

**SCREENING FOR ANTIBACTERIAL ACTIVITY OF 15-MER LINEAR  
SYNTHETIC PEPTIDE PAM-6 AGAINST *Pseudomonas aeruginosa***

By

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## ABSTRACT

### SCREENING FOR ANTIBACTERIAL ACTIVITY OF 15-MER LINEAR SYNTHETIC PEPTIDE PAM-6 AGAINST *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is notorious for its intrinsic and acquired resistance towards many conventional antibiotics. In particular, different resistant mechanisms may occur simultaneously that leads to the emergence of multidrug resistant (MDR) strain of this bacterium and consequently, limiting the therapeutic choices. Antibacterial peptides (ABPs) have been studied extensively as a potential replacement to antibiotics. In short, ABPs are able to kill bacteria rapidly which may prevent the bacteria from conferring resistance towards the ABPs compounds. Therefore, in this study, a linear synthetic ABP namely PAM-6 was modified from its parental peptides which were PAM-1 and PAM-2. Subsequently, the antibacterial potency of PAM-6 was evaluated by using two strains of bacteria which included the reference strain (*P. aeruginosa* ATCC 27853) and clinical isolate MDR *P. aeruginosa* by using microbroth dilution assay. PAM-6 was potent in killing both the reference and clinical strain of bacteria *in vitro* at the titer of  $10^3$  CFU/ml at the MBCs of 4 µg/ml and 32 µg/ml,

respectively. Furthermore, the stability of PAM-6 in *ex vivo* condition was also tested and the MBC obtained was 16 µg/ml and this suggesting that this peptide might be unstable in human plasma. However, the stability of PAM-6 in presence of human plasma is yet to be determined due to the considerable amount of sodium ions present in Alsever's solution that was used as anticoagulant in this assay, which might greatly reduce the antibacterial activity of PAM-6. Lastly, the antibacterial activity of PAM-6 was influenced by bacterial inoculum effect as PAM-6 showed higher MBCs when the initial inoculation titer of the bacteria was increased from  $10^3$  to  $10^5$  CFU/ml. From these findings, PAM-6 is considered as a suitable antibacterial agent in combating *P. aeruginosa*.

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## **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.

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(CHEN PEI FEI)

## APPROVAL SHEET

This project report entitled "**SCREENING FOR ANTIBACTERIAL ACTIVITY OF 15-MER LINEAR SYNTHETIC PEPTIDE PAM-6 AGAINST *Pseudomonas aeruginosa***" was prepared by CHEN PEI FEI 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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**PERMISSION SHEET**

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

Yours truly,

\_\_\_\_\_

(CHEN PEI FEI)

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## LIST OF ABBREVIATIONS

ABP	Antibacterial peptide
ATCC	American Type Culture Collection
CaCl <sub>2</sub>	Calcium chloride
CCSI	Clinical and Laboratory Standard Institute
CFU	Colony forming unit
EDTA	Ethylenediaminetetraacetic acid
F	Phenylalanine
I	Isoleucine
K	Lysine
L	Leucine
LPS	Lipopolysaccharide
LRM	Leucine-arginine-methionine
MBC	Minimum bactericidal concentration
MDR	Multidrug resistant
MH	Mueller-Hinton
MgCl <sub>2</sub>	Magnesium chloride
MIC	Minimum inhibitory concentration
NaCl	Sodium chloride

NHSN	National Healthcare Safety Network
PBS	Phosphate buffered saline
R	Arginine
rpm	Revolutions per minute
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
V	Valine
W	Tryptophan



## CHAPTER 1

### INTRODUCTION

*Pseudomonas aeruginosa* possesses serious health issue because of its resistance towards many conventional antibiotics. Besides the intrinsic resistance, the additional genetic capacity in *P. aeruginosa* enables it to acquire mutational resistance. The cumulative mutational resistance and selective antibiotic pressure due to widely use of antibiotics could lead to the emergence of multidrug resistant strains of bacteria (Lister, Wolter and Hanson, 2009). In addition, the ability of the bacteria to form biofilm may also contribute to the antibiotic resistance.

The rising of these drug-resistant bacterial strains may cause shortage of available antibiotic required to cure infectious diseases caused by this bacteria. Thus, there is an urgent need for alternative treatment. Throughout these years, antibacterial peptides (ABPs) have been proposed as a potential replacement for antibiotics because of their broad spectrum of targeted bacteria. Besides that, ABPs act on multiple target sites of bacteria and kill them rapidly. Subsequently, it is hardly for the bacteria to develop resistance towards ABPs (Cezard, et al., 2011). Therefore, ABPs can be potential therapeutic agents against multidrug resistant bacteria.

Unfortunately, ABPs also possess several drawbacks that may limit their potential as novel antibacterial agent. These peptides may be highly susceptible to proteolysis by circulating proteases that may cause degradation of the peptides before exhibiting their antibacterial effect *in vivo* (Falciani, et al., 2012). Moreover, isolation of natural ABPs for clinical consumption is difficult due to their fragility, time-consuming process and low yield (Haney and Hancock, 2013). Therefore, synthetic ABPs are given prior consideration instead of natural ABPs as synthetic ABPs can be modified to become more potent antibacterial peptides and less toxic towards mammalian cells.

In a previous study, Tan (2014) had synthesized two 12-mer linear synthetic peptides namely PAM-1 and PAM-2 and their antibacterial activity was screened. However, moderate cationicity of these two peptides had dampened their antibacterial potency. Therefore, a newly synthetic peptide which named PAM-6 was designed based on the template of PAM-1 and PAM-2 by increasing the peptide length from 12-mer to 15-mer with enhanced cationicity and moderate hydrophobicity. Thus, this study was carried out to:

1. Screen for antibacterial activity of PAM-6 against reference strain of *Pseudomonas aeruginosa* ATCC 27853 and clinical isolate of multidrug resistant *Pseudomonas aeruginosa*.

2. Determine the minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) of PAM-6 if the antibacterial activity in (1) is present.
3. Determine the stability of PAM-6 in human plasma by using *ex vivo* assay.
4. Investigate the influences of inoculum effect on minimum bactericidal concentration (MBC) of PAM-6.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 *Pseudomonas aeruginosa*

##### 2.1.1 Clinical Significance of *P. aeruginosa*

*Pseudomonas aeruginosa* is an opportunistic pathogen which causes nosocomial infections that are associated with high mortality and morbidity. This gram-negative, rod-shaped bacterium can be straight or slightly-curved under microscopic observation. Morphologically, it is characterized by its blue-green colouration when it is grown on culture media (Lister, Wolter and Hanson, 2009). Besides that, *P. aeruginosa* has a polar, single flagellum that makes it motile (Barrios, et al., 2014).

In addition, *P. aeruginosa* is a ubiquitous organism that can be frequently isolated from diverse environmental settings and living resources such as human, animals and plants. As a biofilm-former, *P. aeruginosa* is able to produce the exopolysaccharide alginate which will then facilitate the bacterial attachment to solid surfaces and hence protect them from environmental changes (Cotton, Graham and Lee, 2009). Particularly, the anionic alginate will bind to the cationic antibiotics and block their diffusion into the bacteria, hence rendering it with antibiotic resistance (Lambert, 2002).

*P. aeruginosa* possesses a number of virulent factors which can be classified into surface virulent factors and secreted virulent factors. The former facilitates the bacterial attachment and colonization on a surface of the infected target while the latter is associated with increased cytokine production and cell damage (Mesaros, et al., 2006). In spite of its wide distribution in the environment, *P. aeruginosa*-associated infections are mainly hospital acquired (Lister, Wolter and Hanson, 2009). *P. aeruginosa* rarely causes disease in healthy individual. However, infectious diseases caused by this pathogen are usually linked to immunodeficiency (Mesaros, et al., 2006). Therefore, *P. aeruginosa* is termed as opportunistic pathogen. National Healthcare Safety Network (NHSN) has reported and ranked *P. aeruginosa* as the sixth most common hospital-associated pathogen which accounts for 8% of total nosocomial infections (Alhazmi, 2015).

