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Isolation and Characterisation of Bacteriophages infecting *Klebsiella pneumoniae* from Sewage Samples in Sarawak, Malaysia

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Isolation and Characterisation of Bacteriophages infecting *Klebsiella pneumoniae* from Sewage Samples in Sarawak, Malaysia

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Jeremiah 29:11

"For I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future.

ABSTRACT

A group of nosocomial multidrug-resistant pathogens that posed threats to immunocompromised patients have previously been referred to by the acronym ESKAPE. The ESKAPE pathogens which include Gram-negative *Klebsiella pneumoniae* are considered a great threat, due to the emergence of strains that are resistant to all or most available antibiotics. Misuse and extensive consumption of broad-spectrum antibiotics in hospitalised patients have allowed the evolution of drug-resistant bacterial strains by producing defense mechanisms such as extended-spectrum β-lactamases and diverse aminoglycoside-inactivating enzymes. Many of these strains are highly virulent and exhibit a strong tendency to propagate. In this study, five lytic *Klebsiella* bacteriophages namely ϕKPaV03, ϕKPaV04, ϕKPaV08, ϕKPaV10, and ϕKPaV12 were isolated from domestic sewage at Universiti Malaysia Sarawak (UNIMAS) and characterised based on their biological properties, including bacteriophage morphology, host range, growth curve, bacteriophage multiplicity of infection (MOI) and structural protein composition. These bacteriophages have large burst size with high titer assay between $10^8 – 10^{12}$ pfu / mL and were predominantly stable under 4 ºC. Two among the five bacteriophages were capable of efficiently lysing more than five *Klebsiella pneumoniae* strains out of 18 clinical and community-acquired isolates from Borneo Medical Centre (BMC) and students of UNIMAS, respectively. These bacteriophages exhibit several properties indicative of potential utility in phage cocktails and phage-antibiotic synergy (PAS) approach.

Keywords: *Klebsiella pneumoniae*, multidrug-resistant, sewage samples, bacteriophages, phage approaches

Pengasingan dan Pengenalpastian Bakteriofaj menjangkiti* Klebsiella pneumoniae *dari Sampel Kumbahan di Sarawak, Malaysia

ABSTRAK

Sekumpulan patogen nosokomial yang resisten terhadap pelbagai jenis antibiotik telah menimbulkan ancaman kepada pesakit yang lemah daya tahan. Patogen nosokomial ini telah dirujuk sebagai akronim ESKAPE. Patogen ESKAPE, termasuklah bakteria Gram-negatif Klebsiella pneumoniae *yang dianggap sebagai ancaman besar, kerana resisten dengan kebanyakkan antibiotik yang sedia ada. Penyalahgunaan antibiotik spektrum luas oleh pesakit-pesakit telah menyebabkan evolusi bakteria yang resisten dengan pelbagai jenis antibiotik melalui mekanisme pertahanan seperti spektrum lanjutan β-laktamase dan enzim yang menyah-aktif aminoglikosida. Kebanyakan strain bakteria* Klebsiella pneumoniae *menunjukkan daya virulen yang tinggi. Kajian ini telah berjaya memencilkan lima bakteriofaj* Klebsiella *iaitu ϕKPaV03, ϕKPaV04, ϕKPaV08, ϕKPaV10, dan ϕKPaV12 dari sampel kumbahan domestik Universiti Malaysia Sarawak (UNIMAS) dan dikategorikan berdasarkan sifat biologi, termasuk morfologi bakteriofaj, kesan jangkitan bakteriofaj terhadap pelbagai jenis* Klebsiella pneumoniae*, lengkung pertumbuhan bakteriofaj, pergandaan jangkitan bakteriofaj (MOI) dan komposisi struktur protein. Kesemua bakteriofaj yang terpilih mempunyai saiz letusan besar dengan kadar titer yang tinggi antara 10^{8}-10^{12} pfu / mL dan kebanyakannya stabil di bawah suhu 4 ˚C. Dua daripada lima bakteriofaj mampu membasmi lebih daripada lima strain* Klebsiella pneumoniae *daripada 18 isolat klinikal dan komuniti dari Pusat Perubatan Borneo (BMC) dan pelajar-pelajar*

UNIMAS. Disamping itu, bakteriofaj ini juga telah mempamerkan beberapa sifat yang menunjukkan potensi kegunaan dalam koktel bakteriofaj dan juga secara pendekatan sinergi bakteriofaj-antibiotik (PAS).

Kata kunci: Klebsiella pneumoniae, *resisten pelbagai antibiotik, sampel kumbahan, bakteriofaj, pendekatan bakteriofaj*

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
blaKPC	*Klebsiella pneumoniae* Carbapenemase
CAP	Community-acquired Pathogens
CO_2	Carbon Dioxide
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
g	Gram
ICTV	International Committee on Taxonomy of Viruses
kb	Kilobase
LB	Luria-Bertani
MDR	Multi-drug Resistant
mL	Milliliter
mm	Millimeter
mg	Milligram
MOI	Multiplicity of Infection
NDM	New Delhi Metallo-β-Lactamase
nm	Nanometer
OD	Optical Density
RNA	Ribonucleic Acid
rpm	Rotation per Minute
ρmol	Picomole
PCR	Polymerase Chain Reaction

PFU	Plaques per Unit
ssDNA	Single-stranded Deoxyribonucleic Acid
ssRNA	Single-stranded Ribonucleic Acid
TAE	Tris-Acetate-EDTA
TEM	Transmission Electron Microscope
V	Voltage
v/v	Volume per Volume
μL	Microlitre

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The bacteriophages were independently discovered by British microbiologist Frederick Twort in 1915 and French-Canadian microbiologist Felix d'Hérelle in 1917 (Carlton, 1999). A discovery which occurred about 20 years before the practical application of penicillin, the first antibiotic (Matsuzaki et al., 2005). At the time of discovery, bacteriophages were regarded as a potential treatment for bacterial infections and have been developed to control bacterial diseases such as dysentery, cholera, and gangrene (Dublanchet & Bourne, 2007).

