INVESTIGATING THE POTENTIALS OF PHAGE THERAPY IN *BURKHOLDERIA PSEUDOMALLEI* INFECTION

ONG GUANG HAN

DISSERTATION SUBMITTED IN FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF MEDICAL SCIENCES

FACULTY OF MEDICINE

UNIVERSITY OF MALAYA

KUALA LUMPUR

2016

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate**: Ong Guang Han**

Registration/Matric No: **MGN 100049**

Name of Degree: **Master of Medical Sciences**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Investigating the Potentials of Phage Therapy in *Bukholderia pseudomallei* Infection

Field of Study: **Medical Microbiology**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before

Witness Signature Date:

Name:

Designation:

ABSTRACT

Melioidosis is a fatal disease caused by the Gram negative bacterium, *Burkholderia pseudomallei*. The routes of infection of melioidosis include ingestion, inhalation and inoculation, with the latter two are believed to be the main routes of infection. The vast clinical manifestations of the infection and the intrinsically antibiotic resistant nature of the bacteria have caused the diagnostic and treatment of the disease difficult. Furthermore, no licensed vaccine for melioidosis has been registered so far. Phage therapy may be the solution for prophylactic prevention and novel antimicrobial agent for melioidosis. Hereby, the potential of phage therapy on *B. pseudomallei* infection was investigated.

Firstly, environmental samples comprising of sewage, soil, fresh and coastal sea water were collected from various locations. These samples were enriched and tested for the presence of *B. pseudomallei* phages. A total of 43 phages were isolated and their host range was determined against 43 strains of clinical isolates of *B. pseudomallei* in the lab's collection. Then, based on their location and host range, five isolates were chosen for propagation and their DNA was extracted for restriction digestion analysis. It was found that they fell under three different restriction profiles. Under transmission election miscroscopy, all three strains were categorised under family Myoviridae. One of the phages, C34 which can constantly propagated to high titre was chosen for time kill curve. The result showed that C34 was able to reduce the bacterial load in liquid culture.

Experimental phage therapy was then carried out in cell culture model and mice model. In A549 human lung epithelial cell lines, C34 successfully protected 41.6 ± 6.5% of A549 cells when administered 24 hours prior to *B. pseudomallei* infection.

Intraperitoneal injection of phage into intranasal-infected BALB/c mice successfully rescued 33.3% infected mice at the end of the 14 days experiment therapy. It was also shown that phage application was able to reduce the bacterial load in the spleen of the infected mice, and that C34 persisted longer in infected mice as compared to healthy mice injected with C34.

In short, C34 can be a potential candidate in phage therapy on *B. pseudomallei* infections.

ABSTRAK

Melioidosis adalah satu penyakit berjangkit merbahaya yang disebabkan oleh *Burkholderia pseudomallei*, sejenis bakteria Gram negatif. Penyakit ini boleh dijangkiti melalui pemakanan, pernafasan, dan pendedahan bahagian luka kepada sumber-sumber yang dicemari bakteria tersebut. Melioidosis sukar untuk didiagnostikkan kerana penyakit ini mempunyai manifestasi klinikal yang agak luas. Ia juga sukar diubati kerana bakteria ini rintang terhadap banyak antibiotik secara semula jadi. Tambahan lagi, setakat ini, tiada vaksin terhadap jangkitan ini telah dihasilkan. Oleh itu, terapi menggunakan bakteriofaj mungkin adalah alternatif untuk mencegah jangkitan melioidosis. Dalam kajian ini, potensi terapi bakteriofaj terhadap jangkitan *B. pseudomallei* telah disiasat.

Sampel merangkumi sisa kumbahan, tanah, air tawar, dan air laut telah dikumpul daripada beberapa lokasi di dalam negara. Kehadiran bakteriofaj di dalam sampel-sampel ini telah disiasat and 43 isolat bakteriofaj telah diasingkan. Keupayaan isolat-isolat ini untuk menjangkiti hos-hos bakteria telah dikaji. Berdasarkan lokasi and keupayaan keberjangkitan bakteriofaj, lima isolat telah dipilih untuk kajian seterusnya. Keputusan analysis restriksi menunjukkan isolat-isolat ini mempunyai tiga profil restriksi yang berasingan. Kesemua isolat ini dikategorikan di dalam famili Myoviridae di bawah permerhatian mikroskop elektron transmisi. C34 telah dipilih untuk eksperiment terapi bakteriofaj kerana ia sentiasa dapat menghasilkan titre yang tinggi dan stabil. Dalam kajian pembunuhan bakteria, C34 berjaya mengurangkan bilangan bakteria dalam cecair kultur.

Eksperiment diteruskan dengan kajian dalam model sel kultur and model tikus BALB/c. Dalam kajian menggunakan sel epithelial A549 manusia, C34 berjaya melindungi sel sejumlah 41.6 ± 6.5% daripada jangkitan maut apabila ia dibekalkan 24 jam sebelum

kajian dimulakan. Apabila C34 disuntik secara intra-peritoneum ke dalam tikus yang dijangkiti melalui salur pernafasan, 33.3% tikus berjaya diselamatkan and hidup sehingga akhir eksperiment sepanjang 14 hari. Applikasi bakteriofaj juga dapat mengurangkan muatan bakteria di dalam limpa tikus yang dijangkiti bakteria. Kajian juga telah menunjukkan bakteriofaj lebih persis dalam sistem tikus yang dijangkiti bakteria berbanding dengan tikus yang sihat.

Kesimpulannya, C34 mempunyai potensi untuk digunakan sebagai calon terapi bakteriofaj dalam jangkitan *B. pseudomallei*.

ACKNOWLEDGEMENTS

First and foremost, I have to thank my research supervisors, **Prof Jamuna Vadivelu** and **Dr Chang Li Yen**. Without their assistance and dedicated involvement throughout the process, this thesis would have never been accomplished. Thank you very much for your support and understanding over these past few years.

Getting through the hardship of research requires more than just academic support. Throughout the process, I have many, many people to thank for listening to and, at times, having to tolerate me over down times. I cannot begin to express my gratitude and appreciation for their friendship especially my labmates (and also ex-labmates), Dr Kumutha Vellasamy, Dr Vanitha Mariappan, Jaikumar Thimma, Dr Loke Mun Fai, Dr Chua Eng Guan, Le Tian Xin, Siew Wei Hong, Choh Leang Chung, Kaveena Kay, Ng Shetlee, and Lee Lynn Fay, who have been unwavering in their personal and professional support during the time I spent at the University.

Most importantly, none of this could have happened without my family and my girlfriend, Miss Tan Cheng Lee. No matter how many words I use or write, they are just not enough to represent even just a bit of how I felt on their immeasurable support and patience. I know deep in their heart, they have always worried that I might get frustrated and give up on the way to the completion of the study. But they have never pressured me for not contributing enough for the family, and always stand by my side. Without this kind of freedom I would have never finished. This dissertation stands as a testament to their unconditional love and encouragement.

Lastly, special thanks to the mice sacrificed in the experimental phage therapy. Your sacrifice will not be in vain and gone unnoticed. The result of the study will most certainly help in the development of a novel treatment strategy on melioidosis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

Melioidosis is a respiratory disease caused by *Burkholderia pseudomallei*, a Gram-negative bacillus bacterium. The bacteria infect both humans and animals through inhalation, ingestion and inoculation. Most of the reported cases of melioidosis were from Australia, Thailand, Singapore, Vietnam and Malaysia (Nasner-Posso *et al.*, 2015). Meliodosis has a high mortality rate, ranging from 30-60% depending on whether the patients are septicemic (Puthucheary, 2009). The National Institute of Allergy and Infectious Disease of United States (NIAID) lists *B. pseudomallei* as a Category B bioterrorism agent due to the severity of infection, aerosol infectivity and worldwide availability of the bacteria.

At present, melioidosis patients are treated with a combination of antibiotics for a period of 20 weeks or longer, but the mortality is still high (Sookpranee *et al.*, 1992; Cheng and Currie, 2005; Lipsitz *et al.*, 2012). Failure to adhere to the complete 20-weeks of therapy may raise the risk of relapse (Chaowagul *et al.*, 1993; Limmathurotsakul *et al.*, 2006). *B. pseudomallei* is resistant to many first and second generation antibiotics including cephalosporins, penicillins, macrolides, colistin, rifamycins, and aminoglycosides (Dance *et al.*, 1989; Jenney *et al.*, 2001; Wiersinga *et al.*, 2012). Currently, there are also no vaccines available for melioidosis (Choh *et al.*, 2013). The intracellular lifestyle of the bacterium following its entry into the host system leads to either an acute or chronic infection, which encompasses latency and recrudescence. This complicates the development of an efficient vaccine. Due to these complications, development of a novel antimicrobial agent against *B. pseudomallei* infections is vital, and the use of phages could be an alternative treatment therapy.

Bacteriophages or phages are bacterial viruses that infect bacteria, disrupt the metabolism and cause the lysis of the bacterial host. Phage as a therapeutic agent fulfills

almost all the criteria listed for a good antimicrobial agent (Hanlon, 2007). The characteristics that make phages an antimicrobial agent of choice as compared to antibiotics include that phages: i) are highly specific, ii) do not cause microbial imbalance, iii) are able to replicate at the site of infection, iv) are able to reach areas with poor blood circulation, and v) do not cause serious side effects (Sulakvelidze *et al.*, 2001; Chan *et al.*, 2013; Nobrega *et al.*, 2015). In conclusion, it is obvious that phages have certain advantages over antibiotics.

Studies on phages of *B. pseudomallei* have reported several phages with broad infectivity on *Burkholderia* species other than *B. pseudomallei* (Sariya *et al.*, 2006; Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011; Kvitko *et al.*, 2012). However, to date, no study on phage therapy for *B. pseudomallei* has been reported. In order to investigate the potential of the phage therapy for melioidosis, this study was performed to determine the effects of a novel phage C34 isolated from seawater on *B. pseudomallei*-infected cells.

Objectives:

1. To isolate and characterize the bacteriophages of *B. pseudomallei* from environmental samples.

2. To examine the treatment efficacy of the isolated bacteriophage on *B. pseudomallei* via in vitro and in vivo experimental therapy.

CHAPTER 2: LITERATURE REVIEW

2.1 *Burkholderia pseudomallei*

Melioidosis, first described by Whitmore (1913) as a 'glander-like'disease, is caused by the gram-negative rod shaped bacterium which is currently known as *Burkholderia pseudomallei*. The bacterium had been previously named as *Bacillus pseudomallei*, *Bacillus whitmorii*, *Pseudomonas pseudomallei*, and was renamed to *Burkholderia pseudomallei* (Yabuuchi *et al.*, 1992). The current nomenclature is based on genetic studies (Lew and Desmarchelier, 1993).

B. pseudomallei appears to be vacuolated and slender with rounded ends under bipolar staining (Cheng and Currie, 2005). It can be differentiated from the closely related non-pathogenic relative, *B. thailandensis*, where the latter has the ability to assimilate arabinose (Wuthiekanun *et al.*, 2002). In addition, although until now there is no standardised test to differentiate *B. pseudomallei* from *B. mallei*, these two bacteria may be distinguished by their motility where *B. mallei* is non-motile (Redfearn *et al.*, 1966).

2.1.1 Geographical Distribution

Geographically, *B. pseudomallei* has been isolated and identified at tropical latitudes between 20° North and 20° South (Leelarasamee and Bovornkitti, 1989) and it has been endemic at southeast Asia and northern Australia (Cheng and Currie, 2005; Nasner-Posso *et al.*, 2015). The bacterium can be found in a wide range of niches, including soil of all temperatures, freshwater systems and even underground waters due to the ability to survive in various hostile conditions, for example in the prolonged absence of nutrients (Wuthiekanun *et al.*, 1995), antiseptic and detergent solutions (Choy *et al.*, 2000; Gal *et al.*, 2004), acidic environments (Dejsirilert *et al.*, 1991), and a wide temperature range (Tong *et al.*, 1996; Chen *et al.*, 2003).

The versatility and adaptability of the bacterium is due to its capability to produce various extracellular enzyme such as protease, lipases, lecithinase, catalase, peroxidase, superoxide dismutase, hemolysins, cytotoxic exolipid and siderophore (White, 2003). *B. pseudomallei* also has a high degree of genomic and phenotypic plasticity and forms seven different morphotypes on Ashdown agar (Chantratita *et al.*, 2007). The morphotype switching was found to be affected by a range of stresses such as heat shock, iron limitation and sub-inhibitory antibiotic concentration. The switching is believed to be caused by the expression of various putative virulence determinants, including secreted enzymes, motility and biofilm. These changes aided the bacterium to survive in the adverse conditions and contributed to the adaptation of *B. pseudomallei* within macrophages and intracellular persistence (Tandhavanant *et al.*, 2010).

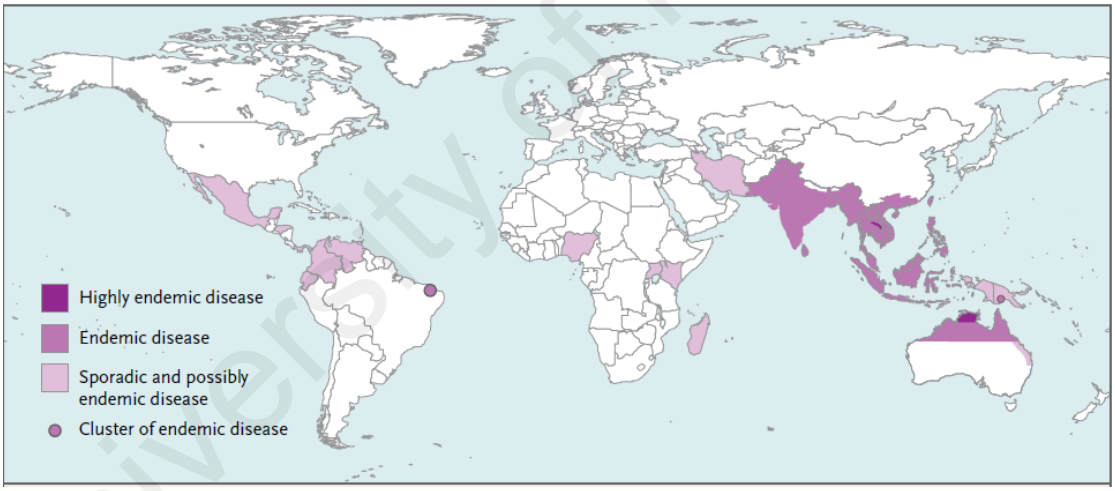


Figure 2.1: Global Distributions of Melioidosis. This figure was adapted from Wiersinga *et. al.* (2012)

2.1.2 Route of Infection

The routes of infection of melioidosis include inhalation, ingestion and inoculation. Despite the fact that there have been reported outbreaks related to ingestion of *B. pseudomallei*-contaminated water (Inglis *et al.*, 2000; Currie *et al.*, 2001; Inglis *et al.*, 2001), inhalation and inoculation are generally believed to be the major route of infection (Cheng and Currie, 2005). The possibility of acquiring the infection via

inhalation route contributes to the potential of the bacteria as a bioweapon, although any biological warfare involving the bacteria was yet to happen. There were evidence that the bacteria's close relative, *B. mallei* was possibly used as a bioweapon during World War 1, World War 2 and conflict at Afghanistan between 1982 and 1984 (Alibek and Handelman, 2000; Kortepeter *et al.*, 2001). This raises the concern that the bacteria might be used as a bioweapon. Consequently this led to the enlistment of *B. pseudomallei* as a Category B bioterrorism agent by the National Institute of Allergy and Infectious Disease of United States (NIAID).

2.1.3 Clinical Manifestations and Identification

Melioidosis presents as a wide range of clinical manifestations, ranging from chronic infections mimicking tuberculosis to fatal septicaemia (Currie *et al.*, 2010; Meumann *et al.*, 2012). It had been nicknamed "The Great Mimicker" due to the wide range of clinical presentations involving multiple organs (Yee *et al.*, 1988). A descriptive study involving 540 melioidosis patients at Australia over 20 years revealed that 51% of patients showed signs of pneumonia, follow by genitourinary infection (14%), skin infection (13%), bacteremia without evident focus (11%), septic arthritis/osteomyelitis (4%) and neurological melioidosis (3%) (Currie *et al.*, 2010). Mortality rate of the disease could be as high as 50% in patients with septic shock and 4% for non-septic shock patient. Recrudescence of the disease has been reported months to years after the initial infection (Ngauy *et al.*, 2005) and it was found that 13% of patient experienced reoccurrence (Limmathurotsakul *et al.*, 2009).

Since the disease is often misidentified as "other disease" due to the vast clinical presentations, identification and diagnosis of melioidosis is important to determine the treatment strategy. Ashdown's selective agar was developed for the isolation and presumptive identification of the bacteria from clinical and soil samples (Ashdown, 1979). Most strains of *B. pseudomallei* form highly wrinkled circular purple colonies on

Ashdown agar by 48 hours while there are six other morphotypes exist as observed (Chantratita *et al.*, 2007). These morphotypes are interchangeable and the switching was found to be induced by a variety of experimental stresses, including heat shock, iron limitation and sub-inhibitory antibiotic concentration.

There are also a few other techniques, namely indirect haemaglutination assay (IHA), latex agglutination and immunofluorescence are currently used for clinical diagnostic purpose (Cheng and Currie, 2005). However, the *sine qua non* of melioidosis diagnostic remained as isolation technique of *B. pseudomallei* from clinical samples.

2.1.4 Bacterial Pathogenesis

B. pseudomallei is a very versatile and resilient soil bacterium which may be attributed to the various virulence factors of the bacteria. However, the pathogenesis of the disease is only poorly defined. One of the more well-studied pathogenicity of *B. pseudomallei* is the ability to form biofilms. Biofilms can be referred to as a community of microorganisms attached to a surface embedded in a layer of extracellular matrix of polymeric substances (O'Toole *et al.*, 2000). Biofilms of *B. pseudomallei* was comprised of glycocalyx polysaccharide capsule (Steinmetz *et al.*, 1995). The capsule acted as a protective shield which offers resistance towards various antimicrobial agents and host defence factors to the bacteria residing within the biofilms (Vorachit *et al.*, 1993; Fux *et al.*, 2003; Fux *et al.*, 2005).

Other than biofilms, *B. pseudomallei* also produces and secretes many immunogenic antigens. These antigens include proteases, phospholipase C, hemolysin, lecithinase and lipase (Ashdown and Koehler, 1990; Sexton *et al.*, 1994; Korbsrisate *et al.*, 1999; Lee and Liu, 2000; Korbsrisate *et al.*, 2007). However, the role of these antigens in the bacterial pathogenesis is still unclear as it was shown that mutations on the secretion pathway which affects the secretion of these molecules did not result in attenuation of

the bacteria (Brett and Woods, 2000). Capsular polysaccharide (CPS), lipopolysaccharide (LPS), and two other surface O-polysaccharides (O-PS; types III O-PS and IV O-PS) are additional putative virulence factors which are also found to be immunogenic in patients with melioidosis (Wiersinga *et al.*, 2012). These antigens help in attachment to the host cells and the evasion from the host immune response.

It was also observed that some *B. pseudomallei* strains form small colony variants by passaging *in vivo* or *in vitro* (Cheng and Currie, 2005). On other bacteria, this differentiation was associated to decreased susceptibility to antibiotic treatment, reduced carbohydrate metabolism, altered virulence factor expression, elevated biofilm formation capacity and prolonged persistence *in vitro* (Kahl *et al.*, 1998; Haussler *et al.*, 2003a; Haussler *et al.*, 2003b; Samuelsen *et al.*, 2005; Anderson *et al.*, 2007). Although small colony variants of *B. pseudomallei* was found to be less virulence than their wild type counterparts, this differentiation was believed to play a role in the persistence of the small colony variants in host, causing chronic infection or relapse.

Similar to other disease-causing gram-negative bacteria, *B. pseudomallei* possess type three secretion system (T3SS) which function to inject various effector proteins into host cell cytosol (Spano and Galan, 2008). T3SS-3 of *B. pseudomallei* shares a high homology to SPI-1 pathogenicity island of *Salmonella enterica* (Inv/Spa/Prg), where SPI-1 facilitates the invasion and survival of the bacteria in phagosomes(Galan, 2001; Rainbow *et al.*, 2002; Stevens *et al.*, 2002; Zaharik *et al.*, 2002; Stevens *et al.*, 2003). It was found that mutations in T3SS-3 had resulted in attenuated virulence in a hamster model (Warawa and Woods, 2005) and knockout studies on *bsa* (where T3SS is encoded) led to redundancy in the bacteria's intracellular lifestyle (Stevens *et al.*, 2002; Cullinane *et al.*, 2008; Gong *et al.*, 2011). As a comparison, the absence of some components of T3SS in *B. thailandensis* rendered the bacteria avirulent (Rainbow *et al.*,

2002). Thus, T3SS was believed to be one of the major virulence factors of *B. pseudomallei* related to intracellular survival.

Its ability to survive intracellularly in both phagocytic and non-phagocytic cells allows the bacteria to evade the host immune responses (Jones *et al.*, 1996). This ability also accounts for various features of melioidosis, for example, latency and recrudescence (Allwood *et al.*, 2011). The intracellular lifestyle starts with adhesion to target cell, followed by internalization in endocytic vesicle. The vesicle membrane is then disrupted, allowing the bacteria escape from the vesicle and multiply in the cytosol. Finally the infection will spread to other adjacent cells via actin-mediated propulsion across cell membrane or released when cell lysis via apoptosis and infects other cells. Upon escaping from the endosomal vesicles, the bacteria are now capable of surviving and replicating intracellularly. Cell-to-cell spread of the bacteria will then follow via actin-based motility, in which causing the development of the infection into a fatal systemic infection.

2.1.5 Current Treatment Strategy

As yet, no licensed vaccine for melioidosis has been registered (Choh *et al.*, 2013). Treatment for the infection is purely dependant on course of antibiotics. However, *B. pseudomallei* is intrinsically resistant to many first and second generation of antibiotics (Wiersinga *et al.*, 2012). The resistance is attributed to a variety of mechanisms including the presence of efflux pumps, bacterial-cell-membrane impermeability, alterations in the antibiotic target site, and amino acid changes in *penA*, the gene encoding the highly conserved class A β-lactamase (Trunck *et al.*, 2009; Rholl *et al.*, 2011).

In 2010, a workshop was conducted to discuss on the treatment strategy and post exposure prophylaxis for *B. pseudomallei* (Lipsitz *et al.*, 2012). It came into agreement

that the current treatment of melioidosis consists of two phases and includes a combination of antibiotics. The first phase of the treatment upon confirmed diagnosis of melioidosis is the initial intensive phase therapy. The patient will be given intravenous administration of ceftazidime, meropenem, or imipenem for 10-14 days. In more severe cases such as septic shock, deep-seated or organ abscesses, extensive lung disease, osteomyelitis, septic arthritis, or neurologic melioidosis, therapy may be extended to more than a month. It is suggested that trimethoprim-sulfamethoxazole (TMP-SMX) is used if the infection involves privileged sites like brain and prostate.

The initial intensive phase therapy is considered successful if the condition of the patient improves with negative blood culture results as the indicator. This is then followed by oral eradication therapy as *B. pseudomallei* is able to avoid host immune clearance via intracellular lifestyle. Patients are at high risk of relapse without the eradication therapy. The therapy involves orally receiving TMP-SMX for 3 to 6 months (Peacock *et al.*, 2008). Amoxicillin-clavulanate combination is an alternative for oral eradication therapy, when dealing with patients who are allergic to sulphonamide, cotrimoxazole intolerance or when use of cotrimoxazole or doxycycline is contraindicated (Cheng *et al.*, 2008). Despite such prolonged eradication therapy, approximately 10% of the patients still suffered relapse or reoccurrence (Limmathurotsakul *et al.*, 2006).

2.2 Phage Therapy

In pre-antibiotic period, only few antibacterial compounds were available to treat bacterial infections. One of the first "magic bullet" is arsenic compound 606, Salvarsan, however the compound, very much like others is high in toxicity (Sulakvelidze and Kutter, 2005). The discovery of bacteriophages brought excitement to the medical world. Felix d'Herelle, the co-discoverer of phages was aware of the importance of phage discovery and among the first to use phages to treat bacterial dysentery. Trials

were carried out at the Hospital des Enfants-Malades in Paris in 1919 and single dose of phage was prove effective in ceasing the symptoms of dysentery (Summers, 1999). However, the results of the trials had not been published immediately. The first ever publication of usage of phage in treating bacterial infection was published by Richard Bruynoghe and Joseph Maisin who successfully treated staphylococcal skin disease in six patients (Bruynoghe and Maisin, 1921).

D'Herelle's work received much attention and soon he was invited to India to conduct a large scale study to examine the efficacy of phage therapy for plague and mainly on cholera. The study, spanning over more than ten years, was subsequently directed by Igor Asheshov, Dr Pasricha and Lt. Col. J. Morison after d'Herelle left for a faculty position at Yale University in 1928. The study observed good results in prophylactic and therapeutic application, reducing the incidents and mortality rate of cholera outbreaks in India (d'Herelle *et al.*, 1928; Summers, 1999). The efficiency of anti-cholera phage was so high until the conventional anti-cholera measures at the time were totally being abandoned and substituted by phage doses. However, this had resulted in the bloom of cholera outbreak in 1944, bringing 150,000 deaths. In addition to that, World War II and the rise of Indian nationalism contributed to the termination of the project (Summers, 1999).