Furthermore, this bacterium possesses another notorious feature as it has very low membrane permeability to foreign compounds. This attributes to their intrinsic resistance to many classes of antibiotics. The bacterial membrane serves as a special barrier that limits the entry of small hydrophilic molecules such as beta-lactams and quinolones (Lambert, 2002). This is one of the major reasons why treatment of *P. aeruginosa*-mediated infections using these antibiotics is usually ineffective.

### **2.1.2 Multidrug Resistant (MDR) *P. aeruginosa***

The rising of multidrug-resistant strains of bacteria is mainly due to the selective pressure created by the inappropriate use of antibiotics. Particularly, *P. aeruginosa* has high tendency to acquire mutational resistance which contributes to its acquired multidrug-resistant property. In most of the literatures, MDR is defined as an isolate that is resistant to at least three classes of antibiotics, which include aminoglycosides, antipseudomonal penicilins, carbapenems, cephalosporins and quinolones (Falagas, Koletsi and Bliziotis, 2006; Podnos, et al., 2001; Roberts, Findlay and Lang, 2001).

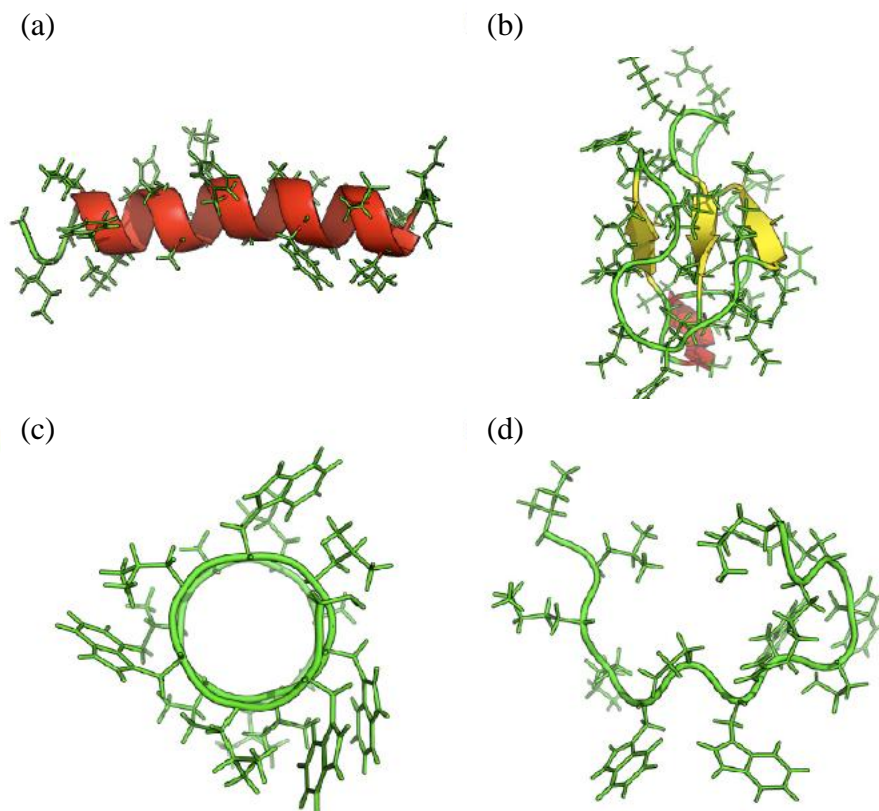
However, not every class of antibiotics will be compromised simultaneously by only a single mutation. In fact, simultaneous emergence of different mutations is unlikely because of its low spontaneous mutation rate. However, sequential emergence of multiple resistance phenotypes is probable due to inappropriate administration of antibiotics (Livermore, 2002). As the result of antibiotic stress, the combination and accumulation of different resistant mechanisms such as target mutation, drug efflux system and enzymatic modification towards conventional antibiotics causes the initially antibiotic-susceptible *P. aeruginosa* to transform into a multidrug-resistant strain (Hirsch and Tam, 2010). Therefore, to overcome this unsolved and growing problem of antibiotic resistance, the development and research for alternative therapeutic drugs against bacterial infections should take precedence in the next decades. Throughout these years,

antibacterial peptides (ABPs) have been intensively studied as a possible candidate of alternative therapeutic agent in replacing conventional antibiotics.

## **2.2 Antibacterial Peptides (ABPs)**

### **2.2.1 Overview of Antibacterial Peptides (ABPs)**

Antibacterial peptides (ABPs) are effector molecules from innate immunity that normally contain less than fifty amino acid residues (Cezard, et al., 2011). ABPs usually contain an excess of cationic amino acids such as arginine (R) and lysine (K), contributing to the net positive charge of the peptides ranging from +2 to +9. Apart from that, most ABPs are rich in hydrophobic residues such as leucine (L), isoleucine (I), valine (V), phenylalanine (F) and tryptophan (W). The presence of these amino acids enables ABPs to adopt four classes of amphipathic structures which are  $\alpha$ -helical,  $\beta$ -sheet, loop and extended peptide as shown in Figure 2.1 (Haney and Hancock, 2013).



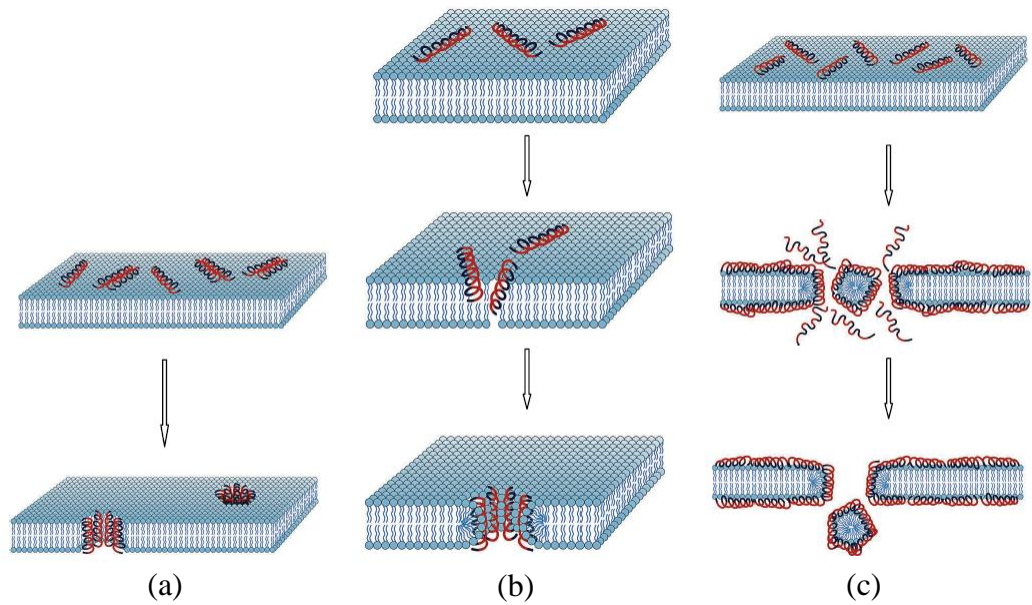
**Figure 2.1:** Three-dimensional structures of the four main classes of ABPs. (a)  $\alpha$ -helical peptide. (b)  $\beta$ -sheet. (c) Loop structure. (d) Extended peptide (Cezard, et al., 2011).

