

**SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE
AGAINST *Klebsiella pneumoniae* USING 12-MER PHAGE-DISPLAYED
PEPTIDE LIBRARY**

By

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ABSTRACT

SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Klebsiella pneumoniae* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY

Lai Kah Nyin

Klebsiella pneumoniae is commonly associated with nosocomial and community-acquired infections. The excessive use of antibiotics to treat these bacterial infections has led to the emergence of multidrug resistant strains. Thus, alternative antibacterial agents like antibacterial peptides are urgently needed to complement the antibiotic treatment. The objective of this study was to screen and isolate antibacterial peptides against *Klebsiella pneumoniae* using phage-displayed peptide library. This study was also aimed to analyze the physiochemical properties of isolated peptides if they show inhibition against the bacteria. Briefly, a 12-mer random peptide library was used to pan against *Klebsiella pneumoniae* ATCC 13883 for four rounds to isolate phage-displayed peptides that bind to the bacteria. Individual phage clones from the fourth round of biopanning that were bound to the bacteria were randomly selected and amplified. The binding selectivity of individual clones towards the bacteria was determined by phage ELISA. These phage-displayed peptides were then tested for antibacterial effect using microtiter broth assay and bacterial titer. The DNA of clones that exhibited observable antibacterial

effect was extracted and sent for sequencing. Four of the 29 selected phage-displayed peptides showed various degree of inhibition against the growth of *Klebsiella pneumoniae* ranging from 18.3% to 31.46%. Sequence analysis revealed four peptides as WPVWQMRPMVQG, FRDTDSLFTGSP, HSNWALYGIAEL and GLHTSATNLYLH. Two peptides contained -His-XXXXX-Leu-Tyr- linear consensus sequence which might serve as critical binding residues to the target bacteria. WPVWQMRPMVQG is cationic; FRDTDSLFTGSP and HSNWALYGIAEL are anionic; while GLHTSATNLYLH is zwitterionic. Low cationicity of the peptides probably reduced the degree of inhibition against the bacteria. These peptide sequences cannot match to any antibacterial peptides that were found in Antimicrobial Peptide Database, suggesting that they might be novel peptides. In conclusion, antibacterial peptides against *Klebsiella pneumoniae* were successfully isolated using 12-mer phage-displayed peptide library. These findings may provide fundamental information for identification and development of novel antibacterial peptides.

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My friends and coursemates for their encouragement and advice. Thank you for all the joy and laughter that we have shared.

DECLARATION

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

(LAI KAH NYIN)

APPROVAL SHEET

This project report entitled “**SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Klebsiella pneumoniae* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY**” was prepared by LAI KAH NYIN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

Yours truly,

(LAI KAH NYIN)

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azinobis (3-ethylbenz-thiazoline sulphonic acid)
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CFU	Colony forming units
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended-spectrum beta-lactamase
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
H ₂ O ₂	Hydrogen peroxide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
LTM	Liquid test medium
MBC	Minimum bactericidal concentration
MIC	Minimum inhibition concentration
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaI	Sodium Iodide
NaN ₃	Sodium azide
NDM-1	New Delhi metallo-beta-lactamase 1
OD	Optical density

Omp	Outer membrane protein
PEG	Polyethylene glycol
PFU	Plaque forming units
Ph.D. TM	Phage Display Library (New England Biolabs)
pI	Isoelectric point
rpm	Revolutions per min
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline+Tween
TE	Tris-EDTA
Tet	Tetracycline
UV	Ultraviolet
Xgal	5-bromo-4-chloro-indolyl- β -D-galactopyranoside
$\times g$	Times gravity

CHAPTER 1

INTRODUCTION

Klebsiella pneumoniae is commonly associated with hospital and community-acquired infections such as pneumonia, urinary tract infections, septicemia and soft tissue infections (Gupta 2002). Extensive use of antibiotics to treat *Klebsiella* infections among hospitalized patients has caused the emergence of multidrug-resistant strains that produce extended-spectrum beta-lactamase (ESBL) (Sikarwar and Batra 2011). ESBL-producing *Klebsiella pneumoniae* develops high level of resistance to beta-lactam antibiotics and this has led to limited choice of antibiotic treatment. Therefore, development of alternative treatment against infection by *Klebsiella pneumoniae* has become increasingly important.

Antibacterial peptide is a new family of antibacterial agent that might serve as a potential alternative treatment over conventional antibiotics for multidrug resistant bacterial infections. Antibacterial peptides are components of the innate immunity that are found universally in all living organisms against bacterial infections (Pasupuleti et al., 2012). Previous studies have demonstrated few antibacterial peptides which are effective against *Klebsiella pneumoniae* including pyrrolicin (Cudic et al., 2002), S-thanatin (Wu et al., 2009) and cathelicidin-related antibacterial peptides (Kovach et al., 2012).

Many scientists throughout the world use different methods to identify and characterize antibacterial peptides from various sources. These include crude extraction of natural antibacterial peptides (Treffers et al., 2005; Wang et al., 2012), chemically synthesized antibacterial peptides using gene screening (Patrzykat et al., 2003) and also molecular manipulation (Pini et al., 2005). However, these methods seem to be time-consuming and required tedious and laborious protocol. On the other hand, screening of antibacterial peptides using phage-displayed peptide library has been applied for antimicrobial drug discovery and design. Scientists have successfully isolated peptides that exhibited antibacterial effect using phage-displayed peptide library (Bishop-Hurley et al., 2005; Pini et al., 2005; Bishop-Hurley et al., 2010; Rao et al., 2013).

In this study, antibacterial peptides against *Klebsiella pneumoniae* were screened and isolated using 12-mer phage-displayed peptide library. The isolated antibacterial peptides were then deduced and characterized for their physiochemical properties against the bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of *Klebsiella pneumoniae*

2.1.1 Characteristic and Morphology

Klebsiella pneumoniae is a member of the family Enterobacteriaceae. It is a non-motile, Gram-negative rod and facultative anaerobe (Public Health Agency of Canada 2011). Most of the strains express fimbriae to adhere to the host tissues (Chart 2007). *Klebsiella pneumoniae* has a prominent polysaccharide capsule which encases the entire cell surface. This capsule accounts for the large appearance of *Klebsiella pneumoniae* on Gram stain, and also provides resistance against many host immune mechanisms (Umeh 2011).

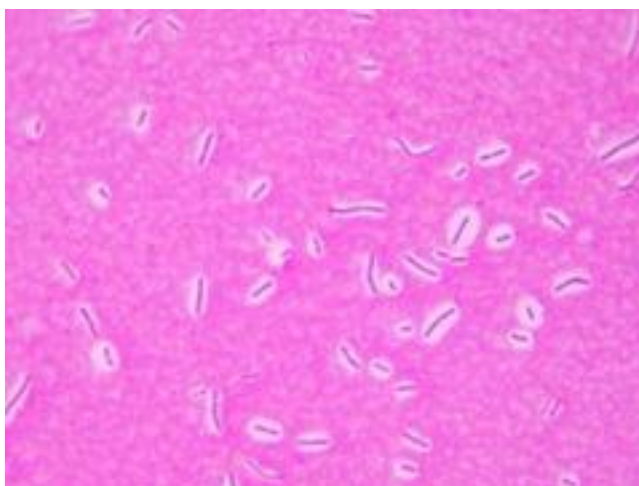


Figure 2.1: Morphology of *Klebsiella pneumoniae* in capsule stain (Quizlet 2012).

Klebsiella pneumoniae typically express two types of antigens on their cell surface: capsular polysaccharide (K antigen) and lipopolysaccharide (O antigen). There are total of 80 types of K antigens and 11 types of O antigens being recognized in Klebsiellae (Behera 2010). Both of these antigens contribute to its pathogenicity. K antigen plays a protective role against serum-induced killing and phagocytosis by human polymorphonuclear leukocytes (Schembri et al., 2005). O antigen has been reported to protect pathogens from complement-mediated killing and it contributes to bacteremia and lethality during murine pneumonia (Shankar-Sinha et al., 2004). The structural variability of the combination of these antigens forms a basis for classification into different serotypes.

Among the species in *Klebsiella* genus, *Klebsiella pneumoniae* is the most medically important species of the group (Jadhav et al., 2012). *Klebsiella pneumoniae* is usually identified and differentiated by simple biochemical tests. It produces lysine decarboxylase but not ornithine decarboxylase, and is generally positive in the Voges-Proskauer test (Public Health Agency of Canada 2011). When the bacteria are grown on media rich in carbohydrate, they produce greater amounts of capsular materials, and the colonies appear luxuriant, greyish white and extremely mucoid (Chart 2007).

2.1.2 Clinical Significance

Klebsiella pneumoniae is significantly associated with numerous community and nosocomial infections. It is a common cause of respiratory infections and ranks fourth as the leading cause of hospital-acquired pneumonia (Bouza and Cercenado 2002). *Klebsiella pneumoniae* are responsible for 1% to 5% of all cases of community-acquired pneumonia and around 19% of cases of nosocomial pneumonia (Muller et al., 2007). The pneumonia caused by *Klebsiella* infection is typically present as lobar pneumonia (Cunha 2010). Although *Klebsiella* pneumonia can be treated by antibiotic, it has a high mortality rate of approximately 50%. The mortality rate can even approach 100% in persons with alcoholism and bacteremia (Umeh 2011).

On the other hand, *Klebsiella pneumoniae* accounts for a significant proportion of urinary tract infections, septicemia and soft tissue infections especially in the immunocompromised host (Gupta 2002). This bacterium normally infects the body parts of hospitalized patients including urinary tract, lower respiratory tract, biliary tract and surgical wound. Other opportunistic infections caused by *Klebsiella pneumoniae* are liver abscess, brain abscess, osteomyelitis, endophthalmitis, and meningitis (Kawai 2006). The risk of *Klebsiella* infection may also increase in individual with debilitating diseases such as malignancy, cirrhosis, biliary tract disorders and diabetes mellitus (Tsai et al., 2010). The gastrointestinal tract of hospitalized patients and the hands of healthcare workers can serve as reservoirs for the transmission of bacteria, and they are responsible for multiple hospital outbreaks (Gupta 2002). There are other factors that increase the likelihood of nosocomial infections

with *Klebsiella pneumoniae* such as the usage of invasive devices and urinary catheters, and also the contamination of respiratory support equipment (Umeh 2011).

2.1.3 Common Treatment

Klebsiella infections are commonly treated with antibiotics. Antibiotics susceptibility test should be carried out along with microbiological diagnosis to determine the choice of specific antimicrobial agents against *Klebsiella pneumoniae*. Examples of these agents are third-generation cephalosporins, carbapenems, aminoglycosides and quinolones. These agents may be used as monotherapy or combination therapy (Umeh 2011).

2.1.4 Multidrug Resistance Problem

Antimicrobial agents such as antibiotics are important to treat patients infected with *Klebsiella pneumoniae* at the early stage of infection. However, extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* and the emergence of multidrug resistant strains (Sikarwar and Batra 2011). Multidrug resistant *Klebsiella pneumoniae* are often isolated from the hospitals, revealing a serious clinical problem (Lim et al., 2009; Mohamudha et al., 2010; Medell et al., 2012). Treatment of this infection is compromised worldwide by the emergence of bacterial strains that develop resistance to multiple drugs (Alekhshun and Levy 2007).

ESBL-producing *Klebsiella pneumoniae* is one of the most problematic multidrug resistant pathogens in the hospital setting (Alekhun and Levy 2007). These strains of *Klebsiella pneumoniae* are able to resist most of the antibiotic classes (Carlet et al., 2012). The first case of ESBL-producing *Klebsiella* was reported in 1983 from a patient in Germany (Knothe et al., 1983). Since then, several outbreaks caused by this pathogen strain have been documented worldwide including United States (Quale et al., 2002), Europe (Webster et al., 2011), Asia (Rastogi et al., 2010), Africa (Ben-Hamouda et al., 2003), South America (Medell et al., 2012), and also Western Pacific region (Winokur et al., 2001). In Malaysia, an association of ESBL-producing *Klebsiella pneumoniae* with a nosocomial outbreak had been reported in a pediatric oncology unit in a Malaysian public hospital (Palasubramaniam et al., 2005). A more recent study by Lim et al. (2009) had characterized the multidrug resistant and ESBL-producing *Klebsiella pneumoniae* strains from five Malaysian public hospitals. Generally, the target of antibiotic resistances that are developed in the ESBL-producing *Klebsiella pneumoniae* include beta-lactams, fluoroquinolones and aminoglycosides (Alekhun and Levy 2007). These pathogens may also resist to many other non-beta-lactam antibiotics, leaving few available therapeutic options (Nordmann et al., 2009).

ESBL-producing *Klebsiella pneumoniae* produces beta-lactamases that are able to hydrolyze antibiotics with beta-lactam structure. Few years ago, a new class B enzyme, New Delhi metallo-beta-lactamase 1 (NDM-1), was characterized from a *Klebsiella pneumoniae* isolated from Sweden, in which the bacterium is believed to be imported from India (Yong et al., 2009). This

bacteria strain resists to a broad range of beta-lactam antibiotics including carbapenems. Unfortunately, carbapenem antibiotics are often the last resort of treatment against Gram-negative bacterial infections that are resistant to other antibiotics (Centers for Disease Control and Prevention 2010). The emergence of this resistant strain of bacteria may lead to very serious clinical problem.

2.1.5 Alternative Treatment for Multidrug Resistant Bacteria

Multidrug resistant *Klebsiella pneumoniae* can be treated with a combination of high-dose tigecycline and colistin (Humphries et al., 2010). However, the emergence of colistin-resistant *Klebsiella pneumoniae* has been reported in few countries including Greece (Antoniadou et al., 2007), Italy (Mezzatesta et al., 2011) and the United State (Marchaim et al., 2011). The use of antibiotics to treat multidrug resistant *Klebsiella* infections is no longer secure and effective. Even though novel antibiotics against the particular drug resistant bacteria may be developed in future, the bacteria may ultimately become resistant to such drugs. To prevent this vicious cycle, there is a significant and urgent need to explore alternative approach to combat these bacterial infections (Matsuzaki et al., 2005). One of such alternative stems up from an old idea is antibacterial peptide, which is now being widely studied.