The downfall of interest in phage therapy was further exacerbated by the discovery of antibiotics in 1940s. Treatment at that period favoured antibiotics as antibiotics have a broader host range and relatively non-specific killing. Most etiologic agent cannot be accurately and rapidly identified at that time and a wide spectrum antimicrobial agent would be excellent for clinical application. Furthermore, the lack of adequate and reliable study on the efficacy of therapeutic phage had prompted the western scientist to be doubtful of phage therapy (Summers, 2001). Since then, phage therapy ceased in the western world, while it has continued to be developed in the Eastern Europe. The phage

biologist in the west then turned their attention to phage biology and molecular biology of phage, contributing to the bloom in the development of modern cloning and genetic studies (Summers, 1999). Phage therapy has only come back to the limelight in the western world due the emergence of antibiotic resistant bacteria in recent years.

2.2.1 Bacteriophages

Bacteriophages (phages) are bacterial viruses which prey on bacteria host. Phages were first observed and reported individually by Frederick W. Twort and Felix d'Herelle (Summers, 1999). The term 'bacteriophages' was introduced by d'Herelle, in which 'phages' was derived from *phagein*, a Greek word for 'to eat'. They are common in all natural environments and can be found in faeces, sewage, soil and water samples. They are found to be the most abundant organism on earth and it was estimated that with every bacterial cell exists, there will be 10-100 phage particles present in the surroundings (Bergh *et al.*, 1989; Wommack and Colwell, 2000; Weinbauer, 2004).

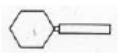
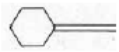
2.2.2 Classification of Phages

Phages are varied in their morphology, physiochemical and biological properties. They also have either double stranded or single stranded DNA or RNA. The great diversity of phages appears to be a huge challenge for their research and understanding. A systematic classification of bacteriophages would be beneficial to the development of therapeutic phage and phage-related industry. The current classification of phages was based on the phage morphology and nucleic acid composition as governed by The International Committee on the Taxonomy of Viruses (ICTV). They are currently being classified into one orders and 10 families (Table 1.1).

To date, at least 5000 bacteriophages had been examined under electron microscopy and characterised based on the classification scheme by ICTV (Ackermann, 2007). Majority of these bacteriophages (96%) are tailed viruses from three families, the

Myoviridae, Siphoviridae and Podoviridae constituting the order Caudovirales. The remaining 200 over phages are either polyhedral, filamentous, or pleomorphic in their shapes. Some of the phages such as those in family Corticoviridae, Tectiviridae, Cystoviridae and Plasmaviridae contain lipid envelope or inner lipid vesicles which make them sensitive to chloroform (Ackermann, 2009). The classification is updated frequently and can be found on ICTV website.

Table 2.1: Overview of phage family. (Ackermann, 2011).

Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

2.2.3 Overview of Infection Process

Phages are obligate intracellular parasites. Without a bacterial host as reproduction machinery, phages are of no difference with any ordinary proteins. They replicate by injecting their genomes into the infected host and overtaking the host's reproduction machinery to their service.

Depending on the type of infection, phages can be classified into two groups, namely lytic and lysogenic or temperate infection. During a lytic infection process, a phage will first attach to a host bacterial cell by binding to specific surface receptors on the host. In general, bacteria may develop resistance through mutation or alteration of phage-targeted receptors. However most of the receptors targeted by phages are crucial for host functions. Resistance through mutations may lead to reduction in competitiveness. In order to counter the resistance, phages may also alter their adsorption structures to recognise the altered receptor proteins or even bind to a new host receptor (Guttman *et al.*, 2005).

After attachment, the phage genome will be injected across the outer membrane, peptidoglycan layers and inner membrane into the host cell. Most phages are equipped with a peptidoglycan degrading enzyme at the tip of the tail for the purpose (Letellier *et al.*, 2004). Upon entry, the phage DNA is susceptible to the insult from host exonucleases and restriction enzymes. Many phages rapidly circularised their DNA by means of sticky ends or terminal repeats or the protection of linear genome ends by proteins. Several other anti-restriction strategies are also evolved such as accumulation of point mutations and odd nucleotide-containing DNA (replacing cytosine with hydroxymethylcytosine) which prevents endonucleases recognition (Labrie *et al.*, 2010). From there, the phage will then hijack the host metabolism for the purpose of producing more phages.

All the components of virions are constructed in a highly regulated process. In brief, the phage head (procapsid) and the pore complex are the first to be assembled. Then, phage DNA is translocated into the procapsid for packaging, transforming it into a mature capsid. Finally, the tail is attached to the pore complex completing the virion. The lytic infection cycle is completed substances such as lysins and holins lysed the host cell to release the newly assembled virions (Wang, 2006).

In a temperate infection, phages are able to choose to initiate a lytic cycle or to enter a lysogenic cycle. In the lysogenic cycle, the phage genome undertakes an inactivated state known as a prophage. Most prophages integrate into the host genome while some are maintained in the host cell as plasmids and being replicated whenever the host cell reproduced. Under certain circumstances or occasionally, prophages are able to restore its lytic capability and thus initiating the lytic infection cycle of assembling new phage particles and lysing host cells to be released. The infection process of bacteriophages was as summarised in Figure 1.3 below.

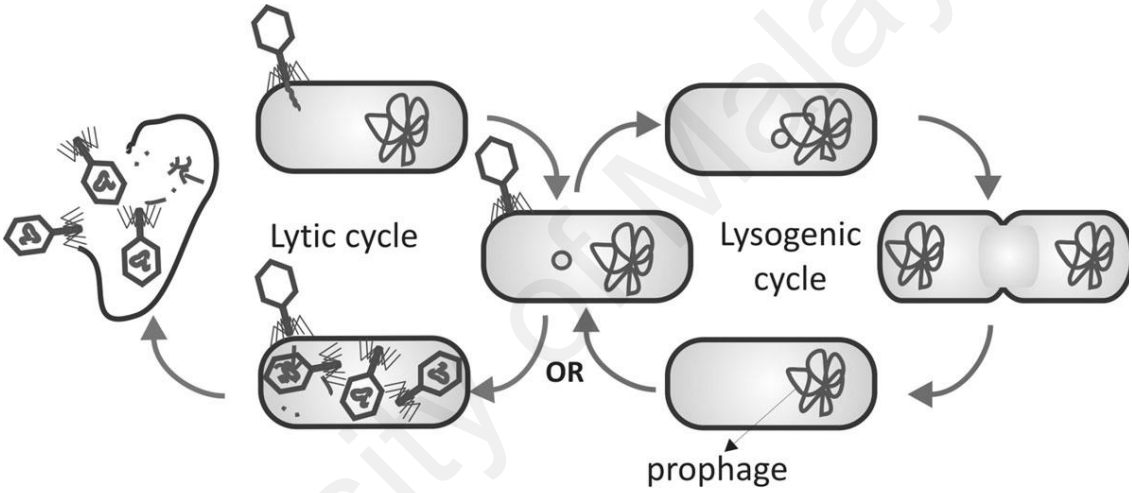


Figure 2.2: Overview of bacteriophages infection process. Figure adopted from Sabour *et al*. (2010).

2.2.4 Features of Phage Therapy

The advantages of phage therapy over the conventional antibiotics lie in the unique biological properties of phages. Various reviews on the advantages and disadvantages of phage therapy have been published and will be summarised as below:

i. Minimum disruption to microflora

Host-specificity- the reason which orchestrated the downfall of phage therapy research in the 1940s had played a major role in the revival of phage therapy. The broad

spectrum and non-selective bacteriocidal effect of antibiotics means that pathogens, together with the normal microflora of the receipient will be affected. In contrast, phages have limited host range. Most phages can only infect a few specific strains of bacteria and only a handful of phages can cause cross-species/genus infection (Hyman and Abedon, 2010). As a result, phage therapy would only bring minimal disruption to patient's normal microflora. This would therefore avoid secondary infection due to dysbiosis caused by the effect of antibiotics (Edlund and Nord, 2000; Rafii *et al.*, 2008). The development of various rapid diagnostic array and commercial microbial identification kits further contributed to the tendency to the use of antimicrobial agents with narrow and specific host range as the disease-causing pathogens can now be identified in a relatively short period of time.

ii. Auto-dosing and single-dose potential

Phages have the ability to multiply. As long as there is presence of suitable bacteria host around, the phage is able to multiply and produce more phages to search and eliminate more host bacteria. This phenomenon is termed auto-dosing as the phages themselves contribute towards the increasing dose of therapeutic agent (Abedon and Thomas-Abedon, 2010). Auto-dosing leads to the possibility of a successful treatment by using only a single dose of phage and a low dosage could be used too. The obvious advantage of this is the convenience of avoiding the trouble of repetitive drug intake and thus at least partially reducing medical cost.

iii. Low toxicity and side effects

Previously it was a concern that bacterial lysis due to phage activity (during phage preparation and therapy) may lead to the release of anaphylactic components such as endotoxins (Abedon *et al.*, 2011). Phages mainly consist of nucleic acids and proteins, which may also cause immunogenic reaction to human. However, recent studies revealed the opposite. Several reviews carefully addressed the safety issue of phage

therapy and apparently no side effect had been described yet (Krylov, 2001; Sulakvelidze and Kutter, 2005; Letkiewicz *et al.*, 2010). One logical explanation to this is because human are exposed to phages since infant as they are abundant in the environment and the immune system became tolerant to their presence. Nevertheless, advancement in technology has also helped in the purification of phages to exclude bacterial components in crude phage lysates (Gill and Hyman, 2010).

iv. Lower occurrence of phage resistance in nature

Resistance to antibiotic is one of the main reasons in the revival of interest towards phage therapy. Antibiotics are used (abused) in a variety of applications such as agriculture, veterinary and medical, resulting in the emergence of more antibiotic resistance bacteria in the environment. In contrast, the narrow spectrum of phage again proved to be an advantage, with the resistance can only arise in the specific host bacteria whilst others are not affected (Hyman and Abedon, 2010). Even though targeted bacteria can confer resistance to phage infection via mutations, the virulence and fitness of the bacteria are often affected (Capparelli *et al.*, 2007; Hall *et al.*, 2012).

Another interesting feature of therapeutic phage is the mechanism of infection and killing of phages is different from that of antibiotics. One mechanism of antibiotic resistance which offers protection against a few classes of antibiotic does not offer cross-protection against phage infection (Loc-Carrillo and Abedon, 2011). Therefore phage therapy is employed to treat some antibiotic-resistant infections, notably the notorious multi-drug-resistant *Staphylococcus aureus* infection on skin burnt patients (Gupta and Prasad, 2011).

v. Rapid discovery for new and effective phage

It was predicted that the occurrence of phage resistance is comparable to that of antibiotics in vitro (Drake *et al.*, 1998). The emergence of resistance would mark the

need of new antibacterial agent. However, the development of a new antibiotic takes many years and several millions (Silver and Bostian, 1993). Ironically, after spending so much effort in getting the new drug approved, resistant bacteria to the drug may have already been identified or emerged shortly after the use of the drug. One classic example is linezolid, which was found to be active against many pathogens. In less than a year after being approved for human therapeutic use, mutants resistance to linezolid had already been reported in clinic (Gonzales *et al.*, 2001; Prystowsky *et al.*, 2001).

Although the same may apply for therapeutic phage, new phages are relatively easier to be discovered due to their abundance in the environment. Therefore, whenever a pathogen was found to be resistant against the phage applied, a new active phage can be identified easily. The rapid discovery of new and effective phage also provides flexibility in responding to new or sudden emerging of infectious disease. An example was demonstrated during the summer of 2011, an outbreak of foodborne enterohemorrhagic *Escherichia coli* O104:H4 infection at Germany which caused 54 deaths. It was demonstrated that potential therapeutic phages can be rapidly isolated from the environment, carefully selected and genetically characterised within three days of an outbreak (Merabishvili *et al.*, 2012). Due to the concern of activating the expression of Shiga toxin, antibiotics were contraindicated in the case and the rapid isolation of phages may prove to be useful in counteracting the outbreak.

2.2.5 Current Applications of Phage Therapy

Even during the antibiotic era, phage therapy continues to be developed in some areas including United States. Many experiments and clinical trials have been carried out in between and the results varied. Despite the massive numbers of trials carried out, only few passed the rigid testing and approved for application.

One of the phage preparations approved for human application is the Phage BioDerm developed by the Centre for Medical Polymers and Biomaterials, Georgian Technical University and Eliava Institute of Bacteriophage, Microbiology and Virology (EIBMV) in Georgia. Phage Bioderm is a biodegradable, non-toxic polymer impregnated with bacteriophages together with antibiotics (ciprofloxacin and benzocaine) (Markoishvili *et al.*, 2002). The bacteriophages contained in the polymers include lytic phages against *P. aeruginosa*, *E. coli*, *S. aureus*, *Steptococcus* and *Proteus*. In a case study, Phage BioDerm was used in treatment of ulcers and wounds, with a successful rate of 70% on patients who failed to respond to conventional therapy (Markoishvili *et al.*, 2002). In another study, it was used to treat two Georgian lumberjacks who developed severe burns that then infected with antibiotic resistant *S. aureus*. Improvement was observed within a 7-day period (Stone, 2002; Jikia *et al.*, 2005). The success also prompted the development of other versions such as "PhageDent" for periodontal applications (Sulakvelidze and Kutter, 2005).

Another product, the Staph Phage Lysate (SPL) was developed and produced in the United States. The preparation was permitted for human therapeutic and veterinary applications after completing the safety trials in 1959 (Salmon and Symonds, 1963; Mudd, 1971). SPL was only found to have a few minor side-effects such as local erythema and swelling observed over a period of 12 years. It was used to treat various staphylococcal infections which had developed resistant against various antibiotics with 80% of recovery rate (Salmon and Symonds, 1963). However in later stages it was suggested that SPL exercised its effect via stimulating the host immune respond rather than the activity of lytic phages in the preparation (Dean *et al.*, 1975; Lee *et al.*, 1982; Lee *et al.*, 1985a; Lee *et al.*, 1985b). Despite the proven therapeutic value on human applications, the production of SPL for human use was suspended in the 1990s while veterinary applications are still extensively used.

Other than medical applications, phages were also used in biocontrol agents in food. For example, Food and Drug Administration of United States (USFDA) approved a few anti-listeria products such as Listex P100 and ListShield^{TM}, a mixture of *Listeria* phage as food additives (Carlton *et al.*, 2005; L., 2007). These products are able to reduce or control the amount of *Listeria monocytogenes* on ready-to-eat food such as cheese, salmon and catfish fillet (Guenther *et al.*, 2009; Soni and Nannapaneni, 2010; Soni *et al.*, 2010; Soni *et al.*, 2012). The approval has been considered as a major breakthrough in human phage applications as modern sciences always questioned the safety of phage therapy. Approval of phage application on ready-to-eat food by USFDA may facilitate the development and marketing of future phage application on human.

2.2.6 Bacteriophages of *Burkholderia* species

Several phages of *B. pseudomallei* have been isolated from the environment and a few of them have demonstrated broad infectivity, which can infect closely-related *Burkholderia* species other than *B. pseudomallei* (Sariya *et al.*, 2006; Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011; Kvitko *et al.*, 2012). However, none of these phages was tested for application in phage therapy.

In contrast, experimental phage therapy against *B. cepacia* complex already been carried out *in vivo*. One of the studies was carried out on *Galleria mellonella* and rescuing 90% of the infected larvae with only a single dose of phage application (Seed and Dennis, 2009). It was also shown that heat inactivated phage application did not rescued the infected larvae. This suggested that the treatment effect was due to phage activity instead of stimulated immune respond by phage. It was demonstrated in another study that systemic phage administration was more effective than inhalational administration using a *B. cenocepacia* pulmonary infected mice model (Carmody *et al.*, 2010). These studies suggested that phage therapy against *B. pseudomallei* is possible. Hence in this study, we will be isolating and characterising bacteriophages from

environmental sources. The therapeutic potential of these isolated phages will be investigated *in vitro* and *in vivo*.

CHAPTER 3: METHOD AND MATERIALS

3.1 Bacterial Strains

A total of 43 *B. pseudomallei* strains used in the study were obtained from the Medical Microbiology Diagnostic Laboratory, University of Malaya Medical Centre (UMMC) Kuala Lumpur and Hospital Tengku Ampuan Afzan (HTAA) Kuantan, Pahang. These strains were These 43 strains were collected from 1997-2013 and identified as *B. pseudomallei* using API 20NE (Biomerieux, France) and PCR assay using an in-house primer (Suppiah *et al.*, 2010). In addition, *Pseudomonas aeruginosa* ATCC 9027, two clinical isolates of *B. cepacia* (CQK and CYH), and *B. thailandensis* E264 were also screened as potential hosts. All the bacterial strains were cultured overnight using Luria-Bertani (LB), agar or broth, at 37 °C unless otherwise specified. For long term storage, overnight cultures of *B. pseudomallei* (16-18 hours) were kept in LB broth added with 30% (v/v) glycerol at -80°C.

3.2 Isolation of Bacteriophages from Water, Soil and Sewage

A total of 43 environmental samples were obtained from 8 areas within Malaysia. The type of samples collected includes soil, river water, coastal seawater and sewage samples (Table 3.1).

Table 3.1: Type and number of samples collected from each location.

Location	Type of sample	No. of samples collected
Palm oil plantation, Selangor	Soil	4
Rubber tree plantation, Selangor	Soil	4
Paddy field, Sekinchan	Soil	6
Maran Waterfall, Pahang	Soil	4
	Fresh water	4
Sewage Treatment Plant, Pantai Dalam	Raw sewage	5
	Aerated sewage	5

Table 3.1 continued:

	Anaerobic sludge	5
Kinchang Waterfall, Rawang	Soil	4
	Fresh water	6
Pulau Ketam, Selangor	Sea water	10
Port Dickson, Negeri Sembilan	Sea water	14
Templer's Park, Selangor	Soil	3
	Fresh water	5
Total		79

3.2.1 Soil Sampling

A total of 24 environmental soil samples were collected from 50 cm deep soil from palm oil plantation, rubber tree plantation, paddy field, Maran waterfall, Kinchang waterfall and Templer's park. Approximately 50 g of soil were collected in sterile tubes. Bacteriophage was isolated according to the method described in the literature (Van Twest and Kropinski, 2009). Five grams of soil were weighed and added with 5 ml of PBS in 50 ml centrifuge tubes. Samples were mixed vigorously on a vortex mixer for few minutes and let to settle. Supernatants were extracted and mixed with equal volume of double strength LB broth inoculated with 0.1 ml of overnight culture of *B. pseudomallei* strain K96243 and CMS. The inoculums were incubated at 37 °C with shaking at 180 RPM. After overnight incubation, the cultures were centrifuged at 10000 RPM for an hour. The supernatants were sterilised using Sartorius Stedim 0.45 µm filter unit with cellulose ester membrane. The filtrates were kept at 4 °C for detection of the presence of bacteriophages (Figure 3.1).

3.2.2 Water Sampling

A total of 39 surface water samples were collected from Maran waterfall, Kinchang waterfall, Pulau Ketam, Port Dickson and Termpler's Park. Approximately 50 ml of water were collected in sterile tubes. Bacteriophage was isolated according to the

method described previously (Van Twest and Kropinski, 2009). Five millilitres of samples were added with equal volume of double strength LB broth inoculated with 0.1 ml of overnight culture of *B. pseudomallei* strain K96243 and CMS. The inoculums were incubated at 37 °C with shaking at 180 RPM. After overnight incubation, the cultures were centrifuged at 10000 RPM for an hour. The supernatants were sterilised using 0.45 µm filter unit (Sartorius Stedim, Germany) with cellulose ester membrane. The filtrates were kept at 4 °C for detection of presence of bacteriophages (Figure 3.1).

3.2.3 Sewage Sampling

Sewage samples were collected from Indah Water Sewage Treatment Plant located at Pantai Dalam, Kuala Lumpur. Five samples were collected from the oxidative pond, anaerobic pond and raw tank respectively. Bacteriophage was isolated according to the method described previously (Van Twest and Kropinski, 2009). The samples were centrifuged at 4000 RPM for 10 minutes to eliminate the larger particles. Five millilitres of the supernatant were added with equal volume of double strength LB broth inoculated with with 0.1 ml of overnight culture of *B. pseudomallei* strain K96243 and CMS. The inoculums were incubated at 37 °C with shaking at 180 RPM. After overnight incubation, the cultures were centrifuged at 10000 RPM for an hour. The supernatants were sterilised using 0.45 µm filter unit with cellulose ester membrane. The filtrates were kept at 4 °C for detection of the presence of bacteriophages (Figure 3.1).

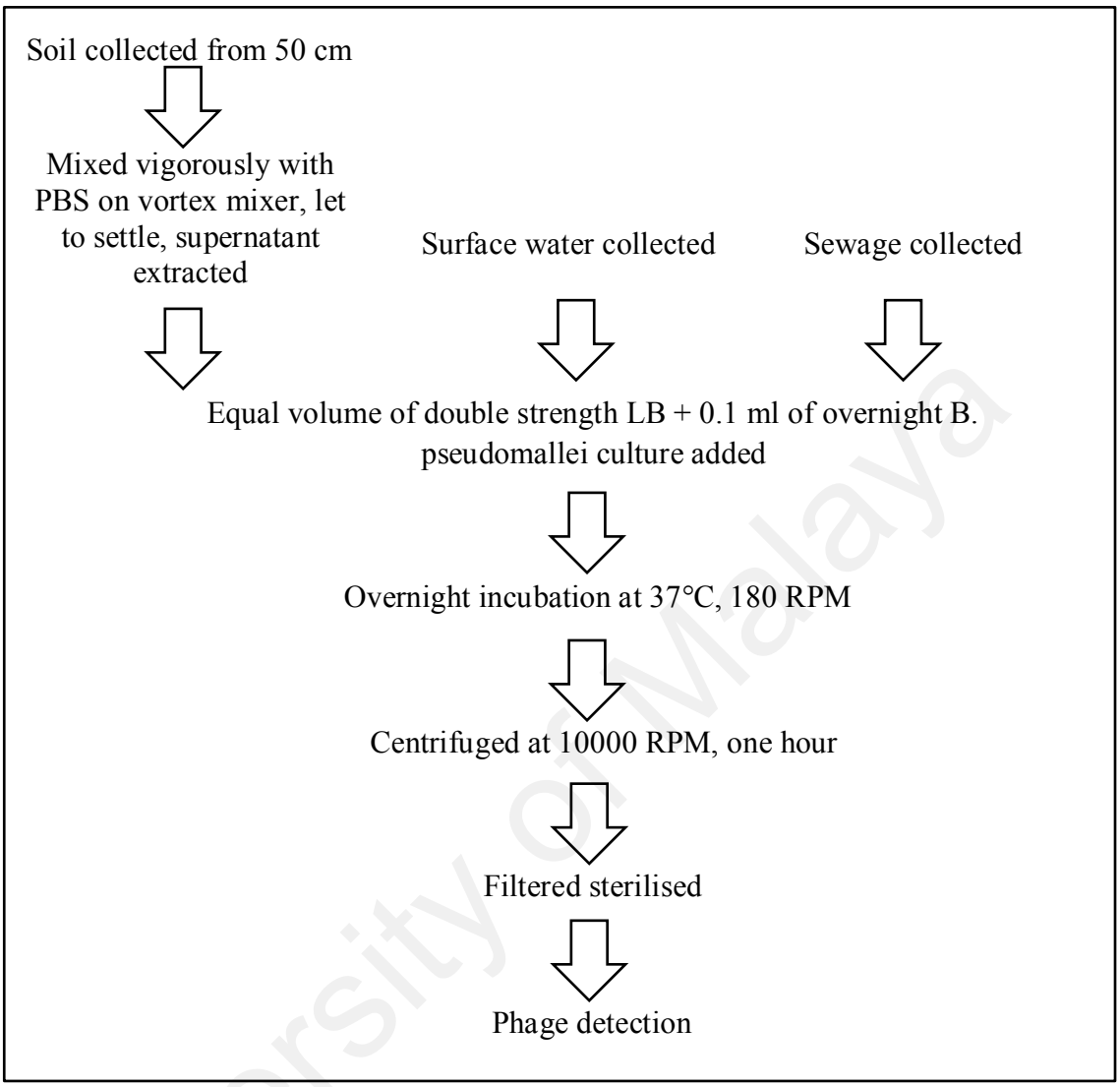


Figure 3.1: Flow chart of procedures for isolation of bacteriophage from soil, water and sewage samples.

3.2.4 Detection of Bacteriophages

Presence of bacteriophages in culture filtrates were detected using double layer agar overlay method (Sambrook *et al.*, 2001). In brief, 100 µl of culture filtrate was mixed with equal volume of overnight *B. pseudomallei* culture (K96243 and CMS, depending on the strains used in screening process) in a sterile tube. Three millilitres of molten soft LB agar was added to the tube and poured onto sterile LB agar plate. The plates were left solidified for 15 minutes prior to incubation at 37 °C for overnight. Formation of clear zones (plaques) on soft LB agar overlay represents presence of bacteriophages.

3.2.5 Isolation and Purification of Bacteriophages

Double agar overlay method was used for isolation and purification of bacteriophages. Well isolated plaques were picked from the soft agar overlay into sterile 1.5 ml centrifuge tubes using sterile toothpick. One hundred microlitres of sterile PBS was added to the centrifuge tube and mixed vigorously on a vortex mixer for 30 seconds to dislodge bacteriophages from the picked agar. The bacteriophage suspensions were mixed with 100 µl of overnight *B. pseudomallei* culture in a sterile tube. Three millilitres of molten soft LB agar was added to the tube and poured onto sterile LB agar. The agar was left to solidify for 15 minutes before overnight incubation at 37 °C. Another plaque was picked from the overnight incubated agar and the process was repeated for another 2 times. Final agar plates were kept at 4 °C.