As mentioned previously, both peptide cationicity and hydrophobicity may facilitate the interaction between the peptide and bacterial membrane. The presence of phosphate groups in lipopolysaccharides of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria confer highly negatively-charged outer membrane to the bacteria (Cezard, et al., 2011). Consequently, cationic ABPs tend to bind to anionic bacterial membrane via electrostatic interaction (Diamond, et al., 2009).



Then, the distribution of polar and hydrophobic residues in ABPs lead to attachment of the peptides to bacterial membrane which then allows the ABPs to aggregate and deposit on bacterial outer membrane (Diamond, et al., 2009). The binding of ABPs to bacterial membrane is favored by self-promoted uptake, in which the accumulation of ABPs on bacterial membrane will increase the binding affinity (Hancock and Scott, 2000). As more and more ABPs are adsorbed into the membrane, the ratio of peptide-to-lipid will be increased. Once the threshold is reached, the structure of the ABPs will be changed and becomes perpendicular to the membrane (Cezard, et al., 2011). Eventually, the hydrophobic side of the ABPs that is rich in tryptophan residues will penetrate into the lipid bilayers (Hancock and Chapple, 1999; Diamond, et al., 2009).

After the insertion of ABPs into the bacterial membrane, several models of peptide downstream actions were proposed via different studies. These include carpet model, barrel-stave model and toroidal pore model as illustrated in Figure 2.2. These models of actions will then contribute to the rapid disruption of membrane and cell death in consequent.



**Figure 2.2:** Three models of peptide mechanisms on bacterial membrane were proposed by different researchers, which are (a) barrel-stave model, (b) toroidal model, and (c) carpet model [Adapted from Ebenhan, et al., 2014].

### 2.2.2 Antibacterial Peptides as an Alternative to Antibiotics

The role of ABPs as host defense effectors in innate immunity was established by the fact that some of the ABPs are inducible in the presence of pathogens while the others are constitutively expressed. Several animal studies demonstrated that ABPs are able to protect the animal from bacterial infection (Otvos, et al., 2014; Chromek, Arvidsson and Karpman, 2012; Wozniak, et al., 2014). Besides antibacterial activity, certain peptides possess other effector functions such as antifungal and antiendotoxin in nature (Hancock and Scott, 2000).

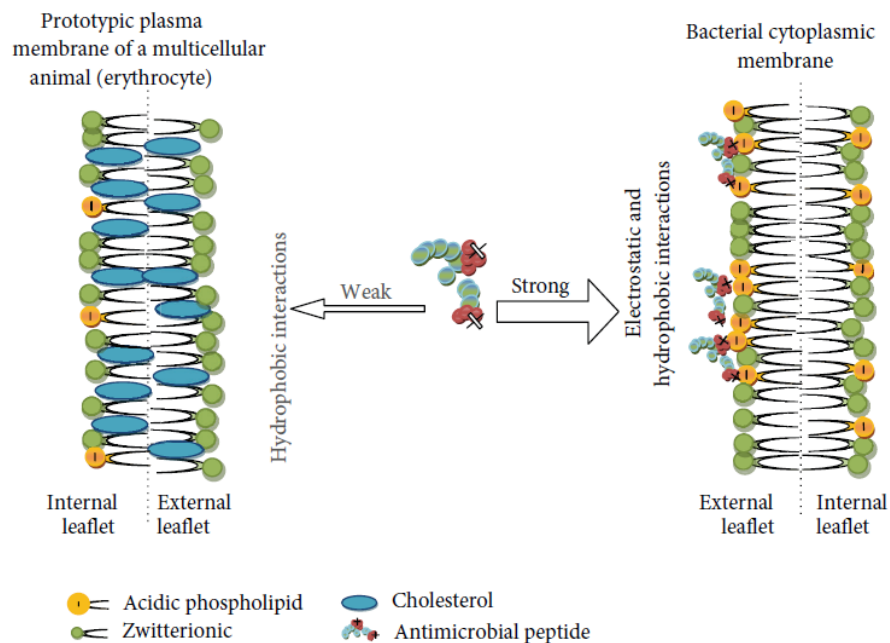
The ability of these peptides to inhibit or kill bacteria has inspired researchers to consider ABPs as a potential alternative to the conventional antibiotics. In fact, as

compared to antibiotics, ABPs exhibit broader spectrum of activity against gram-positive and gram-negative bacteria, yeasts, viruses and protozoa (Cezard, et al., 2011; Ganz, 2003). Most importantly, these peptides remain unaffected by bacterial resistance mechanisms that have been observed for antibiotics (Cezard, et al., 2011).

In addition, many researchers came to a mutual agreement that it is unlikely for bacteria to develop resistance towards ABPs despite their diverse resistance mechanisms (Bahar and Ren 2013). As the bacterial membrane is the primary target for ABPs, the bacteria require a very lengthy procedure to modify their entire membrane, in order to resist the action of ABPs (Cezard, et al., 2011). In addition to that, ABPs are able to kill bacteria rapidly, thus disallowing the bacteria to modify their membrane in time.

Moreover, the selective toxicity of ABPs towards microbial cells but not mammalian cells further suggests ABPs as potential candidate in replacing or complementing antibiotics. The eukaryotic membranes are normally made up of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin which are neutrally-charged. In contrast, the anionic hydroxylated phospholipids phosphatidylglycerol, cardiolipin and phosphatidylserine are components of prokaryotic membrane and these molecules create a net negative charge on the bacterial membrane (Yeaman and Yount, 2003). As illustrated in Figure 2.3, the

fundamental differences mentioned previously allow ABPs to bind selectively to the bacteria. This binding is established by both electrostatic and hydrophobic interactions which contribute to a stronger interaction as compared to the solely hydrophobic interaction between cationic peptide and zwitterionic eukaryotic membrane (Ebenhan, et al., 2014).



**Figure 2.3:** The basis of selectively toxicity exhibited by ABPs [Adapted from Ebenhan, et al., 2014].

On the other hand, ABPs are also capable in counteracting bacterial sepsis. When treating bacterial infections with conventional antibiotics, the antibiotic is known to induce the release of lipopolysaccharide (LPS) by gram-negative bacteria and lipoteichoic acid by gram-positive bacteria in systemic circulation. This may in turn induce elevated cytokine production such as tumor necrosis factor- $\alpha$  (TNF-

$\alpha$ ) and interleukin-6 (IL-6) by macrophages. These pro-inflammatory mediators could cause septic shock which can be fatal. In fact, ABPs have an analogous ability that oppose the capability of the bacterial products in induction of cytokine release and hence prevent the onset of sepsis (Hancock and Scott, 2000).

In conclusions, in terms of pharmacologic and therapeutic applications against bacterial infections, ABPs can be used as single therapeutic agent or in combination with conventional antibiotics or antivirals. Apart from that, ABPs may serve as potential endotoxin-neutralizing agent to prevent septic shock (Gordon and Romanowski, 2005).

### **2.2.3 Limitations of Natural Antibacterial Peptides**

Despite the strength of ABPs mentioned previously, many issues of ABPs are still remain unsolved. In the presence of salt and divalent cations, the action of ABPs tends to be affected (Rotem and Mor, 2008). For example, a study had shown that the antibacterial activities of several ABPs such as LL-37 and human  $\beta$ -defensins (hBDs) were reduced in the presence of low level of ions (50 mM of NaCl). The ionic interactions between ABPs and bacterial membrane is usually affected by monovalent, divalent or polyanions. Anions like chloride ions tend to conjugate with cationic ABPs and hence prevent their binding towards bacterial membrane (Diamond, et al., 2009). Besides that, the anionic bacterial membrane could be masked by cations such as sodium ions and hence hinder the binding of ABPs

towards the membrane (Kandasamy and Larson, 2006). These cations might compete with the cationic ABPs for binding to the membranous anionic target, thus reducing their degree of aggregation on the membrane.