2.2 Antibacterial Peptides

2.2.1 Properties and Functions

Antibacterial peptides are found universally in all living organisms as an ancient type of innate immunity. These peptides provide the first-line of defense against the invading pathogens (Pasupuleti et al., 2012). The potential advantages of antibacterial peptides as antimicrobial drugs are significantly studied (Hancock and Sahl 2006; Hamill et al., 2008). They have been demonstrated to express antibacterial activity against Gram-negative and Gram-positive bacteria, including strains that are resistant to conventional antibiotics (Wimley and Hristova 2011). Antibacterial peptides are diverse in their sequence and structure. They are generally made up of 12–50 amino acids, have a net positive charge provided by arginine and lysine, and also contain approximately 50% hydrophobic amino acids (Hancock 2001). The cationicity and hydrophobicity of the antibacterial peptides allow them to selectively bind to the anionic bacterial surface through an electrostatic interaction, as well as partition into the bacterial membrane lipid bilayer (Yeaman and Yount 2003). On the contrary, the presence of zwitterionic phospholipids and cholesterol on the mammalian membrane can reduce the interaction of antibacterial peptides with the mammalian membrane (Giuliani et al., 2007).

Antibacterial peptides are immunomodulatory molecules in the innate immune system. In mammals, they are expressed in a variety of cell types including monocytes or macrophages, neutrophils, epithelial cells, keratinocytes and mast cells. Some peptides are released constitutively, while others are strongly

inducible in response to stimuli such as microbial signature molecules, inflammation or tissue injury. Although some of these peptides are sufficiently potent to be considered as natural antibiotics with direct bactericidal capacity, increasing evidence suggests that many mammalian antibacterial peptides have limited bactericidal activity under physiologically relevant conditions due to their modest concentrations (Bowdish et al., 2005). For instance, antibacterial peptides that are expressed at mucosal surfaces and under physiological salt concentrations will have reduced bactericidal activity. Nevertheless, the antibacterial peptides may possess other immunomodulatory properties that do not target the pathogens directly, but rather selectively alter and enhance the host defence mechanisms. The immunomodulatory activities of these peptides indicate the potential application of natural and synthetic antibacterial peptides in infection management (Mookherjee and Hancock 2007).

A wide range of functions has been documented for antibacterial peptides from extensive research in the last decade. These functions include the ability to confer protection against diverse pathogens (Bowdish et al., 2005), anti-endotoxin activity (Scott et al., 2002), selective modulation of pro-inflammatory responses (Mookherjee et al., 2006), chemotactic activity (Tjabringa et al., 2006), modulation of cell proliferation and differentiation (Davidson et al., 2004), promotion of wound healing and angiogenesis (Koczulla et al., 2003), induction of gene expression and enhancement of protein secretion in mammalian host cells (Bowdish et al., 2004), as well as initiation and polarization of adaptive immunity (Yang et al., 2002). At their

antibacterial concentrations, antibacterial peptides can directly kill the pathogens by disrupting the integrity of bacterial cytoplasmic membrane or by targeting the bacterial intracellular components that are essential for bacterial survival (Hancock 2001).

Antibacterial peptides are peptides or polypeptides with antibacterial properties that are branched from antimicrobial peptides. According to Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/database/antiB.php>), a total of 1,783 antibacterial peptides has been identified and characterized from various sources including animals, plants, bacteria, fungi and also synthetic peptides.

2.2.2 Classification of Antibacterial Peptides

Antibacterial peptides can be structurally categorized into four classes: 1) amphipathic alpha-helical, 2) beta-sheet, 3) extended, and 4) looped structures.

Antibacterial peptides in amphipathic alpha-helical structure are amongst the most abundant and widespread peptides in nature. These peptides adopt a random structure in aqueous solution, but form alpha-helices in organic solvents and upon contact with phospholipids of cell membrane (Zhang et al., 2008). They are capable of autonomously interacting with different types of membranes (Giangaspero et al., 2001). Depending on the type and their concentration, these peptides can aggregate to form transient pores or channels on membrane, or can induce a more massive membrane disruption, leading to

cellular inactivation (Oren and Shai 1997; Matsuzaki 1998). Beta-sheet antibacterial peptides contain cysteine residues linked by two or three disulphide bonds and adopt either a beta-sheet or beta-hairpin fold. Some researchers believe that most beta-sheet peptides act on intracellular targets, as they are very effective in inducing lipid flip-flop movement and undergoing membrane translocation (Sahl et al., 2005). Antibacterial peptides with extended structure are rich in specific amino acids such as proline, glycine, tryptophan, arginine or histidine. These peptides lack of classical secondary structures because of their high proline and/or glycine contents. These peptides form their final structures through hydrogen bonds and Van der Waals interactions with bacterial membrane phospholipids (Powers and Hancock 2003). The looped peptides are characterized by their loop structures with a single bond of either disulphide, amide or isopeptide (Pasupuleti 2009).

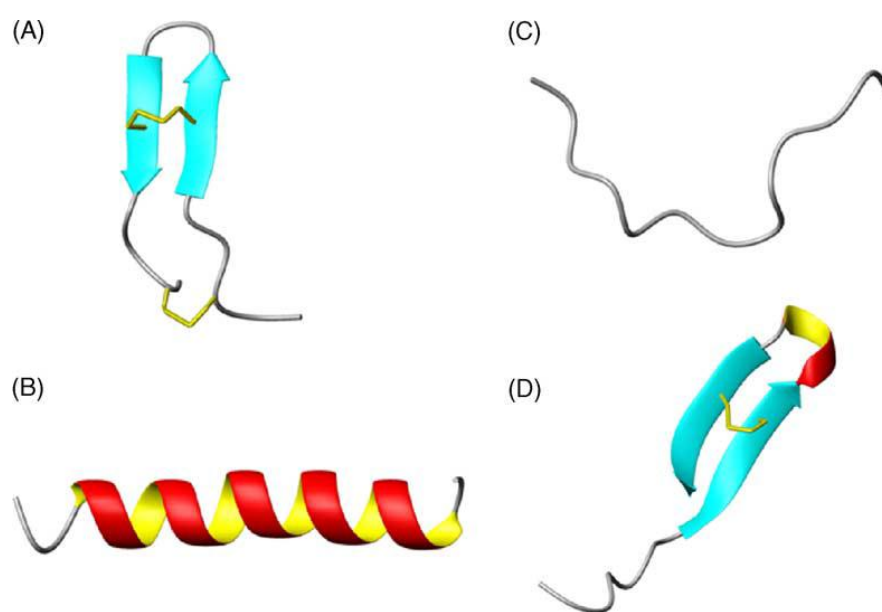


Figure 2.2: Structural classes of antibacterial peptides: (A) beta-sheet; (B) alpha-helical; (C) extended; (D) loop (Powers and Hancock 2003).

2.2.3 Mechanisms of Antibacterial Peptides

In general, antibacterial peptides may act as either membrane disruptors or metabolic inhibitors in their antibacterial activity. However, there is no clear-cut evidence to distinguish their modes of action, due to the fact that a peptide may disrupt the membrane of one bacteria strain and inhibit the metabolite synthesis of another (Hale and Hancock 2007).

Most of the antibacterial peptides inhibit bacteria by compromising the integrity of bacteria membrane. Therefore, antibacterial activity and lipid bilayer permeabilization are correlated (Wimley and Hristova 2011). Antibacterial peptides selectively bind to anionic bacterial membranes rather than mammalian membranes because the mammalian membranes are riched in sterols and zwitterionic phospholipids with neutral net charge. The presence of cholesterol in mammalian cell membranes will reduce the activity of antibacterial peptides and thus protects the cells from attacking by the peptides (Giuliani et al., 2007). In addition, the membrane of Gram-negative bacteria differs from Gram-positive bacteria by having a thinner peptidoglycan layer and an outer membrane. The outer membrane of Gram-negative bacteria contains highly negative-charged lipopolysaccharides which act as permeability barrier. Cationic antibacterial peptides distort the bacterial outer membrane either by strongly binding to the anionic lipopolysaccharides or by neutralizing charge over a patch of the bacterial outer membrane. The peptides are then inserted into or translocated across bacterial membrane lipid bilayer. The interaction between the peptides and bacterial outer membrane allows the peptides to attack discrete areas of the cytoplasmic membrane. Hence, many

antibacterial peptides are selective for Gram-negative bacteria over Gram-positive bacteria (Hancock 2001).

Various models of mechanism have been proposed to describe the action of antibacterial peptides on the bacterial membrane, including barrel stave model, toroidal pore model and carpet model (Wimley and Hristova 2011). However, different studies have revealed that antibacterial peptides can also translocate across the membrane and act on the intracellular components (Gao and Fang 2009).

2.2.4 Antibacterial Peptides against *Klebsiella pneumoniae*

Antibacterial peptides impose a threat to the survival of bacteria, and they have long been proposed as a possible novel class of antibiotics that could be used against bacterial infections. A study has shown that cathelicidin-related antibacterial peptides are required for effective immune response to infection caused by *Klebsiella pneumoniae* on lung mucosa (Kovach et al., 2012). Another antibacterial peptide, namely pyrrolicin, is also effective to kill *Klebsiella pneumoniae* that resists to beta-lactam, tetracycline or aminoglycoside in a low micromolar concentration range (Cudic et al., 2002). According to another study conducted in China, S-thanatin (an analog to thanatin) was inhibited *Klebsiella pneumoniae* ATCC 700603 with the bactericidal rate of more than 99% (Wu et al., 2009).

2.2.5 Methods to Isolate Antibacterial Peptides

There are a number of methods to isolate and identify antibacterial peptides. Crude extraction is one of the conventional methods used to isolate antibacterial peptides from various sources including neutrophils of deer (Treffers et al., 2005), hemolymph of lobster (Battison et al., 2008), plant seeds (Aliahmadi et al., 2011), skin secretions of frog (Wang et al., 2012), and also mucus of snail (Zhong et al., 2013). Homogenization and centrifugation of the collected source are usually involved in this method, and then followed by peptide purification using high-performance liquid chromatography (HPLC).

However, the techniques which are mentioned above are time-consuming and required tedious procedure. In addition, highly skilled labors are required to purify the peptides in crude extraction using HPLC. Therefore, screening of antibacterial peptides from phage-displayed peptide library has been applied for antimicrobial drug discovery and design (Bishop-Hurley et al., 2005; Pini et al., 2005; Bishop-Hurley et al., 2010; Rao et al., 2013). It also provides a more convenient method to isolate different antibacterial peptides from a phage-displayed peptide library that contains diverse peptide sequences.

2.3 Phage-Displayed Peptide

2.3.1 Principle of Phage-Displayed Peptide

Biopanning using phage-displayed peptide library is an *in vitro* selection technique, in which a library of peptides or proteins variants with desired properties is expressed on the surface of a phage virion while the genetic material encoding each variant resides within the virion. This provides a direct linkage between each variant protein sequence (phenotype) and the DNA encoding it (genotype) which allows rapid identification of peptides based on their binding affinity to a given target molecule (New England Biolabs). Peptides that are specifically bound to the target molecule are then selected and amplified to enrich the specific clones of binding peptides, while the unspecific or weak binders are washed away during the washing steps.

2.3.2 M13 Phage-Displayed Peptide Library

Various phage-displayed peptide libraries have been designed using either lytic, filamentous phage or phagemid vectors. The most commonly used phage-displayed peptide libraries are based on filamentous phages in which the peptides are fused to either major (pVIII) or minor (pIII) coat proteins (Molek et al., 2011). M13 is one of the popular filamentous phages being used in phage display technology. It is an *Escherichia coli*-specific filamentous phage that contains five different coat proteins on its surface.

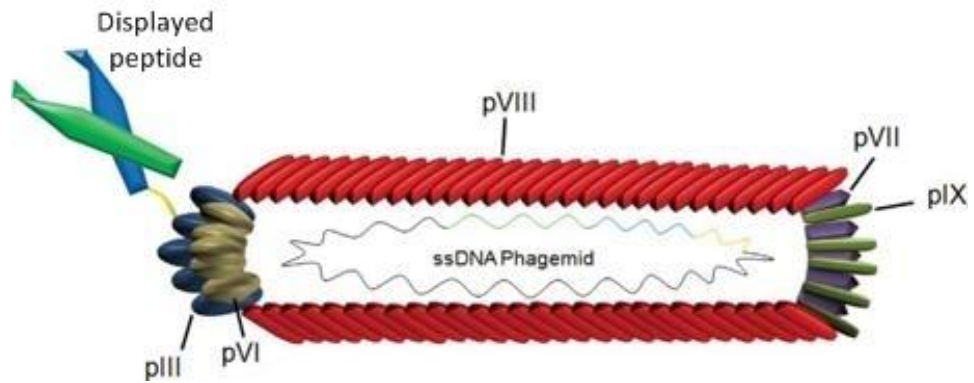


Figure 2.3: M13 filamentous phage used in phage display (Kierny et al., 2012).

The Ph.D.-12 phage-displayed peptide library from New England Biolabs is a combinatorial library of random dodecapeptides fused to the N-terminus of the M13 minor (pIII) coat protein, which is expressed in five copies at one end of the phage virion. The pentavalent display of each 12-mer peptide on a M13 virion does not measurably affect the infectivity of the phage to the host bacteria. This library has a complexity on the order of 10^9 independent clones and sufficient to encode less than one millionth of the 4.1×10^{15} possible 12-mer peptide sequences (New England Biolabs).

2.3.3 Advantages of M13 Phage-Displayed Peptide Library

The minor (pIII) coat protein of M13 is the protein of choice for most phage display fusions due to its tolerance for the insertion of large protein, its compatibility with monovalent display, and the wide availability of suitable vectors (Russel et al., 2004). The monovalent display of pIII libraries has made the Ph.D. system suitable for the discovery of higher affinity ligands.

M13 is preferable used to display short peptides because peptides longer than 30-50 amino acids may have a deleterious effect on the infectivity of pIII coat protein (New England Biolabs).