3.2.6 Propagation of Bacteriophages

Plates from 3.2.5 were used for propagation of bacteriophages. Single plaque was picked from the plate into 1 ml of LB broth inoculated with 100 µl of overnight *B. pseudomallei* culture in a 1.5 ml centrifuge tube. The tube was incubated horizontally at 37 °C with shaking at 180 RPM for 8 hours. The culture was then transferred into 10 ml of *B. pseudomallei* culture at OD_{600nm} of 0.1 (~10^7 CFU/ml) and incubated at 37 °C with shaking at 180 RPM until lysis took place. A few drops of chloroform were added to complete the lysis process before the lysate was centrifuged at 13000 RPM for one hour. The lysate was then filter sterilised using Sartorius Stedim 0.22 µm filter unit. The filtered lysate was stored at 4 °C for further use.

3.2.7 Preparation of High Titre Phage Lysate

Phages C34, C38 and K43 were used for preparation of high titre lysate. Each phage was inoculated into 100ml of *B. pseudomallei* strain CMS culture at OD_{600nm} of 0.6. The culture was incubated at 37 °C with shaking at 180 RPM until lysis had occurred. A few drops of chloroform were added to complete the lysis. The lysate was centrifuged (10000 RPM, 30 minutes) and sterilised using 0.22 µm filter membrane. The phages were precipitated with polyethylene glycol (PEG-6000) (10%, w/v) and centrifuged at 13000 RPM for one hour (Yamamoto et al., 1970). The pellets were resuspended in phosphate buffered saline (PBS). Chloroform was used to extract PEG from the suspension. A few drops of chloroform were added to the suspension and mixed vigorously on a vortex mixer for a few seconds. The mixture was centrifuged at 3000 RPM for 5 minutes. The supernatant was transferred to a new sterile tube. The extraction was carried out for a four rounds and the PEG-free supernatant was stored at 4 °C.

3.2.8 Determination of Phage Titre and Long Term Storage of Phages

Titre of bacteriophages was determined using Miles and Misra assay on double agar overlay plates (Miles *et al.*, 1938). In brief, molten soft LB agar inoculated with overnight culture of *B. pseudomallei* strain were poured onto fresh LB agar and let to solidify for 15 minutes. A series of 10 fold serial dilution of phage lysate were prepared in sterile PBS. Ten microlitres of lysate from each dilution were spotted in triplicate on the solidified top agar overlay and left for air dry. The agar plate was then incubated overnight at 37 °C and plaque formation on each spotted dilution was counted. For long term storage of bacteriophages, glycerol was added to the lysate to a final concentration of 20% and kept at -80°C.

3.3 Characterization of Bacteriophages

3.3.1 Determination of Host Range of Bacteriophages

The host range of all bacteriophages was tested against 43 strains of *B. pseudomallei* clinical isolates from the laboratory archive collection. Overnight cultures of the test bacteria strains were prepared and 100 µl of the culture was added to 3 ml of molten soft LB agar prior to overlaying onto fresh LB agar. The agar overlay was left to solidiy for 15 minutes and 10 µl of phage lysate was spotted onto the overlay. The agar plate was air dried for 10 minutes and incubated overnight at 37 °C. A clear zone around the spotted area indicates that the phage was able to lyse the test bacteria.

3.3.2 DNA Extraction of Bacteriophages

HiYield^{TM} Viral Nucleic Acid Extraction Kit II was used for the extraction of phage DNA. Extraction of DNA was carried out according to the manufacturer's instruction. Bacteriophages were concentrated by mixing 150 µl of PP buffer with the phage lysate and incubated for 30 minutes under room temperature. The mixture was centrifuged at 12000 RPM for 15 minutes and the supernatant was discarded. The resulting phage pellet was lysed with 100 µl of LS buffer and incubated for 15 minutes to lyse the phage particles. A volume of 234 µl of absolute ethanol was added to the lysate and mixed by invert shaking for 10 times. The mixture was transferred to the binding column and centrifuged at 12000 RPM for 30 seconds. The flow through was discarded and the column was washed with Wash Buffer for 2 times. The column was centrifuged again at 12000 RPM for 2 minutes to completely remove the ethanol residue. Following this, 50 µl of Released Buffer preheated at 65 °C was added to the column matrix to release the phage DNA. The column was transferred to a new collection tube and incubated at 65 C° for 5 minutes before centrifuging at 12000 RPM for one minute to elute the phage DNA into collection tube. The concentration of the phage DNA was measured using Nanospec and stored at -20 °C.

3.3.3 Restriction Digestion and Analysis of Bacteriophages DNA

Bacteriophage DNA was restrict-digested using 5 FastDigest restriction enzymes (Thermo Scientific), namely Apa1, BamHI, EcoRI, Pst1, and Xba1. Digesting reaction was prepared as below:

10x FastDigest buffer	2 µl
Restriction Enzyme	1 µl
Phage DNA	200 ng
Ultrapure water	Top up to 20 µl

The sample was incubated overnight at 37 °C and electrophoresed on 0.8% agarose gel stained with the Sybersafe dye. The gel was visualised using a Geldoc.

3.3.4 Transmission Electron Microscopy Observation

Concentrated high titre phage ($\geq 10^8$ PFU/ml) was deposited on carbon-coated copper grid for 5 minutes and stained with 2% phosphotungstic acid for 2 minutes. Excessive solution was drained using filter paper and air dried. The grids were then observed using LEO-Libra 120 at a magnification of 50000x and digital image of the bacteriophages were recorded (Ackermann, 2009).

3.3.5 Temperature Stability Test

The temperature stability of phage C34 at 4°C, 37°C, 65°C and 90°C was examined using method as described by Capra (2004) with minor modifications. Approximately 1ml of PBS containing 1×10^8 PFU/ml of phage was aliquoted into 1.5ml centrifuge tubes and incubated at temperatures stated above. At predetermined intervals (five minutes interval for 37°C, 65°C and 90°C, up to 30 minutes; one week interval for 4°C, up to 8 weeks) , the viable count of phage in the tubes was titred as method described in Section 3.2.8.

3.3.6 Time Kill Curve

The time kill curve of phages on bacteria was constructed using method described by Kanthawong (2012) with minor modifications. Briefly, approximately 1×10^8 colony forming unit (CFU)/ml of *B. pseudomallei* strain CMS was aliquoted into assay plates and infected with phage C34 at a multiplicity of infection (MOI) of 10, 1 and 0.1 (phage: bacteria). Viable bacterial counts were determined at every hour (Miles *et al.*, 1938). In addition, growth curves were generated using absorbance readings at 570 nm that was recorded at every hour. Uninfected CMS was used as control and CMS treated with 500 µg/ml of kanamycin was used as the antibiotic control.

3.4 Experimental Phage Therapy

The human lung epithelial cell line A549 and specific pathogen free BALB/C mice were used in the experiments described as below. A549 epithelial cells were routinely maintained in complete growth medium (RPMI 1640 medium supplemented with 10% (v/v) fetal bovine, 2mM of L-glutamine, with/without 1mM penicillin-streptomycin) at 37°C in 5% CO_2 atmosphere.

The specific pathogen free BALB/C mice (aged six to eight weeks, female) were purchased from Monash University, Malaysia. These mice were maintained under specific-pathogen-free conditions and housed in sterile cages with a bedding of paper shavings, subjected to a 12-hour light/dark cycle, and fed a diet of commercial pellets, with water provided *ad libitum*. The animal work was performed with approval from University of Malaya Institutional Animal Care and Use Committee (File no: PAT/05/11/2007/0912/WKT).

3.4.1 Experimental Phage Therapy in Cell Culture Model

Prior to experimental phage therapy, a range of MOIs (1, 5, 10 and 50) and concentration of kanamycin used (39-5000 µg/ml) were optimised for the infection

assay. Approximately 2×10^4 human lung epithelial cells, A549 were infected at MOI of 10 with *B. pseudomallei* strain CMS grown to mid-log phase in 96-well assay plates. The infection of A549 cells was performed for two hours at 37 ℃. Concurrently, 2×10^4 A549 cells were pre-infection treated using 2×10^7 phage C34 phage particles overnight before proceeding with the infection assay. In order to evaluate the efficacy of phage C34 against intracellular infection, the infected cells were washed three times with PBS and then treated with phage (2×10^7 PFU) in RPMI complete medium supplemented with 500 µg/ml of kanamycin to eliminate extracellular *B. pseudomallei.* The assay plates were incubated in the presence of 5% CO_2 at 37°C overnight. The cells were then washed three times with PBS to eliminate the dead cells and the viability of A549 cells were determined using modified crystal violet cell viability assays (Alegado *et al.*, 2011). Briefly, crystal violet solution (0.1%) was added to assay plates for three minutes, removed, and the cells washed three times with distilled water and air-dried. Absolute ethanol was added to resolubilize the stain and the absorbance was measured at 570 nm. Untreated but infected A549 cells were used as positive control while uninfected A549 cells were used as negative control. The cytotoxicity of different phage titres was also determined using the same method as mentioned above by exposing the A549 cells to a series of 10 fold dilution of phage lysate diluted with complete growth medium for 24 hours.

Viability of A549 cells was calculated using the formula below:

$$\frac{B}{A} \times 100\%$$

Where

A = Absorbance of negative control,

B = Absorbance of sample

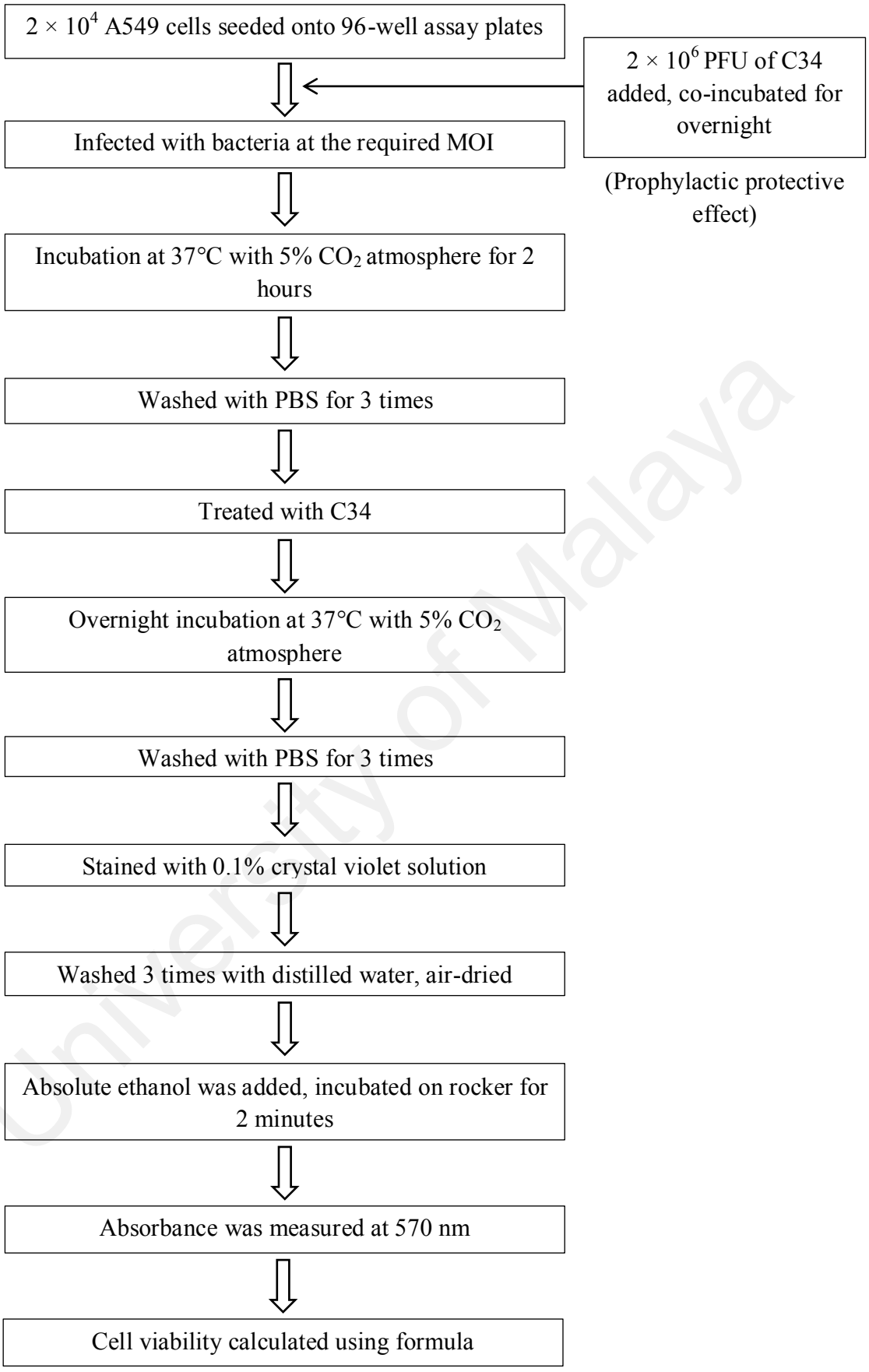


Figure 3.2: Flow chart of experimental phage therapy on A549 cells.

3.4.2 Experimental Phage Therapy in Mice Model

BALB/C mice were infected intranasally (i.n.) with *B. pseudomallei* CMS using method as described by Conejero *et al.* (2011) with modifications. Briefly, bacteria were grown to OD_{600nm} of 0.6 (~2.5×10^8 CFU/ml). Ten millilitres of bacterial culture was then spun down at 8000 RPM for 15minutes and the supernatant was discarded. The bacteria pellet was resuspended in 10 ml of sterile PBS. The process was repeated for another round and then diluted to the desired bacteria titre in sterile PBS. Mice were anesthetised with diethyl ether prior to i.n. injection, i.e. by delivering 10µl of the diluted bacteria culture in PBS into the mice nostril. For experimental phage therapy on mice, 2×10^8 PFU of C34 in 100 µl of PBS was administered via intraperitoneal (i.p.) route to the infected mice two hours post infection. In order to examine the prophylactic protective effect of phage, phage was administered 24 hours prior to infection via i.p. route. Sterile PBS was administered to the infected mice to serve as control. The mice were then monitored for disease symptoms daily and were euthanized according to predetermined humane end points (Figure 3.3).

The bacterial burden at mice tissues was enumerated by euthanizing the mice with ether at day 1, 2 and 3 post-infection. Blood was collected from the mice via cardiac puncture using syringe rinsed with EDTA. Lung, liver and spleen of the mice were obtained aseptically and homogenised in 1 ml of sterile PBS using a tissue homogeniser. Blood and homogenised tissue samples were plated onto Ashdown's agar and incubated at 37°C for 48 hours.

In order to study the presence of phage in mice system, 2×10^8 PFU of C34 in 100 µl of PBS was administered to uninfected mice via i.p. route, 24 hours prior to infection and 2 hours post infection. Blood, lung, liver and spleen samples were obtained and homogenized at the designated time points (day 1, 2 and 3 post-injection). Mock-

infected mice were used as the control. Titre of bacteriophages was then determined as method described in Section 3.2.8.

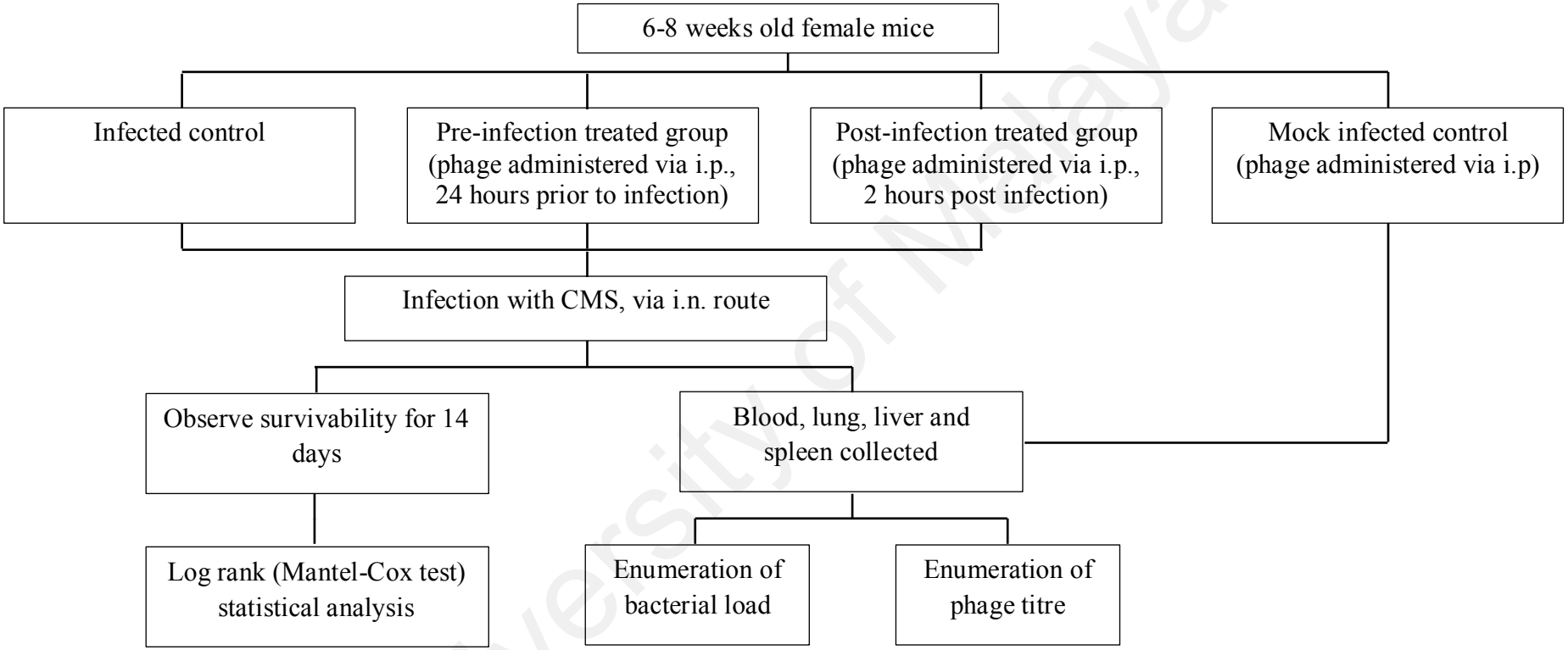


Figure 3.3: Flow chart of experimental therapy on BALB/C mice.

CHAPTER 4: RESULTS

4.1 Isolation of Bacteriophages

A total of 79 samples were collected from nine different locations in Malaysia and tested for the presence of bacteriophages (Table 4.1). Twenty nine samples were tested positive for the presence of bacteriophages. All 24 seawater samples collected from coastal area of Pulau Ketam (14) and Port Dickson (10) yielded bacteriophages (24/24). Only 2 in 25 of the soil samples and 2 in 15 of the freshwater samples collected were positive for presence of bacteriophages. Of the 15 samples collected from different sewage conditions of the sewage treatment plant, only one sample obtained from the aerated sewage was positive. One or more plaques from positive plates were picked, purified and stocked at 4°C as working stock or at -80°C for long term storage.

Table 4.1: Location of samples collected, type of samples collected and the number of isolates obtained from each site.

Location	Type of sample	No. of samples collected	No. of samples with phage	No. of phages isolated
Palm oil plantation, Selangor	Soil	4	0	0
Rubber tree plantation, Selangor	Soil	4	0	0
Paddy field, Sekinchan	Soil	6	0	0
Maran Waterfall, Pahang	Soil	4	0	0
	Fresh water	4	0	0
Sewage Treatment Plant, Pantai Dalam	Raw sewage	5	0	0
	Aerated sewage	5	1	1
	Anaerobic sludge	5	0	0
Kinchang Waterfall, Rawang	Soil	4	1	1
	Fresh water	6	0	0
Pulau Ketam, Selangor	Sea water	10	10	20
Port Dickson, Negeri Sembilan	Sea water	14	14	15
Templer's Park, Selangor	Soil	3	1	3
	Fresh water	5	2	3
Total		79	29	43

4.2 Characterisation of Bacteriophages

4.2.1 Determination of Host Range of Bacteriophages

The host range of the bacteriophages isolated were determined using 43 strains of clinical isolates of *B pseudomallei*. Results were scored as a positive result (Score 1) when indicated by formation of plaque or a clearing zone on LB agar plate and negative when there was no clearance zone (score 0) (Appendix B). Most of the phages tested had a similar host range and were able to lyse 19 to 32 of the 43 strains of *B. pseudomallei* in the culture collection included in the study. Phage C38 demonstrated the broadest host range with an ability to lyse 32 of the 43 strains, followed by phages C2, C12 and K17 which were able to lyse 30 of the 43 strains. Phage C30 demonstrated the most narrow host range (19/43). Phage C34 with a host range of 23/43 was the only phage that was able to lyse *B. pseudomallei* #63. Different *B. pseudomallei* strains demonstrated varied levels of sensitivity or resistance towards infection with the different phages. However, some of the bacterial strains were sensitive and some were resistant to all phages (Table 4.2).

Table 4.2: List of *B. pseudomallei* which were sensitive and resistant to all phages

	B. pseudomallei strains
Sensitive to all phages	2, 4, 14, 17, 19, 44, 46
Resistant to all phages	11, 12, 13, 18, 16, 33, 64, 68

4.2.2 Restriction Digestion and Analysis of Bacteriophages DNA

Phages were chosen for further characterisation based on their host range and location of isolation. Based on these criteria, when phages were isolated from the same location and possessed similar host range, only one representative phage was selected. Using restriction enzymes Apa1, BamHI, EcoRI, PstI, and XbaI on DNA samples of phages K2, K43, C5, C34 and C38 were found to be resistant to digestion by BamHI, EcoRI and XbaI, but were digested using ApaI and PstI (Figure 4.1). Based on the RFLP analysis, all the five phages were categorised into three different profiles, where K2 and K43, showed the same restriction pattern. Similarly, C5 and C38 showed the same restriction pattern, while C34 showed a distinct restriction pattern.

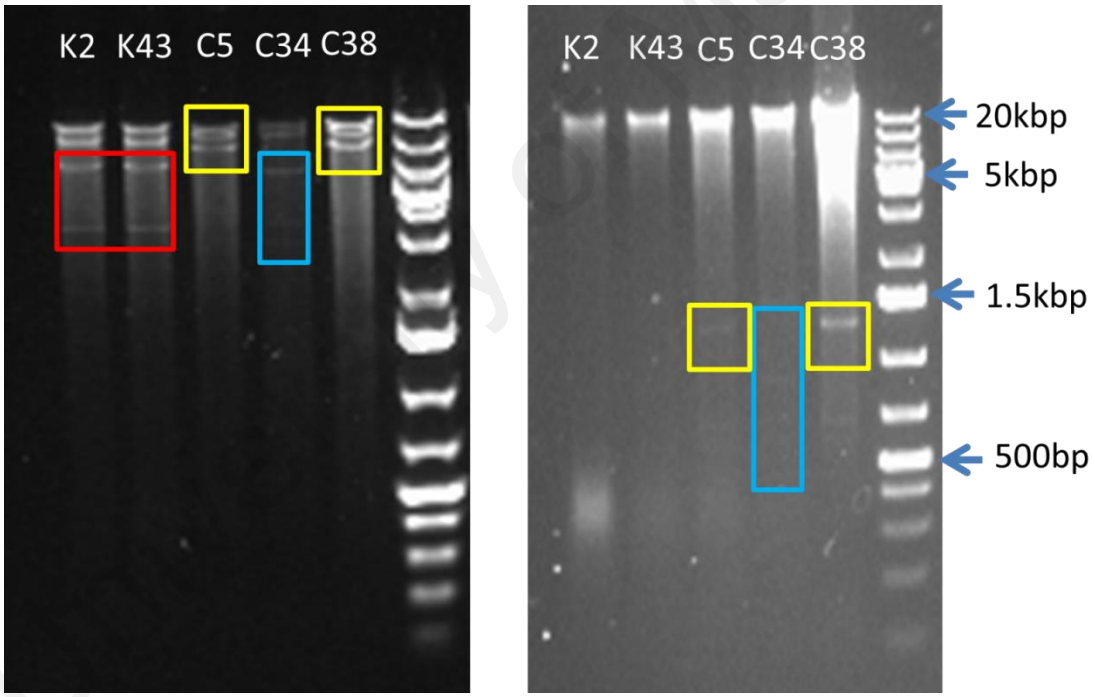


Figure 4.1: Gel photos of bacteriophages DNA following restriction digestion using Apa1 (left) and Pst1(right)

4.2.3 Transmission Electron Microscopy Observation

Further TEM analysis of the representative phage (C34, C38 and K43) from each of the restriction-digestion profile group revealed that all the three phages were from the same family. These phages were classified as belonging to the family Myoviridae based on the presence of an icosahedral head, a neck/collar region and a contractile tail (Figure 4.2).