Besides that, ABPs are also vulnerable to pH changes, proteases and plasma components. These factors greatly reduce the bioavailability of ABPs and may limit their pharmacokinetic properties. When only D-amino acids are present in ABPs or liposomal incorporation may confer high resistance towards proteolysis (Hancock and Scott, 2000). However, this may significantly increase the cost of ABPs production (Rotem and Mor, 2008). The toxicity issue of ABPs is also another major concern. In fact, some cationic ABPs such as bee venom melittin are very toxic towards mammalian cells (Hancock and Scott, 2000).

Although bacteria do not develop resistance easily towards ABPs, indeed there are some bacterial strains which are naturally resistant to ABPs' activity (Diamond, et al., 2009). For instance, *Porphyromonas gingivalis* secretes arginine- and lysine-specific proteases which target and destroy many highly cationic ABPs (Diamond, et al., 2009). *Staphylococcus aureus* secretes adhesion molecules that facilitate their adhesion on its target cells or other surface. These adhesion molecules are positively-charged molecules and tend to adsorb into the bacterial membrane. The anionic bacterial membrane is shielded by the adhesin and thus, hinders the binding of ABPs (Bahar and Ren 2013).

Apart from that, the antibacterial action of antibacterial agents such as ABPs and conventional antibiotics are always associated with a common laboratory phenomenon namely inoculum effect (IE). IE is described as the decreasing efficacy of an antibacterial agent due to increasing initial bacterial inoculation titer. The presence of IE in clinical setting may lead to the administration of insufficient dose of antibiotics and hence decrease the survival rates of infected patients (Tan, et al., 2012). Moreover, Bulitta and colleagues (2010) had found that the efficacy of colistin (Polymyxin E), a peptide antibiotic, was drastically reduced in killing *P. aeruginosa* with high inoculum size and higher concentration of colistin was required to achieve its desired bactericidal activity. However, an increased dose of antibiotic may lead to toxicity effect and increased economic burden of patient. Thus, the antibacterial agents which cause inoculum effect in its action should be studied extensively to achieve a balance in between the dosage and bactericidal effect of the particular antibacterial agent, and hence prevent the above mentioned side effect.

Therefore, the ABPs need to be engineered and modified to obtain a better antibacterial effect in order to overcome bacterial resistance towards ABPs mentioned earlier.

#### 2.2.4 Previous Findings on Synthetic Antibacterial Peptides

Antibacterial peptides can be classified into two major types based on their source of origin, which are natural ABPs and synthetic ABPs. As mentioned earlier, the components of natural ABPs are first line defense which are usually found in the host tissues that are exposed to pathogenic microorganisms in environment (Bahar and Ren, 2013). Before the antibacterial potency of these natural ABPs can be evaluated, the ABPs have to be extracted. However, the extraction and purification of large amount of ABPs from natural resources are usually lengthy, laborious and time-consuming. Thus, synthetic ABPs are recommended as the peptides can be modified to become less fragile, with better antibacterial efficacy and less toxicity to mammalian cells (Haney and Hancock, 2013).

A number of studies on synthetic ABPs were carried out by different group of researchers, and majority of them demonstrated promising outcomes. León-Calvijo and colleagues (2014) had designed a total number of 22 synthetic antibacterial peptides based on the template of human and bovine lactoferrin. The potency of these peptides was tested against both *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212. It was found that the peptides RWQWRWQWR and  $(RRWQWR)_4K_2Ahx_2C_2$  were very potent to the target bacteria. According to them, the enhanced antibacterial effect was contributed by the RWQWR motif which possesses tryptophan (W) and arginine (R) residues in alternating arrangement.



In another study, a nine-mer tetrabranched synthetic peptide named as M33 (KKIRVRLSA) synthesized by Pini and colleagues (2010) was found to be very potent against gram-negative bacteria such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. Moreover, this peptide was also active against the clinical isolates of multidrug resistant strains. Besides that, M33 was also able to neutralize lipopolysaccharides (LPS) produced by the bacteria and prevent the LPS-mediated discharge of cytokine TNF- $\alpha$  from macrophages.

#### **2.2.5 Previous Findings on Synthetic Antibacterial Peptides against *P. aeruginosa***

Deslouches and colleagues (2005) had composed a 13-mer synthetic ABP *de novo* named as WLBU2 (RRWVRRVRRWVRRVVRVRRWVRR). This peptide was active against *P. aeruginosa* at low MBC. Besides that, despite of the presence of CaCl<sub>2</sub>, NaCl and MgCl<sub>2</sub> at their physiological concentrations, the bactericidal effect of WLBU2 was not affected. WLBU2 also exhibited rapid killing on *P. aeruginosa* (within 20 min). Moreover, the efficacy of WLBU2 remained unaltered despite the presence of human whole blood. More importantly, this peptide did not cause any adverse effect to mammalian cells such as human monocytes or skin fibroblasts. These findings were almost consistent to his another finding when the peptide was tested *in vivo* by using animal model, where the peptide also demonstrated similar efficacy (Deslouches, et al., 2007). This

suggests the therapeutic potential of the peptide against *P. aeruginosa* in clinical application.

Sánchez-Gómez and colleagues (2015) evaluated the potency of a series of synthetic peptides which were analogs to human lactoferrin, LF11. These peptides were acylated by adding octanoyl, 2,2-dimethylbutanoyl and 6-methyloctanoyl groups to the parental peptide LF11. Consequently, acylated synthetic peptides showed better potency towards *P. aeruginosa* reflected by a lower MIC value. Among these peptides, LF11-324 (PFFWRIRIRR) was the most potent peptide which inhibited both the reference and the clinical strains of multidrug resistant *P. aeruginosa* at MIC of 8 µg/ml. The additional of proline (P), phenylalanine (F) and tryptophan (W) residues to the N-terminal of LF11-324 was shown to form hydrophobic core segment in the peptide and facilitated its penetration into the bacterial membrane. Thus, this greatly enhanced the antibacterial activity of LF11-324. Besides that, all the peptides were able to display rapid killing in less than 21 min at concentration of two times of their MIC value.

In a nutshell, many synthetic ABPs are potential to exhibit antibacterial effect on *P. aeruginosa*. Therefore, in this study, a synthetic linear peptide which named as PAM-6 was screened for its antibacterial activity against both *P. aeruginosa* ATCC 27853 and clinical isolate of multidrug-resistant *P. aeruginosa*.

### 2.2.6 Linear Synthetic Antibacterial Peptides PAM-6

In this study, the antibacterial activity of PAM-6 (RPRGKLRWKLRVLRM) on *P. aeruginosa* ATCC 27853 and multidrug-resistant *P. aeruginosa* was screened. This peptide was not synthesized *de novo* but designed by rational modification of two phage displayed-peptides, which showed high binding affinity and specificity towards *P. aeruginosa*. These two phage displayed-peptides, namely PAI-20 (YNHKTDKNA**LRM**) and PA-26 (GPVNKSSTIL**LRM**) were isolated by Gwee (2011) and Lee (2012), respectively, via phage displayed-peptide affinity selection. In spite of the varied peptide sequences of these two phage displayed-peptides, a common motif of LRM (leucine-arginine-methionine) was found in these peptides, suggesting that this motif might contribute to the binding affinity of the peptides towards *P. aeruginosa*. However, both of these peptides showed low antibacterial potency towards *P. aeruginosa*. This might be due to the steric hindrance from the phage particles which prevent the accumulation of the peptides on bacterial membrane to disrupt the membrane (Chauchan and Varma, 2009).