2.3.4 Application of Phage-Displayed Peptide

According to New England Biolabs, phage-displayed peptide library has been used for a wide range of applications such as epitope mapping (Rowley et al., 2004), searching for antibacterial or antiviral peptides (Ramanujam et al., 2002; Lavilla et al., 2009; Rao et al., 2013), screening for material-specific peptides (Estephan et al., 2009), small molecule binders (Qi et al., 2008), and novel enzyme substrates (Lavi et al., 2008). Furthermore, the library is useful for the discovery of bioactive peptides through *in vivo* and *in vitro* panning approaches. For instance, peptide antagonists of VEGF-mediated angiogenesis (Binetruy-Tournaire et al., 2000), plasmodesmal trafficking peptides (Kragler et al., 2000), and cell targeting peptides (Kelly et al., 2008) have all been identified from the Ph.D. library. Also, the library has been extensively used to analyze protein-protein interaction in proteomics (Serasinghe et al., 2010).

2.3.5 Isolation of Antibacterial Peptide using Phage-Displayed Peptide

Phage-displayed peptide is recognized as a powerful tool for selecting novel peptides that express antibacterial activity. Numerous studies on isolation of antibacterial peptides using phage-displayed peptide library have been published. Bishop-Hurley et al. (2005 and 2010) had isolated antibacterial

peptides against *Haemophilus influenzae* and *Campylobacter jejuni*. Pini et al. (2005) had used phage-displayed peptide library to pan against *Escherichia coli*, and the isolated peptides were able to inhibit multidrug resistant *Pseudomonas aeruginosa* and members of the *Enterobacteriaceae*. Tanaka et al. (2008) had used phage-displayed peptide library to pan against bacterial magnetic particles that obtained from *Magnetospirillum magneticum*, and the isolated peptide was able to inhibit *Bacillus subtilis*. Rao et al. (2013) had used phage-displayed peptide library to pan against *Escherichia coli*, and the isolated peptide was able to inhibit *Escherichia coli* and *Pseudomonas aeruginosa*.

Nevertheless, no previous study on the screening of antibacterial peptides against *Klebsiella pneumoniae* using phage-displayed peptide library has been reported, thus leading to the objective of this study to screen and isolate antibacterial peptide against *Klebsiella pneumoniae*.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Plan of the Experiment Work

Ph.D.-12 phage-displayed peptide library (New England Biolabs) was used to select phage-displayed peptides binding to *Klebsiella pneumoniae* strain ATCC 13883. The biopanning process was repeated for four times. Individual selected clones of phage-displayed peptides from the fourth round of biopanning were amplified and subjected to subsequent analysis. The binding selectivity of phage-displayed peptides towards *Klebsiella pneumoniae* was tested by using phage ELISA. Microtiter broth assay and bacteria titer were used to test for antibacterial activity of the selected phage-displayed peptides against *Klebsiella pneumoniae*. DNAs of the phage clones that exhibited antibacterial effect were then extracted and sent for sequencing by 1st BASE. Lastly, those successfully sequenced DNAs were deduced into peptide sequence and analyzed using bioinformatics tools. The general flow of experiment was summarized in Figure 3.1.

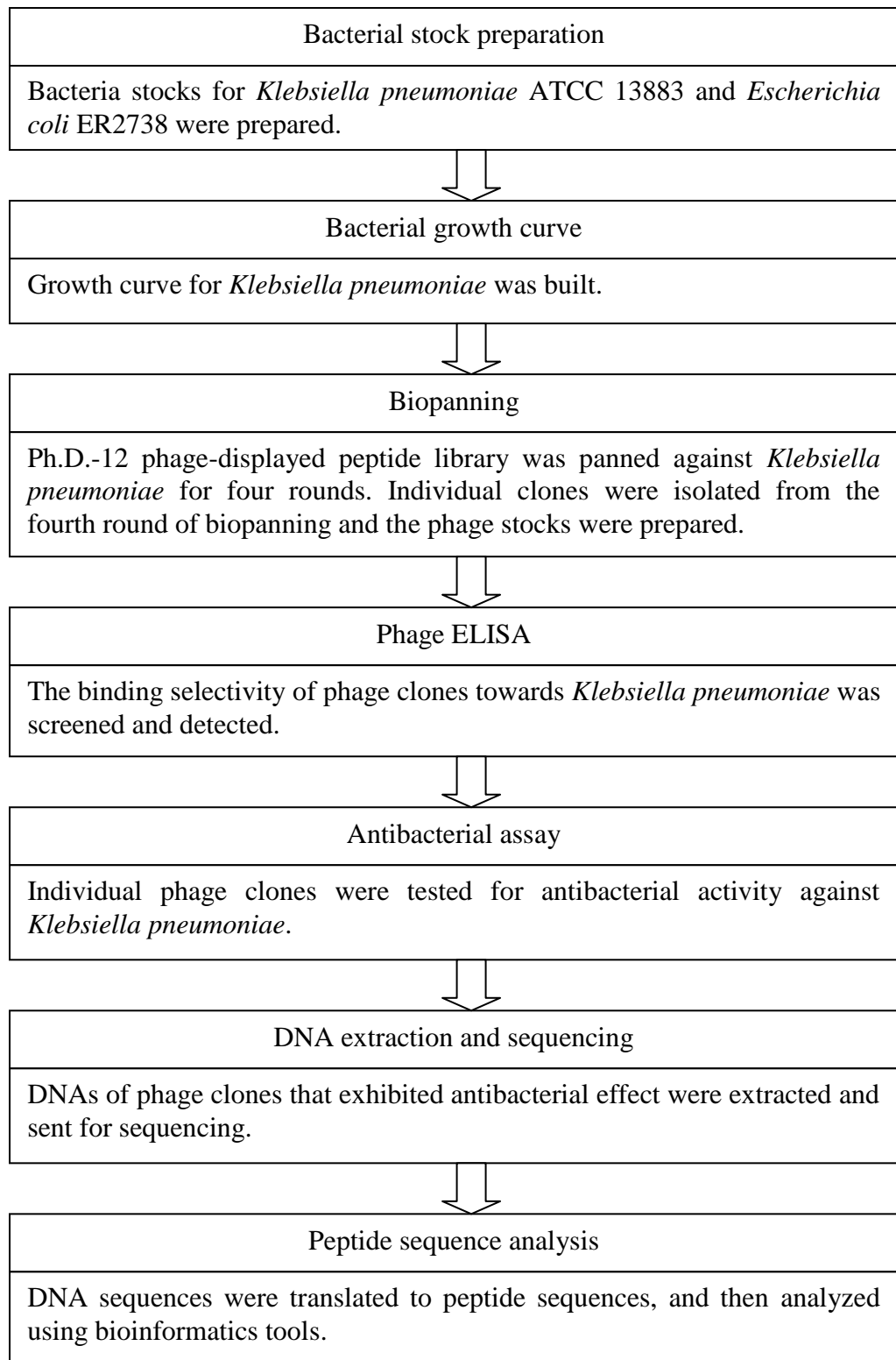


Figure 3.1: The flow chart of experimental work.

3.2 Materials

3.2.1 Bacteria Strains

Klebsiella pneumoniae strain ATCC 13883 which was provided by Universiti Tunku Abdul Rahman (UTAR) was the main bacteria that being used as the target of biopanning in this project. This bacterium was propagated in Luria Bertani (LB) broth and grown on MacConkey agar. Microgen™ GnA+B-ID System, a bacterium identification kit, was used to further confirm this bacterium. *Escherichia coli* ER2738 supplied by New England Biolabs was used as the host bacteria for amplification of phage clones. *Escherichia coli* ER2738 is grown on LB+Tet agar (Luria Bertani agar supplemented with 20 µg/mL of tetracycline) as the F-factor of ER2738 contained a mini-transposon which conferred tetracycline resistance. All bacteria stocks were maintained in LB broth containing 80% (v/v) glycerol at -80°C.

3.2.2 Phage-Displayed Peptide Library

Ph.D.-12 phage-displayed peptide library (New England Biolabs) was used in this study. This library utilizes M13 phage vector that is modified for pentavalent display of peptides as N-terminal fusions to the minor coat protein pIII. It contained a complexity of 10^9 independent clones and sufficient to encode less than one millionth of the 4.1×10^{15} possible 12-mer peptide sequences. The library cloning vector M13KE is derived from the common cloning vector M13mp19, which carries the *lacZα* gene. When the phages are plated on LB agar containing 50 µg/mL of IPTG (isopropyl β-D-1-thiogalactopyranoside) and 40 µg/mL of Xgal (5-bromo-4-chloro-indolyl-β-D-

galactopyranoside), the phages form blue plaques. The individual phage clone displaying the desired peptides were propagated in *Escherichia coli* ER2738 using LB+Tet broth (Luria Bertani broth supplemented with 20 µg/mL of tetracycline).

3.3 Construction of Bacterial Growth Curve

Klebsiella pneumoniae strain ATCC 13883 was grown in LB broth and incubated at 200 rpm at 37°C. For every 1 hour interval, 1 mL of the bacteria culture was used to measure the absorbance at OD₆₀₀ using a spectrophotometer (Genesys 20, Thermo Scientific). Same volume of LB broth was set as the blank. 100 µL of the bacteria culture was serially diluted and then plated on MacConkey agar to determine the bacterial titer in colony forming units per mL (CFU/mL). The procedure was repeated at second hour, third hour, fourth hour as well as fourth and half hour. The OD₆₀₀ reading and bacterial titer were then used to plot the bacterial growth curve.

3.4 Selection of Phage-Displayed Peptides Binding to *Klebsiella pneumoniae*

3.4.1 Solution Biopanning

Four rounds of solution biopanning were performed to select phage-displayed peptides that were bound to *Klebsiella pneumoniae*. In the first round of biopanning, 1×10^{12} plaque forming units per mL (PFU/mL) of phages from the library were incubated with 10^9 CFU/mL of *Klebsiella pneumoniae* strain ATCC 13883 in Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5], 150

mM NaCl) for 60 minutes at room temperature with gentle agitation. The mixture of bacteria and phages were precipitated by centrifuging at $5,000 \times g$ for 5 minutes at 4°C using a centrifuge (Velocity 14R Centrifuge, Dynamica). In the first round of affinity selection, 0.1% (v/v) of Tween-20 in TBS (TBST 0.1%) was used to wash away the unbound phages. The bound phages were pelleted with bacterial cells and finally eluted with 200 μL of elution buffer (0.2 M Glycine-HCl [pH 2.2], 1 mg/mL bovine serum albumin [BSA]) with gentle shaking at room temperature for 10 minutes. The eluted phages were then neutralized immediately with 30 μL of neutralization buffer (1 M Tris-HCl [pH 9.1]). 10 μL of the eluate were used to determine the titer of the selected phages while the rest of the eluate was amplified by infecting *Escherichia coli* ER2738 before proceeding to subsequent round of biopanning. The level of specific phage enrichment was determined by calculating the yield of selection. The biopanning procedure was repeated for another three rounds, with a gradual increase of the Tween-20 concentration in the washing buffer to 0.3% (v/v) in the second round of selection and then 0.5% (v/v) in the third and fourth rounds of selection. After four rounds of biopanning, individual phage clones appeared as blue plaques on the titer LB/IPTG/Xgal plate were randomly selected and propagated for subsequent analysis.

3.4.2 Phage Titering

Escherichia coli ER2738 was inoculated into LB+Tet broth and grown to mid-log phase (OD₆₀₀ approximately 0.5). The eluted phage in every round of biopanning as mentioned in 3.4.1 was serially diluted to the desired dilution factor using TBS. For each diluted phage, 10 µL of the phage suspension was added into 200 µL of ER2738. The phages were allowed to infect ER2738 for 5 minutes at room temperature. The infected cells were then transferred to culture tubes containing 45°C molten top agar. The culture was mixed briefly and quickly poured onto a LB/IPTG/Xgal plate. After the top agar had solidified, the plate was inverted and incubated overnight at 37°C. The number of blue plaques that formed on the LB/IPTG/Xgal plate was counted and the phage titer in PFU/mL was determined.

3.4.3 Phage Amplification and Purification

An overnight culture of *Escherichia coli* ER2738 was diluted according to 1:100 in LB broth and grown until the OD₆₀₀ of between 0.01 to 0.05. The remaining of the eluted phages after titering were added into the diluted ER2738 culture and incubated at 200 rpm for 4.5 hours at 37°C. After incubation, the culture was centrifuged at 12,000 × g for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and re-spun. The upper 80% of the supernatant was transferred again to another fresh tube and 1/6 volume of PEG/NaCl (20% [w/v] polyethylene glycol-8000, 2.5 M NaCl) was added to precipitate the phages overnight at 4°C.

The next day, the PEG precipitation was spun at $12,000 \times g$ for 15 minutes at 4°C . The supernatant was discarded completely and the phage pellet was resuspended with 1 mL of TBS. The phage suspension was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 5 minutes at 4°C . The upper 80% of the supernatant was precipitated again by PEG/NaCl for 1 hour on ice. After that, the precipitate was spun at 14,000 rpm for 10 minutes at 4°C and the supernatant was discarded. The phage pellet was resuspended with 200 μL of TBS and then spun for 1 minute to pellet any remaining insoluble material. The supernatant was transferred to a fresh tube and stored at 4°C for up to 3 weeks. This amplified eluate was titered according to the protocol as described in 3.4.2 and used for subsequent round of biopanning.

3.4.4 Plaque Amplification

An overnight culture of *Escherichia coli* ER2738 was diluted according to 1:100 in LB broth. Individual blue plaques that were formed on the LB/IPTG/Xgal plate were randomly picked and transferred to a microcentrifuge tube containing 1 mL of the diluted ER2738 culture. The culture was incubated at 200 rpm for 4.5 hours at 37°C . After incubation, the culture was centrifuged at 14,000 rpm for 1 minute. The supernatant containing the amplified phage was transferred to a fresh tube and re-spun. The upper 600 μL of the supernatant was transferred to a fresh tube using a pipette. This amplified phage stock was then diluted according to 1:1 with sterile 50% (v/v) glycerol and stored at -20°C .

3.4.5 Large Scale Phage Amplification

The procedure of large scale phage amplification for subsequent analysis in phage ELISA and antibacterial assay was identical to the protocol as described in 3.4.3 by amplifying the phage stock prepared in plaque amplification (3.4.4).