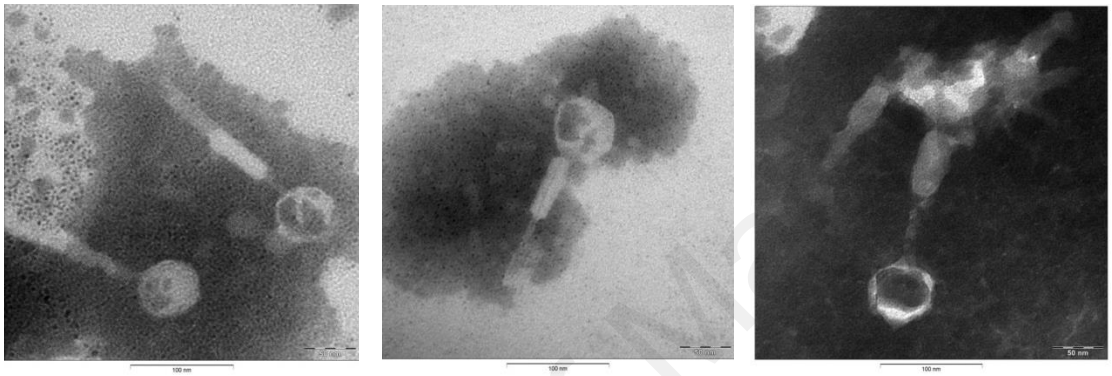


Figure 4.2: Transmission Electron Micrography revealed icosahedral head with contractile tail, the typical morphology of family Myoviridae. Magnification: x50000. From left: K43, C38, C34

4.2.4 Phage Propagation and Preparation of High Titre Phage

In order to determine the efficacy of phage antibacterial activity, a high titre of bacteriophage up to 10^9 PFU/ml was needed. Of the five phages, only C34, C38 and K43 phages representing the three different restriction-digestion profiles were propagated in triplicates and phage titre was compared following PEG-precipitation and chloroform extraction (Table 4.3). It was found that C34 had the highest phage titre (2.3×10^9 PFU/ml) in the propagation process and the only phage which could achieve the required titre (at least 10^9 PFU/ml). Phage K2 and C5 were not used for preparation of high titre phage lysate as they had the same restriction profile with C38 and K43. PEG-

precipitation of C38 and K43 failed to produce the phage titre required for experimental therapy. Thus, C34 was selected for further study.

Table 4.3: Phage titre of C34, C38 and K43 after the PEG-precipitation and chloroform extraction process.

Phage	Phage titre 1 (PFU/ml)	Phage titre 2 (PFU/ml)	Phage titre 3 (PFU/ml)	Average (PFU/ml)
C34	2.3×10^9	1.8×10^9	2.8×10^9	2.3×10^9
C38	3.4×10^7	6.5×10^7	6.0×10^7	5.3×10^7
K43	2.0×10^8	9.3×10^7	1.3×10^8	1.4×10^8

4.2.5 Time Kill Curve

Approximately 1×10^8 CFU/ml of *B. pseudomallei* strain CMS were challenged by phage C34 with the MOI of 10, 1 and 0.1. The result showed that MOI of 10 was the most potent MOI, with killing of bacteria occurred as early as the first hour after the addition of phage and a reduction of 4 log CFU as compared to the initial inoculum (Figure 3.3). At lower MOIs (1 and 0.1), the number of bacteria was reduced to 4.95 ± 0.33 log CFU/ml at 3 hours post-challenge and 5 ± 0.08 log CFU/ml at 4 hours post-challenge, respectively. In all cases, the number of bacterial count gradually increased after the killing/reduction point. The growth curve generated using optical density was found to compliment the time kill curve, with the application of phage C34 resulted in the reduction of bacterial growth, represented by the drop in the OD reading. The phage-infected *B. pseudomallei* strain CMS was growing again after the drop, albeit at a slower rate as compared to the uninfected control (Figure 4.4).

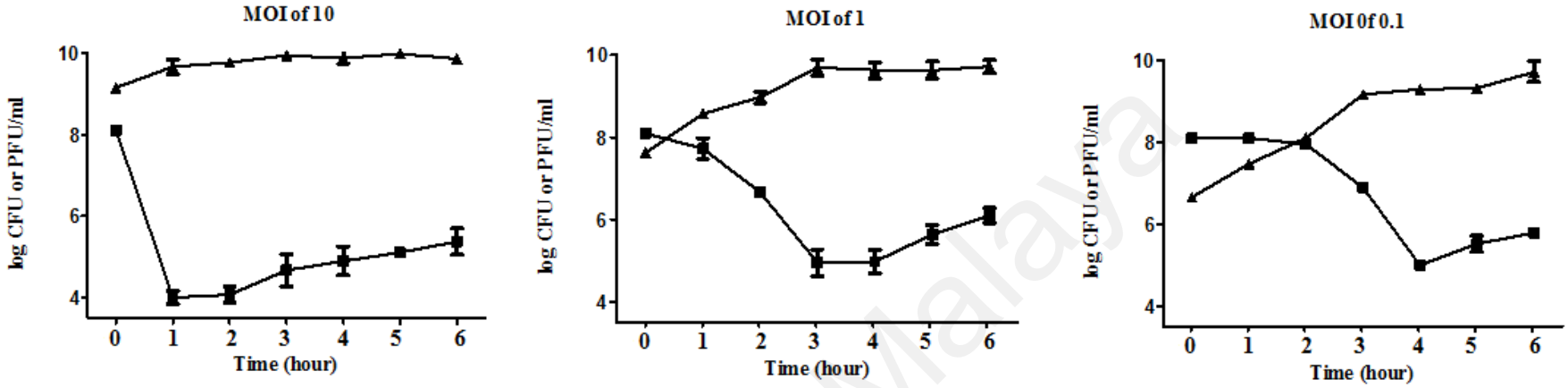


Figure 4.3: Bacterial count of *B. pseudomallei* infected by different MOIs (■) and the corresponding phage titre of C34 (▲) over the course of 6 hours. The results are the averages of three independent assays.

The growth curve generated (Figure 4.4) was found to compliment the time kill curve, with the application of phage C34 resulted in the reduction of bacterial growth, represented by the drop in the OD reading. The phage-infected *B. pseudomallei* strain CMS was growing again after the drop, albeit at a slower rate as compared to the uninfected control.

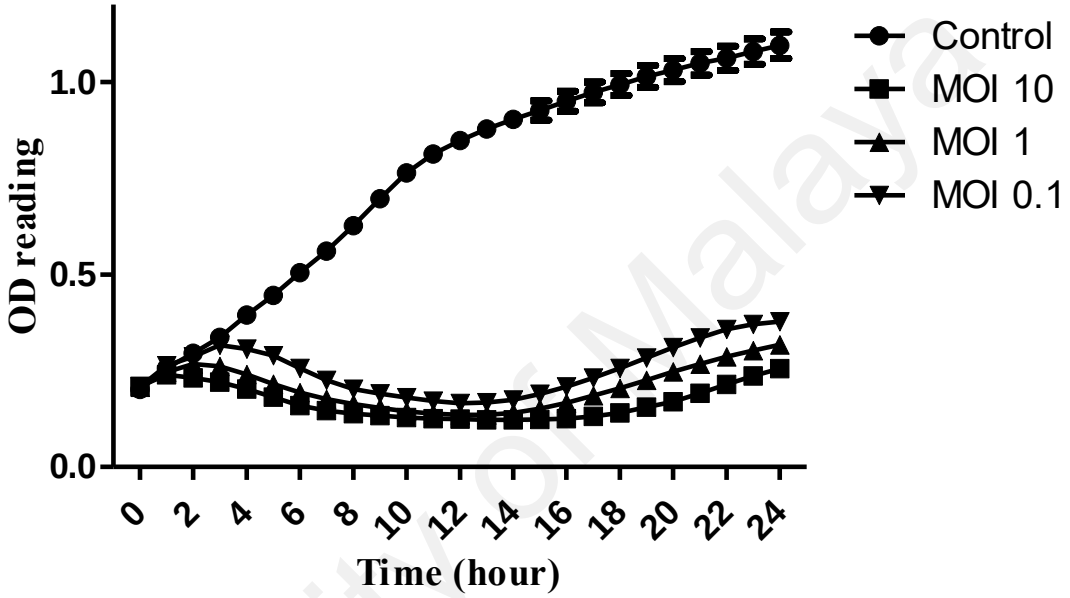


Figure 4.4: Growth curve of *B. pseudomallei* generated using absorbance at 570nm over the course of 24 hours. ●: Control (growth of uninfected *B. pseudomallei* strain CMS). ■: *B. pseudomallei* strain CMS infected by phage C34 (MOI of 10). ▲: *B. pseudomallei* strain CMS infected by phage C34 (MOI of 1). ▼: *B. pseudomallei* strain CMS infected by phage C34 (MOI of 0.1).

4.2.6 Temperature Stability Assay

Stability of phage C34 at different temperatures was tested by incubating the phage at different temperatures and measuring the phage titre at pre-determined time points. The test was performed in duplicate and three readings were obtained for each replicate, It was found that C34 was relatively stable at 37°C. At 65°C, the phage titre demonstrated a near linear regression (R^2 = 0.9430) and the rate of regression was -0.142 log

PFU/ml/min. However, at 90°C, the phage titre became too low to be detected after 5 minutes, indicating that C34 is very heat labile (Figure 4.5). C34 was also stable when stored at 4°C in the refrigerator up to a period of 2 months with a reduction of less than 1 log PFU/ml (Figure 4.6).

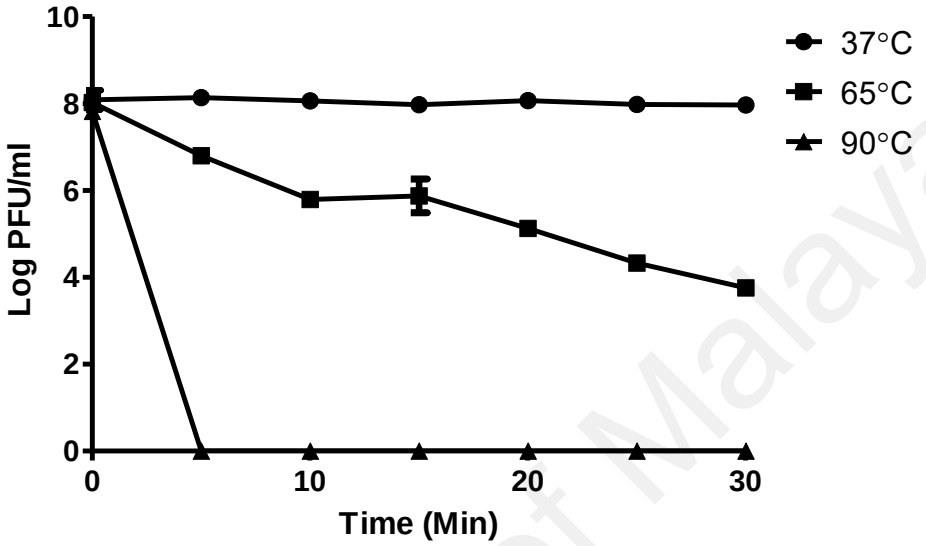


Figure 4.5: Temperature stability test of C34 at 37°C (●), 65°C (■) and 90°C (▲).The results are the averages of three independent assays.

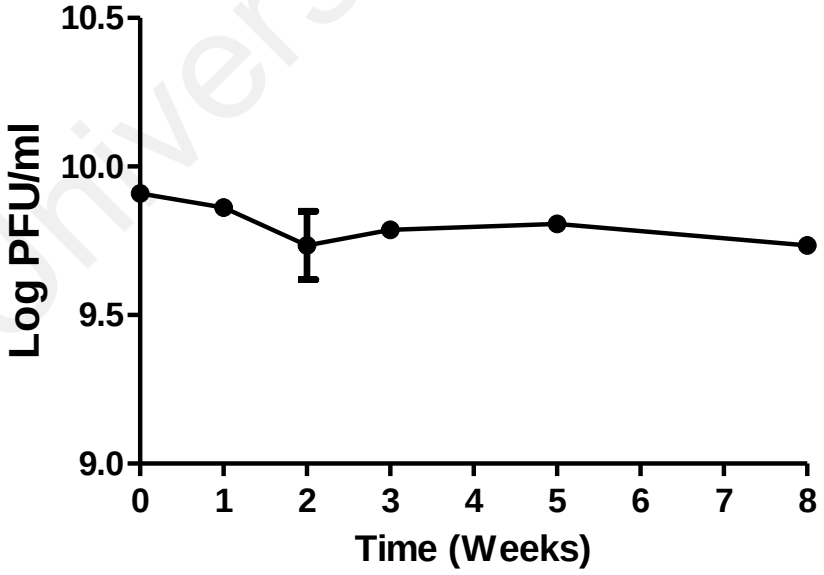


Figure 4.6: Temperature stability test of C34 at 4°C over the course of 8 weeks. The results are the averages of three independent assays.

4.3 Experimental Phage Therapy

4.3.1 Experimental Phage Therapy in Cell Culture Model

The objective of the experimental phage therapy was to examine the ability of phage C34 to treat or increase the viability of A549 cells infected with *B. pseudomallei*. Prior to the experiment, the MOI of *B. pseudomallei* strain (CMS) and concentration of kanamycin used in the experiment was optimised. The four different MOIs (1, 5, 10 and 50) tested in the infection assay showed different levels of cell viability (Figure 4.7). The viability of A549 cells infected with CMS at a MOI of 1 was 84.2 ± 3.3%. However, at the MOI of 5, there was a vast decrease in the viability of the cells to 38.0 ± 1.7%. A549 cells infected with CMS at a MOI of 10 and 50 demonstrated similar viability of 19.3 ± 1.0% and 21.8 ± 1.1 %, respectively. The difference between these two MOIs used was statistically not significant ($p>0.05$). Examination of the test wells of MOI 10 and 50 under inverted microscope revealed that despite a viability of approximately 20% post infection, nearly all cells appeared to be dead. From a range of the MOIs tested, MOI 10 was selected for further analysis.

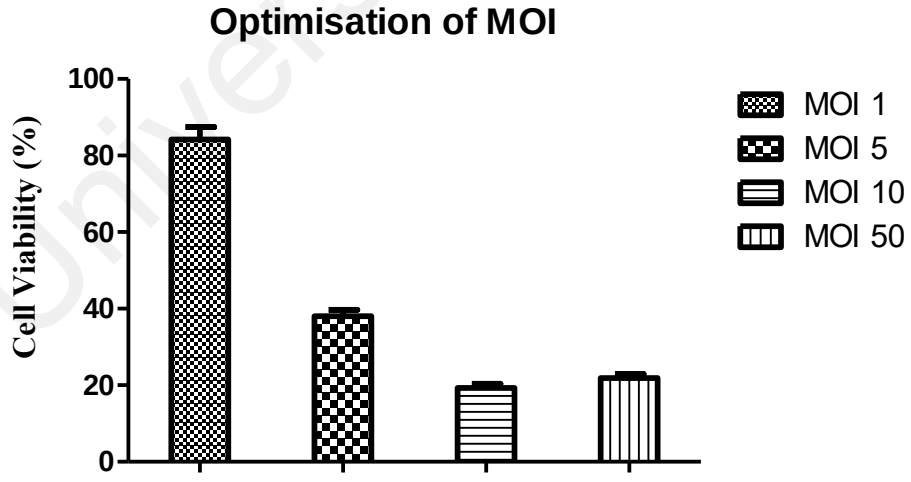


Figure 4.7: Viability of A549 cells infected at MOI 1 to 50 (left to right).

Kanamycin used in the experimental phage therapy functions to kill the extracellular bacteria released or excreted from the infected cells. This is to prevent the applied phage from infecting extracellular bacteria present thus affecting the PFU of the phage applied. The appropriate concentration of antibiotic used in experimental therapy was determined in order to completely kill any extracellular bacteria, and avoiding a concentration too high to the extent of being toxic to the A549 cells. Two-fold serially diluted stock kanamycin solution with concentrations ranging from 39 µg/ml to 5000 µg/ml was tested on A549 cells infected by *B. pseudomallei* at a MOI of 10. There was no significant difference between the viability of A549 cells supplemented with concentrations of kanamycin from 39 µg/ml to 5000 µg/ml (Figure 4.8). However, the concentration of 500 µg/ml was selected as it represented the mid-range of concentrations tested and it was also the minimum inhibitory concentration (MIC) of kanamycin for CMS as tested in the laboratory (unpublished data).

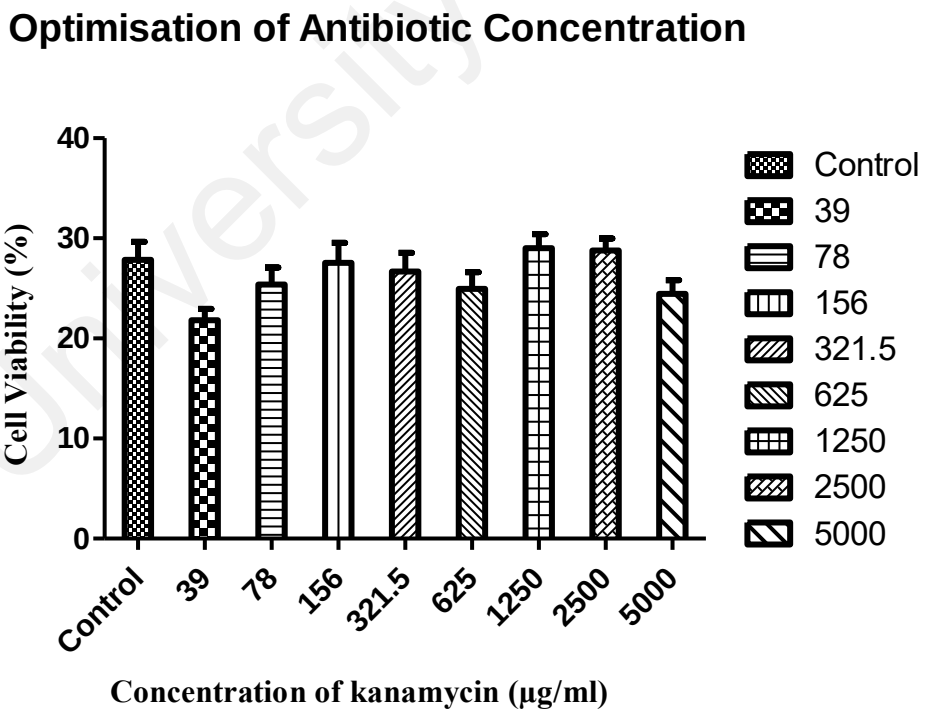


Figure 4.8: Viability of infected A549 cells at different concentration of kanamycin in the cell culture media.

The experimental phage therapy performed using the kanamycin concentration of 500 µg/ml and MOI of 10 from the optimisation showed an average of 22.8 ± 6.0% viability of A549 cells infected by CMS after 18 hours of infection. A range of PFUs tested, from 10 – 10^7, demonstrated that there was no increase in the viability of A549 cells in relative to the control group at any of the PFUs used.(Figure 4.9).

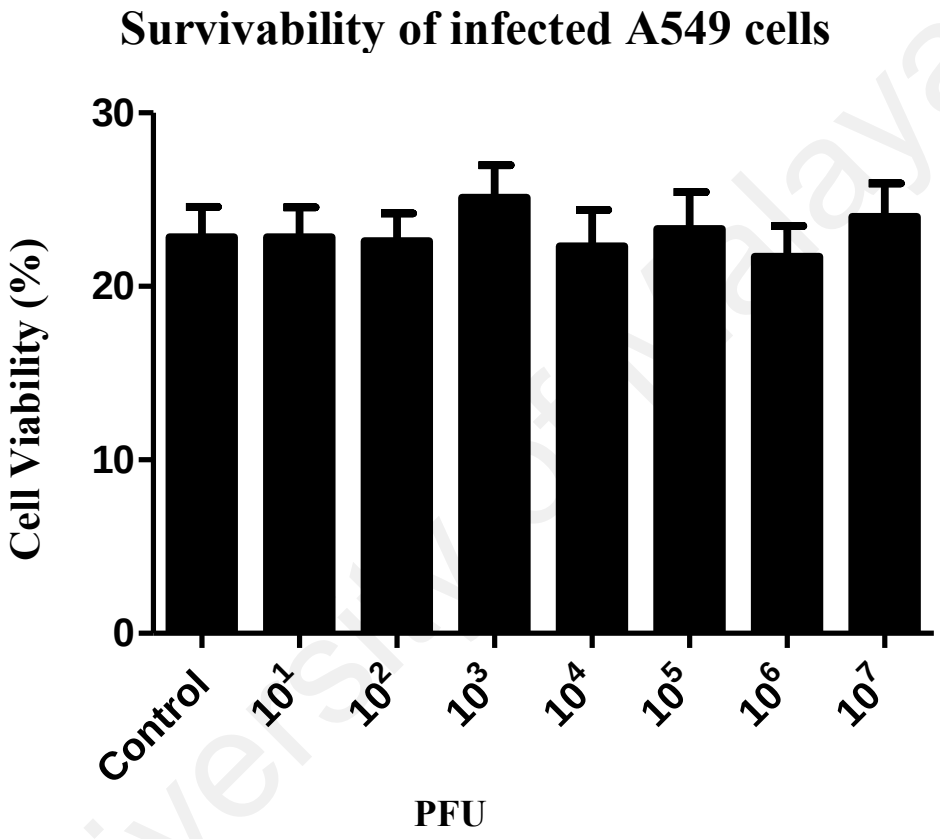


Figure 4.9: Viability of infected A549 cells treated with different PFUs of C34 phage.

4.3.2 Prophylactic Protective Effect of Bacteriophage in Cell Culture Model

A pre-treatment experiment was performed to examine whether the application of phages prior to infection (Pre) and pre + post-infection treatment (Pre+post) had any prophylactic effect on the A549 cells. It was demonstrated that the viability of pre-infection treated A549 control cells was 41.6 ± 6.5% compared to 22.8 ± 6.0% of the post-infection treated cells (Post) (Figure 4.10). The result suggested that additional administration of post-infection treatment to the pre-infection treated cells (Pre+post) did not increase the viability of infected A549 cells. Paired T-test analysis was performed and it was shown that the viability of A549 cells in pre-infection treated group is very significantly higher than that of the post-infection treated only group ($p<0.001$). These results suggested that phage C34 could provide prophylactic protection against *B. pseudomallei* infection.

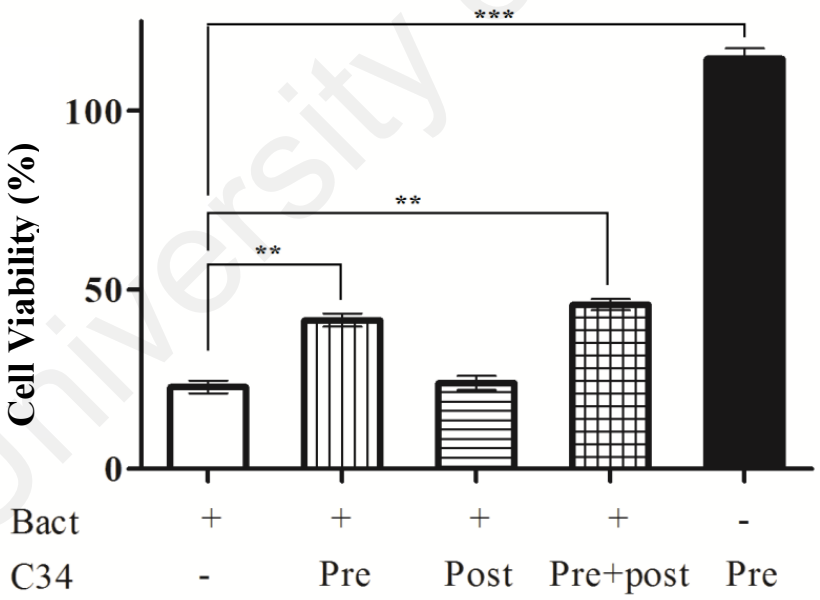


Figure 4.10: Viability of infected A549 cells. Viability of non-infected A549 cells was calculated as 100% and thus not shown in the figure. The viability of infected cells which received pre-infection and pre + post-infection treatment was significantly higher than the control. The bar chart shows averages for three independent assays. The results are the averages of three independent assays.

4.3.3 Experimental Phage Therapy in Mice Model

4.3.3.1 Selection of Infection Dose

It was shown in the *in vitro* model using A549 cells that phage C34 was able to provide partial protection towards infection by *B. pseudomallei* CMS. In order to verify this result in an *in vivo* model, the experimental phage therapy was performed in a mice model. The suitable infection dose was determined by infecting 6-8 weeks old female BALB/C mice with different dose of CFU of CMS injected via intranasal (i.n.) route (n = 10 per group). It was found that upon infection, the mice displayed symptoms of disease such as onset of fever and lethargy upon three days of infection. The time to achieve 100% death from 10^2-10^5 CFU was 11 days, 8 days, 4 days and 3 days, respectively, while 90% of the mice injected with 10 CFU survived until the end of the observation period (Figure 4.11). The median time to death was found to be 7.5 days for 10^2 CFU, 5.5 days for 10^3 CFU and 3 days for both 10^4 and 10^5 CFU. Infection dose of 10^2 and 10^3 CFU was chosen for experimental therapy as it was believed that infection 10^4 and 10^5 CFU were too virulent.

