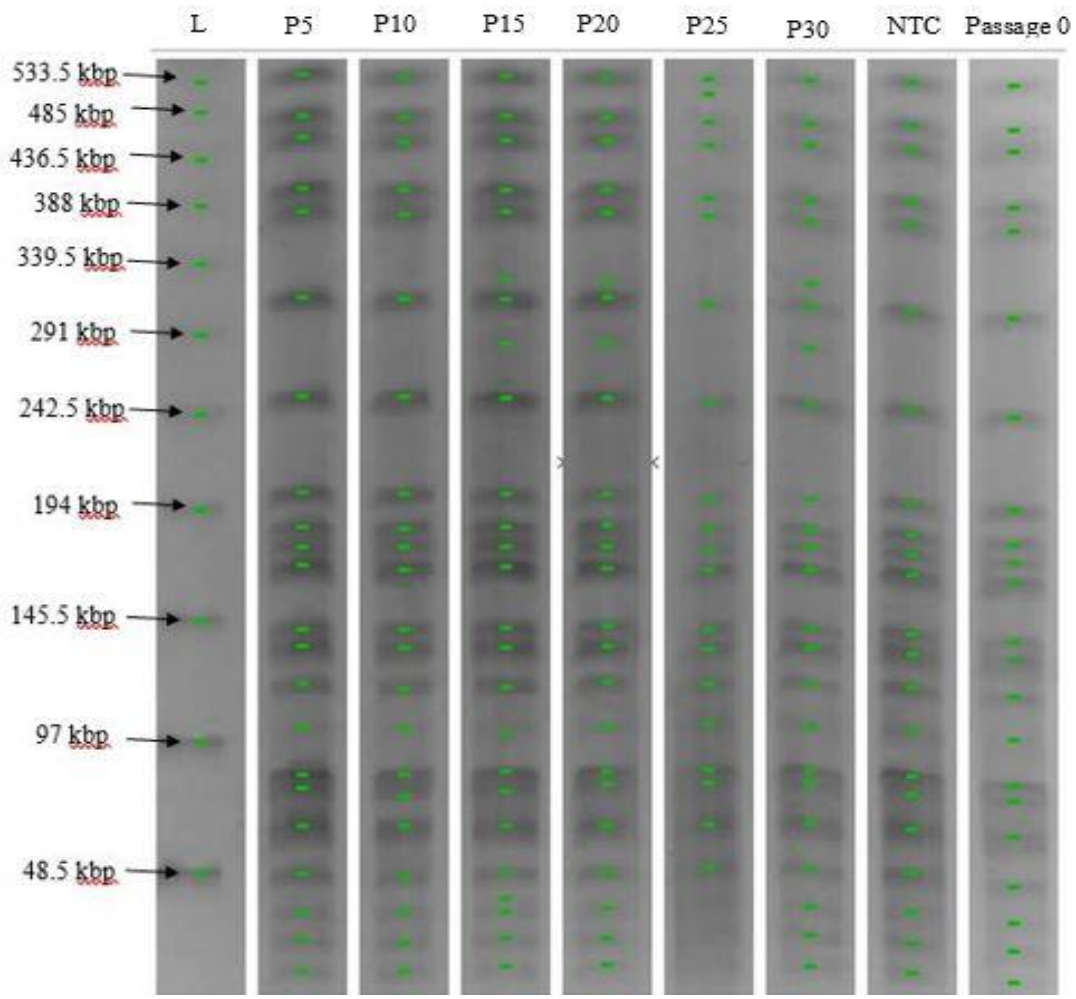

Figure 4.7: Result analysis of Pulsed Field Gel Electrophoresis photo for combined phage-ampicillin treated *S. flexneri* culture from passage 5 to passage 30. "P" represents the passage number; "NTC" represents no treatment control.

CHAPTER 5

DISCUSSION

5.1 Colony PCR Screening

Negative detection in the colony PCR screening suggests the presence of SFN6B bacteriophage-resistant bacteria. However, the reappearance of lysogenic-converted bacteria at the later passage showed that the bacteriophage resistance did not persist throughout the passages. In order to understand this event, the mechanism of the immune response in bacterial cells need to be understood as well.