3.5 Phage ELISA

Phage enzyme-linked immunosorbent assay (ELISA) was performed by coating 150 μL of a suspension of 3.15×10^8 CFU/mL *Klebsiella pneumoniae* strain ATCC 13883 in the 96-wells plate overnight at 4°C using 0.1 M NaHCO_3 (pH 8.6). As a negative control, a separate set of wells was treated with blocking buffer (0.1 M NaHCO_3 [pH 8.6], 5 mg/mL BSA, 0.02% NaN_3) without bacteria coating. On the next day, the bacterial suspension and blocking buffer in the plate were discarded. After that, the wells were filled completely with blocking buffer and incubated for 1 hour at 4°C. The wells were washed six times with TBST 0.5% (v/v). The selected amplified phage clones with a titer of 10^{12} PFU/mL were serially diluted four-fold using TBS. 150 μL of each diluted phages were added into the bacteria-coated wells and the correspondent negative control wells. The plate was then incubated at room temperature for 1 hour with gentle agitation. After that, the wells were washed six times with TBST. HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare) was diluted according to 1:1000 in blocking buffer. 200 μL of the diluted conjugated antibody was added into each well. After 1 hour of incubation at room temperature with gentle agitation, the wells were washed again six times with TBST. The positive binding of phage to the target

bacteria was detected by adding HRP substrate solution, ABTS (2, 2'-azinobis [3-ethylbenz-thiazoline sulfonic acid] diammonium salt in 50 mM sodium citrate [pH 4.0] with 30% H₂O₂) to each well. The plate was incubated for 60 minutes at room temperature with gentle agitation. After incubation, the absorbance of ELISA reactivity was read at OD₄₀₅ using a microplate reader (FLUOstar Omega, BMG LabTech).

3.6 Antibacterial Assay

Antibacterial assay was performed according to the method previously described by Rathinakumar et al. (2009) with slight modification. *Klebsiella pneumoniae* strain ATCC 13883 was grown for 4.5 hours in LB broth and then diluted to 10³ CFU/mL with minimal liquid test medium (LTM; 1% LB in TBS). In a 96-wells plate, 50 µL of the diluted bacterial cell suspension was added into 150 µL of 10¹² PFU/mL of individual phage clone in TBS. As a negative control, a separate set of wells was loaded with bacterial cell suspension of similar titer in TBS without phage. The plate was incubated at 37°C for up to 4 hours and then 50 µL of LB broth was added into each well. The bacterial cells were allowed to recover overnight. After 16 to 18 hours of incubation, the absorbance of bacteria culture in both the sample and negative control wells was read at OD₆₀₀ using a microplate reader (Infinite M200, Tecan). Antibacterial effect was defined as OD₆₀₀ of the sample was lower than the correspondent negative control. Those bacterial cultures coincubated with phage clones that exhibited antibacterial effect were spread on MacConkey agar to determine the bacterial titer.

3.7 Phage Genomic DNA Extraction and Sequencing

An overnight culture of *Escherichia coli* ER2738 was diluted according to 1:100 in LB broth. 100 μ L of the phage stock was added into a microcentrifuge tube containing 1 mL of the diluted ER2738 culture. The culture was then incubated at 200 rpm for 4.5 hours at 37°C. After incubation, the phage clones were purified twice by precipitation with PEG/NaCl. The phage pellet was suspended thoroughly in 100 μ L of Iodide buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 4 M NaI) by vigorously tapping the tube. 250 μ L of absolute ethanol was added into the tube and incubated at room temperature for 20 minutes. After incubation, the suspension was spun at 14,000 rpm for 10 minutes at 4°C and the supernatant was discarded. The pellet was washed again with 0.5 mL of 70% cold ethanol and the pellet was dried under vacuum for 30 minutes. The small DNA pellet was suspended in 30 μ L of TE buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA).

The extracted phage DNA was then analyzed by agarose gel electrophoresis. 1% agarose gel was prepared by adding 0.15 g of agarose powder into 15 mL of 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). 5 μ L of phage DNA was mixed with 1 μ L of 6X DNA loading dye (Fermentas). Each DNA sample was then loaded carefully into the wells of the gel which was placed in the 1X TAE electrophoresis buffer. The samples were electrophorezed at 60 V for 60 minutes. After electrophoresis, the gel was stained with 0.5 μ g/mL ethidium bromide for 5 minutes and then destained with distilled water for 10 minutes. The DNA samples were detected by viewing the gel under UV light in a gel imager (InGenius Syngene Bioimaging, Syngene).

The concentration of the extracted phage genomic DNA was measured by using a spectrophotometer (NanoDrop 2000, Thermo Scientific). The phage DNA was stored at -20°C before sending out for sequencing by 1st BASE. The phage DNA was sequenced using -96 gIII sequencing primer (5' – ^{HO}CCC TCA TAG TTA GCG TAA CG – 3') and also -28 gIII sequencing primer (5' – ^{HO}GTA TGG GAT TTT GCT AAA CAA C – 3') (New England Biolabs).

3.8 Peptide Sequence Analysis

The oligonucleotide inserts of the phage clones were analyzed from the sequenced phage genomic DNA using MEGA 5.1. The reverse complement sequences of the sequenced oligonucleotides were then translated into peptide sequences using ExPASy Translate Tool (<http://web.expasy.org/translate/>).

The peptides were examined for their isoelectric point (pI) and net charge through Innovagen Peptide Property Calculator (<http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp>). The hydrophobicity of peptides was predicted using Antimicrobial Peptide Predictor (http://aps.unmc.edu/AP/prediction/prediction_main.php). Besides, the peptide sequences were aligned to search for consensus sequence using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and to characterize the physiochemical properties of the amino acid residues using GeneDoc. Also, the peptide sequences were blasted for similar antibacterial peptide sequence through Antimicrobial Peptide Database (http://aps.unmc.edu/AP/database/query_input.php).

CHAPTER 4

RESULTS

4.1 Bacterial Growth Curve

The growth rate of *Klebsiella pneumoniae* strain ATCC 13883 was assessed by OD₆₀₀ and the correspondent bacterial titer of different time intervals throughout the 4.5 hours incubation period. The data tabulated in Table 4.1 demonstrates the growth of *Klebsiella pneumoniae* and these values had been used to construct the bacterial growth curves. Both the bacterial growth curves indicated by OD₆₀₀ and bacterial titer were increased across lag phase, exponential phase and the beginning of stationary phase (Figure 4.1 and Figure 4.2). The lag phase of the bacteria growth occurred at the first two hours of the incubation period; while the exponential phase occurred from the second to the fourth hour; and the early stationary phase was observed at the 4.5 hour of the incubation period. This bacterial growth curve can be used to determine the time required for the bacteria to grow to the expected titer. The bacterial incubation was stopped at the 4.5 hour after reaching the expected titer, which was 10⁹ CFU/mL.

Table 4.1: Growth of *Klebsiella pneumoniae* strain ATCC 13883 indicated by OD₆₀₀ and bacterial titer.

Time (hours)	OD ₆₀₀	Bacterial titer (CFU/mL)
1	0.015	1.18×10^6
2	0.093	1.7×10^6
3	0.486	1.2×10^7
4	1.174	6.7×10^8
4.5	1.316	3.1×10^9

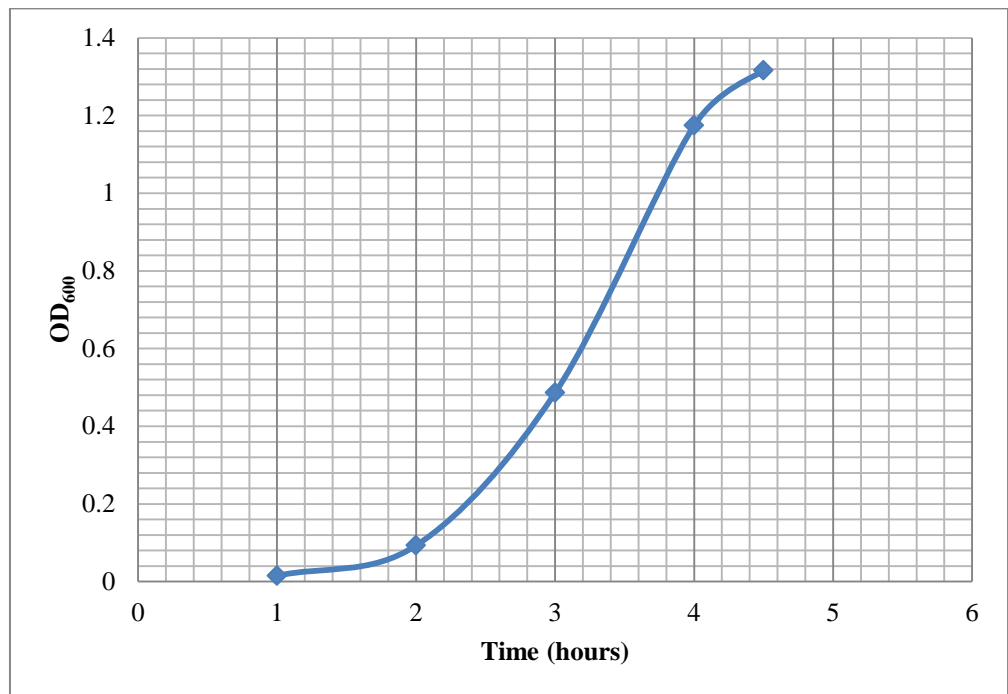


Figure 4.1: Growth curve of *Klebsiella pneumoniae* throughout the 4.5 hours incubation period as measured in OD₆₀₀.

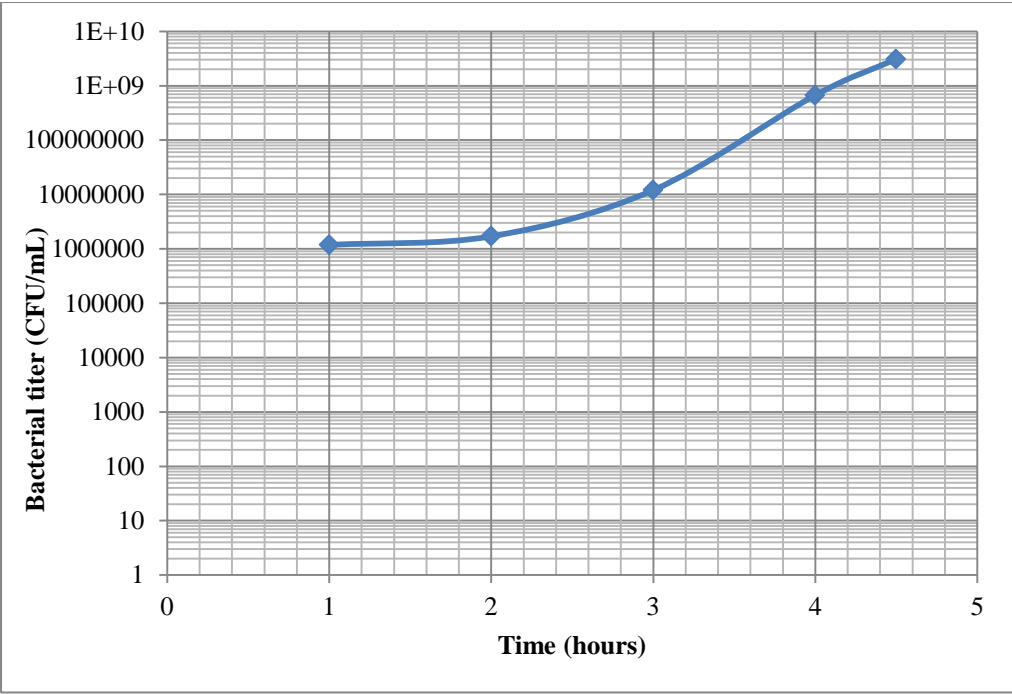


Figure 4.2: Growth curve of *Klebsiella pneumoniae* throughout the 4.5 hours incubation period as measured in bacterial titer.

4.2 Isolation of Phage-Displayed Peptides Binding to *Klebsiella pneumoniae*

In biopanning, phage titer was determined by counting the number of blue plaques formed by individual phage on LB/IPTG/Xgal plate (Figure 4.3). The input phage titer was the phage titer initially used to pan against *Klebsiella pneumoniae* while the output phage titer was the eluted phage titer from a particular round of biopanning. From the phage-displayed peptide library, the titer of input phages that was panned against *Klebsiella pneumoniae* during the first round of biopanning consists of 10^{12} PFU/mL. After the first selection, the eluted phages yielded between 10^6 and 10^7 PFU/mL in each round of subsequent biopanning. The input phage titer in the subsequent round of biopanning was then recovered to 10^{12} PFU/mL through amplification of the eluted phages following each round of biopanning. The yield of selection can be calculated from the input and output phage titer, which was tabulated in Table 4.2. First round of biopanning had selected $3.1 \times 10^{-4}\%$ of phages from the library. On the other hand, $2.34 \times 10^{-4}\%$ of phages were selected in the second round of biopanning. While in the third round of biopanning, $3.16 \times 10^{-4}\%$ of phages were obtained. $7.73 \times 10^{-4}\%$ of phages were selected in the fourth round of biopanning. Based on the graph shown in Figure 4.4, the yield of selection decreased from the first to the second round of biopanning, and then increased from the second to the fourth round of biopanning. The yield of selection in the fourth round of biopanning was increased 2.4-fold as compared to the third round of biopanning. In short, the highest yield of selection was observed at the fourth round of biopanning. Twenty-nine individual phage clones were randomly selected from the fourth round of biopanning and independently propagated for further screening.