Then, Tan (2014) had used PAI-20 and PA-26 as the template to design two 12-mer linear synthetic peptides named as PAM-1(WPVKKWKWAL**LRM**) and PAM-2 (RPVKKWKRI**LRM**). The consensus motif LRM was preserved. Besides that, tryptophan (W) which enhances membrane perturbation and arginine (R) which retains the peptide cationicity were substituted into the peptide (Chan, Prenner

and Vogel, 2006). This modification had contributed certain bactericidal activity by the peptides. However, the minimum bactericidal concentrations (MBCs) for PAM-1 and PAM-2 were very high, which were at 512 µg/ml. It is commonly known that ABPs at high concentration may lead to their nonselective action targeting on both the bacterial and mammalian cells (Lavery and Gilmore, 2014). In addition, the moderate cationicity of PAM-1 (+4) and PAM-2 (+6) may not confer strong electrostatic binding towards bacterial membrane and this might be a possible factor of their low antibacterial potency.

Therefore, based on the PAM-1 and PAM-2, a novel 15-mer linear synthetic peptide named as PAM-6 was designed with enhanced cationicity of +7 by substituting and adding more arginine (R) residues and lysine (K) residues with the LRM motif preserved. Apart from that, the hydrophobicity of PAM-6 was maintained at a moderate level (40%) which was relatively lower than PAM-1 (58%) and PAM-2 (41%). By fulfilling the two major criteria of ABP, which are enhanced cationicity and moderate hydrophobicity, it is hypothesized that PAM-6 would demonstrate potent antibacterial effect against *P. aeruginosa*.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 General Experimental Design

A novel 15-mer synthetic peptide named as PAM-6 (RPRGKLRWKLRVLRM) was designed based on the templates as mentioned in Chapter 2. The peptide was synthesized by Bio Basic Inc. in Canada. To evaluate the antibacterial potency of PAM-6 against the reference strain of *P. aeruginosa* ATCC 27853 and clinical strain of multidrug resistant (MDR) *P. aeruginosa*, *in vitro* microbroth dilution assay was carried out in order to determine the minimum bactericidal concentrations (MBCs) of PAM-6. In addition, to evaluate the *in vivo* potency of PAM-6, the antibacterial assay was also conducted by simulating an *in vivo* condition via *ex vivo* microbroth dilution assay. Lastly, the MBCs of PAM-6 against different initial bacterial inoculation titer was determined to study the bacterial inoculums effect on the peptide efficacy. All the assays were carried out in triplicates to ensure the result was reproducible.

#### 3.2 Materials

##### 3.2.1 Bacterial Strains

Both reference strain of *P. aeruginosa* ATCC 27853 and clinical strain of MDR *P. aeruginosa* were used as the target bacteria in the assays. The reference strain was kindly provided by Dr Sit Nam Weng, Department of Biomedical Science, Universiti Tunku Abdul Rahman (UTAR), which the clinical strain of MDR *P. aeruginosa* was

obtained from Gleneagles Medical Center, Penang. These two strains of bacteria were cultured on Mueller-Hinton (MH) agar and propagated in MH broth. MH broth with 20% of glycerol was used to preserve the bacteria and the bacterial glycerol stock was stored at -80°C. Prior to the assay, the frozen bacterium was thaw and inoculated on MH agar as master culture plate.

### **3.2.2 Peptide Design, Synthesis and Preparation**

PAM-6 (RPRGKLRWKLRVLRM), a novel 15-mer linear synthetic peptide which was designed based on the template as mentioned in Section 2.2.6. The modification of peptide was conducted by using two online softwares, which were Antimicrobial Peptide Calculator and Predictor ([http://aps.unmc.edu/AP/prediction/prediction\\_main.php](http://aps.unmc.edu/AP/prediction/prediction_main.php)) and Antimicrobial Peptide Designer ([http://aps.unmc.edu/AP/design/design\\_improve.php](http://aps.unmc.edu/AP/design/design_improve.php)). Particularly, the hydrophobicity ratio, molecular weight and formula, total net charge, ratio of the particular amino acid in the peptide sequence as well as the tendency to form  $\alpha$ -helix structure can be predicted by these online softwares.

Then, the peptide was synthesized by Bio Basic Inc. in Canada and arrived as lyophilized form. The peptide was stored in a sealed and dry container at -20°C with silica gels. Before dissolving the peptide, the peptide was equilibrated to room temperature for one hour in drawer to protect it from light to minimize the peptide degradation. Degassed sterile distilled water was used to dissolve PAM-6 since the side chain of methionine residue is susceptible to oxidation. Then, the peptide was

serially diluted by two-fold in degassed sterile PBS and stored in silica bottles for maximal seven days at 4°C.

### **3.2.3 Buffers and Reagents**

Refer to Appendix A.

### **3.3 Lab Wares and Equipments**

Refer to Appendix B.

## **3.4 Protocols**

### **3.4.1 Bacterial Glycerol Stock Preparation**

A loop-full of bacterial colonies was transferred into 20 ml of MH broth and incubated for 4.5 h at 37°C with agitation of 200 rpm. A final concentration of 20% (v/v) of glycerol was added into the culture. After mixing well, 500 µl of the bacterial suspension was aliquoted into 1.5 ml of microcentrifuge tubes. The bacteria were stored overnight at -20°C and after that, stored at -80°C. Prior to preparing the master culture, the frozen bacteria were thawed on ice for at least half an hour. Then, the bacterial culture was inoculated onto MH agar and incubated overnight at 37°C. After overnight incubation, the master culture plate was stored at 4°C for a maximal period of one week.

### 3.4.2 Bacterial Killing and Growth Inhibition Assay

The antibacterial potency of PAM-6 on both reference and clinical strains of *P. aeruginosa* was tested by microbroth dilution assay as recommended by Clinical and Laboratory Standard Institute (CCSI) with slight modification. A loop-full of three to four bacterial colonies was picked from the master culture and inoculated into 20 ml of MH broth, which was subsequently incubated at 37°C with 200 rpm overnight. On the following day, a portion of the overnight bacterial culture was diluted by 100 folds with 20 ml of fresh MH broth. The diluted culture was then incubated at 37°C with 200 rpm until the mid-log phase of bacterial growth (absorbance of 0.500 to 0.600 at OD<sub>600</sub>). The bacteria were then separated through centrifugation at 5000×g and washed for two times with PBS (pH 7.4). After removing the PBS, the bacterial pellet was resuspended in 1 ml of fresh PBS and serially diluted with PBS to obtain bacteria at the titer of 10<sup>3</sup> CFU/ml.

After that, 100 µl of the bacterial suspension in PBS was loaded into the wells of 96-well microplate followed by 100 µl of two-fold serially diluted PAM-6 at the concentrations ranging from 2 µg/ml to 256 µg/ml. The microplate was pre-incubated for 1 h at 37°C before 50 µl of MH broth was added into each well. Then, the microplate was incubated overnight at 37°C. After 16 to 18 h of incubation, 10 µl of the culture suspension from each well was plated onto MH agar plate for colony counting. Besides that, based on visual inspection, culture which developed turbidity in the wells was serially diluted and inoculated onto



MH agar for titer determination. After another overnight incubation of the culture on MH agar, the bacterial colonies were counted to determine the bacterial viability and the minimum bactericidal concentration (MBC) of PAM-6 against both strains of bacteria. The average MBC was obtained from triplicate results.

For positive control, two-fold serially diluted Polymyxin B at concentrations ranging from 2 µg/ml to 256 µg/ml was inoculated with both strains of the bacteria. Bacterial suspension in PBS was used as the negative control. In Table 3.1, the contents loaded into each well were tabulated.