Clustered regularly interspaced short palindromic regions (CRISPR) and CRISPR-associated (Cas) proteins is a system found in bacteria that is similar to the RNA interference system found in eukaryotic organisms (Makarova, et al., 2006). The CRISPR-Cas system is suggested to play a very important role in the development of bacteriophage resistance, which resulted in the negative result in colony PCR screening. It acts like a form of adaptive immunity for the host bacteria to prevent the invasion of bacterial viruses. The bacteriophage DNA can be acquired by this CRISPR-Cas system and the DNA will be incorporated into the CRISPR locus as a “spacer” sequence. This spacer sequence is heritable to the subsequent progeny of the bacterial cell. The spacer sequence will eventually be transcribed into RNA to form complex with the Cas proteins. The complex

will then bind to the invading bacteriophage DNA and degrade them (van der Oost, et al., 2009).

It was noted that even a single point mutation within the spacer sequence will eliminate the resistance ability of the host (Deveau, et al., 2008; Mojica, et al., 2009). Another interesting finding is that older spacer sequences often undergo rapid changes in their sequences. It was proposed that the rapid changes in the spacer is an essential ability of the bacteria to generate new resistance type in an environment where evolutionary changes of bacteriophage are rapid. Thus, the immunity mediated by the CRISPR-Cas system is only short term. After many subsequent generations, the immunity towards a particular bacteriophage can fade. This explains the inconsistencies of the colony PCR results throughout the 30 passages (Levin, 2010).

5.2 Initial Hypothesis of Bacteriophage Effect on Antibiotic Resistance Evolution

Initially, combined phage and antibiotic treatment was expected to increase the development of antibiotic resistance in the bacteria. This is because bacteriophage can act as a mobile genetic elements (MGEs) that helps to carry antibiotic resistance genotype from one bacteria to another. This occurs in the event of specialized transduction or generalized transduction as mentioned in the literature review. One study demonstrated that bacteriophage carries large

reservoir of antibiotic resistance genes and thus can help to spread antibiotic resistance in a bacterial population (Lekunberri, Subirats, Borrego, and Balcazar, 2016). Another study also observed that bacteriophage was capable of transducing antibiotic resistance genes which lead to the rapid spread of antibiotic resistance genes in the *Staphylococcus aureus* population (Haaber, et al., 2016). Based on these findings and the knowledge on virus-mediated transduction, the hypothesis that bacteriophage can increase the evolution of antibiotic resistance in bacteria was made in this study.

5.2.1 Contradiction to Hypothesis

Despite the initial hypothesis, the findings in this study contradicts with the expected result. Nonetheless, there are several other studies that actually supported the findings in the study. In a study by Chan, et al. (2016), it was shown that the minimal inhibitory concentrations (MICs) of antibiotics such as ciprofloxacin, tetracycline, ceftazidime, and erythromycin is lower in the phage-resistant *Pseudomonas aeruginosa* than the phage-sensitive type. The selection for phage resistance during the process of evolution in *P. aeruginosa* resulted in a trade-off, which increased the bacteria's sensitivity towards the antibiotic antimicrobial actions while granting them the resistance towards bacteriophage infection. While looking into the mechanism of this trade-off, it was observed that the *P. aeruginosa*-specific bacteriophage binds to the efflux pump during the infection. The development of phage resistance in the bacteria caused alteration to the efflux pump, resulting in phage-resistant, but also reducing the

function of efflux pump to remove antibiotics out from the bacterial cells. A study by Zhang and Buckling (2012) also showed that the bacteriophage can decrease the kanamycin resistance level in *Pseudomonas fluorescens* bacteria. Besides, another study also discovered that bacteriophage was able to slow down the increase in MICs of trimethoprim and carbenicillin in *P. aeruginosa* bacteria that were given combined bacteriophage and antibiotic treatments. However, the presence of bacteriophage treatment was not able to slow down the increase in MIC for gentamicin, an aminoglycoside class of antibiotics. The gentamicin resistance level was the same in *P. aeruginosa* with or without bacteriophage treatment (Torres-Barcelo, et al., 2016). This indicates a possibility whereby not all types of antibiotic resistance are affected by the presence of phage treatment. This possibility is further confirmed in another study by Zhang (2013). It was found out that bacteriophage did not reduce the level of resistance towards antibiotics such as cefotaxime, chloramphenicol and kanamycin in *P. fluorescens* bacteria. The level of resistance for these antibiotics were similar with or without bacteriophage treatments.