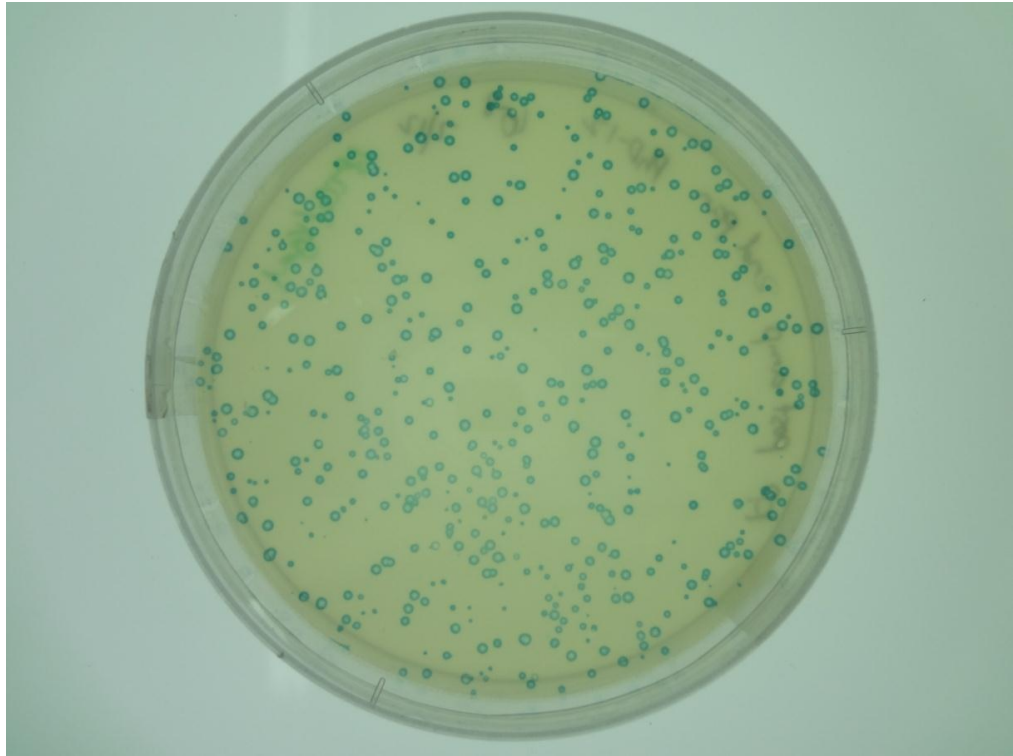


Figure 4.3: Blue plaques formed by individual phage appeared on LB/IPTG/Xgal plate in phage titering. The blue plaques indicate the selected phages from the biopanning were derived from the library with *lacZ α* gene insertion.

Table 4.2: Yield of selection of phage-displayed peptides from four rounds of biopanning.

Round of biopanning	Input phage titer (PFU/mL)	Output phage titer (PFU/mL)	Yield of selection (%) [*]
1	1×10^{12}	3.1×10^6	3.1×10^{-4}
2	1.6×10^{12}	3.75×10^6	2.34×10^{-4}
3	3.8×10^{12}	1.2×10^7	3.16×10^{-4}
4	2.2×10^{12}	1.7×10^7	7.73×10^{-4}

^{*}The yield of selection in percentage was calculated by the formula: [(Output phage titer / Input phage titer) \times 100].

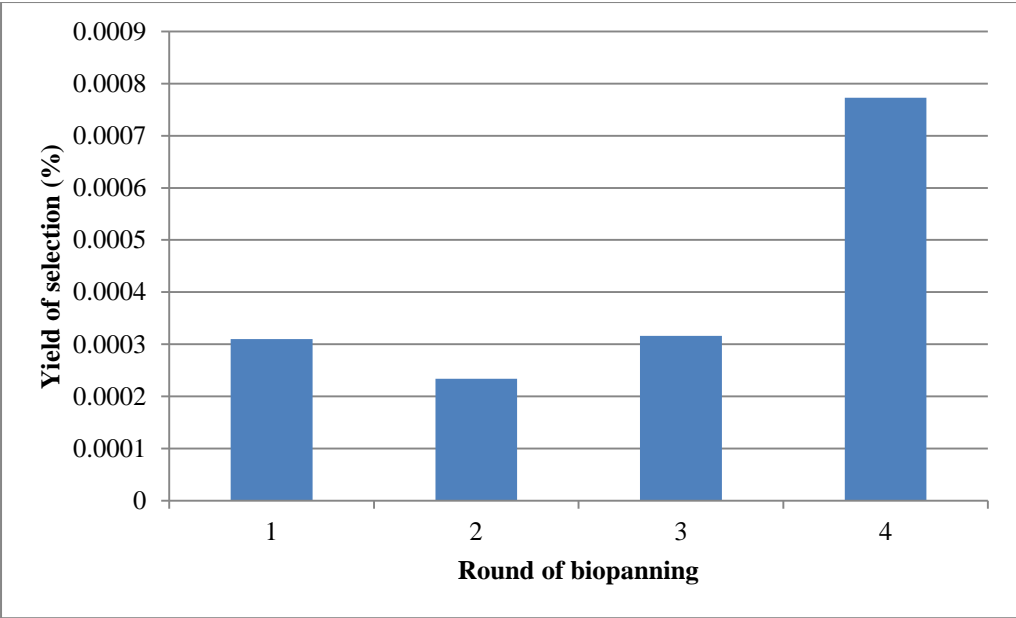


Figure 4.4: Yield of selection calculated from the four rounds of biopanning.

4.3 Binding Selectivity of the Selected Phage-Displayed Peptides towards *Klebsiella pneumoniae*

Phage ELISA was performed to determine the binding selectivity of the selected phage-displayed peptides towards *Klebsiella pneumoniae*. Figure 4.5 showed the development of ABTS substrate color in the test wells in which the color intensity could be measured in OD₄₀₅. Figure 4.6 illustrates the ELISA reactivity measured as OD₄₀₅ for each phage-displayed peptide corresponding to their binding strength to *Klebsiella pneumoniae*. The sample wells were wells coated with *Klebsiella pneumoniae* while the negative control wells were wells filled with blocking buffer without coating bacteria. The OD₄₀₅ readings of the sample wells were much higher than their correspondent negative control indicating that the selected phage-displayed peptides were bound to the target bacteria. The data tabulated in Table 4.3 demonstrates that all the 29 isolated phage clones had the OD₄₀₅ reading above 1.0 in the ELISA reactivity. Clone 4KP29 showed the highest OD₄₀₅ reading among the sample wells, which was 3.124. Clone 4KP10 showed the lowest OD₄₀₅ reading among the sample wells, which was 1.058. The ratio of binding selectivity was then calculated by dividing the OD₄₀₅ reading of the sample to the negative control, and the ratios were ranging from 17.93 (4KP10) to 46.88 (4KP25). Most of the isolated phage clones demonstrated the ratio of binding selectivity more than 20, except clone 4KP8 (19.61), 4KP10 (17.93), 4KP23 (18.51) and 4KP28 (19.28). Those isolated phage clones demonstrated the ratio more than 40 were 4KP25 (46.88) and 4KP27 (44.18).

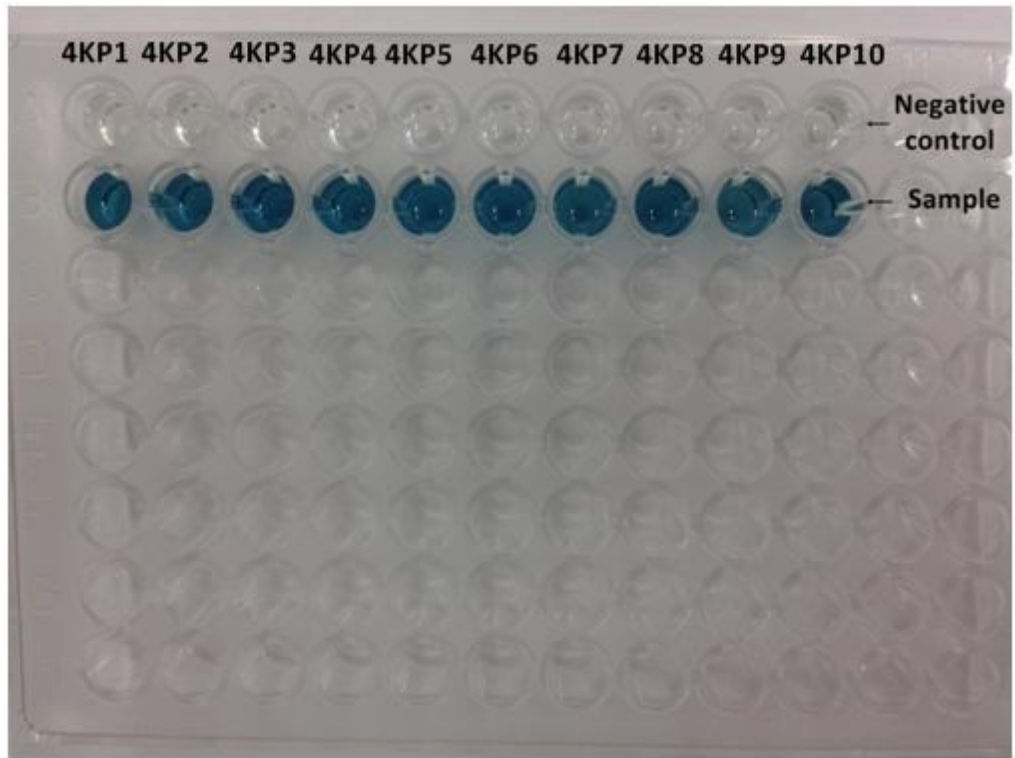


Figure 4.5: The color development of ABTS substrate in ELISA showing the color intensity of the sample was much higher than the negative control.

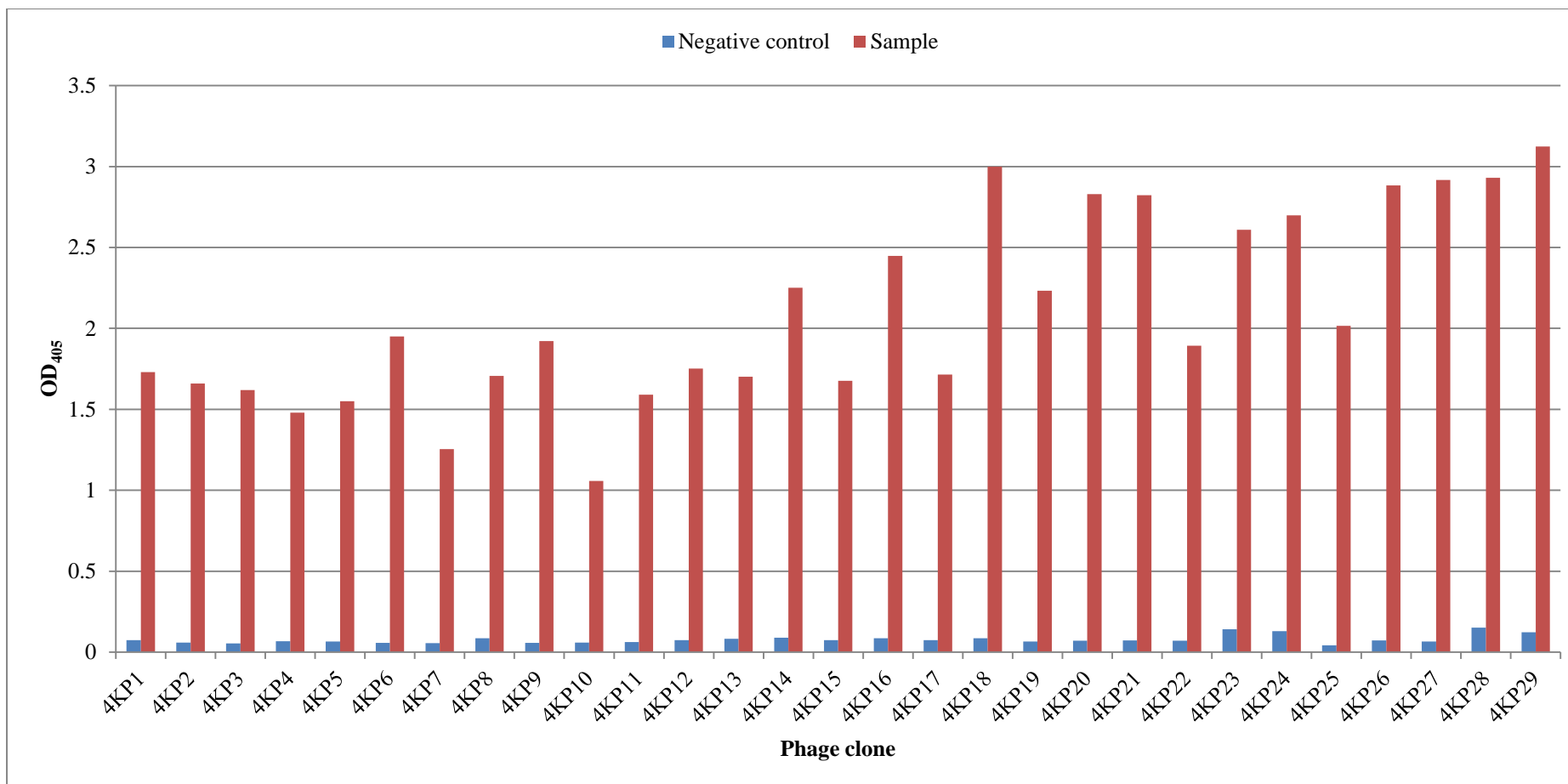


Figure 4.6: OD₄₀₅ reading of ELISA reactivity for the selected phage-displayed peptides binding to *Klebsiella pneumoniae*.

Table 4.3: OD₄₀₅ reading of ELISA reactivity for the selected phage-displayed peptides and the correspondent ratio of binding selectivity.