**Table 3.1:** Contents of wells (sample, positive control and negative control) of microplate in microbroth dilution assay. The hyphen in table indicating the particular variable was not present.

<b>Contents</b>	<b>Sample</b>	<b>Positive control</b>	<b>Negative control</b>
Diluted bacteria in PBS	100µl	100µl	100µl
PAM-6	100µl	-	-
Polymyxin B	-	100µl	-
PBS (pH 7.4)	-	-	100µl
MH broth	50µl	50µl	50µl

### **3.4.3 Relationship between Bacterial Inoculation Effect on the Efficacy of PAM-6**

In order to study the influences of initial inoculating bacterial titer on the MBCs of PAM-6, three similar antibacterial assays of PAM-6 against different bacterial inoculation titer with different orders of magnitude ( $10^3$ ,  $10^4$  and  $10^5$  CFU/ml) were conducted as explained in Section 3.4.2.

### **3.4.4 Preparation of Fresh Human Plasma**

Ten milliliter of fresh blood was drawn from a volunteer and aliquoted into two centrifuge tube containing 5 ml of Alsever's solution in a ratio of 1:1. The tubes were then inverted for several times to ensure the blood was completely mixed with the Alsever's solution. Subsequently, the tubes were centrifuged at 3000 rpm for 10 min. Finally, the supernatant which was the plasma was carefully aspirated into a clean tube by using a Pasteur pipette.

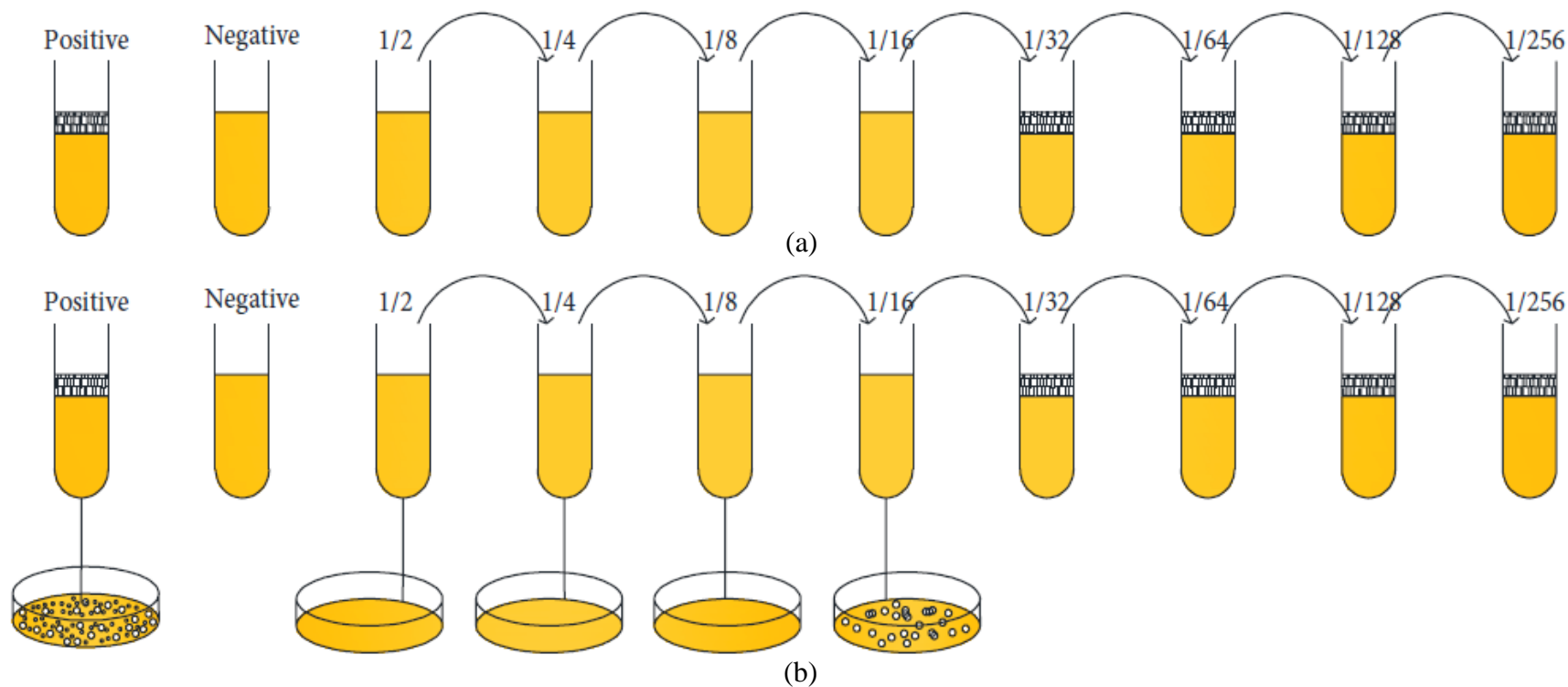
### **3.4.5 Stability of PAM-6 in Fresh Human Plasma (*ex vivo* Microbroth Dilution Assay)**

As described in Section 3.4.2, similar antibacterial assay was carried out with certain modification. The antibacterial activity of PAM-6 was evaluated in the presence of human plasma. In this assay, the bacterial suspension was diluted with human fresh plasma to  $10^3$  CFU/ml and the incubation time for the treatment

of PAM-6 on the target bacteria was extended to 44 h instead of overnight incubation. Also, the bacteria treated with PAM-6 were not supplemented with nutrient enrichment medium (MH broth). After the incubation period, the bacterial culture was inoculated onto MH media for the purpose of titer determination.

#### **3.4.6 Determination of MBC and MIC by Using Microbroth Dilution Assay**

Figure 3.1 illustrated how minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by using microbroth dilution assay. The lowest concentration of PAM-6 that inhibits the visible growth of target bacteria after overnight incubation was considered as MIC. On the other hand, MBC for PAM-6 was defined as the lowest concentration of PAM-6 that will prevent the formation of bacterial colony after culturing the treated bacterial culture on a media free from antibiotic.



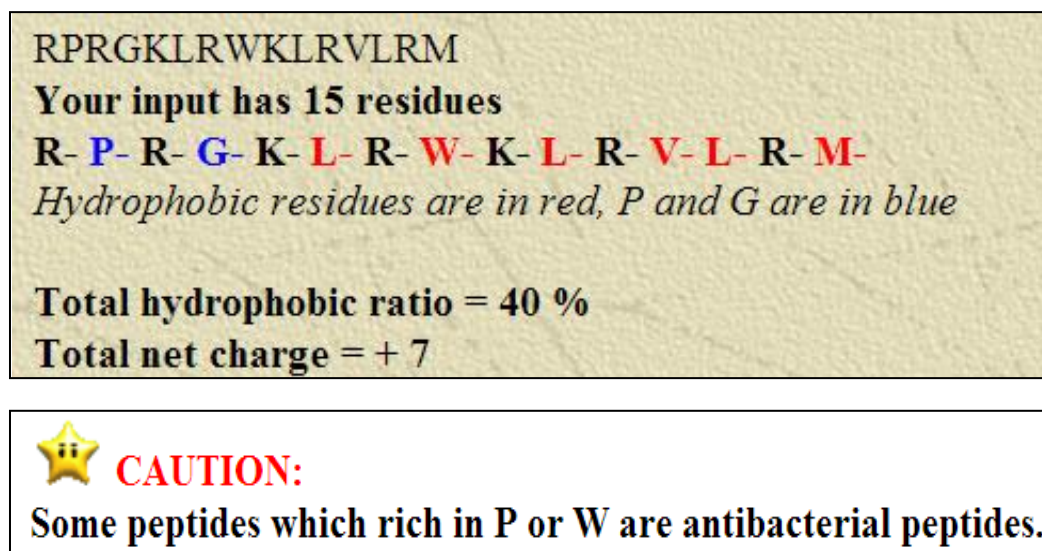
**Figure 3.1: Microbroth dilution tube method.** In (a) MIC assay, based on visual inspection, the lowest concentration of antibacterial agent that yield no growth of bacteria in tube is defined as MIC. The dilution that yielded the MIC of this agent shown in this figure is 1/16. In (b) MBC assay, the lowest concentration of antibacterial agent that prevent the formation of bacterial colony after culturing to an antibiotic-free media is considered as MBC. In this figure, the dilution that produced the MBC is 1/8 [Adapted from Yilmaz, 2012].