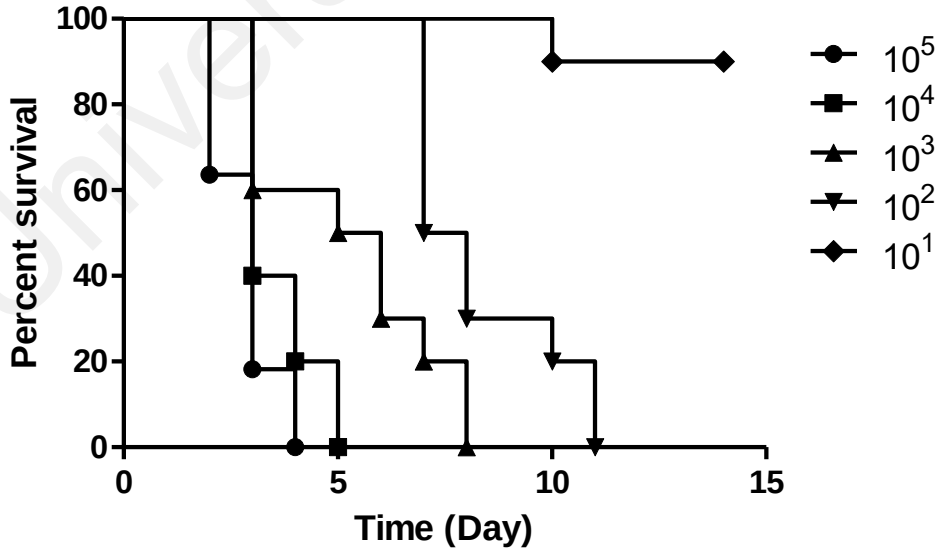


Figure 4.11: Survival plot of mice infected by different infection dose of CMS via i.n. route.

4.3.3.2 Experimental Phage Therapy at 10^3 CFU

Groups of mice (n = 10 per group) were infected with 1×10^3 CFU of CMS via i.n. route. Mice were then treated for 24 hours prior to infection or two hours post infection with 2 x 10^8 PFU of C34 in 100 µl of PBS via i.p. injection. The survivability of mice was observed over the course of 14 days and presented in Kaplan-Meier survival plot (Figure 4.12). Log rank (Mantel-Cox test) statistics was used to determine the statistical significance of the differences between the control and the treatment groups.

All mice displayed symptoms of disease such as onset of fever and lethargy upon three days of infection. It was found that both pre- and post-infection treatment of C34 did not provide protection to the infected mice. The survival plot of both groups did not differ significantly from the survival plot of the control mice ($p > 0.05$). The median time to death of control, pre- and post-infection treated groups were 5.5 days, 7 days and 6 days, respectively.

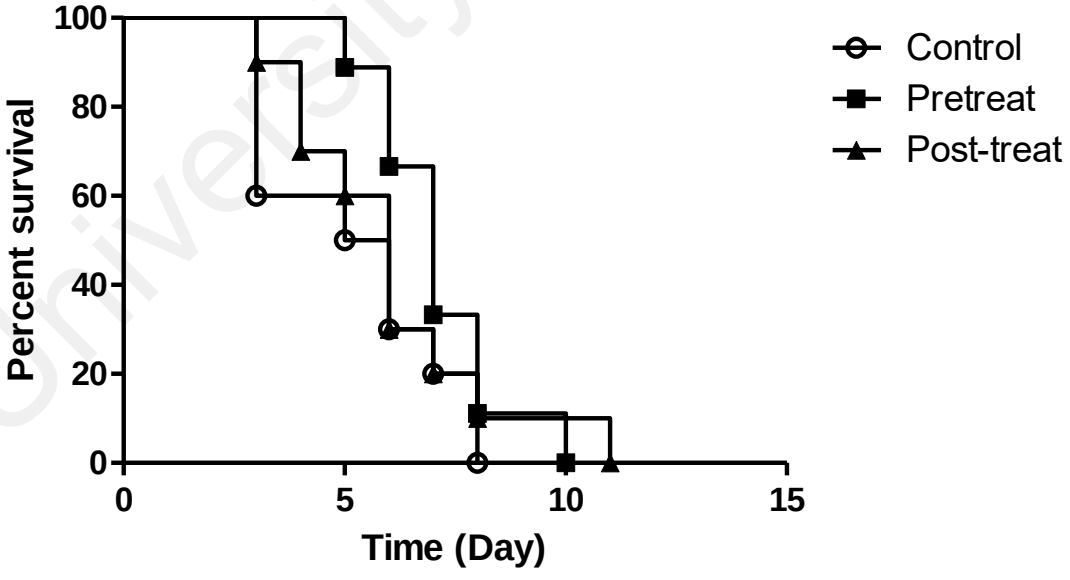


Figure 4.12: Mortality of mice infected with 1×10^3 CFU via i.n. route and received a single dose of 2×10^8 PFU of phage C34, which were administered via i.p. route, 24 hours before the infection (■) or 2 hours post-infection (▲). Control mice without any treatment (○) were 100% moribund at day 8.

4.3.3.3 Experimental Phage Therapy at 10^2 CFU

Groups of mice (n=15 per group) were infected with 1 x 10^2 CFU of CMS via i.n. route. Mice were then treated 24 hours prior to infection or two hours post infection with 2 x 10^8 PFU of C34 in 100 µl of PBS via i.p. injection. The survivability of mice was observed over the course of 14 days and presented in Kaplan-Meier survival plot (Figure 4.13). Log rank (Mantel-Cox test) statistics was used to determine the statistical significance of the differences between the control and the treatment groups.

All untreated mice displayed symptoms of disease such as onset of fever and lethargy upon three days of infection while the treated mice only started to show symptoms at day five. The application of C34, both pre- and post-infection treatment significantly protected the infected mice compared to the untreated controls ($p<0.001$). Both of the treatment group demonstrated similar percentage of survival (33.3%) with 5 mice surviving at the end of the experiment, respectively. Statistical analysis also revealed that there was no significant difference between the survival curves of pre- and post-infection treated groups ($p = 0.7006$). All of the untreated mice were moribund at day 11. The median of survival was 8 days for untreated mice, 13 days for pre-infection treated mice and 11 days for post-infection treated mice. In the process of organ harvesting, it was found that there was presence of macroscopic lesions on the spleen of the mice and signs of splenomegaly (spleen enlargement) (Figure 4.14). The results suggested that phage treatment using C34, regardless to the time of application, was able to provide partial protection towards *B. pseudomallei* infection.

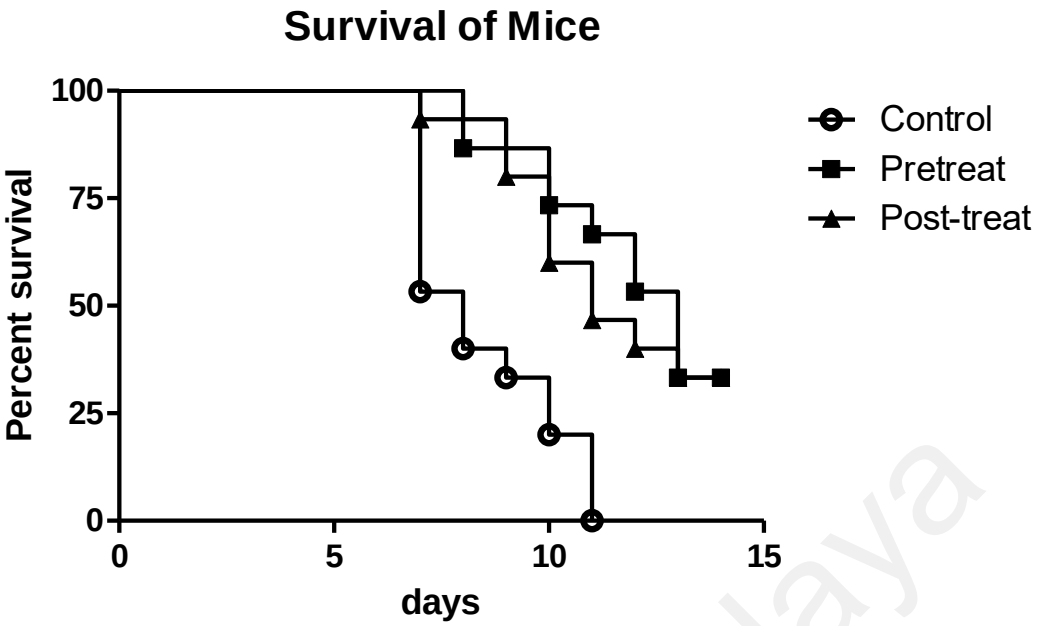


Figure 4.13: Mortality of CMS-infected mice which received a single dose of 2×10^8 PFU of phage C34, which were administered via i.p. route, 24 hours before the infection (■) or 2 hours post-infection (▲). Control mice without any treatment (○) were 100% moribund at day 11.

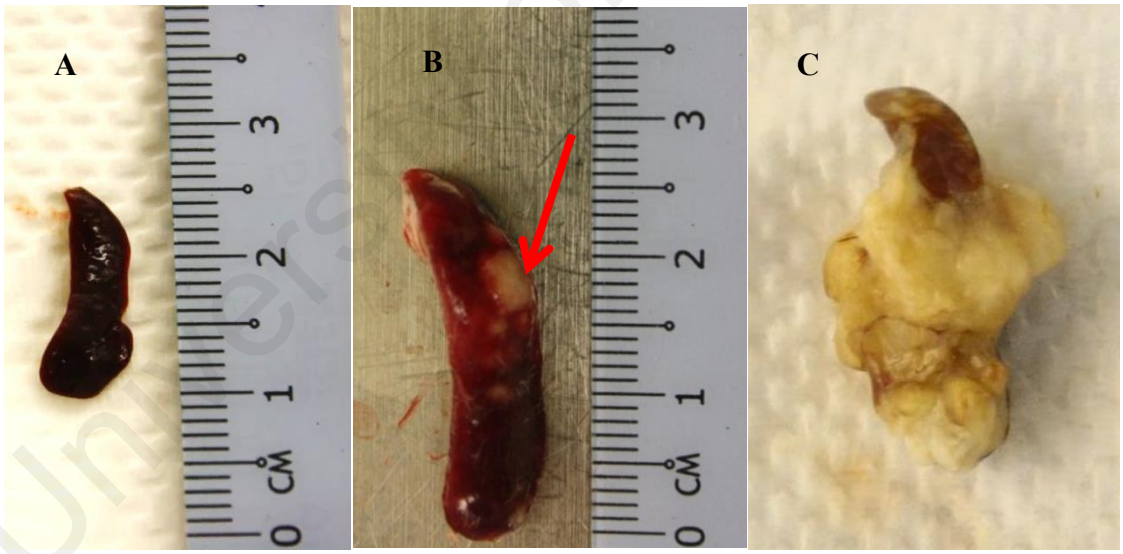


Figure 4.14: Comparison of the spleen of a healthy control mouse (A), infected mouse which survived for 14 days after receiving phage treatment (B) and spleen of an infected mice at day 5 (C). The arrow indicates macroscopic lesion on the spleen.

4.3.3.4 Bacterial Load in Mice Tissue

In order to confirm whether the application of C34 into infected mice was able to inhibit or reduce the bacterial load in mice tissue, blood, lung, liver and spleen of the mice were harvested one, two and three days post infection (n = 6 mice per group per day) and grind to check for viable bacteria count by diluting and plating on Ashdown's agar. The plates were incubated at 37°C for two days and the CFU/ml is counted.

No viable bacterium was detected from the blood of the mice throughout the three days period. The bacterial load in the lung of the infected mice was less detectable one day and three days post infection, where viable bacteria were observed on Ashdown's agar in less than 50% of the mice (Figure 4.15). During day two, it was found that the average bacterial load in the lungs of the control mice was higher than that of the pre- and post-infection treated group (log 2.71 ± 1.92 CFU/ml vs log 1.51 ± 1.47 and log 1.42 ± 1.59 CFU/ml). However, the differences between these averages were not statistically significant ($p>0.05$). In the liver, all three groups showed similar average bacterial load in day one, with log 1.11 ± 1.22, 1.37 ± 1.14 and 1.19 ± 0.97 CFU/ml, respectively. The bacterial load of control group was higher than the bacterial load of both treated group for day two and three. However, the differences between the averages were again not statistically significant ($p>0.05$).

In the spleen, all three groups again showed similar average bacterial load in day one. The bacterial load of control group was higher than that of both treated group for day two and three, with at least one log of inhibition observed. Statistical analysis revealed that the differences between the groups at day two were not significant. For day three, the average of bacterial load in the spleen of the post-infection treated mice is significantly lower than that of the control mice ($p<0.001$) whereas it is insignificant for the pre-infection treated group ($p>0.05$).

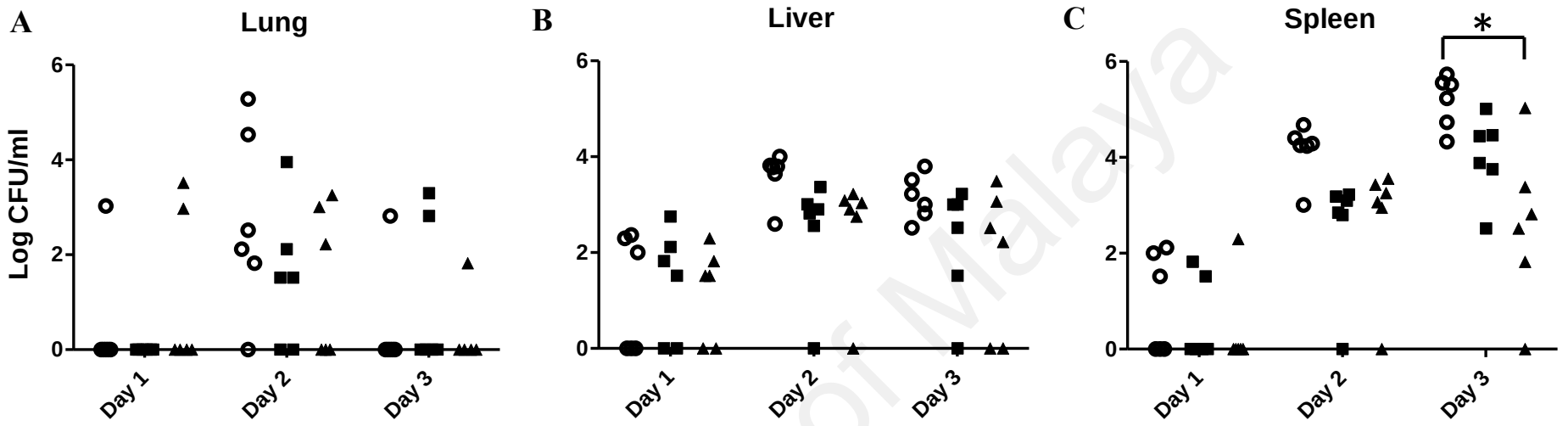


Figure 4.15: Bacterial burdens in lung, spleen, and liver of control mice (○) and mice treated with i.p. phage treatment (2×10^8 PFU of phage C34), administered 24 hours before the infection (■) or 2 hours post-infection (▲). The numbers of viable bacteria (CFU) were enumerated from the organs of mice on day 1, 2 and 3 post-infection (A-C).

4.3.3.5 Persistence of Phage in Mice Tissues

In order to examine the persistence and circulation of C34 in mice system, a single phage dose of 2×10^8 PFU in 100 µl of PBS was injected via i.p. route into mock-infected mice and mice infected with 1×10^2 CFU of CMS via i.n. route, 24 hours pre-infection and 2 hours post infection. Blood, lung, liver and spleen of the mice were harvested one, two and three days post-infection (n = 6 mice per group per day) for detection of phage using double agar overlay method.

No phage was detected in the blood of mice in every group throughout the three days of experiment. For control mice (mock-infected), an average of log 4.35 ±0.69 PFU/ml of C34 was recovered from the spleen of the mice (n = 5) while none was detected from lung and liver in day one post infection (Figure 4.16). No phage was detected from all tissues for day two and three post injection. As for the pre-infection treated group, no phage was recovered from all tissues for all three days. In the post-infection treated group, phage was present in lung, liver and spleen of the infected mice one day post infection. However, only half of the mice (n = 3) injected were found to be carrying the phage in their lung and liver, with an average of log 2.29 ± 0.61 and 3.69 ± 0.77 PFU/ml, respectively. Phage was detected from the spleen of five of the mice with an average of log 3.83 ±1.19 PFU/ml. Statistical analysis revealed that there was no difference on the number of phage found at the spleen between the control and treated group ($p > 0.05$). Similar to the control group, no phage was recovered at day two and three post-infection.

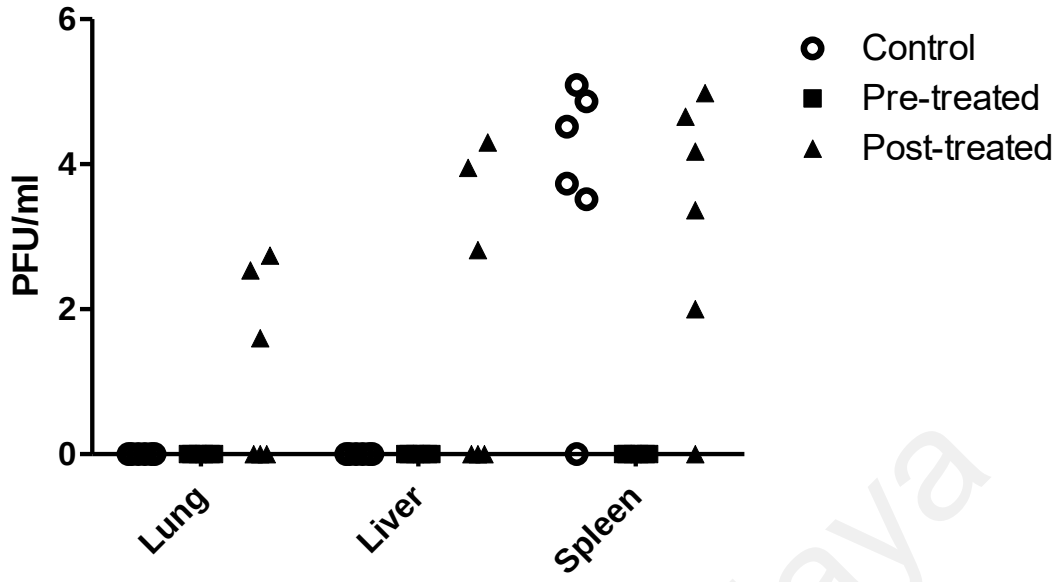


Figure 4.16: Recovery of phages from the mice tissues after i.p. administration of 2×10^8 PFU phage C34. Presence of phages were examined at 24 hours post-injection in the mock-infected mice (○), at 24 hours after the infection in pre-infection treated mice (■) and 24 after the infection in post-infection treated mice (▲).

CHAPTER 5: DISCUSSION

Bacteriophages are often found to be the contributors of bacterial virulence and pathogenicity (Boyd *et al.*, 2001; Boyd, 2012). However, the history on the use of bacteriophages was unfolded by d'Herelle with the intention of using them as therapeutic agents for dysentery (Summers, 1999). The discovery of antibiotics later, stumped the development of phage therapy. Nevertheless, with the development of antibiotic resistance posing to be a therapeutic dilemma for the clinician, the interest in phage therapy has been revived, eg phage preparation approved for human application against multiple drug resistant *Staphylococcus aureus* infection (Markoishvili *et al.*, 2002). Experimental phage therapy on other antibiotic resistant bacteria such as *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* has also shown promising results in mouse models (Broxmeyer *et al.*, 2002; Hall *et al.*, 2012).

B. pseudomallei is intrinsically resistant to many antibiotics (Wiersinga *et al.*, 2012). The treatment strategy for *B. pseudomallei* infection often requires a period up to 6 months (Peacock *et al.*, 2008). Despite such prolonged eradication therapy, some of the patients still suffered from relapse or reoccurrence (Limmathurotsakul *et al.*, 2006). Thus, phage therapy on *B. pseudomallei* infection may be an alternative to conventional antibiotic therapy. To date, several bacteriophages of *B. pseudomallei* have been isolated and characterised. However, none of these phages was tested for application in phage therapy. In this study, bacteriophages of *B. pseudomallei* were isolated and characterised. The potential of these phages on *B. pseudomallei* infection was then evaluated in the *in vitro* and *in vivo* model.

5.1 Isolation of Bacteriophages from Environmental Samples

Bacteriophages are normally found at places where their host are present. However, phages of *B. pseudomallei* were unable to be isolated from the first few samples sites

(paddy field, Maran waterfall, oil palm and rubber tree plantation) by directly testing the sample suspension for the presence of phage. This may have been due to the low occurrence of these phages in the samples. In order to increase the number of phages, an enrichment method was used (Van Twest and Kropinski, 2009). In this enrichment method, overnight culture of *B. pseudomallei* was added to the sewage samples to serve as host for phage multiplication. Following overnight incubation, the culture was centrifuged and sterilised using a 0.45µm filter unit. The resulting supernatant was used for detection of bacteriophages. Observation of plaques only following enrichment indicated that enrichment is required for isolation of phages and thus, was used throughout the remaining isolation process.

Using the enrichment method, all samples collected from the seawater (from both Pulau Ketam and Port Dickson) were positive for the presence of bacteriophages. However, the enrichment method could not be used for the samples collected from paddy field, Maran waterfall, oil palm and rubber tree plantation as recollection of samples were unavailable. Similar to previous phage isolations reported in Thailand (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011), phages were also isolated from soil samples collected at the Kanching waterfall (one out of four soil samples) and Templer's Park (one out of three soil samples). In total, 43 phages were isolated and purified from these samples. Phages isolated from the seawater was believed to have originated from soil erosion, which may have brought *B. pseudomallei*, the soil bacterium together with *Burkholderia* phages into the river and subsequently to the seawater. Naturally, many bacteria (and thus, their phage) can also be found in the sewage system. Due to its diverse ecosystems (raw input, aerobic and anaerobic condition), sewage treatment plants were thought to be ideal sources for isolation of bacteriophages. One phage was isolated successfully from the sewage samples collected from the aerobic tank.

The downside of using the enrichment is that this method can introduce bias towards isolation of phages which are well adapted to the bacterial strains added. As a result of the selection, the fast growing phages may dominate the culture and thus, mask the presence of other slower-growing phages, possibly with broader host range (Gill and Hyman, 2010).

5.2 Characterisation of Bacteriophages:

5.2.1 Determination of Host Range of Bacteriophages

The basic characterisation of phages includes determining the host range of the phage. Host range of a phage is described as the breadth of bacteria it is capable of infecting (Kutter, 2009). While some phages are able to infect host of different genera or species, many of the phages only have a narrow host range and sometimes are unable to lyse some bacterial strains within the same species. The differences in infectivity of the phages is the result of a variety of factors related to the phage and host, such as phage-host receptor recognition, bacterial restriction-modification systems, abortive infection mechanisms and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) mechanisms (Hyman and Abedon, 2010). In terms of phage therapy in clinical application, determination of host range is particularly important as the success of a therapy is dependent on the selection of a suitable therapeutic phage.

In order to determine the host range, the isolated phages were tested against 43 clinical *B. pseudomallei* isolates from the laboratory's culture collection. It was discovered that most of the phage isolates can form plaques on 19 to 32 strains (44% - 74%) of the *B. pseudomallei* isolates tested. This result was comparable with the report by Yordpratum *et al*. (2011), whereby the phages isolated were found to have the ability to lyse 41% - 78% of *B. pseudomallei* strains in their collection. In contrast, Gatedee *et al.* (2011) reported that the phage isolated was able to lyse all tested *B. pseudomallei* strains. This

result needs to be treated with caution since Gatedee and colleagues have only tested their phage on 11 strains of *B. pseudomallei.* However, both present and the study by Yordpratum *et al.* (2011) tested the phages against 43 and 63 *B. pseudomallei* strains, respectively.

It was interesting to note that the efficacy of phage infection varies geographically. Gatedee *et al*. (2011) reported that their phage have a higher efficiency of plating on the *B. pseudomallei* from Thailand (local strains) as compared to the *B. pseudomallei* strains originated from Australia. Despite the lack of evidence hitherto to support this finding, it was suggested that phage therapy, if applicable, should be tailored to the needs of each patient to achieve the maximum treatment efficacy. The presence of bacterial isolates which are resistant to all phage infections in this study, further highlighted the importance of personalised therapy. Fortunately, one of the advantages of phage therapy is that new phages can be easily isolated from the environment and the isolation process can be achieved in a relatively short period (Merabishvili *et al.*, 2012). Therefore, phages with narrow host range should not be excluded in the attempt of searching for a suitable candidate for experimental therapy.

5.2.2 Restriction Digestion Analysis and TEM Observation

In order to further characterise and select suitable phages for experimental therapy, DNA was extracted from five of the phage isolates (K2, K43, C5, C34 and C38) and restriction digestion analysis was performed. DNA extracted from the selected phage strains were restrict-digested using five different REs, namely Apa1, BamHI, EcoRI, PstI, and XbaI. The phage DNAs were found to be sensitive to restrict-digestion by ApaI and PstI. Analysis of the digestion patterns revealed that all the five phages tested belonged to three different profiles. Being vulnerable to restrict-digestion by REs indicated that the phages tested were indeed double-stranded DNA viruses. TEM observation revealed that all the five phages were tailed viruses, categorised under the

family Myoviridae. The finding was a surprise, as to date most of the tailed phages discovered are from the family Siphoviridae, which constituted 61% of the total discovered tailed phages. Myoviridae have been reported to account for approximately 24.5% of the total tailed phages while the rest were Podoviridae (14%) (Ackermann, 2012). Excluding our isolates, of seven other environmental phage isolated by the Thai group, six of them are myoviruses and the remaining one is podovirus (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011). Based on a study by Suttle *et al* (2005), it was found that myoviruses normally have a broader host range than other tailed phages. However, it was assumed that the podovirus isolated would possess a different mechanism of infection or attach to a different set of host receptors as compared to the myoviruses and thus, would complement the antibacterial activity of myoviruses. Yet, the assumption will require further examinations.