Phage clone	Negative control (OD₄₀₅)	Sample (OD₄₀₅)	Ratio of binding selectivity *
4KP1	0.074	1.73	23.38
4KP2	0.06	1.659	27.65
4KP3	0.054	1.619	29.98
4KP4	0.068	1.479	21.75
4KP5	0.066	1.55	23.48
4KP6	0.058	1.95	33.62
4KP7	0.056	1.254	22.39
4KP8	0.087	1.706	19.61
4KP9	0.057	1.921	33.7
4KP10	0.059	1.058	17.93
4KP11	0.062	1.591	25.66
4KP12	0.074	1.752	23.68
4KP13	0.083	1.701	20.49
4KP14	0.09	2.251	25.01
4KP15	0.074	1.677	22.66
4KP16	0.086	2.448	28.47
4KP17	0.075	1.715	22.87
4KP18	0.087	3	34.48
4KP19	0.066	2.233	33.83
4KP20	0.071	2.83	39.86
4KP21	0.073	2.822	38.66
4KP22	0.071	1.893	26.66

Table 4.3 continued:

Phage clone	Negative control (OD₄₀₅)	Sample (OD₄₀₅)	Ratio of binding selectivity*
4KP23	0.141	2.61	18.51
4KP24	0.13	2.699	20.76
4KP25	0.043	2.016	46.88
4KP26	0.073	2.884	39.51
4KP27	0.066	2.916	44.18
4KP28	0.152	2.93	19.28
4KP29	0.124	3.124	25.19

*The ratio of binding selectivity was calculated by the formula: [Sample / Negative control].

4.4 Antibacterial Activity of the Selected Phage-Displayed Peptides

The antibacterial activity of the selected phage-displayed peptides against *Klebsiella pneumoniae* was assessed by their ability to inhibit the bacterial growth in LB broth. The sample wells represent the wells which harbored phage-displayed peptides and *Klebsiella pneumoniae* while the negative control wells represent the wells containing only the bacteria. Although 29 clones of phage-displayed peptides were isolated from the fourth round of biopanning, there were only four clones, namely 4KP3, 4KP5, 4KP11 and 4KP29 showed antibacterial effects by inhibiting the growth of *Klebsiella pneumoniae* (Figure 4.7). The antibacterial effect was indicated by lower OD₆₀₀ reading of the sample well which harbored phage-displayed peptides and bacteria compared to the correspondent negative control containing only the bacteria. Higher OD₆₀₀ reading of the sample well indicates those clones have no antibacterial activity. Only the four clones that exhibited observable antibacterial effect in the first antibacterial assay were then subjected to the repeated assay, and the results were reproducible by demonstrating lower OD₆₀₀ reading in the sample wells (Figure 4.8). The degree of inhibition was calculated by comparing the OD₆₀₀ reading of the sample well to their correspondent negative control. The data tabulated in Table 4.4 and 4.5 demonstrates the calculated degree of inhibition of the selected phage-displayed peptides in the first and repeated antibacterial assay, respectively. These data were then used to calculate the average degree of inhibition for the selected phage-displayed peptides. The data shown in Figure 4.9 illustrates various degree of inhibition for the four clones of phage-displayed peptides that exhibited antibacterial effect against *Klebsiella pneumoniae*. As shown in

the graph, phage-displayed peptide 4KP3, 4KP5, 4KP11 and 4KP29 averagely produced the degree of inhibition at 26.96%, 18.3%, 22.26% and 31.46%, respectively. Also, the antibacterial effect of the selected clones was further checked by bacterial titer as determined by plating the culture of the post-antibacterial assay on MacConkey agar (Figure 4.10). The bacterial titers from the culture coincubated with the phage-displayed peptides were lower than the negative control. Although the bacteria culture in both sample wells and negative control wells were grown until 10^8 or 10^9 CFU/mL, the number of bacteria colonies grown on the agar plate for the sample wells were lesser than the negative control wells (Table 4.6). Nevertheless, the phage-displayed peptides were unable to inhibit the bacteria completely since the OD_{600} reading of the sample well was higher than 0.005 as defined by Bishop-Hurley et al. (2005) (Figure 4.7 and 4.8), and the bacteria culture was able to grow until 10^8 CFU/mL on agar plate even though after coincubating with the phage-displayed peptides (Figure 4.10).

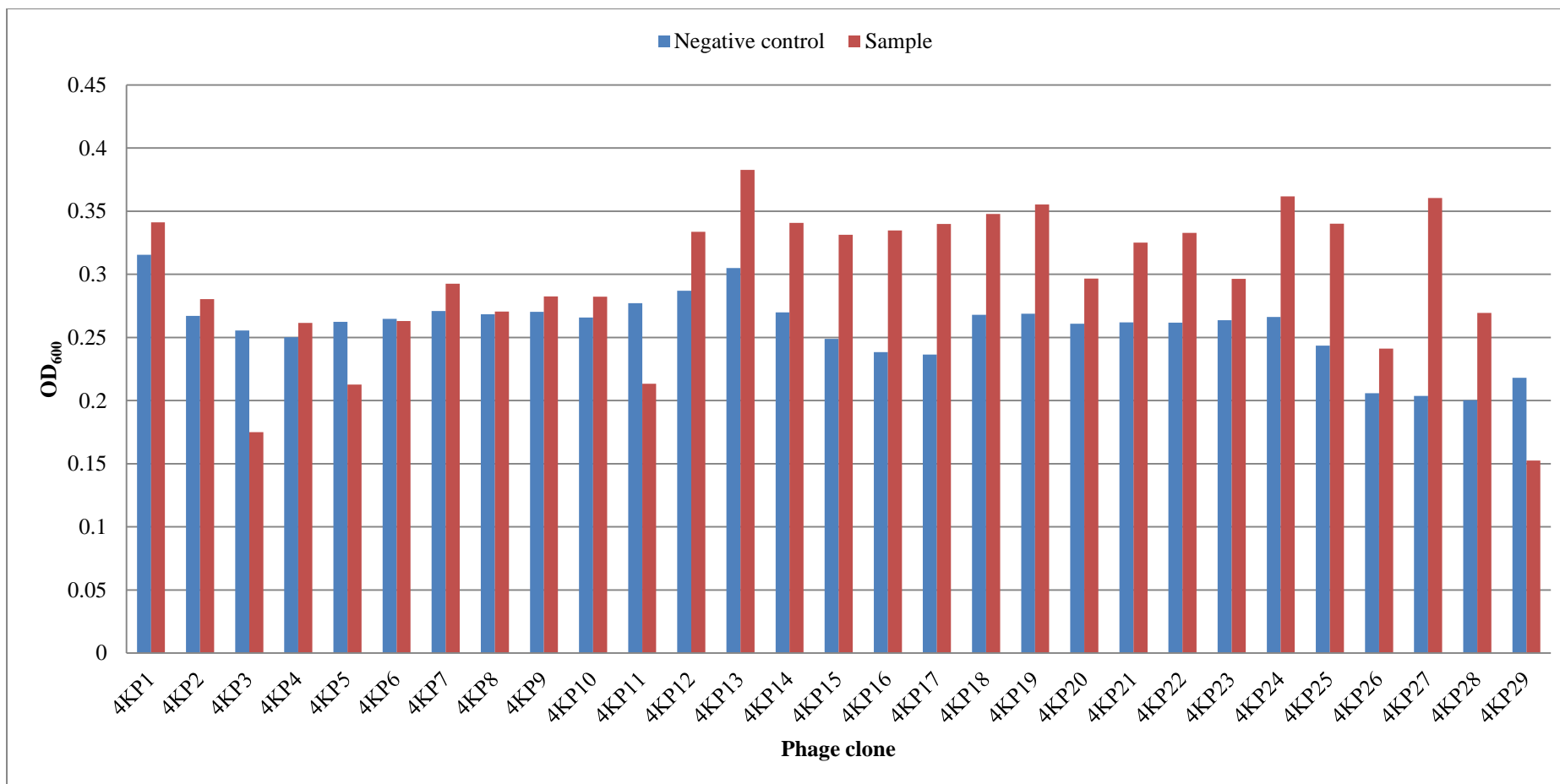


Figure 4.7: OD₆₀₀ reading of bacteria culture in the first antibacterial assay.

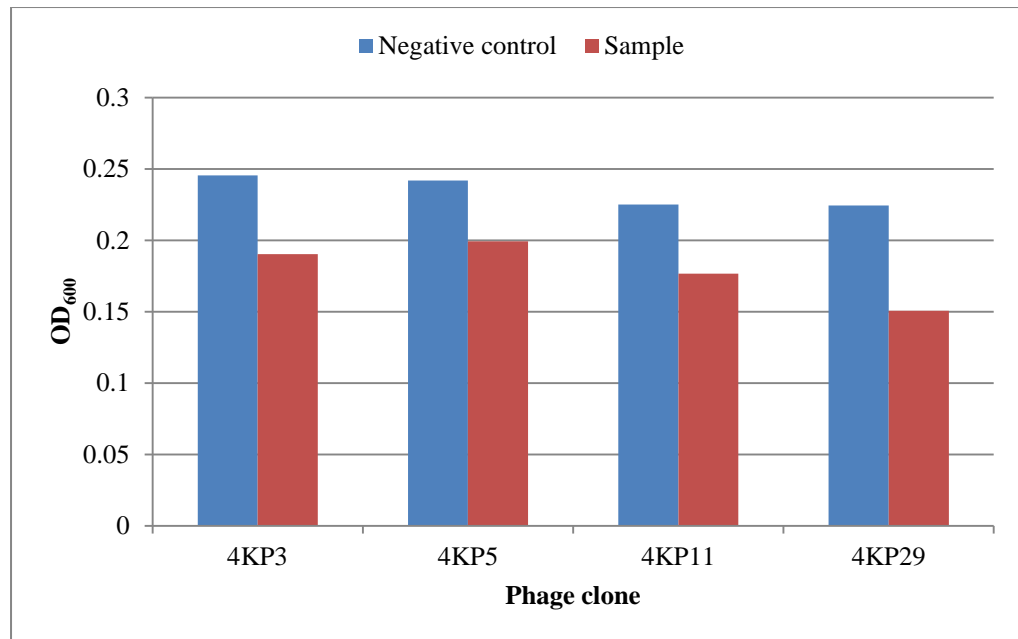


Figure 4.8: OD₆₀₀ reading of bacteria culture in the repeated antibacterial assay for the four clones that exhibited antibacterial effect in the first assay.

Table 4.4: OD₆₀₀ reading of bacteria culture in the first antibacterial assay and the correspondent degree of inhibition of the selected phage-displayed peptide clones.

Phage clone	Negative control (OD ₆₀₀)	Sample (OD ₆₀₀)	Degree of inhibition*
4KP3	0.2555	0.1751	31.47
4KP5	0.2624	0.2127	18.94
4KP11	0.2771	0.2133	23.02
4KP29	0.218	0.1526	30

*The degree of inhibition in percentage was calculated by the formula: $[100 - \{(Sample / Negative\ control) \times 100\}]$.

Table 4.5: OD₆₀₀ reading of bacteria culture in the repeated antibacterial assay and the correspondent degree of inhibition of the selected phage-displayed peptide clones.

Phage clone	Negative control (OD ₆₀₀)	Sample (OD ₆₀₀)	Degree of inhibition*
4KP3	0.2455	0.1904	22.44
4KP5	0.2419	0.1992	17.65
4KP11	0.2251	0.1767	21.5
4KP29	0.2245	0.1506	32.92

*The degree of inhibition in percentage was calculated by the formula: $[100 - \{(Sample / Negative\ control) \times 100\}]$.

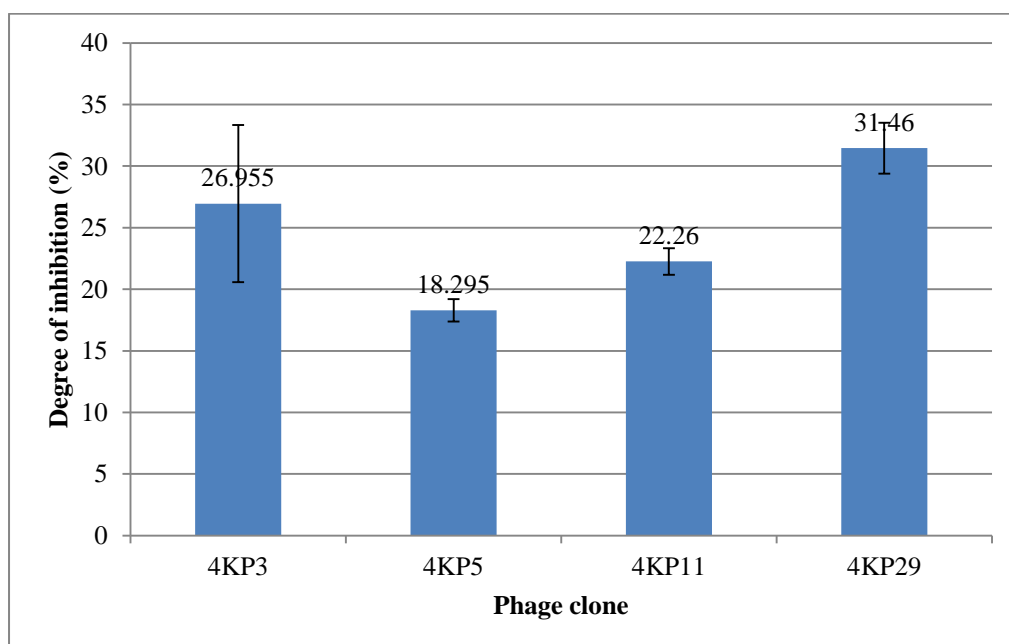


Figure 4.9: The average degree of inhibition of the selected clones of phage-displayed peptides against *Klebsiella pneumoniae*.

Error bars indicate standard deviations for the first and repeated antibacterial assay.