## CHAPTER 4

### RESULTS

#### 4.1 Characteristics of PAM-6

As mentioned in Chapter 2, a novel 15-mer synthetic linear peptide, PAM-6 was designed via modification of two hypothetical ABPs, namely PAM-1 and PAM-2. This design was done by using two online softwares, Antimicrobial Peptide Calculator and Predictor and Antimicrobial Peptide Designer – Improve your peptides. By using this software, some of the properties of PAM-6 including total hydrophobicity and total net charge can be predicted. The analysis of the peptide is shown in Figure 4.1.



**Figure 4.1:** The analysis of PAM-6 by Antimicrobial Peptide Calculator and Predictor.

After the modification, PAM-6 with increased peptide length conferred higher cationicity (+7) than PAM-1 (+4) and PAM-2 (+6) with moderate hydrophobicity of 40%. The modification of PAM-6 from its parental peptides was demonstrated in Table 4.1. Besides that, based on the analysis by Antimicrobial Peptide Calculator and Predictor, PAM-6 is likely an antibacterial peptide as it consists of proline (P) and tryptophan (W) residues, as shown in Figure 4.1.

**Table 4.1:** Comparison of the physiochemical characteristics of PAM-6 with its parental peptides, PAM-1 and PAM-2.

Peptide	Peptide sequence	Cationicity	Percentage of Hydrophobicity	Length
<b>PAM-1</b>	WPVKKWKWALRM	+4	58%	12-mer
<b>PAM-2</b>	RPVKKWKRIILRM	+6	41%	12-mer
<b>PAM-6</b>	RPRGKLRWKLRVLRM	+7	40%	15-mer

PAM-6 can be compared to the common established ABPs which can be archived from the online software database mentioned earlier. The archived peptide which shows the highest similarity to PAM-6 (38.09%) is an engineered ABP, namely A3-APO (RPDKPRPYLPRPRPPRPVR) with ID number of AP00707. It was designed to interfere with the bacterial membrane and heat-shock protein in bacterial cells (Szabo, et al., 2009). Another four ABPs, namely Pep39 (AP02430), Temporin-1RNb (AP02297), Astacidin 2 (AP01720) and S-dodecapeptide

(AP00450) showed 37.5% of similarity to PAM-6. The antimicrobial peptide Pep39 (RLFRHAFKAVLRL) was derived from the anchovy hydrolysate while Temporin-1RNb (FLPLKKLRFGLL) is a 12-mer peptide that isolated from black-spotted frog (Tang, et al., 2013; Li, et al., 2013). Next, Astacidin 2 (RPRPNYRPRPIYRP) is a proline- and arginine-rich peptide in hemocytes of crayfish that confers a net positive charge of +6 (Jiravanichpaisal, et al., 2006). Moreover, S-dodecapeptide (RICRIIFLRVCR) is the sheep myeloid cathelicidins (Bagella, Scocchi and Zanetti, 1995). All the comparisons are shown in Figure 4.2.

Your input sequence is: RPRGKLRWKLRVLRM

---- Alignment results ----

Number 1, Similarity percentage: 38.09 %  
AP00707  
 -Alignment Result-: R P D K P R P Y L P R P R P P R P V + R +  
 --Input Sequence--: R + + + P R G K L + R + W K L R + V L R M

Number 2, Similarity percentage: 37.5 %  
AP02430  
 -Alignment Result-: R L F R + + H A F K + A V L R L  
 --Input Sequence--: R + P R G K L R W K L R V L R M

Number 3, Similarity percentage: 37.5 %  
AP02297  
 -Alignment Result-: F L P L K K L R F G L + + L + +  
 --Input Sequence--: + R P R G K L R W K L R V L R M

Number 4, Similarity percentage: 37.5 %  
AP01720  
 -Alignment Result-: R P R P N Y R + + P R P I Y R P  
 --Input Sequence--: R P R G K L R W K L R + V L R M

Number 5, Similarity percentage: 37.5 %  
AP00450  
 -Alignment Result-: R I C R + + + I I F L R V C R +  
 --Input Sequence--: R + P R G K L R W K L R V L R M

**Figure 4.2: Comparison of PAM-6 with the five most similar archived ABPs.** These five archived ABPs with ID number of AP00707, AP02430, AP02297, AP01720 and AP00450 showed highest similarity to PAM-6 in peptide sequences.



## **4.2 Antibacterial Effect of PAM-6**

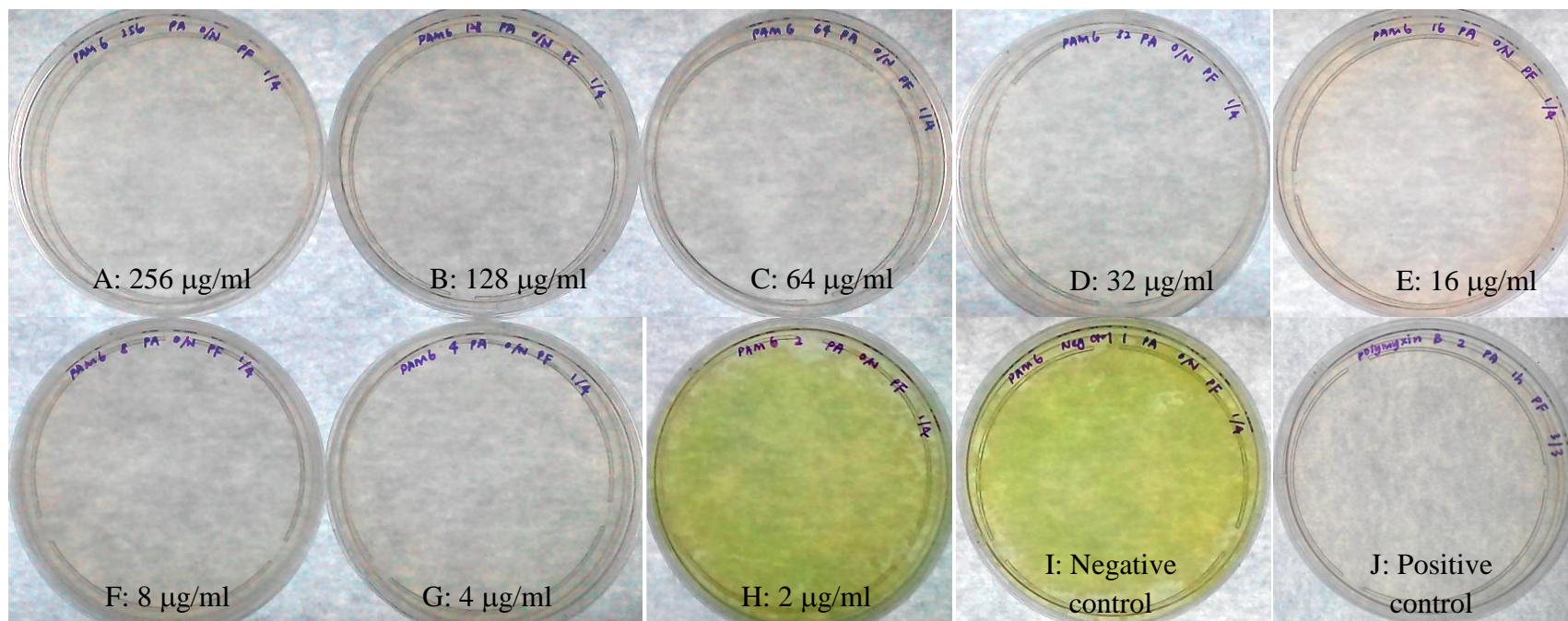
In this project, microbroth dilution assay was used to evaluate the antibacterial potency of PAM-6 against two strains of *P. aeruginosa*, which were reference strain *P. aeruginosa* ATCC 27853 and clinical strain of MDR *P. aeruginosa*. The antibacterial potency of the peptide is reflected by its minimum inhibitory concentration (MIC) if the peptide action is bacteriostatic or minimum bactericidal concentration (MBC) if the peptide action is bactericidal.