5.3 Effect of Phage Resistance to Antibiotic Resistance Level

The treatment of SFN6B bacteriophage in this study resulted in lysis of susceptible bacteria. As a result, there were two remaining categories of bacteria that survived in the population with one that was resistant to bacteriophage infection, and another one that was lysogenic-converted bacteria. The presence of bacteriophage resistance could result in slower increase in the antibiotic

resistance level due to evolutionary trade-off. When the bacteria develop a resistance mechanism, they will suffer a fitness cost. The fitness cost of bacteriophage resistance is significantly higher than the fitness cost of antibiotic resistance (Zhang, 2013). Besides, it was shown that treatment of antibiotic using higher sub-MIC will cause the bacteria to have lower fitness than the bacteria growing in antibiotic of lower sub-MIC. In other words, higher level of resistance is associated with higher level of fitness cost (Melynk, Wong and Kassen, 2014).

For this reason, double resistance towards both bacteriophage and antibiotic is very rare. Bacteria with double resistance will have very low fitness and grow very slowly, which slow down the antibiotic resistance evolution as demonstrated by Zhang (2013). The bacteriophage-resistant bacteria that have already suffered high fitness cost which can only develop lower level of antibiotic resistance. The bacteriophage-sensitive bacteria have relatively higher fitness and thus can develop higher level of antibiotic resistance. This is one of the reasons that explains the slower development of antibiotic resistance during combined antibiotic and bacteriophage treatment as compared to treatment of antibiotic only in this study. Further studies need to be carried out to look into the mechanism of the evolutionary trade-off. It is possible that the SFN6B bacteriophage resistance mechanism can somehow interfere with the development of antibiotic resistance in the *S. flexneri* bacteria.

5.4 Antibiotic Resistance Level in Lysogenic Converted Bacteria

As mentioned earlier, another remaining population that survived after lysis by SFN6B bacteriophage were those lysogenic-converted bacteria. Presence of bacteriophage in the bacteria can sometimes affect certain phenotype. This was demonstrated in bacteria such as *Vibrio cholerae* that was infected by filamentous phage, which increases the bacterial virulence by encoding cholera toxin (Waldor and Mekalanos, 1996). In this study, the presence of SFN6B bacteriophage inside the *S. flexneri* bacteria seemed to reduce the antibiotic resistance level. This could be probably due to certain interactions between the mechanism of antibiotic resistance and SFN6B activity inside the bacterial cells.

5.5 Changes in Minimal Inhibitory Concentration of Trimethoprim

The result in Figure 4.3 demonstrated that the presence of bacteriophage can decrease the resistance level of trimethoprim. This result is consistent with the findings from the study by Torres-Barcelo, et al. (2016), which also showed that the bacteriophage treatment can significantly decrease the MIC of trimethoprim as compared to no phage treatment.

5.5.1 Trimethoprim Mechanism of Action

Trimethoprim is a folate antagonist that targets the dihydrofolate reductase enzyme in the bacteria. It is an analog of dihydrofolic acid that can act as competitive inhibitor of the dihydrofolate reductase enzyme. Due to this, the production of tetrahydrofolic acid is reduced and the DNA synthesis in the bacteria is affected (Avery, 1971).

5.5.2 Trimethoprim Resistance Mechanism and Possible Interaction with Lysogenic Phage

The trimethoprim resistance mechanism includes alteration of antibiotic target and increasing the target production. The alteration of antibiotic target occurs by the production of a high molecular weight dihydrofolate reductase enzyme that has reduced sensitivity towards the trimethoprim action. The production of normal dihydrofolate reductase can also increase the counter the action of trimethoprim (Brogden, et al., 1982).