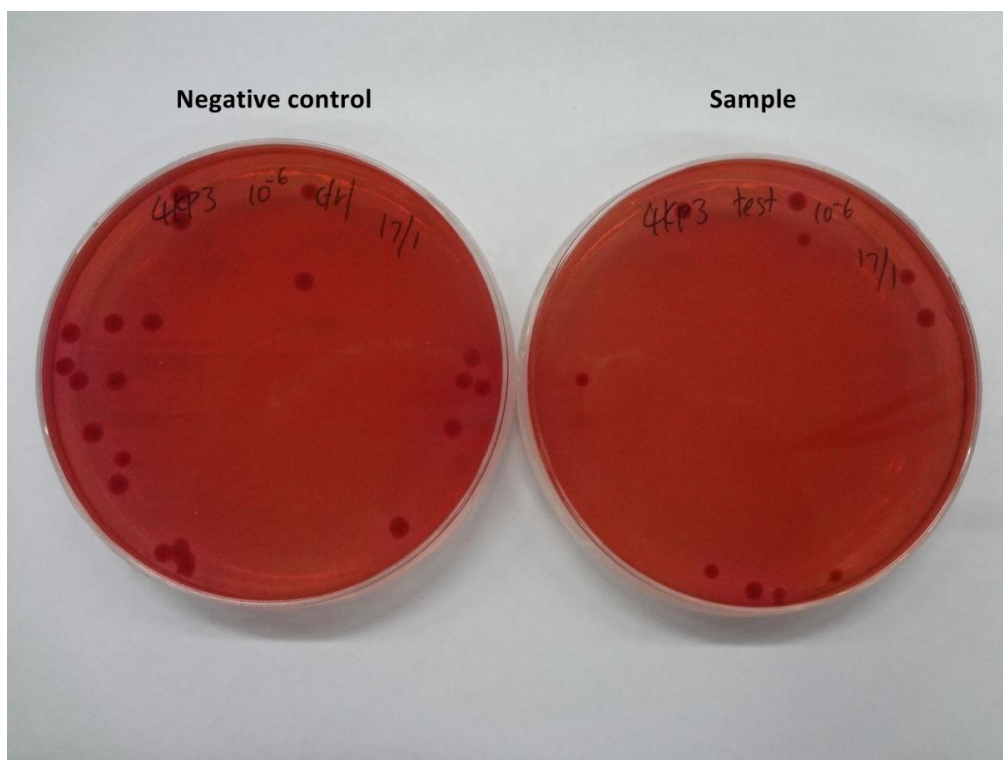


Figure 4.10: Lower bacteria titer was counted in the sample (1×10^8 CFU/mL) compared to the negative control (2×10^8 CFU/mL) for antibacterial assay.

Table 4.6: Bacterial titer of the antibacterial assay in the first and repeated test.

Phage clone	First test (CFU/mL)		Repeated test (CFU/mL)	
	Negative control	Sample	Negative control	Sample
4KP3	2×10^8	1×10^8	1.1×10^9	7×10^8
4KP5	2.6×10^8	1.4×10^8	7×10^8	5×10^8
4KP11	2.6×10^8	1.3×10^8	3.1×10^8	2.7×10^8
4KP29	2.3×10^8	1.5×10^8	1.9×10^8	1.8×10^8

4.5 Purity of the Extracted Phage Genomic DNA

Phage genomic DNAs were successfully extracted from the four clones that shown observable antibacterial activity against *Klebsiella pneumoniae*, namely 4KP3, 4KP5, 4KP11 and 4KP29 (Figure 4.11). The purity of the DNA was assessed by using 1% agarose gel electrophoresis before sending out for sequencing. 1 kb DNA ladder (PhileKorea) was used as a marker to estimate the size of the DNA. According to the instruction manual of phage-displayed peptide library (New England Biolabs), the size of the single-stranded M13 phage genomic DNA is approximately 7,250 nucleotides. However, the DNA bands shown in Figure 4.11 were observed at the position of 6,000 bp. A clear band could be observed at the lanes which are labeled 4KP3, 4KP11 and 4KP29 while the DNA loaded at the lane labeled 4KP5 was not observed. Multiple banding could also be observed on the agarose gel. However, these multiple bandings were not shown as clear bands. The concentrations of the extracted phage genomic DNA for clone 4KP3, 4KP5, 4KP11 and 4KP29 were 26.5 ng/ μ L, 86.2 ng/ μ L, 43 ng/ μ L and 66 ng/ μ L, respectively (Table 4.7).



Figure 4.11: Gel electrophoresis of the extracted phage genomic DNA (indicated by red arrow) before sequencing.

Table 4.7: Concentration of the extracted phage genomic DNA.

Phage clone	DNA concentration (ng/ μ L)
4KP3	26.5
4KP5	86.2
4KP11	43
4KP29	66

4.6 Analysis of Peptide Sequences

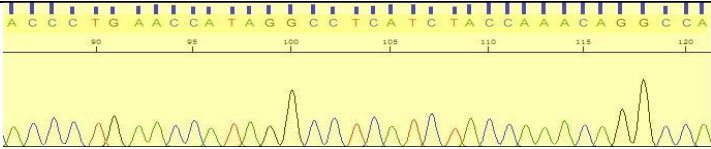
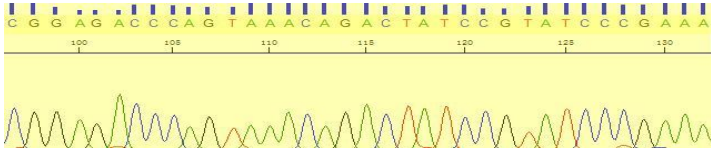
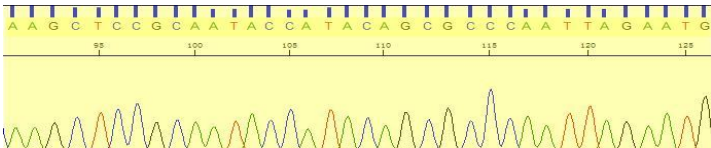
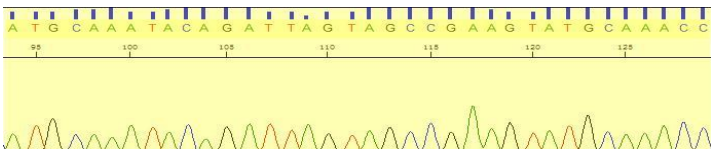
In order to identify the oligonucleotide insert of the selected phage clones, the presence of the conserved leader sequence (ATGGGATTTTGCTAAACAACTTTCAACAGTTTCGGCCGA) must be determined within the sequenced phage genomic DNA (Figure 4.12). The sequenced oligonucleotide was then identified after the +9 conserved nucleotides (ACCTCCACC) that connected with the leader sequence. According to the electropherogram shown in Table 4.8, the oligonucleotides of the clone 4KP3, 4KP5, 4KP11 and 4KP29 were successfully sequenced as ACCCTGAACCATAGGCCTCATCTACCAAA-CAGGCCA, CGGAGACCCAGTAAACAGACTATCCGTATCCCGAAA, AAGCTCCGCAATACCATACAGCGCCCAATTAGAATG and ATGCAA-ATACAGATTAGTAGCCGAAGTATGCAAACC, respectively. The coding oligonucleotide sequences were then obtained from the reverse complement sequences of the sequenced oligonucleotide. As shown in Table 4.8, the coding oligonucleotide sequences of the clone 4KP3, 4KP5, 4KP11 and 4KP29 are TGGCCTGTTTGGTAGATGAGGCCTATGGTTCAGGGT, TTTCGGGATACGGATAGTCTGTTTACTGGGTCTCCG, CATTCTAATTGGGCGCTGTATGGTATTGCGGAGCTT and GGTTTGCATACTTCGGCTACTAATCTGTATTTGCAT, respectively. From these coding oligonucleotides, the correspondent displayed 12-mer peptides were deduced and their physiochemical properties were analyzed by using bioinformatic tools.

Table 4.9 shows the deduced peptide sequences and their respective physiochemical properties. Sequence analysis revealed each of these

oligonucleotides encodes unique peptide. 4KP3 displayed WPVWQMRPMVQG; 4KP5 displayed FRDTDSLFTGSP; 4KP11 displayed HSNWALYGIAEL; and 4KP29 displayed GLHTSATNLYLH. Although these peptides are not identical, a linear consensus motif, -His-XXXXX-Leu-Tyr- (-H-XXXXX-L-Y-, X = any amino acid) was observed in both peptides of 4KP11 and 4KP29 (Figure 4.13). The isoelectric point (pI) values of the peptide WPVWQMRPMVQG, FRDTDSLFTGSP, HSNWALYGIAEL and GLHTSATNLYLH are 11.04, 3.88, 5.13 and 7.91, respectively. 4KP3-displayed peptide (WPVWQMRPMVQG) carried a net positive charge of +1; FRDTDSLFTGSP displayed by 4KP5 and HSNWALYGIAEL displayed by 4KP11 carried net negative charge of -1 and -0.9, respectively; and 4KP29-displayed peptide (GLHTSATNLYLH) carried a neutral charge of 0.2. The hydrophobicity of the peptides that are displayed by 4KP3, 4KP5, 4KP11 and 4KP29 were calculated as 50%, 25%, 50% and 33%, respectively.

In Figure 4.14, the peptides were aligned and shaded according to their physiochemical properties. Specific groups of amino acid residues were selected during the affinity selection process. For instance, all the peptides contained a polar amino acid at position 4 of the aligned peptides. A strong preference for hydrophobic amino acids occurred at position 1, 9, 10 and 12 of the peptides. These peptide sequences cannot match to any of the antibacterial peptides that are found in Antimicrobial Peptide Database (http://aps.unmc.edu/AP/database/query_input.php), suggesting that they might be novel peptides.

Table 4.8: Sequenced and coding oligonucleotide sequences of each clone, as well as the correspondent deduced peptide sequences.

Phage clone	Sequenced oligonucleotide electropherogram ^a	Coding oligonucleotide sequence ^a	Peptide sequence ^b
4KP3		TGGCCTGTTTGGTAGATGAGGCCTA TGGTTCAGGGT	WPVWQMRPMVQG
4KP5		TTTCGGGATACGGATAGTCTGTTTA CTGGGTCTCCG	FRDSDLFTGSP
4KP11		CATTCTAATTGGGCGCTGTATGGTA TTGCGGAGCTT	HSNWALYGIAEL
4KP29		GGTTTGCATACTTCGGCTACTAATC TGTATTTGCAT	GLHTSATNLYLH

^aSingle letter abbreviations of nucleotides: A, Adenine; C, Cytosine; G, Guanine; T, Thymine.

^bSingle letter abbreviations of amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Table 4.9: Sequence and physiochemical properties of the isolated phage-displayed peptides with observable antibacterial activity.

Phage clone	Peptide sequence ^a	pI ^b	Net charge	Hydrophobicity ^c
4KP3	WPVWQMRPMVQG	11.04	+1	50
4KP5	FRDTDSLFTGSP	3.88	-1	25
4KP11	HSNWALYGIAEL	5.13	-0.9	50
4KP29	GLHTSATNLYLH	7.91	0.2	33

^aSingle letter abbreviations of amino acids: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

^bpI was the calculated isoelectric point.

^cHydrophobicity was calculated in percentage.

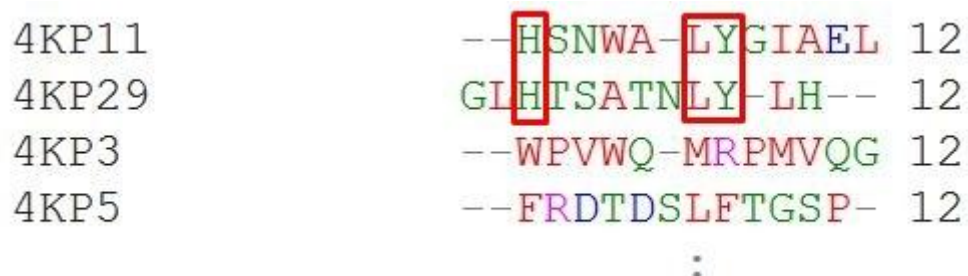


Figure 4.13: Alignment of the peptide sequences to search for consensus sequence using ClustalW.

Dashes indicate gap used to maximize the alignment.

		1	2	3	4	5	6	7	8	9	10	11	12		
4KP3	:	W	P	V	Q	M	R	F	M	V	Q	G		:	12
4KP5	:	E	R	D	T	D	S	L	F	T	G	S	E	:	12
4KP11	:	H	S	N	W	A	L	Y	G	I	A	E	L	:	12
4KP29	:	G	L	H	T	S	A	T	N	L	Y	L	H	:	12

Figure 4.14: Alignment of the peptide sequences to characterize their physiochemical properties using GeneDoc.

The physiochemical mode was used to assign each column of the alignment to one of the 12 pre-defined groups of physiochemical properties. White writing on a black background indicates hydrophobic amino acids; black writing on a green background indicates polar amino acids.

CHAPTER 5

DISCUSSION

Klebsiella pneumoniae is a well-known nosocomial pathogen and the occurrence of multidrug resistant strain *Klebsiella pneumoniae* has been increasing. This has led to the widespread study of alternative antimicrobial agents, such as antibacterial peptides, to complement the antibiotic treatment. Although antibacterial peptides can be isolated using conventional methods like crude extraction, the process are tedious and time-consuming. On the other hand, phage-displayed peptide approach can serve as an advanced tool for selecting peptides with specific binding properties (Carnazza et al., 2008; Lunder et al., 2008). Previous studies have demonstrated the successful screening and isolation of novel peptides using phage-displayed peptide library that allow the selection of bacterial membrane ligands with antibacterial activity (Bishop-Hurley et al., 2005; Pini et al., 2005; Bishop-Hurley et al., 2010; Rao et al., 2013). In this study, a 12-mer random peptide library was used to screen and isolate novel peptides that could inhibit the growth of *Klebsiella pneumoniae*.

5.1 Biopanning against *Klebsiella pneumoniae*

Ph.D.-12 phage-displayed peptide library (New England Biolabs) was used to pan against *Klebsiella pneumoniae* strain ATCC 13883 for four rounds to screen and isolate any phage-displayed peptides that might exhibit antibacterial activity against the bacteria. It is assumed that phage-displayed peptides will be isolated based on the ability of the peptides to bind to the bacterial cell surface epitopes (Bishop-Hurley et al., 2005).

In the process of biopanning, Tris-buffered saline+Tween (TBST) was used as a washing buffer to wash away the unbound phages. Tween-20 is a nonionic detergent that is commonly added into TBS buffer to reduce the non-specific hydrophobic interactions between binding ligands and panning target (Traunmuller et al., 2005). The concentration of Tween-20 in the washing buffer used in each round of biopanning was increased gradually to increase the stringency of selection, particularly during the later rounds of selection. An increasing stringency of selection in biopanning might be able to select more phages that bind specifically to the target bacteria (Kabir et al., 2009).