5.2.3 Preparation of High Titre Phage Lysate

Phage titre is crucial in an experimental therapy. In 2014, a group of researcher working in phage therapy came out with a conclusion that a guideline is needed to ensure the sustainability of phage therapy research. From the discussion, they suggested that active substances (viable phage) for phage study or therapeutic purpose typically contains 10^8 to 10^{10} PFU/ml (Pirnay *et al.*, 2015). High titre lysate of three phage strains (C34, C38 and K43) were prepared and their ability to constantly produce high titre lysate was evaluated. Among the three, phage C34 was able to constantly produce lysate up to 10^9 PFU/ml and thus selected for further study.

5.2.4 Thermal Stability of Bacteriophage

Temperature stability of the phage preparation is among the factors that need to be taken into consideration in order to industrialise and mass produce a phage for phage therapy. The phage preparation needs to be stable and remain viable for the purpose of transportation and storage. It is important that the storage method is made easy and cost

efficient too. Although it was reported that phages survive thawing and freezing at -80°C or in liquid nitrogen with cryo-protectant, the method was regarded as not practical as such facilities may not be available to most end users (Skurnik *et al.*, 2007). Thus, for short-term storage, the stability of phage C34 was tested at 4°C in a refrigerator as refrigerators are available in most pharmacies and hospitals. Over the course of 2 months, there was only a slight decrease in the viability of the phage suspended in PBS and stored at 4°C. However, the decrease was not statistically significant. Based on this observation, it was postulated that phage C34 is suitable to be transported and stored at 4°C. This would be an advantage as mentioned before, most clinical facilities and pharmacies have refrigerators and its stability at 4°C will make it easy for storage and transportation.

A long term storage method commonly used in microbiology is the freeze-drying or lyophilisation method. Lyophilisation has the following advantages (Fortier and Moineau, 2009):

i) proven efficacy in bacteriology

ii) eliminate the need for refrigerators, freezers or liquid nitrogen tank

iii) less space required for the storage for vials

iv) ease in transportation

However, the use of lyophilisation for bacteriophages produced mixed results. Studies by American Type Culture Collection (ATCC) on a variety of phage strains revealed that phages of the *Myoviridae* family were labile while smaller phages such as those of *Siphoviridae* family were more resistant to freeze-drying (Clark, 1962; Clark and Geary, 1973). Other reports revealed otherwise that freeze-dried phages can be revived with minimal loss (less than 1 log PFU/ml reduction) even after 8 months to 18 years of storage (Carne and Greaves, 1974; Engel *et al.*, 1974; Zierdt, 1988). More recently,

Ackermann et al. (Ackermann *et al.*, 2004) observed that the quality of the vacuum in the storage ampoules was the most crucial parameter which kept the phages viable. Lyophilised ampoules with intact vacuum successfully preserved phages for over 20 years, while no viable phage can be revived from those which lost their vacuum, although only after one year in storage. As a conclusion, lyophilisation can be a viable method for long-term preservation of phage C34, however, utilisation of this method needs to be carefully tested in advance.

The effect of heating on phage C34 was also tested by incubating the phage suspension at three different temperatures (namely 37°C, 65°C and 90°C) for 30 minutes in water bath. It was found that incubation of C34 at 37°C has no effect on the viability of the phage but incubation at higher temperature significantly reduced the viability of the phage. This finding was similar to the report on phage ϕ Bp-AMP1 where the podovirus was not detectable after being incubated at 60°C for one hour (Gatedee *et al.*, 2011). It was not clear whether the same applies to the *Burkholderia* phages isolated by Yordpratum *et al.* (2011) as the temperature stability of these phages was not tested. Coupled with the findings that the phages which infect *Lactobacillus delbrueckii* also lose viability at this temperature (Quiberoni *et al.*, 2003), heating of 60°C and above may kill most phages.

5.2.5 Time Killing Curve

Time killing curve analysis demonstrated that phage C34 was able to reduce the amount of *B. pseudomallei* in the early stages of exposure. However, the bacteria started to grow again after six hours upon exposure to the phage suggesting the possible development of resistance towards C34 infection. A growth curve was also constructed using the optical density readings of bacteria cultures and complimented the time killing curve. The growth curve dropped initially and started to rise again. Both of the time killing and growth curves clearly demonstrated that the bacteria started to grow again.

This study could have been enhanced if knowledge on the receptors of the phages on *B. pseudomallei* was known. However it was not the objective of this study. Phages target specific receptors for attachment and binding before an infection begins. As such, it is possible to carefully choose and formulate a cocktail of lytic phages which can avoid the rise of phage resistance and completely eliminate the pathogen. A few studies have shown that treatment with cocktails comprising of at least two different types of phage is more effective than the use of single phage (Brussow, 2005; Cairns and Payne, 2008; Callaway *et al.*, 2008; Alemayehu *et al.*, 2012; Jaiswal *et al.*, 2013). One of the study reported complete clearance of bacteria in milk, using a phage cocktail (Garcia *et al.*, 2007). However, it is important to note that not all cocktails demonstrate better efficacy compared to the single phage treatment (O'Flynn *et al.*, 2004). It is thus advisable to conduct detailed studies on the efficacy of the selected single or phage cocktails prior to treatment.

Despite knowing that cocktails have the potential to decrease the occurrence of resistance and improve the treatment efficacy, there are not many reports on the effect of simultaneous and sequential application of phages. A study by Hall and colleagues (2012) on the effect of simultaneous and sequential application of different bacteriophages on bacterial population and resistance development found that some phage sequences are more efficient than other sequences and simultaneous application of cocktail performed equal or better than the sequential application of the phages. Neither of the simultaneous nor sequential application was able to minimise the occurrence of phage resistance. These observations suggested that phage therapy requires "sur-mesure" approach for maximum efficacy and may be more responsive than the use of "prêt-a`-porter" drug such as currently available antibiotics (Pirnay *et al.*, 2011).

5.3 Experimental Phage Therapy

5.3.1 Experimental Phage Therapy on Cell Culture Model

In order to determine the efficacy of the isolated bacteriophage, C34, against infected cells, treatment of human lung epithelial cell, A549, infected with *B. pseudomallei* CMS was performed.

During optimisation for the infection assay, the effect of different MOIs and concentrations of antibiotics on the survivability of infected A549 cells were tested. It was demonstrated that at the bacteria to cell MOI of 10 and 50, all the infected cells were found to be dead with some of the cell debris still attached to the wells. This was further confirmed under the microscope. The remaining cell debris also absorbed the added crystal violet solution, giving a baseline 'noise' of around 20%. Since all the infected cells were killed at both the MOIs used, MOI of 10 was selected for further studies in infection assay. Kanamycin was used at a concentration of 500 µg/ml in this assay to eliminate any extracellular bacterial cells attaching on the surface of A549 cells. This concentration of kanamycin has been used previously in preliminary studies and showed that it did not affect the viability of the infected cells and that the concentration was also close to the minimum inhibitory concentration for *B. pseudomallei* CMS.

Following optimisation, the therapeutic effect of phage C34 on infected A549 cells was determined. It was found that the application of phage post-infection did not significantly increase the viability of infected A549 cells despite addition of phage titres as high as 10^7 PFU to the infected cells. Pre-exposure of the A549 cells to 2×10^6 PFU of phage C34, 24 hours prior to infection was also attempted followed by treatment with phage using 10 to 1×10^7 PFU post infection. It was found that pre-infection treatment of the cells with phage C34 successfully increased the viability of infected A549 cells. Additional post infection treatment of pre-infection treated cells did not further increase

the viability of infected cells indicating that pre-infection treatment alone contributes to the increased viability.

Burkholderia pseudomallei is a well-known notorious pathogen which causes intracellular infection. Previously, it was shown that phage therapy is effective against several intracellular pathogens such as *Mycobacterium tuberculosis* (Broxmeyer *et al.*, 2002; Danelishvili *et al.*, 2006), *Yersinia pestis* (Filippov *et al.*, 2012) and *Staphylococcus aureus* (Capparelli *et al.*, 2007) during *in vitro* and *in vivo* experiments. However, all of these studies report the use of phagocytic cells (macrophages model), in which the phage will be internalised naturally by the macrophages. In addition, in order to deliver phages more effectively into the cells, closely-related but avirulent strains of bacteria was transiently infected with the phages and used as a vehicle to carry the phages into target cells when the avirulent bacteria invade the cells. For example, *M. smegtis* the avirulent counterpart of *M. tuberculosis* and *M. avium*, was transiently infected and used as the vehicle. It was found that the treatment caused a 100-fold reduction in the amount of *M. tuberculosis in vitro* and similar reduction in the amount of bacteria (approximately 100-fold) was also observed in the spleen of mice infected by *M. tuberculosis* (Broxmeyer *et al.*, 2002; Danelishvili *et al.*, 2006). Likewise, up to 70% of intracellular *S. aureus* was killed when phage M^{SA} was delivered into the infected macrophages using phage-infected *S. aureus*; whereas no killing is observed if treatment was carried out using only the phage (Capparelli *et al.*, 2007).

In present study, the results were in agreement with the observations above, suggesting that phage therapy may be a viable alternative treatment against intracellular pathogen. It was found that the phage C34 is able to provide partial protection to intracellular infection by *B. pseudomallei* CMS. In addition, this was accomplished without the use of any delivery vehicle. To the best of our knowledge, this is the first report of successful experimental phage therapy of *B. pseudomallei* infection in a non-phagocytic

cell culture model (A549 epithelial cell) in the absence of a phage-delivery vehicle. Undeniably this was due to the lack of avirulent host for phage C34 as it is unable to infect *B. thailandensis*, the avirulent counterpart of *B. pseudomallei*. It is suggested that the availability of a delivery vehicle to transport the phage into cells may increase the intake of phage C34 into infected cells and thus further increase the viability of the cells.

It was noticed that the viability of infected cells did not increase when higher doses of phages were applied post-infection in both settings of pre + post-infection treated and only post-infection treated. In another study by our group, it was shown that upon infection by CMS, several pathways involving endocytosis and phagocytosis were down-regulated in A549 cells (unpublished data). Combining the two observations, it was thus postulated that infection by *B. pseudomallei* would reduce permeabilisation or internalisation of phage C34 into A549 cells. Promoting the intake or internalisation of phage into targeted cells would most probably be the key factor in increasing the protective effect of phage. Methods such as genetically modified phage and phage display can be incorporated to improve the efficiency of phage intake and thus, increase the efficacy of phage therapy (Hart *et al.*, 1994; Piersanti *et al.*, 2004).

5.3.2 Experimental Phage Therapy on Mice Model

Inhalation is among the main route of infection of melioidosis (Cheng and Currie, 2005). However, there are only a few animal models available for the experimental therapy. Previously, a series of *B. pseudomallei* infection experiments have been conducted on BALB/c and C57BL/6 mice. It was found that the course of infection in BALB/c mice mimics acute human infection (Leakey *et al.*, 1998). C57BL/6 mice were more resistance to *B. pseudomallei* infections and would normally develop into chronic infection (Leakey *et al.*, 1998; Barnes *et al.*, 2001). It was suggested that the difference in susceptibility was due to the differential inflammatory responses between these mice. The moderate increase in the production of proinflammatory cytokines in infected

C57/BL6 mice (as compared to hyperproduction in infected BALB/c mice) was believed to contribute to the efficient clearing of *B. pseudomallei* from the mice system (Tan *et al.*, 2008). It was also found that *B. pseudomallei* was highly virulent when mice were challenged via intranasal as compared to intraperitoneal challenge (Brett and Woods, 2000; Haque *et al.*, 2006; Titball *et al.*, 2008). Further histopathological examination on the lung of the pulmonary infected animals revealed that the pathology of the infection was similar to that in clinical studies (van Schaik *et al.*, 2008). Thus, BALB/c mice and infection via intranasal route were selected for that purpose of simulating *B. pseudomallei* infection in human.

Administration of phage was performed via the intraperitoneal route based on a study on *B. cenocepacia* by Carmody *et al* (2010). In the experiment, phage which was active against *B. cenocepacia* was administered via both intranasal and intraperitoneal route into the intratrachaelly infected BALB/c mice. It was found that higher titre of phage was recovered in the lung of the mice treated via intranasal inhalation compared to the mice treated via intraperitoneal injection. However, despite having lower phage titre, the mice treated via intraperitoneal injection showed a better treatment efficacy, with a significantly lowered bacterial load in the lung of the infected mice. A reduction in the bacterial load in the lung of the mice treated via intranasal inhalation was also observed but the difference was not significant as compared to the infected controls. The report was in agreement with several studies, suggesting that systemic administration via intraperitoneal injection was more effective in terms of treatment efficacy in comparison with topical or *in situ* administration (McVay *et al.*, 2007; Watanabe *et al.*, 2007; Chhibber *et al.*, 2008).

In this study, from a range of CFU ($10 – 10^5$) used for the infection of the mice in order to determine the suitable infection dose for experimental therapy, 10^2 and 10^3 CFU were selected as the infection dose. Experimental phage therapy of the mice infected with 10^3

CFU of CMS demonstrated that the survival of mice which received pre- and post-infection treatment did not differ significantly from the control mice, despite a slight delay in the median of survival time. However, when the experimental therapy was conducted on mice infected by 10^2 CFU, 33% of the pre- and post-infection treated mice survived at the end of the 14-days experiment period whereas 100% of the infected controls were dead at day 11. The median of survival time also increased from 8 days (for the control) to 13 days for pre-infection treated mice and 11 days for post-infection treated mice. These results suggest that phage C34 was able to provide protection against infection by *B. pseudomallei* CMS. Sterilising immunity was not achieved in both the treatment methods as splenomegaly and macroscopic lesions were found on the spleen and liver of the surviving mice (Dannenberg and Scott, 1958; Davis and Ramakrishnan, 2009).

Recent research on experimental phage therapy against fatal infections using animal models showed varied outcome (Table 4.1). Some of the studies merely extended the mean to time of death (MTTD) of the infected animals (Tiwari *et al.*, 2011; Hall *et al.*, 2012), while some of the treatments successfully protected all of the infected animals (Heo *et al.*, 2009; Tiwari *et al.*, 2011). However to date, there is no similar experimental therapy on *B. pseudomallei* infection, and thus, comparison of this study with previous studies was unable to be performed. In this study, the survival rate of 33% and increased median of survival time can be considered as a moderate success and serves as a stepping stone for future studies. Despite not showing any treatment efficacy in the *in vitro* model, administration of phage two hours post-infection protected 33% of the infected mice.

The prophylactic effect exerted by C34 on A549 epithelial cells when applied overnight prior to infection also prompted this study to investigate the potential prophylactic protective effect of C34 *in vivo*. The phage was administered to the mice 24 hours prior

to infection via the intraperitoneal route and the survival of these pre-infection treated mice was similar to the post-infection treated mice. However, the median survival time of the pre-infection treated mice was slightly longer (13 days) compared to the post-infection treated mice (11 days) and the controls (8 days). The reason behind the delay is still unknown, but it was postulated that the immune-modulator properties of the phage may play a role in the delay. As suggested by Tan (2008), a moderate production of pro-inflammatory factors is more effective in the control of *B. pseudomallei* infection as compared to hyperproduction of these factors. Further studies involving monitoring the changes of chemokines and cytokines produced during the course of infection may be able to address this postulation.

Nevertheless, this study clearly indicates that the antibacterial property of the administered phage contributed to the survival of the infected mice, which is evident through the protective effect shown by phage C34 in the *in vitro* model. In this *in vitro* model, the A549 epithelial cells do not possess any immune system; however, pre-infection treatment rescued around 40% of the infected cells. Thus, in combination with both the observation from the *in vitro* and *in vivo* model, it can be suggested that the activity of phage C34 did contribute to the therapeutic effect against *B. pseudomallei* infection. This hypothesis was further supported by the fact that administration of bacteriophage also significantly reduced the bacterial load in the spleen of the infected mice. The average bacterial load in the spleen of the post-infection treated mice at day three was approximately two fold lower than that of the untreated controls. Albeit not statistically significant, the bacterial load in the liver of mice from both treatment groups was also lower than the untreated controls. It is believed that despite not completely clearing the infection, the reduction of bacterial load in the organs of the mice prolonged the survival of the treated groups.

To date, it is still a challenge to achieve sterile immunity against *B. pseudomallei* (Conejero *et al.*, 2011). It is common for the infection to develop into a chronic localised infection with the presence of splenomegaly and macroscopic lesions as the common features (Dannenberg and Scott, 1958). The observation of lesions and splenomegaly signified the presence of degenerated and viable macrophages and neutrophils. These observations were always accompanied by the deposition of collagen and formation of fibrosis, which act as the barrier between healthy tissues and areas of necrosis (Conejero *et al.*, 2011). Macroscopic lesions were also normally associated with high bacterial load in the organs. The bacteria were confined in these pyogranulomas, which enabled it to persist in the host via these modified niches (Lever *et al.*, 2003). It was suggested that these fibrotic response, though limiting the spread of the bacteria, may also shield the bacteria from penetration of antibiotics and access of new inflammatory cells into the confined area, similar to that in tuberculosis (Davis and Ramakrishnan, 2009). In this study, even though 33% of the infected mice were rescued with the single injection of phage, *B. pseudomallei* still persisted in the organs of the surviving mice. Thus, similar to antibiotic treatment on melioidosis patients, it is believed that an eradication therapy is required for total clearance of the bacteria.

Table 5.1: Published results on experimental therapy of fatal bacterial infections. SC: subcutaneous; IP: intraperitoneal; IN: intranasal; IV: intravenous.

Animal Model	Bacteria	Infection route	Infection dose	Treatment dose	Treatment route	Time of administer	Observation period	Results	Literature
BALB/c	*Yersinia pestis*	SC	$2 – 2 \times 10^4$ CFU	5 x $10^{8,9}$ PFU	IP	1 hour	14 days	20-40% survival, 84% increase in MTTD	(Filippov *et al.*, 2012)
ICR	*P. aeruginosa*	IP	5 x 10^6 CFU	MOI of 1, 10, 100	IP	Immediate	100 hours	Normal: 80-100% survival Neutropenic: Survived 12 hours longer than control	(Tiwari *et al.*, 2011)
ICR	*P. aeruginosa*	Oral (drinking water)	10^8 in drinking water	1 x 10^{10} PFU	Oral	1 day before, 1 days after, 6 days after	21 days	1 day after: 67% survival	(Watanabe *et al.*, 2007)
		IP	2.4 x 10^6 to 300 x10^6 CFU	1 x 10^{10} PFU	IP	Immediate	6 days	92.3% (treated) vs 41.7% (untreated)	
ICR	*P. aeruginosa*	IP	2 x10^6 CFU	2 x $10^{6,7}$ PFU	IP IM	6 hours	48h	50-100% 20-80%	(Heo *et al.*, 2009)
Drosophila		Pricking	10^7 cfu/ml	5 x 10^7 PFU	In medium	Immediate	48h	Extended MTTD	

Table 5.1 continued:

Waxmoth	*P. aeruginosa*	Between abdomen	5 x 10^5 CFU	10^5 PFU	Between abdomen	Immediate and reapplied every 12h	48h	Increased MTTD	(Hall *et al.*, 2012)
ICR	*S. aureus*	IN	6.4 x 10^8 cfu/ml	1 x 10^{10} PFU	IP	6h	14 days	67% survival	(Takemura-Uchiyama *et al.*, 2014)
BALB/c	*S. aureus*	IV	10^8 CFU	10^{7-9} PFU	IV	Immediate	4 days	10^9: 97%	(Capparelli *et al.*, 2007)
Galleria mellonella	*B. cepacia*	Injection	K56-2: 2.5 x 10^3 CFU	KS4-M: MOI 1-1000 KS12: 50-5000	Injection	Immediate, 6h, 12h	48h	27-93%	(Seed and Dennis, 2009)
			C6433: 1 x 10^5 CFU	KS14: 0.001-0.1					
BALB/c	*Vibrio parahaemolyticus*	IP	2.0 × 10^7 CFU	2.0 × 10^8 PFU	IP	1 hour	72 hours	92%	(Jun *et al.*, 2014)
		Oral	2.0 × 10^7 CFU	2.0 × 10^8 PFU	Oral			84%	

5.3.3 Recovery of Phage from Mice Tissues

Phages administered to test animals have been known to be sequestered in the spleen and cleared from the circulation in a short period (Dubos *et al.*, 1943; Smith and Huggins, 1982; Brussow, 2005). In this study, administration of phage C34 into healthy mice via the i.p. route also showed similar results where phage can only be detected in the spleen of the mice. When the phage was administered into *B. pseudomallei*-infected mice, it was recovered from the spleen as well as the lung and liver of half (50%) of the mice, one day post infection. In both groups (mock-infected and infected mice), phage was only detected one day post infection and was not able to be detected on the following days.

This result is in agreement with previous studies by Carmody *et al.* (2010) and Takemura-Uchiyama *et al.* (2014). In these studies, it has been reported that phage titre in the lungs of the infected mice was significantly higher than that of the control mice and this observation was attributed to the *in vivo* replication of the phages. Likewise, in this study, the phages could be circulated and retained (temporarily) in the infected organs in the presence of host bacteria. For the pre-infection treated group, no phage was recovered at all. The explanation for this observation is that phage was only titred from one to three days post infection, where the one day post infection was actually already day two post-phage injection. The result complimented the results of the mock-infected and post-infection treated group where no phage can be recovered at day two post injection. In the absence of host bacteria, the phages would be sequestered in the spleen and cleared from the mice system thereof. Nevertheless, observation of the multiplication of phage in the infected organs or calculation of the kinetics of phage clearance was unable to be performed as the phage titre was only examined one day post infection. Such information would be crucial in the understanding of phage pharmacokinetics and would most certainly be useful in improving the efficacy of

phage therapy. In future studies, time points with a smaller gap would be useful to model the transport and clearance of phage in the circulatory system.

In the past years, many studies have addressed the rapid clearing of phage from the mice circulatory system. In 1996, a method of selection was devised to select for mutant phages with prolonged circulation in mice system (Merril *et al.*, 1996). This involves injection of the target phage into the mice circulatory system, and then the phages remaining in the system after a designated time point were isolated and propagated. The propagated phages were then re-introduced into the mice and serial passage was performed for nine times or more. It was found that the titre of the selected mutants in the mice system was of 10000 folds higher than the wild type. The selection method also significantly increased the retention time of the phage in mice system.

Capparelli *et al*. (Capparelli *et al.*, 2006; 2007) successfully used this selection method to isolate mutant phages against *E. coli* O157:H7 and *S. aureus* with prolonged circulatory ability up to 38 and 21 days, respectively. Apart from persistence in the mice system, the mutant phage isolates also outperformed the wild type phages in their treatment efficacy. For example, φD, a persistent phage against *E. coli* was found to be more potent than its wild type counterpart, φW. The persistent mutant was able to completely clear the bacteria from the mice organs within 48 hours of infection while bacterial load up to 7.0×10^5 CFU/g was still detected in the organs of mice treated with φW. These studies suggest that the use of similar selection method may be able to improve the persistence of phage C34 in the circulatory system, and thus improving its efficacy of the phage therapy.

CHAPTER 6: CONCLUSION

In this study, the prospect of using phage therapy as an alternative treatment for *B. pseudomallei* infection was examined. Bacteriophages of *B. pseudomallei* was isolated from environmental sources and characterised by their host range, DNA restrict-digestion profiles, transmission electron microscopy and temperature stability. However, further studies such as genomic analysis are necessary to increase our understanding of the phages isolated.

Previously, similar studies on isolation and characterisation of *Burkholderia* phages were carried out (Gatedee *et al.*, 2011; Yordpratum *et al.*, 2011). However, the efficacy of those phages in phage therapy was not examined. In the present study, a total of 43 phages were isolated and one of the phages, C34 was chosen for experimental therapy in cell culture and mice model. In this study, C34 showed potential of prophylactic protection in the human lung epithelial cell, A549 cell culture model. It also successfully rescued 33.3% of the mice infected by *B. pseudomallei* when administered into the infected mice via i.p. route. These findings suggested that phage C34 is a potential candidate for phage therapy on *B. pseudomallei* infection. Nonetheless, more studies are needed to evaluate the pharmacokinetics of C34 and the effect of C34 on immune response against *B. pseudomallei*.

As suggested in the study, several strategies may be able to improve the treatment efficacy of the phage candidate. These strategies includes increasing the internalisation of phages into targeted cells via genetically modifying the phage to increase the internalisation of phages into targeted cells (Rajotte *et al.*, 1998; Piersanti *et al.*, 2004) and selecting mutant phages with prolonged circulatory ability (Capparelli *et al.*, 2006; Capparelli *et al.*, 2007). In the study, it was discovered that sterile immunity was not

achieved. Thus, it was proposed that an eradication therapy is required for total clearance of the bacteria.

REFERENCES

REFERENCES:

Abedon, S. T., Kuhl, S. J., Blasdel, B. G. and Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage* **1**(2): 66-85.

Abedon, S. T. and Thomas-Abedon, C. (2010). Phage therapy pharmacology. *Current pharmaceutical biotechnology* **11**(1): 28-47.

Ackermann, H.-W., Tremblay, D. and Moineau, S. (2004). Long-term bacteriophage preservation. *World Federation for Culture Collections Newsletter* **38**: 6.

Ackermann, H. W. (2007). 5500 Phages examined in the electron microscope. *Archives of virology* **152**(2): 227-243.

Ackermann, H. W. (2009). Phage classification and characterization. *Methods in molecular biology* **501**: 127-140.

Ackermann, H. W. (2011). Bacteriophage Taxonomy. *Microbiology Australia*: 5.