Regardless of the attributes and unique properties of bacteriophages being able to fight bacterial infections, it was then soon abandoned in the 1940s by the West with the arrival of the antibiotic era (Matsuzaki et al., 2005). With the rise of pharmaceutical antibiotics in the mid-20th century, along with a better understanding of diseases and sanitation, both quality of life and life expectancy in the industrialized world drastically improved (Lin et al., 2017). Antibiotics rapidly became an indispensable medical tool with millions of kilograms used globally each year in the prophylaxis and treatment of people, animal, and agriculture (Levy & Bonnie, 2004).

However, the excessive usage and prescription of antibiotics leads to the emergence of pathogenic bacteria resistant to multiple antimicrobial agents such as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) (Weber-

Dabrowska et al., 2016). Antibiotic-resistant genes encoding for resistance to common antibiotics, including β-lactams, aminoglycosides, and tetracycline, are posing a major threat to current medical treatment of common diseases, and these genes now appear to be abundant in the environment (Lin et al., 2017).

The growing of multidrug-resistant pathogens threatens to make the revolutionary achievements of modern medicine obsolete, posing a concern of re-entering the "pre-antibiotics" era (Jeney, 2012). For example, Lin et al., (2017) mentioned the lack of treatment options for hospital-acquired carbapenem-resistant *K. pneumoniae* infections caused a 40 % - 50 % mortality rate in the United States beginning of 2000. Carbapenem resistance in *K. pneumoniae*, mainly attributed to the production of *K. pneumoniae* carbapenemase (KPC) enzyme which is able to destroy carbapenems and causes resistance against a wide spectrum of antibiotics.

Rekindling phage therapy can be a good option for solving the problem of multidrug-resistant pathogens such as *K. pneumoniae*. Major advantages of phage therapy compared to conventional antibiotic treatment are such that phages are highly specific and easy to obtain. Thus, it can be used in cases of sudden bacterial disease outbreaks. Moreover, they do not have chemical side effects like antibiotics (Baharuddin et al., 2017).

Therefore, the objectives of this study were to isolate and characterise lytic bacteriophages with therapeutic potential against clinical- and community-acquired *K. pneumoniae* strains. It is hoped that this study will be able to contribute to future phage therapy studies and applications.

1.2 The History of Therapeutic Bacteriophages

Bacteriophage, or simply called as phages, are viruses that specifically infect and lyse bacteria. The term bacteriophage was coined by Felix d'Herelle in 1916, who derived it from the word 'bacteria' and a Greek word 'phagein' which means 'to devour'. Phages, like all other viruses, are absolute parasites as they have no machinery to generate energy and ribosomes for making proteins even though they carry all the information to direct their own reproduction in an appropriate host. (Kutter & Sulakvelidze, 2005).

In 1910, d'Herelle first observed the bacteriophage phenomenon while studying microbiologic means of controlling an epizootic locusts in Mexico which he then applied his knowledge on phage to treat the dysentery outbreak among several French soldiers (Sulakvelidze, Alavidze, & Morris Jr., 2001). Following the success, the usage of bacteriophage as a therapeutic agent to treat bacterial infections has been widely applied circa the 1930s and 1940s in Eastern Europe and the former Soviet Union. However, it was abandoned by Western Medicine in the 1940s after the discovery of penicillin which soon became widely available (Matsuzaki et al., 2005).

The reason for abandoning the therapeutic usage of phages in the West was due to the lack of appropriate controls and inconsistent results (Wittebole et al., 2014). Moreover, at that time, the biological viral nature and mechanism of phage has yet to be determined and thoroughly studied until they were visualised in the 1940s after the invention of electron microscopy (Cisek et al., 2017).

Merril et. al., (2003) stated that the applications of phage as practiced in the Soviet Union including Poland have been extensively evaluated in which according to one of the review papers from 1998, only 27 papers dealing with bacteriophage therapy were published

between 1966 and 1996. Nineteen of these were from laboratories in Poland and Russia, where research on bacteriophage therapy had never dimmed, and where patients infected with *Staphylococcus, Streptococcus, Klebsiella, Escherichia, Proteus, Pseudomonas, Shigella,* and *Salmonella* were reportedly treated with 80 to 85 percent success (Ho, 2001). Likewise, Bull et al., (2002) has reviewed on the earlier experimental work on phage therapy and prophylaxis and mentioned several impressive studies using phage to treat and prevent bacterial infections in animals including work done by Williams S. H. and team in 1987.

However, details such as the primary qualitative data such as phage dosages and clinical criteria were not properly reported and documented. Hence, most of the studies from Eastern Europe are unable to meet the present standards for pharmaceutical approval in countries that require certification based on the results of efficacy and pharmacokinetic studies in animals and humans.

1.3 Emergence of Antibiotic-Resistant Bacteria

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotics were successfully used to treat bacterial infections in humans and animals, as well as in agriculture. However, the effectiveness of antibiotics is challenged by the increasing number of antibiotic-resistant bacteria. Most of the available antibiotics, including β-lactams, are becoming less effective and in some cases, resistance rates exceed 98 % (Akinkunmi et al., 2014). For instance, El-Shibiny & El-Sahhar (2017) have reported on few multidrug-resistant strains such as *Escherichia coli O104:H4* and some *Salmonella* isolates from poultry which have been found to be resistant to at least 14 different antibiotics, and about 90 % of the *Salmonella* isolates were found to be resistant to at least one or more antibiotics tested.