Based on the trimethoprim resistance mechanism mentioned above, one suggestion can be made to explain how presence of SFN6B bacteriophage in the bacterial cell can reduce the trimethoprim resistance level. SFN6B bacteriophage in the host bacterial cell may hijack the cellular machinery, which includes the protein translation activity of the host. Due to this, the host cell can have reduced production of host protein. This could affect the production of dihydrofolate

reductase enzyme in the host. As a result, the host cell could not increase the synthesis of normal dihydrofolate reductase enzyme. The host cell also could only produce lesser high molecular weight dihydrofolate reductase. Hence, the lysogenic-converted host cells have lesser ability to counter the mechanism of actions of trimethoprim as compared to the uninfected bacteria. Further studies will need to be carried out to investigate and confirm this interaction between trimethoprim resistance mechanism and SFN6B activity in the bacterial cell.

5.6 Changes in Minimal Inhibitory Concentration of Ampicillin

Based on the result in Figure 4.4, it shows that the presence of bacteriophage treatment could reduce the development of ampicillin resistance. However, the effect of bacteriophage on ampicillin resistance is slower as compared to trimethoprim. This was observed in passage 10, where the MIC for both ampicillin-only treatment and combined ampicillin-bacteriophage treatment are the same. The MICs of these two cultures only began to differ in passage 20, where the ampicillin-only treatment have higher MIC while the MIC from combined ampicillin-bacteriophage treatment remains. The study by Torres-Barcelo, et al. (2016) also demonstrated that the MIC of carbenicillin, which is also a beta lactam antibiotic like ampicillin, have smaller variation between presence and absence of bacteriophage treatment as compared to trimethoprim.

5.6.1 Ampicillin Mechanism of Action

Ampicillin is one of the antibiotics from beta lactam class. Its antibacterial action involves the inhibition of cell wall synthesis. It binds to the penicillin-binding proteins (PBPs) in the bacteria, which is an essential enzyme for the cross-linking of peptidoglycan during bacterial cell wall synthesis to give a rigid cell structure. The binding of ampicillin to the active site of PBP will affect the enzyme binding to peptidoglycans, impairing its function (Kotra and Mobashery, 1998).

5.6.2 Ampicillin Resistance Mechanism and Possible Interaction with Lysogenic Phage

The ampicillin resistance mechanisms include production of PBP with low affinity to ampicillin, increasing production of PBP, and also production of beta lactamase enzyme (Miller, Munita and Arias, 2014). Similar to trimethoprim resistance mechanisms, the ampicillin resistance mechanism requires the production of host proteins to allow the host cell to continue cell wall synthesis without interference by the ampicillin action. Hence, the same suggestion can be made, where the SFN6B bacteriophage in the host cell could hijack the translation machinery that reduces the host protein productions. The reduced synthesis of PBP and beta lactamase will cause the host cell to be less tolerating the ampicillin antimicrobial action. As a result, the MIC of ampicillin did not increase as much in the cultures treated with both bacteriophage and ampicillin as compared to the cultures with ampicillin-only treatment. Further studies need

to be carried out to confirm the interaction of SFN6B bacteriophage with ampicillin resistance mechanisms in the bacteria.

5.7 Changes in Minimal Inhibitory Concentration of Kanamycin

Unlike the previous two antibiotics, presence of SFN6B bacteriophage in the bacterial cell did not affect the development of kanamycin resistance as shown in Figure 4.5. This result is similar to the findings in a study by Zhang (2013), which found out that bacteriophage has little impact on the kanamycin resistance development. Another antibiotic with the same aminoglycoside class, gentamicin, also gave similar result according to a study by Torress-Barcelo, et al. (2016).

5.7.1 Kanamycin Mechanism of Action

Kanamycin is one of the aminoglycoside class of antibiotic. It inhibits translation and protein synthesis by targeting the 30S subunit of ribosomal protein in bacteria. The inhibition of 30S initiation complex formation will lead to subsequent inhibition of 70S initiation complex, resulting in the inhibition of protein synthesis (Imran Bashir and Cho, 2016).