Ackermann, H. W. (2012). Bacteriophage Electron Microscopy. *Advances in Virus Research, Vol 82: Bacteriophages, Pt A* **82**: 1-32.

Alegado, R. A., Chin, C. Y., Monack, D. M. and Tan, M. W. (2011). The two-component sensor kinase KdpD is required for *Salmonella typhimurium* colonization of *Caenorhabditis elegans* and survival in macrophages. *Cellular microbiology* **13**(10): 1618-1637.

Alemayehu, D., Casey, P. G., McAuliffe, O., Guinane, C. M., Martin, J. G., Shanahan, F., Coffey, A., Ross, R. P. and Hill, C. (2012). Bacteriophages phiMR299-2 and phiNH-4 can eliminate *Pseudomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway cells. *mBio* **3**(2): e00029-00012.

Alibek, K. and Handelman, S. (2000). *Biohazard: the chilling true story of the largest covert biological weapons program in the world-told from the inside by the man who ran it.* New York: Random House.

Allwood, E. M., Devenish, R. J., Prescott, M., Adler, B. and Boyce, J. D. (2011). Strategies for Intracellular Survival of *Burkholderia pseudomallei*. *Frontiers in microbiology* **2**: 170.

Anderson, S. W., Stapp, J. R., Burns, J. L. and Qin, X. (2007). Characterization of small-colony-variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *Journal of clinical microbiology* **45**(2): 529-535.

Ashdown, L. R. (1979). An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathology* **11**(2): 293-297.

Ashdown, L. R. and Koehler, J. M. (1990). Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei*. *Journal of clinical microbiology* **28**(10): 2331-2334.

Barnes, J. L., Ulett, G. C., Ketheesan, N., Clair, T., Summers, P. M. and Hirst, R. G. (2001). Induction of multiple chemokine and colony-stimulating factor genes in experimental *Burkholderia pseudomallei* infection. *Immunology and cell biology* **79**(5): 490-501.

Bergh, O., Borsheim, K. Y., Bratbak, G. and Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature* **340**(6233): 467-468.

Boyd, E. F. (2012). Bacteriophage-Encoded Bacterial Virulence Factors and Phage-Pathogenicity Island Interactions. *Advances in Virus Research, Vol 82: Bacteriophages, Pt A* **82**: 91-118.

Boyd, E. F., Davis, B. M. and Hochhut, B. (2001). Bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria. *Trends in microbiology* **9**(3): 137-144.

Brett, P. J. and Woods, D. E. (2000). Pathogenesis of and immunity to melioidosis. *Acta tropica* **74**(2-3): 201-210.

Broxmeyer, L., Sosnowska, D., Miltner, E., Chacon, O., Wagner, D., McGarvey, J., Barletta, R. G. and Bermudez, L. E. (2002). Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent mycobacterium: a model for phage therapy of intracellular bacterial pathogens. *The Journal of infectious diseases* **186**(8): 1155-1160.

Brussow, H. (2005). Phage therapy: the *Escherichia coli* experience. *Microbiology* **151**(Pt 7): 2133-2140.

Bruynoghe, R. and Maisin, J. (1921). Essais de the´rapeutique au moyen du. *C. R. Soc. Biol. 85*: 1120–1121.

Cairns, B. J. and Payne, R. J. (2008). Bacteriophage therapy and the mutant selection window. *Antimicrobial agents and chemotherapy* **52**(12): 4344-4350.

Callaway, T. R., Edrington, T. S., Brabban, A. D., Anderson, R. C., Rossman, M. L., Engler, M. J., Carr, M. A., Genovese, K. J., Keen, J. E., Looper, M. L., Kutter, E. M. and Nisbet, D. J. (2008). Bacteriophage isolated from feedlot cattle can reduce *Escherichia coli* O157:H7 populations in ruminant gastrointestinal tracts. *Foodborne pathogens and disease* **5**(2): 183-191.

Capparelli, R., Parlato, M., Borriello, G., Salvatore, P. and Iannelli, D. (2007). Experimental phage therapy against *Staphylococcus aureus* in mice. *Antimicrobial agents and chemotherapy* **51**(8): 2765-2773.

Capparelli, R., Ventimiglia, I., Roperto, S., Fenizia, D. and Iannelli, D. (2006). Selection of an *Escherichia coli* O157:H7 bacteriophage for persistence in the circulatory system of mice infected experimentally. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **12**(3): 248-253.

Capra, M. L., Quiberoni, A. and Reinheimer, J. A. (2004). Thermal and chemical resistance of *Lactobacillus casei* and *Lactobacillus paracasei* bacteriophages. *Letters in applied microbiology* **38**(6): 499-504.

Carlton, R. M., Noordman, W. H., Biswas, B., de Meester, E. D. and Loessner, M. J. (2005). Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory toxicology and pharmacology : RTP* **43**(3): 301-312.

Carmody, L. A., Gill, J. J., Summer, E. J., Sajjan, U. S., Gonzalez, C. F., Young, R. F. and LiPuma, J. J. (2010). Efficacy of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection. *The Journal of infectious diseases* **201**(2): 264-271.

Carne, H. R. and Greaves, R. I. (1974). Preservation of corynebacteriophages by freeze-drying. *The Journal of hygiene* **72**(3): 467-470.

Chan, B. K., Abedon, S. T. and Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future microbiology* **8**(6): 769-783.

Chantratita, N., Wuthiekanun, V., Boonbumrung, K., Tiyawisutsri, R., Vesaratchavest, M., Limmathurotsakul, D., Chierakul, W., Wongratanacheewin, S., Pukritiyakamee, S., White, N. J., Day, N. P. and Peacock, S. J. (2007). Biological relevance of colony morphology and phenotypic switching by *Burkholderia pseudomallei*. *Journal of bacteriology* **189**(3): 807-817.

Chaowagul, W., Suputtamongkol, Y., Dance, D. A., Rajchanuvong, A., Pattara-arechachai, J. and White, N. J. (1993). Relapse in melioidosis: incidence and risk factors. *The Journal of infectious diseases* **168**(5): 1181-1185.

Chen, Y. S., Chen, S. C., Kao, C. M. and Chen, Y. L. (2003). Effects of soil pH, temperature and water content on the growth of *Burkholderia pseudomallei*. *Folia microbiologica* **48**(2): 253-256.

Cheng, A. C., Chierakul, W., Chaowagul, W., Chetchotisakd, P., Limmathurotsakul, D., Dance, D. A., Peacock, S. J. and Currie, B. J. (2008). Consensus guidelines for dosing of amoxicillin-clavulanate in melioidosis. *The American journal of tropical medicine and hygiene* **78**(2): 208-209.

Cheng, A. C. and Currie, B. J. (2005). Melioidosis: epidemiology, pathophysiology, and management. *Clinical microbiology reviews* **18**(2): 383-416.

Chhibber, S., Kaur, S. and Kumari, S. (2008). Therapeutic potential of bacteriophage in treating *Klebsiella pneumoniae* B5055-mediated lobar pneumonia in mice. *Journal of medical microbiology* **57**(Pt 12): 1508-1513.

Choh, L. C., Ong, G. H., Vellasamy, K. M., Kalaiselvam, K., Kang, W. T., Al-Maleki, A. R., Mariappan, V. and Vadivelu, J. (2013). *Burkholderia* vaccines: are we moving forward? *Frontiers in cellular and infection microbiology* **3**: 5.

Choy, J. L., Mayo, M., Janmaat, A. and Currie, B. J. (2000). Animal melioidosis in Australia. *Acta tropica* **74**(2-3): 153-158.

Clark, W. A. (1962). Comparison of several methods for preserving bacteriophages. *Applied microbiology* **10**: 466-471.

Clark, W. A. and Geary, D. (1973). Proceedings: Preservation of bacteriophages by freezing and freeze-drying. *Cryobiology* **10**(5): 351-360.

Conejero, L., Patel, N., de Reynal, M., Oberdorf, S., Prior, J., Felgner, P. L., Titball, R. W., Salguero, F. J. and Bancroft, G. J. (2011). Low-dose exposure of C57BL/6 mice to *Burkholderia pseudomallei* mimics chronic human melioidosis. *The American journal of pathology* **179**(1): 270-280.

Cullinane, M., Gong, L., Li, X., Lazar-Adler, N., Tra, T., Wolvetang, E., Prescott, M., Boyce, J. D., Devenish, R. J. and Adler, B. (2008). Stimulation of autophagy suppresses the intracellular survival of Burkholderia pseudomallei in mammalian cell lines. *Autophagy* **4**(6): 744-753.

Currie, B. J., Mayo, M., Anstey, N. M., Donohoe, P., Haase, A. and Kemp, D. J. (2001). A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. *The American journal of tropical medicine and hygiene* **65**(3): 177-179.

Currie, B. J., Ward, L. and Cheng, A. C. (2010). The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. *PLoS neglected tropical diseases* **4**(11): e900.

d'Herelle, F., Malone, R. H. and Lahiri, M. (1928). *The treatment and prophylaxis of infectious diseases of the intestinal tract and of cholera in particular.* Paper presented at the Transaction of the 7th Congress, British India.

Dance, D. A., Wuthiekanun, V., Chaowagul, W. and White, N. J. (1989). The antimicrobial susceptibility of *Pseudomonas pseudomallei*. Emergence of resistance in vitro and during treatment. *The Journal of antimicrobial chemotherapy* **24**(3): 295-309.

Danelishvili, L., Young, L. S. and Bermudez, L. E. (2006). In vivo efficacy of phage therapy for *Mycobacterium avium* infection as delivered by a nonvirulent mycobacterium. *Microbial drug resistance* **12**(1): 1-6.

Dannenberg, A. M., Jr. and Scott, E. M. (1958). Melioidosis: pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei*. *The Journal of experimental medicine* **107**(1): 153-166.

Davis, J. M. and Ramakrishnan, L. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* **136**(1): 37-49.

Dean, J. H., Silva, J. S., McCoy, J. L., Chan, S. P., Baker, J. J., Leonard, C. and Herberman, R. B. (1975). In vitro human reactivity to staphylococcal phage lysate. *Journal of immunology* **115**(4): 1060-1064.

Dejsirilert, S., Kondo, E., Chiewsilp, D. and Kanai, K. (1991). Growth and survival of *Pseudomonas pseudomallei* in acidic environments. *Japanese journal of medical science & biology* **44**(2): 63-74.

Drake, J. W., Charlesworth, B., Charlesworth, D. and Crow, J. F. (1998). Rates of spontaneous mutation. *Genetics* **148**(4): 1667-1686.

Dubos, R. J., Straus, J. H. and Pierce, C. (1943). The Multiplication of Bacteriophage in Vivo and Its Protective Effect against an Experimental Infection with *Shigella Dysenteriae*. *The Journal of experimental medicine* **78**(3): 161-168.

Edlund, C. and Nord, C. E. (2000). Effect on the human normal microflora of oral antibiotics for treatment of urinary tract infections. *The Journal of antimicrobial chemotherapy* **46 Suppl A**: 41-48.

Engel, H. W., Smith, L. and Berwald, L. G. (1974). The preservation of mycobacteriophages by means of freeze drying. *The American review of respiratory disease* **109**(5): 561-566.

Filippov, A. A., Sergueev, K. V., He, Y., Huang, X. Z., Gnade, B. T., Mueller, A. J., Fernandez-Prada, C. M. and Nikolich, M. P. (2012). Bacteriophage therapy of experimental bubonic plague in mice. *Advances in experimental medicine and biology* **954**: 337-348.

Fortier, L. C. and Moineau, S. (2009). Phage production and maintenance of stocks, including expected stock lifetimes. *Methods in molecular biology* **501**: 203-219.

Fux, C. A., Costerton, J. W., Stewart, P. S. and Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends in microbiology* **13**(1): 34-40.

Fux, C. A., Stoodley, P., Hall-Stoodley, L. and Costerton, J. W. (2003). Bacterial biofilms: a diagnostic and therapeutic challenge. *Expert review of anti-infective therapy* **1**(4): 667-683.

Gal, D., Mayo, M., Smith-Vaughan, H., Dasari, P., McKinnon, M., Jacups, S. P., Urquhart, A. I., Hassell, M. and Currie, B. J. (2004). Contamination of hand wash detergent linked to occupationally acquired melioidosis. *The American journal of tropical medicine and hygiene* **71**(3): 360-362.

Galan, J. E. (2001). Salmonella interactions with host cells: type III secretion at work. *Annual review of cell and developmental biology* **17**: 53-86.

Garcia, P., Madera, C., Martinez, B. and Rodriguez, A. (2007). Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages. *International Dairy Journal* **17**(10): 1232-1239.

Gatedee, J., Kritsiriwuthinan, K., Galyov, E. E., Shan, J., Dubinina, E., Intarak, N., Clokie, M. R. and Korbsrisate, S. (2011). Isolation and characterization of a novel podovirus which infects *Burkholderia pseudomallei*. *Virology journal* **8**: 366.

Gill, J. J. and Hyman, P. (2010). Phage choice, isolation, and preparation for phage therapy. *Current pharmaceutical biotechnology* **11**(1): 2-14.

Gong, L., Cullinane, M., Treerat, P., Ramm, G., Prescott, M., Adler, B., Boyce, J. D. and Devenish, R. J. (2011). The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. *PloS one* **6**(3): e17852.

Gonzales, R. D., Schreckenberger, P. C., Graham, M. B., Kelkar, S., DenBesten, K. and Quinn, J. P. (2001). Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet* **357**(9263): 1179.

Guenther, S., Huwyler, D., Richard, S. and Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and environmental microbiology* **75**(1): 93-100.

Gupta, R. and Prasad, Y. (2011). Efficacy of polyvalent bacteriophage P-27/HP to control multidrug resistant *Staphylococcus aureus* associated with human infections. *Current microbiology* **62**(1): 255-260.

Guttman, B., Raya, R. and Kutter, E. (2005). Basic phage biology *Bacteriophages: biology and applications*. pp. 29-66. (CRC Press: Boca Raton)

Hall, A. R., De Vos, D., Friman, V. P., Pirnay, J. P. and Buckling, A. (2012). Effects of sequential and simultaneous applications of bacteriophages on populations of *Pseudomonas aeruginosa in vitro* and in wax moth larvae. *Applied and environmental microbiology* **78**(16): 5646-5652.

Hanlon, G. W. (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International journal of antimicrobial agents* **30**(2): 118-128.

Haque, A., Chu, K., Easton, A., Stevens, M. P., Galyov, E. E., Atkins, T., Titball, R. and Bancroft, G. J. (2006). A live experimental vaccine against *Burkholderia pseudomallei* elicits CD4+ T cell-mediated immunity, priming T cells specific for 2 type III secretion system proteins. *The Journal of infectious diseases* **194**(9): 1241-1248.

Hart, S. L., Knight, A. M., Harbottle, R. P., Mistry, A., Hunger, H. D., Cutler, D. F., Williamson, R. and Coutelle, C. (1994). Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *The Journal of biological chemistry* **269**(17): 12468-12474.

Haussler, S., Lehmann, C., Breselge, C., Rohde, M., Classen, M., Tummler, B., Vandamme, P. and Steinmetz, I. (2003a). Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* **22**(4): 249-253.

Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B. and Steinmetz, I. (2003b). Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Journal of medical microbiology* **52**(Pt 4): 295-301.

Heo, Y. J., Lee, Y. R., Jung, H. H., Lee, J., Ko, G. and Cho, Y. H. (2009). Antibacterial efficacy of phages against *Pseudomonas aeruginosa* infections in mice and *Drosophila melanogaster*. *Antimicrobial agents and chemotherapy* **53**(6): 2469-2474.

Hyman, P. and Abedon, S. T. (2010). Bacteriophage host range and bacterial resistance. *Advances in applied microbiology* **70**: 217-248.

Inglis, T. J., Garrow, S. C., Henderson, M., Clair, A., Sampson, J., O'Reilly, L. and Cameron, B. (2000). *Burkholderia pseudomallei* traced to water treatment plant in Australia. *Emerging infectious diseases* **6**(1): 56-59.

Inglis, T. J., Golledge, C. L., Clair, A. and Harvey, J. (2001). Case report: recovery from persistent septicemic melioidosis. *The American journal of tropical medicine and hygiene* **65**(1): 76-82.

Jaiswal, A., Koley, H., Ghosh, A., Palit, A. and Sarkar, B. (2013). Efficacy of cocktail phage therapy in treating *Vibrio cholerae* infection in rabbit model. *Microbes and infection / Institut Pasteur* **15**(2): 152-156.

Jenney, A. W., Lum, G., Fisher, D. A. and Currie, B. J. (2001). Antibiotic susceptibility of *Burkholderia pseudomallei* from tropical northern Australia and implications for therapy of melioidosis. *International journal of antimicrobial agents* **17**(2): 109-113.

Jikia, D., Chkhaidze, N., Imedashvili, E., Mgaloblishvili, I., Tsitlanadze, G., Katsarava, R., Glenn Morris, J., Jr. and Sulakvelidze, A. (2005). The use of a novel

biodegradable preparation capable of the sustained release of bacteriophages and ciprofloxacin, in the complex treatment of multidrug-resistant *Staphylococcus aureus*-infected local radiation injuries caused by exposure to Sr90. *Clinical and experimental dermatology* **30**(1): 23-26.

Jones, A. L., Beveridge, T. J. and Woods, D. E. (1996). Intracellular survival of *Burkholderia pseudomallei*. *Infection and immunity* **64**(3): 782-790.

Jun, J. W., Shin, T. H., Kim, J. H., Shin, S. P., Han, J. E., Heo, G. J., De Zoysa, M., Shin, G. W., Chai, J. Y. and Park, S. C. (2014). Bacteriophage Therapy of a *Vibrio parahaemolyticus* Infection Caused by a Multiple-Antibiotic-Resistant O3:K6 Pandemic Clinical Strain. *Journal of Infectious Diseases* **210**(1): 72-78.

Kahl, B., Herrmann, M., Everding, A. S., Koch, H. G., Becker, K., Harms, E., Proctor, R. A. and Peters, G. (1998). Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *The Journal of infectious diseases* **177**(4): 1023-1029.

Kanthawong, S., Bolscher, J. G., Veerman, E. C., van Marle, J., de Soet, H. J., Nazmi, K., Wongratanacheewin, S. and Taweechaisupapong, S. (2012). Antimicrobial and antibiofilm activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*. *International journal of antimicrobial agents* **39**(1): 39-44.

Korbsrisate, S., Suwanasai, N., Leelaporn, A., Ezaki, T., Kawamura, Y. and Sarasombath, S. (1999). Cloning and characterization of a nonhemolytic phospholipase C gene from *Burkholderia pseudomallei*. *Journal of clinical microbiology* **37**(11): 3742-3745.

Korbsrisate, S., Tomaras, A. P., Damnin, S., Ckumdee, J., Srinon, V., Lengwehasatit, I., Vasil, M. L. and Suparak, S. (2007). Characterization of two distinct phospholipase C enzymes from *Burkholderia pseudomallei*. *Microbiology* **153**(Pt 6): 1907-1915.

Kortepeter, M. G., Christopher, T., Cieslak, R., Culpepper, R., Darling, J., Pavlin, J. and John Rowe, K. (2001). In K. McKee & E. Eitzen (Eds.), *Medical management of biological casualties handbook*. pp. 23-27. (U.S. Army Medical Research Institute of Infectious Diseases: Fort Detrick)

Krylov, V. N. (2001). Phage therapy in terms of bacteriophage genetics: Hopes, prospects, safety, limitations. *Russian Journal of Genetics* **37**(7): 715-730.

Kutter, E. (2009). Phage host range and efficiency of plating. *Methods in molecular biology* **501**: 141-149.

Kvitko, B. H., Cox, C. R., DeShazer, D., Johnson, S. L., Voorhees, K. J. and Schweizer, H. P. (2012). phiX216, a P2-like bacteriophage with broad *Burkholderia pseudomallei* and *B. mallei* strain infectivity. *BMC microbiology* **12**: 289.

L., B. (2007). Bacteria-eating virus approved as food additive. *FDA Consum.* **41**(1): 3.

Labrie, S. J., Samson, J. E. and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nature Reviews Microbiology* **8**(5): 317-327.

Leakey, A. K., Ulett, G. C. and Hirst, R. G. (1998). BALB/c and C57Bl/6 mice infected with virulent *Burkholderia pseudomallei* provide contrasting animal models for the acute and chronic forms of human melioidosis. *Microbial pathogenesis* **24**(5): 269-275.

Lee, B., Murakami, M. and Mizukoshi, M. (1985a). [Polyclonal antibody secretion in mouse spleen cells induced by staphylococcal phage lysate (SPL)]. *Yakugaku zasshi : Journal of the Pharmaceutical Society of Japan* **105**(8): 747-750.

Lee, B., Murakami, M., Mizukoshi, M., Shinomiya, N. and Yada, J. (1985b). [Effects of staphylococcal phage lysate (SPL) on immunoglobulin production in human peripheral lymphocyte cultures]. *Yakugaku zasshi : Journal of the Pharmaceutical Society of Japan* **105**(6): 574-579.

Lee, B., Yamaguchi, K., Murakami, M. and Mizukoshi, M. (1982). [Effect of staphylococcal phage lysate(SPL) on polyclonal B cell activation in mouse spleen cells in vitro]. *Nihon saikingaku zasshi. Japanese journal of bacteriology* **37**(4): 787-788.

Lee, M. A. and Liu, Y. (2000). Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*. *FEMS microbiology letters* **192**(1): 67-72.

Leelarasamee, A. and Bovornkitti, S. (1989). Melioidosis: review and update. *Reviews of infectious diseases* **11**(3): 413-425.

Letellier, L., Boulanger, P., Plancon, L., Jacquot, P. and Santamaria, M. (2004). Main features on tailed phage, host recognition and DNA uptake. *Frontiers in Bioscience* **9**: 1228-1239.

Letkiewicz, S., Miedzybrodzki, R., Klak, M., Jonczyk, E., Weber-Dabrowska, B. and Gorski, A. (2010). The perspectives of the application of phage therapy in chronic bacterial prostatitis. *FEMS immunology and medical microbiology* **60**(2): 99-112.

Lever, M. S., Nelson, M., Ireland, P. I., Stagg, A. J., Beedham, R. J., Hall, G. A., Knight, G. and Titball, R. W. (2003). Experimental aerogenic *Burkholderia mallei* (glanders) infection in the BALB/c mouse. *Journal of medical microbiology* **52**(Pt 12): 1109-1115.

Lew, A. E. and Desmarchelier, P. M. (1993). Molecular typing of *Pseudomonas pseudomallei*: restriction fragment length polymorphisms of rRNA genes. *Journal of clinical microbiology* **31**(3): 533-539.

Limmathurotsakul, D., Chaowagul, W., Chierakul, W., Stepniewska, K., Maharjan, B., Wuthiekanun, V., White, N. J., Day, N. P. and Peacock, S. J. (2006). Risk factors for recurrent melioidosis in northeast Thailand. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **43**(8): 979-986.

Limmathurotsakul, D., Chaowagul, W., Day, N. P. and Peacock, S. J. (2009). Patterns of organ involvement in recurrent melioidosis. *The American journal of tropical medicine and hygiene* **81**(2): 335-337.

Lipsitz, R., Garges, S., Aurigemma, R., Baccam, P., Blaney, D. D., Cheng, A. C., Currie, B. J., Dance, D., Gee, J. E., Larsen, J., Limmathurotsakul, D., Morrow, M. G., Norton, R., O'Mara, E., Peacock, S. J., Pesik, N., Rogers, L. P., Schweizer, H. P., Steinmetz, I., Tan, G., Tan, P., Wiersinga, W. J., Wuthiekanun, V. and Smith, T. L. (2012). Workshop on treatment of and postexposure prophylaxis for *Burkholderia pseudomallei* and *B. mallei* Infection, 2010. *Emerging infectious diseases* **18**(12): e2.

Loc-Carrillo, C. and Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage* **1**(2): 111-114.

Markoishvili, K., Tsitlanadze, G., Katsarava, R., Morris, J. G., Jr. and Sulakvelidze, A. (2002). A novel sustained-release matrix based on biodegradable poly(ester amide)s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. *International journal of dermatology* **41**(7): 453-458.

McVay, C. S., Velasquez, M. and Fralick, J. A. (2007). Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrobial agents and chemotherapy* **51**(6): 1934-1938.

Merabishvili, M., De Vos, D., Verbeken, G., Kropinski, A. M., Vandenheuvel, D., Lavigne, R., Wattiau, P., Mast, J., Ragimbeau, C., Mossong, J., Scheres, J., Chanishvili, N., Vaneechoutte, M. and Pirnay, J. P. (2012). Selection and characterization of a candidate therapeutic bacteriophage that lyses the

Escherichia coli O104:H4 strain from the 2011 outbreak in Germany. *PloS one* **7**(12): e52709.

Merril, C. R., Biswas, B., Carlton, R., Jensen, N. C., Creed, G. J., Zullo, S. and Adhya, S. (1996). Long-circulating bacteriophage as antibacterial agents. *Proceedings of the National Academy of Sciences of the United States of America* **93**(8): 3188-3192.

Meumann, E. M., Cheng, A. C., Ward, L. and Currie, B. J. (2012). Clinical features and epidemiology of melioidosis pneumonia: results from a 21-year study and review of the literature. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **54**(3): 362-369.

Miles, A. A., Misra, S. S. and Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *The Journal of hygiene* **38**(6): 732-749.

Mudd, S. (1971). Resistance against *Staphylococcus aureus*. *JAMA : the journal of the American Medical Association* **218**(11): 1671-1673.