Besides, the titer of phages after each round of biopanning was determined to check the enrichment of phages binding towards the target bacteria. A decreasing in titer from the input phages to the output phages indicates the selection of phages. The weak and non-specific binding phages which contribute mostly to the portion of input phages were being washed away during the biopanning. As shown in Figure 4.3, a decreasing in selection from the first to the second round of biopanning could be explained by the high

portion of non-specific binding phages were being washed away using the washing buffer with higher concentration of Tween-20 in the second round of biopanning. Upon each round of selection, the eluted phages were amplified prior to the subsequent round of biopanning, and this could increase the titer of specific binding phages that was used in later round of biopanning. The yield of selection that was increased from the second to the fourth round of biopanning indicates more phages were bound to the target bacteria in the later round of biopanning. Thus, it is assumed that the specific binding phage clones were enriched. As a result, the clones of phage-displayed peptides that were isolated from the fourth round of biopanning probably had the highest binding affinity and specificity towards the target bacteria.

5.2 Phage-Displayed Peptides Binding to *Klebsiella pneumoniae*

All the 29 isolated clones of phage-displayed peptides were able to bind to *Klebsiella pneumoniae* as determined by the phage ELISA. These phage-displayed peptides probably carried some positively-charged amino acids that conferred the electrostatic interaction to the negatively-charged bacterial membrane. Since the artifacts of the biopanning process such as the impurities of buffer solution and plastic tubes that might be the non-specific selector present in the phage-displayed peptides, phage ELISA was performed to distinguish the specific binding phages towards the target bacteria from those target-unrelated phages which bound to the artifacts (New England Biolabs). Moreover, confirming the binding properties of the selected phage-displayed

peptides might avoid unnecessary sequencing and subsequent peptide analysis (Lunder et al., 2008).

5.3 Antibacterial Activity of Phage-Displayed Peptides against *Klebsiella pneumoniae*

Four selected phage-displayed peptides, namely 4KP3, 4KP5, 4KP11 and 4KP29 showed inhibition against the growth of *Klebsiella pneumoniae*. Although all the 29 selected phage-displayed peptides were able to bind towards the bacteria, only some of them exhibited antibacterial effect. The binding property is essential for peptides to induce antibacterial activity. However, the binding affinity of peptides might not directly correlate to their antibacterial activity (Soares et al., 2004). This is also supported by a previous study that demonstrated no antibacterial activity by peptides that interact with bacteria (Steiner et al., 1988). It is assumed that the isolated peptides exhibited a broader binding selectivity towards the target bacteria as compared to their antibacterial activity (Soares et al., 2004).

The four phage-displayed peptides with observable antibacterial activity, namely 4KP3, 4KP5, 4KP11 and 4KP29, showed various degree of inhibition against *Klebsiella pneumoniae* ranging from 18.3% to 31.46%. This antibacterial strength of the phage-displayed peptides is considered low as compare to the study by Bishop-Hurley et al. (2010) in which their isolated peptides inhibited *Campylobacter jejuni* up to 99%. The phage-displayed peptides that were isolated in this study only showed bacteriostatic rather than bactericidal effect.

Several possible reasons might contribute to the low degree of inhibition of the selected phage-displayed peptides against *Klebsiella pneumoniae* in this study. The low degree of inhibition might be due to lesser copies of peptides that are displayed by pIII minor coat protein display system in the Ph.D.-12 phage-displayed peptide library. This phage display system could only display five copies of peptides at one end of the mature M13 virion (New England Biolabs). In contrast to another phage-displayed peptide library used by Bishop-Hurley and colleagues (2005 and 2010), which utilized pVIII major coat protein as the display system, the phages were able to display 150 to 300 copies of peptides on the surface of the virion. These pVIII phage-displayed peptides showed higher degree of inhibition against the bacterial growth. The major advantage of using pVIII major coat protein display system is that more copies of peptides can be displayed on the surface of virion and this might improve the binding avidity of peptides towards the bacterial membrane (Gulig et al., 2008). Also, a threshold peptide density might be easily reached to disrupt the bacterial membrane when more copies of peptides are binding to the bacterial membrane, thus expressing a greater antibacterial activity (Giuliani et al., 2007).

The orientation of phages binding to the bacterial membrane could affect the degree of inhibition. M13 virion contains around 2,700 copies of pVIII major coat protein which compose nearly 87% of the total virion mass (Huang et al., 2012). It is assumed that the possibility of the phages binding to the target bacteria with pVIII major coat protein is higher than pIII minor coat protein. Furthermore, the use of pIII minor coat protein display system might also lead

to steric hindrance due to the display of large peptides on the pIII minor coat protein, thus decreasing the binding of peptides with the target bacteria (Wang et al., 2009).

On the other hand, the surface structure of *Klebsiella pneumoniae* might affect the degree of inhibition by the phage-displayed peptides. Campos et al. (2004) and Llobet et al. (2009) reported that capsular polysaccharide and outer membrane proteins (Omp) of *Klebsiella pneumoniae* are able to resist antibacterial peptides. In order to induce antibacterial effect, most of the antibacterial peptides would target on the bacterial cytoplasmic membrane (Nizet 2006). However, capsular polysaccharide might limit the interaction between antibacterial peptides and bacterial membrane (Campos et al., 2004). Moreover, capsular polysaccharide might neutralize the bacteriostatic or bactericidal activity of antibacterial peptides by binding them, thus reducing the amount of antibacterial peptides reaching the bacterial surface (Llobet et al., 2008). The presence of OmpA on the cell surface of *Klebsiella pneumoniae* might also reduce the susceptibility of the bacteria towards the antibacterial peptides. However, the detail of this mechanism is presently still unknown (Llobet et al., 2009).

The cationicity of antibacterial peptides is important for the initial electrostatic attraction of the peptides to the anionic bacterial membrane (Lavery et al., 2011). Generally, most of the antibacterial peptides possess a net positive charge ranging from +2 to +9 (Jenssen et al., 2006). In this study, the isolated peptides only carried the net charge ranging from -1 to +1, which are

considered as low cationicity. This may reduce their antibacterial effect by limiting their interaction with the bacterial membrane due to less electrostatic attraction. A study conducted by Dathe et al. (2001) had shown that increasing in cationicity of magainin II analogs from +3 to +5 resulted in increasing antibacterial activities against Gram-positive and Gram-negative bacteria.

5.4 DNA Extraction and Gel Electrophoresis

The size of M13KE phage genomic single-stranded DNA is approximately 7,250 nucleotides (New England Biolabs). The DNA band appeared on the agarose gel would not line up with 1 kb DNA ladder (PhileKorea) at the same size because double-stranded DNA marker had been used to estimate the size of phage genomic single-stranded DNA in this study. Furthermore, the position of DNA band would vary depending on the applied voltage, agarose concentration in the gel, and also the choice of running buffer used in gel electrophoresis (New England Biolabs). In order to estimate the size of DNA band, M13 single-stranded DNA marker should be used together with the extracted phage DNA samples.

Besides, the multiple banding on the agarose gel is probably caused by different folding patterns of the phage genomic single-stranded DNA (New England Biolabs). The DNA band that was not observed at the lane labeled 4KP5 on the agarose gel might be due to the low concentration of DNA that was being loaded into the well.

5.5 Sequences and Physiochemical Properties of Peptides

Sequence analysis revealed the four peptides that exhibited antibacterial effect as WPVWQMRPMVQG (4KP3), FRDTDSLFTGSP (4KP5), HSNWALYGIAEL (4KP11) and GLHTSATNLYLH (4KP29). Although all the four peptides were different from each other, two peptides carried a linear consensus motif of -His-XXXXX-Leu-Tyr-, suggesting that this motif might serve as critical binding residues to the target bacteria. According to Antimicrobial Peptide Database (http://aps.unmc.edu/AP/database/query_input.php), the -Leu-Tyr- motif could be found in a total of 56 different antimicrobial peptides including gramicidin C (Townsend et al., 2001), RP-1 (Yeaman et al., 2002), microcin L (Pons et al., 2004) and pleurain-A1 (Wang et al., 2007). In addition, the finding in this study also in concordance with the study by Bishop-Hurley (2010), in which two isolated antibacterial peptides against *Campylobacter jejuni* also carried the -Leu-Tyr- motif. Besides, most of the peptides in that study contained hydrophobic and polar amino acid residues. However, they are located at different position compared to the peptides of this study. Furthermore, leucine is the most frequently appeared amino acid in the isolated peptides of this study. The presence of leucine might increase the hydrophobicity of the peptides (Huang et al., 2010), thus contributing to the antibacterial effect by partitioning into the lipid bilayer of bacterial membrane (Yeaman and Yount 2003). This was further supported by Ma et al. (2013) stating that leucine-rich repeats might enhance the killing mechanism of peptides towards bacteria such as *Escherichia coli*.

The ionic properties of the isolated peptides in this study are different. WPVWQMRPMVQG displayed by 4KP3 is cationic; FRDTDSLFTGSP and HSNWALYGIAEL displayed by 4KP5 and 4KP11, respectively, are anionic; while GLHTSATNLYLH displayed by 4KP29 is zwitterionic. Most of the antibacterial peptides are cationic, in which they may bind selectively to the anionic bacterial membrane. However, there are also anionic and zwitterionic peptides which serve as antibacterial peptides as found in Antimicrobial Peptide Database. In this study, two anionic peptides (FRDTDSLFTGSP and HSNWALYGIAEL) and one zwitterionic peptide (GLHTSATNLYLH) were successfully isolated.

On the other hand, the hydrophobicity of the peptides is essential for effective membrane permeabilization (Jenssen et al., 2006). However, increasing levels of hydrophobicity are associated with stronger hemolytic activity and decreased antibacterial activity (Chen et al., 2007). Most of the antibacterial peptides with optimized antibacterial activity are moderately hydrophobic with 50% of hydrophobic residues within their structure (Yeaman and Yount 2003). The hydrophobicity of the isolated peptides in this study ranges from 25% to 50%. It is assumed that the hydrophobicity of these peptides might contribute to the antibacterial effect against *Klebsiella pneumoniae* with less hemolytic complication. However, further study is needed to confirm this hypothesis.

5.6 Future Studies

The peptide sequences obtained from this study could be served as templates for chemical modification and production of synthetic peptides. These synthetic peptides can be tested for antibacterial effect against *Klebsiella pneumoniae* to determine the minimum inhibition concentration (MIC) or minimum bactericidal concentration (MBC). The relative antibacterial effect of the synthetic peptides can be compared with their phage-displayed counterpart.

Also, outer membrane permeabilization assay could be used to study the killing mechanism of peptides. It is important to study the killing mechanism of peptides in order to improve their antibacterial effect against the target bacteria.

The toxicity of the peptides against mammalian cells might affect the future development of the peptides as antimicrobial agents. The selectivity of the peptides acts towards mammalian cells and erythrocytes could be tested using cytotoxic assay and hemolytic assay. These findings may provide fundamental information for future improvement of existing antibacterial peptides or development of novel antibacterial peptides against *Klebsiella pneumoniae*.

CHAPTER 6

CONCLUSION

In conclusion, 12-mer random phage-displayed peptide library is a convenient tool for screening and isolation of peptides with antibacterial effect against *Klebsiella pneumoniae* strain ATCC 13883. The peptides that were isolated from this study were WPVWQMRPMVQG, FRDTDSLFTGSP, HSNWALYGIAEL and GLHTSATNLYLH. However, the degree of inhibition of these isolated peptides was considered low. Both the cationicity and hydrophobicity of peptides are important factors that contribute to the binding of peptides towards anionic bacterial membrane, as well as expressing the antibacterial effect against the target bacteria. Furthermore, the linear consensus sequence of -His-XXXXX-Leu-Tyr- that was found in two peptides might serve as critical binding residues to the target bacteria.

These peptide sequences cannot match to any antibacterial peptides that were found in Antimicrobial Peptide Database, therefore suggesting that they might be novel peptides. Although these peptides might serve as potential antimicrobial agents, future studies are needed to improve the selectivity and stability of peptides in order to develop as drugs that fight bacterial infection.

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APPENDIX A

LIST OF MATERIALS

Acetic acid glacial (Synerlab)
Agarose (1st BASE)
Albumin fraction V (from bovine serum) for biochemistry (Merck)
Dimethylformamide (Lab-Scan)
Ethanol absolute (HmbG Chemicals)
Ethidium bromide (100 mg/mL solution) (Bio Basic)
Ethylenediaminetetraacetic acid (EDTA free acid) (R&M Chemicals)
Glycerol 99.5% (QRec)
Glycine (Fisher Scientific)
HRP/Anti-M13 monoclonal conjugate (GE Healthcare)
Hydrochloric acid (fuming 37%) (R&M Chemicals)
Hydrogen peroxide 30% (ChemAR)
IPTG (Bio Basic)
Luria agar (Pronadisa)
Luria broth (Pronadisa)
MacConkey agar (Oxoid)
MicrogenTM GnA+B-ID System
PEG 8000 (Bio Basic)
Ph.D.-12 phage-displayed peptide library (New England Biolabs)
Sodium azide (Sigma-Aldrich)
Sodium chloride (Merck)
Sodium hydrogen carbonate (Bendosen)
Sodium hydroxide, beads (Bio Basic)
Sodium iodide (QRec)
Tetracycline hydrochloride (Bio Basic)
Tris (Bio Basic)
Tri-sodium citrate-dihydrate (ChemPur)
Tween 20 (ChemPur)
X-gal (Bio Basic)
1 kb DNA ladder (PhileKorea)
2, 2'-azinobis (3-ethylbenz-thiazoline sulphonic acid) diammonium salt
(Sigma)
6X DNA loading dye (Fermentas)

APPENDIX B

LIST OF ELECTRONIC INSTRUMENTS

Avanti J-E Centrifuge (Beckman Coulter)
FLUOstar Omega (BMG Labtech)
Genesys 20 (Thermo Scientific)
Infinite M200 (Tecan)
InGenius Syngene Bioimaging (Syngene)
LST 3016R Shaking Incubator (LabTech)
Microfuge® 22R Centrifuge (Beckman Coulter)
NanoDrop 2000 (Thermo Scientific)
ScanSpeed MiniVac Beta (Neutec)
Velocity 14R Centrifuge (Dynamica)