5.7.2 Kanamycin Resistance Mechanism and Possible Interaction with Lysogenic Phage

One of the resistance mechanism for kanamycin is methylation of 16S rRNA, which is part of the 30S ribosomal subunit structure. The methylated 16S rRNA can prevent the binding of aminoglycoside and thus the translation process and protein synthesis will not be inhibited by the antibiotics (Lioy, et al., 2013; Doi and Arakawa, 2007; Moric, et al., 2010). Other mechanisms of kanamycin resistance include reduced antibiotic uptake by decreasing cell wall permeability, which is commonly seen in the *Pseudomonas spp.* (Chambers and Sande, 1995). Aminoglycoside-modifying enzyme is also one of the resistance mechanism that can help bacteria to counter the kanamycin antimicrobial actions (Davies and Wright, 1997).

As suggested earlier, the SFN6B bacteriophage infection could probably hijack the translation machinery in the host cell and reduce host protein production. Since kanamycin resistance level was not affected in the presence of bacteriophage infection in this case, it can be proposed that the kanamycin resistance mechanism was not affected by the SFN6B bacteriophage activity in the host bacterial cell. One of the resistance mechanism, methylation of 16S rRNA could be affecting the SFN6B bacteriophage action in hijacking the translation machinery. Another possibility is that the reduced host protein production caused by SFN6B bacteriophage did not interfere with the mechanism of 16S rRNA methylation. As a consequence, the development of kanamycin resistance in the host bacteria was not affected. Therefore, the

kanamycin resistance level in the presence of SFN6B bacteriophage infection remained the same as the resistance level in the absence of SFN6B bacteriophage infection. Further studies need to be carried out to find out why SFN6B bacteriophage did not interfere with the kanamycin resistance mechanisms in the bacteria.

5.8 Genomic Changes in Lysogenic Converted Bacteria

Due to time constraint, pulsed-field gel electrophoresis (PFGE) was only carried out for two times, one for the passage 30 cultures, another for the cultures treated with SFN6B bacteriophage and ampicillin throughout all the passages. Overall, there was no significant genomic changes in *S. flexneri* cultures throughout the treatment. However, there were still additional bands that present in the lane containing the bacteriophage-ampicillin treated cultures. The two additional bands were estimated to be 325 kbp and 285 kbp in size. Based on calculation of the band sizes, these two additional bands were probably due to incomplete digestions. This speculation is due to lower intensity for the 200 kbp band as compared to the other lanes. Complete digestion of 325 kbp should produce another two bands, 125 kbp and 200 kbp in size, which are the same size as the existing bands. As for the additional 285 kbp band, complete digestion should produce another two bands, 200 kbp and 85 kbp in size, which can also be found as the existing bands. Thus, incomplete digestion was suggested to be the reason for the presence of additional bands. In order to confirm this, PFGE needs to be further repeated with adequate optimization in the PFGE procedures. Incomplete

digestion can be ruled out by extending the incubation time during restriction digestion, using higher concentration of restriction enzyme, and increasing the number of plug washing to remove proteinase K (CDC, 2011).

CHAPTER 6

CONCLUSION

In a nutshell, SFN6B lysogenic converted *Shigella flexneri* was able to be obtained under antibiotic stress. Nonetheless, SFN6B bacteriophage was not able to stay inside the bacterial cells permanently as evident by occasional negative result in the colony PCR screening throughout the 30 passages. These indicated that the bacteria could exhibit immunity towards the bacteriophage. Re-infection in the later passage after the negative result was successful, which shows that the bacteriophage resistant was not permanent.

Moreover, SFN6B bacteriophage was able to slow down the development of trimethoprim and ampicillin resistance in lysogenic-converted *S. flexneri*. This suggests that bacteriophage can be used as a strategy to slow down the emergence of antibiotic-resistant bacteria in the clinical settings. However, SFN6B bacteriophage did not have any influence on the development of kanamycin resistance in lysogenic-converted *S. flexneri*. More studies are needed to be carried out to study the interaction of antibiotic resistance mechanism and bacteriophage activity in the host cells.

Overall, SFN6B lysogenic converted *S. flexneri* did not have any significant genome changes based on the PFGE results. Only two additional bands in the size of 325 kbp and 285 kbp were observed in the *S. flexneri* culture treated with ampicillin and SFN6B bacteriophage. The presence of addition bands was probably due to incomplete digestion. Further studies need to be carried out to confirm this possibility.

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