Nasner-Posso, K. M., Cruz-Calderon, S., Montufar-Andrade, F. E., Dance, D. A. and Rodriguez-Morales, A. J. (2015). Human melioidosis reported by ProMED. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **35**: 103-106.

Ngauy, V., Lemeshev, Y., Sadkowski, L. and Crawford, G. (2005). Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. *Journal of clinical microbiology* **43**(2): 970-972.

Nobrega, F. L., Costa, A. R., Kluskens, L. D. and Azeredo, J. (2015). Revisiting phage therapy: new applications for old resources. *Trends in microbiology* **23**(4): 185-191.

O'Flynn, G., Ross, R. P., Fitzgerald, G. F. and Coffey, A. (2004). Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Applied and environmental microbiology* **70**(6): 3417-3424.

O'Toole, G., Kaplan, H. B. and Kolter, R. (2000). Biofilm formation as microbial development. *Annual review of microbiology* **54**: 49-79.

Peacock, S. J., Schweizer, H. P., Dance, D. A., Smith, T. L., Gee, J. E., Wuthiekanun, V., DeShazer, D., Steinmetz, I., Tan, P. and Currie, B. J. (2008). Management of accidental laboratory exposure to *Burkholderia pseudomallei* and *B. mallei*. *Emerging infectious diseases* **14**(7): e2.

Piersanti, S., Cherubini, G., Martina, Y., Salone, B., Avitabile, D., Grosso, F., Cundari, E., Di Zenzo, G. and Saggio, I. (2004). Mammalian cell transduction and internalization properties of lambda phages displaying the full-length adenoviral penton base or its central domain. *Journal of molecular medicine* **82**(7): 467-476.

Pirnay, J. P., Blasdel, B. G., Bretaudeau, L., Buckling, A., Chanishvili, N., Clark, J. R., Corte-Real, S., Debarbieux, L., Dublanchet, A., De Vos, D., Gabard, J., Garcia, M., Goderdzishvili, M., Gorski, A., Hardcastle, J., Huys, I., Kutter, E., Lavigne, R., Merabishvili, M., Olchawa, E., Parikka, K. J., Patey, O., Pouilot, F., Resch, G., Rohde, C., Scheres, J., Skurnik, M., Vaneechoutte, M., Van Parys, L., Verbeken, G., Zizi, M. and Van den Eede, G. (2015). Quality and safety requirements for sustainable phage therapy products. *Pharmaceutical research* **32**(7): 2173-2179.

Pirnay, J. P., De Vos, D., Verbeken, G., Merabishvili, M., Chanishvili, N., Vaneechoutte, M., Zizi, M., Laire, G., Lavigne, R., Huys, I., Van den Mooter, G., Buckling, A., Debarbieux, L., Pouillot, F., Azeredo, J., Kutter, E., Dublanchet, A., Gorski, A. and Adamia, R. (2011). The phage therapy paradigm: pret-a-porter or sur-mesure? *Pharmaceutical research* **28**(4): 934-937.

Prystowsky, J., Siddiqui, F., Chosay, J., Shinabarger, D. L., Millichap, J., Peterson, L. R. and Noskin, G. A. (2001). Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. *Antimicrobial agents and chemotherapy* **45**(7): 2154-2156.

Puthucheary, S. D. (2009). Melioidosis in Malaysia. *The Medical journal of Malaysia* **64**(4): 266-274.

Quiberoni, A., Guglielmotti, D. M. and Reinheimer, J. A. (2003). Inactivation of *Lactobacillus delbrueckii* bacteriophages by heat and biocides. *International journal of food microbiology* **84**(1): 51-62.

Rafii, F., Sutherland, J. B. and Cerniglia, C. E. (2008). Effects of treatment with antimicrobial agents on the human colonic microflora. *Therapeutics and clinical risk management* **4**(6): 1343-1358.

Rainbow, L., Hart, C. A. and Winstanley, C. (2002). Distribution of type III secretion gene clusters in *Burkholderia pseudomallei*, *B. thailandensis* and *B. mallei*. *Journal of medical microbiology* **51**(5): 374-384.

Rajotte, D., Arap, W., Hagedorn, M., Koivunen, E., Pasqualini, R. and Ruoslahti, E. (1998). Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. *The Journal of clinical investigation* **102**(2): 430-437.

Redfearn, M. S., Palleroni, N. J. and Stanier, R. Y. (1966). A comparative study of *Pseudomonas pseudomallei* and *Bacillus mallei*. *Journal of general microbiology* **43**(2): 293-313.

Rholl, D. A., Papp-Wallace, K. M., Tomaras, A. P., Vasil, M. L., Bonomo, R. A. and Schweizer, H. P. (2011). Molecular Investigations of PenA-mediated beta-lactam Resistance in *Burkholderia pseudomallei*. *Frontiers in microbiology* **2**: 139.

Sabour, P. M. and Griffiths, M. W. (Eds.). (2010). *Bacteriophages in the control of food-and waterborne pathogens.*: ASM Press.

Salmon, G. G., Jr. and Symonds, M. (1963). Staphage Lysate Therapy in Chronic Staphylococcal Infections. *The Journal of the Medical Society of New Jersey* **60**: 188-193.

Sambrook, J., Russell, D. W. and (2001). *Molecular cloning: a laboratory manual.* (Vol. 1-3).

Samuelsen, O., Haukland, H. H., Kahl, B. C., von Eiff, C., Proctor, R. A., Ulvatne, H., Sandvik, K. and Vorland, L. H. (2005). *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B. *The Journal of antimicrobial chemotherapy* **56**(6): 1126-1129.

Sariya, L., Prempracha, N., Keelapan, P. and Chittasophon, N. (2006). Bacteriophage isolated from *Burkholderia pseudomallei* causes phenotypic changes in *Burkholderia thailandensis*. *Science asia* **32**: 9.

Seed, K. D. and Dennis, J. J. (2009). Experimental bacteriophage therapy increases survival of *Galleria mellonella* larvae infected with clinically relevant strains of the *Burkholderia cepacia* complex. *Antimicrobial agents and chemotherapy* **53**(5): 2205-2208.

Sexton, M. M., Jones, A. L., Chaowagul, W. and Woods, D. E. (1994). Purification and characterization of a protease from *Pseudomonas pseudomallei*. *Canadian journal of microbiology* **40**(11): 903-910.

Silver, L. L. and Bostian, K. A. (1993). Discovery and development of new antibiotics: the problem of antibiotic resistance. *Antimicrobial agents and chemotherapy* **37**(3): 377-383.

Skurnik, M., Pajunen, M. and Kiljunen, S. (2007). Biotechnological challenges of phage therapy. *Biotechnology letters* **29**(7): 995-1003.

Smith, H. W. and Huggins, M. B. (1982). Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *Journal of general microbiology* **128**(2): 307-318.

Soni, K. A., Desai, M., Oladunjoye, A., Skrobot, F. and Nannapaneni, R. (2012). Reduction of *Listeria monocytogenes* in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. *International journal of food microbiology* **155**(1-2): 82-88.

Soni, K. A. and Nannapaneni, R. (2010). Bacteriophage significantly reduces *Listeria monocytogenes* on raw salmon fillet tissue. *Journal of food protection* **73**(1): 32-38.

Soni, K. A., Nannapaneni, R. and Hagens, S. (2010). Reduction of *Listeria monocytogenes* on the surface of fresh channel catfish fillets by bacteriophage Listex P100. *Foodborne pathogens and disease* **7**(4): 427-434.

Sookpranee, M., Boonma, P., Susaengrat, W., Bhuripanyo, K. and Punyagupta, S. (1992). Multicenter prospective randomized trial comparing ceftazidime plus co-trimoxazole with chloramphenicol plus doxycycline and co-trimoxazole for treatment of severe melioidosis. *Antimicrobial agents and chemotherapy* **36**(1): 158-162.

Spano, S. and Galan, J. E. (2008). A novel pathway for exotoxin delivery by an intracellular pathogen. *Current opinion in microbiology* **11**(1): 15-20.

Steinmetz, I., Rohde, M. and Brenneke, B. (1995). Purification and characterization of an exopolysaccharide of *Burkholderia (Pseudomonas) pseudomallei*. *Infection and immunity* **63**(10): 3959-3965.

Stevens, M. P., Friebel, A., Taylor, L. A., Wood, M. W., Brown, P. J., Hardt, W. D. and Galyov, E. E. (2003). A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. *Journal of bacteriology* **185**(16): 4992-4996.

Stevens, M. P., Wood, M. W., Taylor, L. A., Monaghan, P., Hawes, P., Jones, P. W., Wallis, T. S. and Galyov, E. E. (2002). An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Molecular microbiology* **46**(3): 649-659.

Stone, R. (2002). Bacteriophage therapy. Stalin's forgotten cure. *Science* **298**(5594): 728-731.

Sulakvelidze, A., Alavidze, Z. and Morris, J. G., Jr. (2001). Bacteriophage therapy. *Antimicrobial agents and chemotherapy* **45**(3): 649-659.

Sulakvelidze, A. and Kutter, E. (2005). Bacteriophage therapy in Humans. In E. Kutter & A. Sulakvelidze (Eds.), *Bacteriophages: Biology and Applications*. pp. 381-436. (CRC Press: Florida)

Summers, W. C. (1999). *Félix d'Herelle and the origins of molecular biology*. Connecticut: Yale University Press.

Summers, W. C. (2001). Bacteriophage therapy. *Annual review of microbiology* **55**: 437-451.

Suppiah, J., Thimma, J. S., Cheah, S. H. and Vadivelu, J. (2010). Development and evaluation of polymerase chain reaction assay to detect *Burkholderia* genus and to differentiate the species in clinical specimens. *FEMS microbiology letters* **306**(1): 9-14.

Suttle, C. A. (2005). Viruses in the sea. *Nature* **437**(7057): 356-361.

Takemura-Uchiyama, I., Uchiyama, J., Osanai, M., Morimoto, N., Asagiri, T., Ujihara, T., Daibata, M., Sugiura, T. and Matsuzaki, S. (2014). Experimental phage therapy against lethal lung-derived septicemia caused by *Staphylococcus aureus* in mice. *Microbes and infection / Institut Pasteur*.

Tan, G. Y., Liu, Y., Sivalingam, S. P., Sim, S. H., Wang, D., Paucod, J. C., Gauthier, Y. and Ooi, E. E. (2008). *Burkholderia pseudomallei* aerosol infection results in differential inflammatory responses in BALB/c and C57Bl/6 mice. *Journal of medical microbiology* **57**(Pt 4): 508-515.

Tandhavanant, S., Thanwisai, A., Limmathurotsakul, D., Korbsrisate, S., Day, N. P., Peacock, S. J. and Chantratita, N. (2010). Effect of colony morphology variation of *Burkholderia pseudomallei* on intracellular survival and resistance to antimicrobial environments in human macrophages in vitro. *BMC microbiology* **10**: 303.

Titball, R. W., Russell, P., Cuccui, J., Easton, A., Haque, A., Atkins, T., Sarkar-Tyson, M., Harley, V., Wren, B. and Bancroft, G. J. (2008). *Burkholderia pseudomallei*: animal models of infection. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102 Suppl 1**: S111-116.

Tiwari, B. R., Kim, S., Rahman, M. and Kim, J. (2011). Antibacterial efficacy of lytic *Pseudomonas* bacteriophage in normal and neutropenic mice models. *Journal of microbiology* **49**(6): 994-999.

Tong, S., Yang, S., Lu, Z. and He, W. (1996). Laboratory investigation of ecological factors influencing the environmental presence of *Burkholderia pseudomallei*. *Microbiology and immunology* **40**(6): 451-453.

Trunck, L. A., Propst, K. L., Wuthiekanun, V., Tuanyok, A., Beckstrom-Sternberg, S. M., Beckstrom-Sternberg, J. S., Peacock, S. J., Keim, P., Dow, S. W. and Schweizer, H. P. (2009). Molecular basis of rare aminoglycoside susceptibility and pathogenesis of *Burkholderia pseudomallei* clinical isolates from Thailand. *PLoS neglected tropical diseases* **3**(9): e519.

van Schaik, E., Tom, M., DeVinney, R. and Woods, D. E. (2008). Development of novel animal infection models for the study of acute and chronic *Burkholderia pseudomallei* pulmonary infections. *Microbes and infection / Institut Pasteur* **10**(12-13): 1291-1299.

Van Twest, R. and Kropinski, A. M. (2009). Bacteriophage enrichment from water and soil. *Methods in molecular biology* **501**: 15-21.

Vorachit, M., Lam, K., Jayanetra, P. and Costerton, J. W. (1993). Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole. *Antimicrobial agents and chemotherapy* **37**(9): 2000-2002.

Wang, I. N. (2006). Lysis timing and bacteriophage fitness. *Genetics* **172**(1): 17-26.

Warawa, J. and Woods, D. E. (2005). Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. *FEMS microbiology letters* **242**(1): 101-108.

Watanabe, R., Matsumoto, T., Sano, G., Ishii, Y., Tateda, K., Sumiyama, Y., Uchiyama, J., Sakurai, S., Matsuzaki, S., Imai, S. and Yamaguchi, K. (2007). Efficacy of bacteriophage therapy against gut-derived sepsis caused by *Pseudomonas aeruginosa* in mice. *Antimicrobial agents and chemotherapy* **51**(2): 446-452.

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS microbiology reviews* **28**(2): 127-181.

White, N. J. (2003). Melioidosis. *The Lancet* **361**(9370): 1715-1722.

Whitmore, A. (1913). An Account of a Glanders-like Disease occurring in Rangoon. *The Journal of hygiene* **13**(1): 1-34 31.

Wiersinga, W. J., Currie, B. J. and Peacock, S. J. (2012). Melioidosis. *The New England journal of medicine* **367**(11): 1035-1044.

Wommack, K. E. and Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiology and molecular biology reviews : MMBR* **64**(1): 69-114.

Wuthiekanun, V., Anuntagool, N., White, N. J. and Sirisinha, S. (2002). Short report: a rapid method for the differentiation of *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *The American journal of tropical medicine and hygiene* **66**(6): 759-761.

Wuthiekanun, V., Smith, M. D. and White, N. J. (1995). Survival of *Burkholderia pseudomallei* in the absence of nutrients. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **89**(5): 491.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiology and immunology* **36**(12): 1251-1275.

Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L. and Treiber, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**(3): 734-744.

Yee, K. C., Lee, M. K., Chua, C. T. and Puthucheary, S. D. (1988). Melioidosis, the great mimicker: a report of 10 cases from Malaysia. *The Journal of tropical medicine and hygiene* **91**(5): 249-254.

Yordpratum, U., Tattawasart, U., Wongratanacheewin, S. and Sermswan, R. W. (2011). Novel lytic bacteriophages from soil that lyse *Burkholderia pseudomallei*. *FEMS microbiology letters* **314**(1): 81-88.

Zaharik, M. L., Gruenheid, S., Perrin, A. J. and Finlay, B. B. (2002). Delivery of dangerous goods: type III secretion in enteric pathogens. *International journal of medical microbiology : IJMM* **291**(8): 593-603.

Zierdt, C. H. (1988). Stabilities of lyophilized *Staphylococcus aureus* typing bacteriophages. *Applied and environmental microbiology* **54**(10): 2590.

APPENDIXES

APPENDIX A: MEDIA PREPARATION

Preparation of Bacteriological Media:

A. Luria Bertani (LB) Broth (Difco, USA)
- Suspend 25g of LB broth powder in 1000 ml of double-distilled water, autoclaved at 121°C for 15 mins.

B. Double strength LB broth
- Suspend 50g of LB broth powder in 100 ml of double-distilled water, autoclaved at 121°C for 15 mins.

C. Soft LB agar
- Suspend 2.5g of LB broth powder and 0.5g of bacteriological agar powder in 100 ml of double-distilled water, autoclaved at 121°C for 15 mins. Stored at 56°C for no more than one week.

D. Nutrient Agar (NA) (Oxoid, UK)
- Suspend 28g of NA powder in 1000 ml of double distilled water, autoclaved at 121°C for 15 mins. Cooled to 60°C and poured into petri dishes.

E. Ashdown Agar
- Bacteriological agar 7.5 g
- Tryptone Soy Broth 5 g
- Glycerol 20 ml
- 0.1 % Crystal violet solution 2.5 ml
- 1% Neutral red 2.5 ml
- Distilled water 475 ml
- Mix well and autoclaved at 121°C for 15 mins. Cooled to 56°C, 1 ml of 100 µg/ml of gentamycin added, poured into petri dishes.

F. 30% glycerol stock
- 50% sterile glycerol (Sigma, USA) 60 ml
- LB broth 40 ml

Preparation of Tissue Culture Media:

A. 10× Roswell Park Memorial Institute (RPMI) stock solution (Sigma, USA)
- 10× RPMI powder
- $NaHCO_3$ 2 g
- Ultra-pure water 100 ml
- Stirred on magnetic stirrer until completely dissolved, filter sterilised and stored at 4°C.

B. RPMI complete growth medium
- 10× RPMI stock solution 100 ml
- Fetal Calf Serum (FCS) 100 ml
- 2mM L-glutamine 10 ml
- Sterile Ultra-pure water Top up bottle to 1000 ml
- 1mM of penicillin-streptomycin may be added for cell line propagation and maintenance purpose.

APPENDIX B: PHAGE DESIGNATION AND LOCATIONS OF ORIGIN

Table : Location, type of sample, host used for phage enrichment and designation.

No.	Location	Host	Sample	Designation
1	Pantai Dalam Sewage Treatment Plant	CMS	Sewage	C1
2	Kinchang Waterfall	K9	Soil	K2
3	Pulau Ketam	CMS	Coastal Sea-Water	C3
4				C4
5				C5
6				C6
7				C7
8				C8
9				C9
10				C10
11				C11
12				C12
13				C13
14		K9		K14
15				K15
16				K16
17				K17
18				K18
19				K19
20				K20
21				K21
22				K22
23	Port Dickson	CMS	Coastal Sea-Water	C23
24				C24
25				C25
26				C26
27				C27
28				C28
29				C29
30				C30
31				C31
32				C32
33				C33
34				C34
35				C35
36				C36
37				C37
38	Templer's Park	CMS	Soil	C38
39		K9	Soil	K39
40				K40
41			Water	K41
42				K42
43				K43

APPENDIX C: HOST RANGE OF ISOLATED PHAGE

Table: Host range of 43 isolates against *B. pseudomallei*. 1 = plaque formation; 0 = no plaque.

Phage	Clinical Isolates of *B. pseudomallei*																
	1	2	3	4	6	7	8	9	10	11	12	13	14	16	17	18	19
C1	1	1	0	1	1	1	1	1	1	0	0	0	1	0	1	0	1
K2	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C3	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C4	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C5	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C6	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C7	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C8	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C9	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C10	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C11	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C12	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C13	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K14	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K15	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K16	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K17	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K18	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K19	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K20	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1
K21	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K22	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C23	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C24	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C25	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C26	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C27	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C28	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C29	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C30	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1	0	1
C31	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C32	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C33	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C34	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C35	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C36	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
C37	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	1
C38	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K39	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K40	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K41	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K42	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1
K43	1	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1

Table continued from previous page.

Phage	Clinical Isolate of *B. pseudomallei*															
	19	21	22	23	24	25	26	27	28	33	41	42	44	45	49	50
C1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0
K2	1	0	1	0	1	1	0	0	1	0	1	1	1	1	1	1
C3	1	0	1	0	1	0	0	0	1	0	1	1	1	1	1	1
C4	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	0
C5	1	0	1	1	1	0	0	0	1	0	1	1	1	1	1	1
C6	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
C7	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	0
C8	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0
C9	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0
C10	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
C11	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0
C12	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1
C13	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0
K14	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1
K15	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1
K16	1	0	0	1	1	1	0	0	1	0	1	1	1	1	1	1
K17	1	1	0	0	0	1	0	1	0	0	0	1	1	1	1	1
K18	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1
K19	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1
K20	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1
K21	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1
K22	1	1	0	0	0	1	0	1	1	0	0	1	1	1	1	1
C23	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	1
C24	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	1
C25	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
C26	1	0	1	0	1	0	0	0	1	0	0	0	1	1	1	1
C27	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
C28	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0
C29	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0
C30	1	1	1	1	0	1	0	1	0	0	0	0	1	0	0	0
C31	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	1
C32	1	1	1	1	0	1	0	1	0	0	0	1	1	1	1	0
C33	1	1	1	1	0	1	0	1	1	0	0	1	1	1	1	0
C34	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	0
C35	1	1	0	1	0	1	0	1	0	0	0	1	1	1	1	0
C36	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	0
C37	1	1	1	1	0	0	0	0	1	0	0	1	1	1	1	1
C38	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1
K39	1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1
K40	1	0	0	0	1	0	0	0	1	0	0	1	1	1	1	1
K41	1	0	0	0	1	0	0	0	1	0	0	1	1	1	1	1
K42	1	0	0	0	1	0	0	0	1	0	0	1	1	1	1	1
K43	1	0	0	0	1	1	0	0	1	0	1	1	1	1	1	1

Table continued from previous page.

Phage	Clinical Isolate of *B. pseudomallei*											
	51	52	53	54	56	57	58	63	64	67	68	Score
C1	0	1	1	1	1	0	0	0	0	0	0	20
K2	1	1	1	1	1	1	1	0	0	1	0	30
C3	1	1	1	1	1	0	1	0	0	1	0	28
C4	1	1	1	1	1	0	1	0	0	1	0	27
C5	1	1	1	1	1	0	1	0	0	1	0	29
C6	0	1	1	1	1	0	1	0	0	1	0	27
C7	0	1	1	1	1	0	1	0	0	1	0	27
C8	0	1	1	1	1	0	1	0	0	1	0	28
C9	1	1	1	1	1	0	1	0	0	1	0	28
C10	0	1	1	1	1	0	1	0	0	1	0	26
C11	0	1	1	1	1	0	1	0	0	1	0	28
C12	1	1	1	1	1	1	1	0	0	0	0	30
C13	0	1	1	1	1	0	1	0	0	1	0	26
K14	1	1	1	1	1	0	0	0	0	0	0	23
K15	1	1	1	1	1	0	1	0	0	1	0	27
K16	1	1	1	1	1	1	1	0	0	1	0	30
K17	1	1	1	1	1	0	1	0	0	1	0	26
K18	1	1	1	1	1	0	1	0	0	1	0	28
K19	1	1	1	1	1	0	1	0	0	1	0	28
K20	1	1	1	1	1	0	1	0	0	1	0	21
K21	1	1	1	1	1	0	1	0	0	1	0	28
K22	0	1	1	1	1	0	1	0	0	1	0	26
C23	1	1	1	1	1	0	1	0	0	1	0	27
C24	1	1	1	1	1	0	1	0	0	1	0	27
C25	1	1	1	1	1	0	0	0	0	1	0	27
C26	1	1	1	1	1	0	0	0	0	0	0	24
C27	0	1	1	0	1	0	0	0	0	1	0	25
C28	0	1	1	0	1	0	0	0	0	1	0	24
C29	0	1	1	0	1	0	0	0	0	1	0	25
C30	0	0	0	0	1	0	0	0	0	0	0	19
C31	1	1	1	0	1	0	0	0	0	1	0	28
C32	0	1	1	0	1	0	0	0	0	0	0	24
C33	0	1	1	1	1	0	1	0	0	0	0	28
C34	0	1	1	1	1	0	0	1	0	0	0	23
C35	1	1	1	1	1	0	0	0	0	0	0	25
C36	1	1	1	1	1	0	1	0	0	1	0	28
C37	1	1	1	1	1	0	1	0	0	1	0	24
C38	1	1	1	1	1	1	1	0	0	1	0	32
K39	1	1	1	1	1	0	1	0	0	1	0	24
K40	1	1	1	1	1	0	1	0	0	1	0	26
K41	1	1	1	1	1	0	1	0	0	1	0	26
K42	1	1	1	1	1	0	1	0	0	1	0	26
K43	1	1	1	1	1	0	1	0	0	1	0	28

LIST OF PUBLICATIONS AND PAPERS PRESENTED

Scientific Publication:

1. Choh, L. C., **Ong, G. H.**, Vellasamy, K. M., Kalaiselvam, K., Kang, W. T., Al-Maleki, A. R., Mariappan, V. and Vadivelu, J. (2013). Burkholderia vaccines: are we moving forward? *Frontiers in cellular and infection microbiology* **3**: 5.

Manuscripts Submitted:

1. **Ong, G. H.**, Choh, L. C., Vellasamy, K. M., Mariappan, V., Chang, L.Y., Vadivelu, J.

Experimental Phage Therapy on *Burkholderia pseudomallei* Infection.

Manuscript submitted to *PLOS ONE.*

2. Choh, L. C., **Ong, G. H.,** Chua, E. G., Vellasamy, K. M., Khan, A.M., Wise, M. J., Wong, K. T., Vadivelu, J.

Absence of BapA Type III Effector Protein Affects *Burkholderia pseudomallei* Intracellular Lifecycle in Human Host Cells

Manuscript submitted to *Fronties in Cellular and Infection Microbiology*.

Posters Presented:

1. **Ong, G. H.,** Chang, L.Y., Vadivelu, J.

Isolation and Characterisation of Environmental *Burkholderia pseudomallei* Bacteriophages.

International Congress of the Malaysian Society for Microbiology, 8th -11th December 2011. Penang, Malaysia.

2. **Ong, G. H.,** Chang, L.Y., Vadivelu, J.

Isolation and Characterisation of Environmental phages of *Burkholderia pseudomallei*.

17th Biological Science Graduate Conference (BSGC), December 2012. Chulalongkorn University, Bangkok, Thailand.