### **4.2.1 Determination of MBC for PAM-6 against *P. aeruginosa* ATCC 27853**

As mentioned earlier, microbroth dilution assay was used to determine the MBC of PAM-6. Figure 4.4 shows the cultures of *P. aeruginosa* ATCC 27853 treated with two-fold serially diluted PAM-6 with concentrations ranging from 2 µg/ml to 256 µg/ml (Plate A to Plate H), as well as the negative control (Plate I) and positive control (Plate J).

As shown in the figure 4.3, PAM-6 at concentrations ranging from 4 µg/ml to 256 µg/ml (Plate A to Plate G) exhibited complete bactericidal action towards the target bacteria. This was indicated by the total absence of bacterial colony on the media culture as compared to both the negative and positive control. A reduced or absence of antibacterial efficacy was observed when the target bacteria was treated with PAM-6 at concentrations of 2 µg/ml (Plate H) as indicated by the

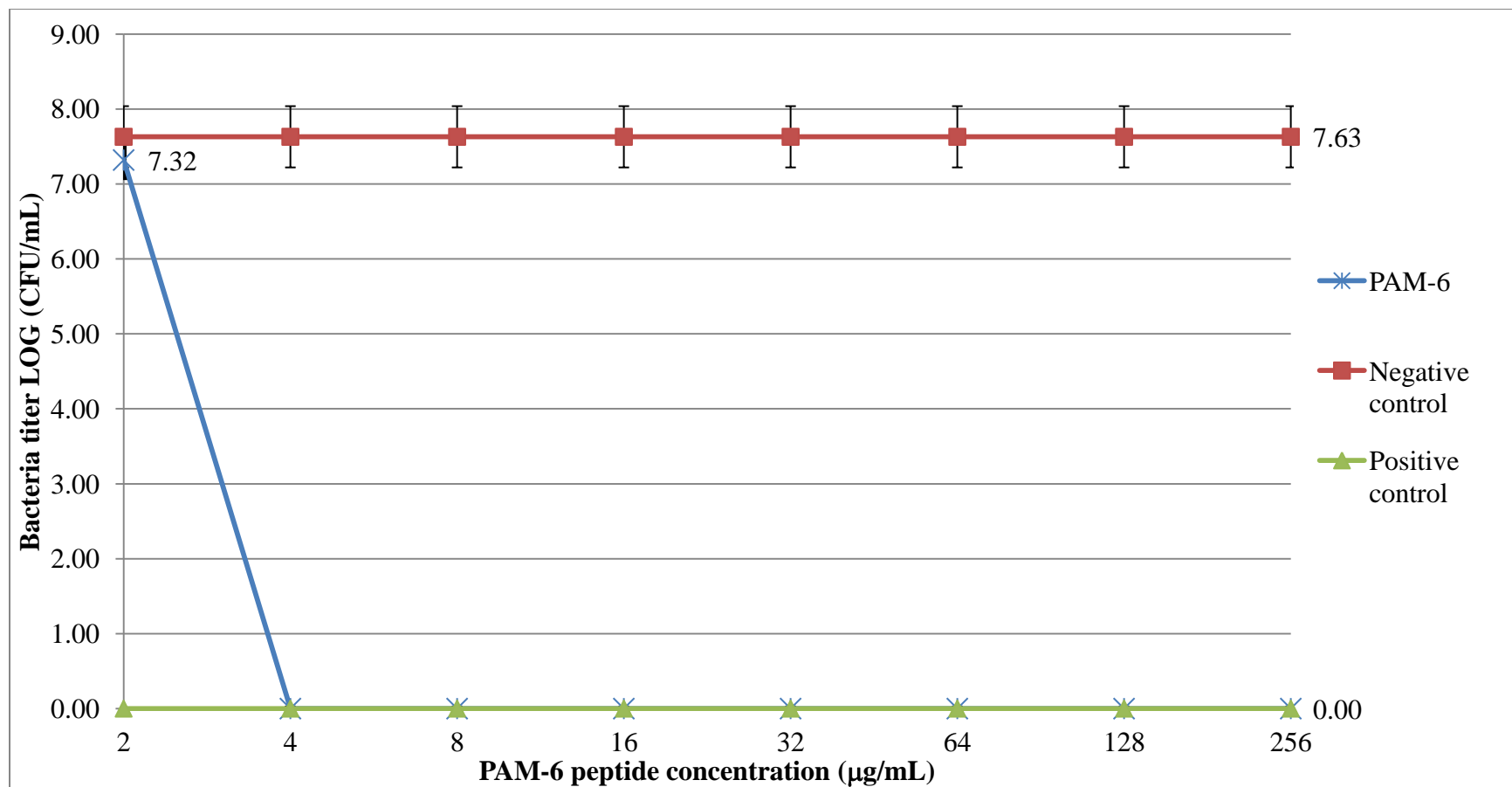
fully grown of bacterial lawn on the media plate. The MBC of PAM-6 at this bacterial input titer ( $2.71 \times 10^3$  CFU/ml) was determined at 4 µg/ml after repeated the assay for three rounds. Polymyxin B which ranged from 2 µg/ml to 256 µg/ml was used as the positive control and it was able to kill the target bacteria at the lowest treated concentration which was indicated by Plate J.



Bacterial inoculation titer:  $2.71 \times 10^3$  CFU/ml

**Figure 4.3: Cultures of *P. aeruginosa* ATCC 27853 treated with PAM-6.** PAM-6 at concentrations ranging from 4 µg/ml to 256 µg/ml (Plate A to Plate G) demonstrated complete bactericidal effect towards the target bacteria. Bacteria which were treated with 2 µg/ml of PAM-6 (Plate H) as well as negative control (Plate I) fully grown on the media. Positive control (Plate J) was inoculated with the bacteria treated with 2 µg/ml of Polymyxin B.

The antibacterial assay was repeated for three times with the same initial inoculation bacteria titer maintained at  $10^3$  CFU/ml. The results were reproducible throughout the three assays. The average data were calculated and demonstrated in Figure 4.4. Based on the graph, PAM-6 at concentrations ranging from 4 µg/ml to 256 µg/ml displayed complete bactericidal effect on the reference strain of *P. aeruginosa*, whereas poor antibacterial activity was shown at concentrations lower than 4 µg/ml. This was reflected by the increased bacterial viability after treating with PAM-6 at concentration lower than 4 µg/ml, which was quite close to the bacterial titer of negative control (without treatment). Thus, the MBC of PAM-6 against *P. aeruginosa* ATCC 27853 was determined at 4 µg/ml, which was the lowest concentration of PAM-6 that displayed complete bactericidal action towards the target bacteria.



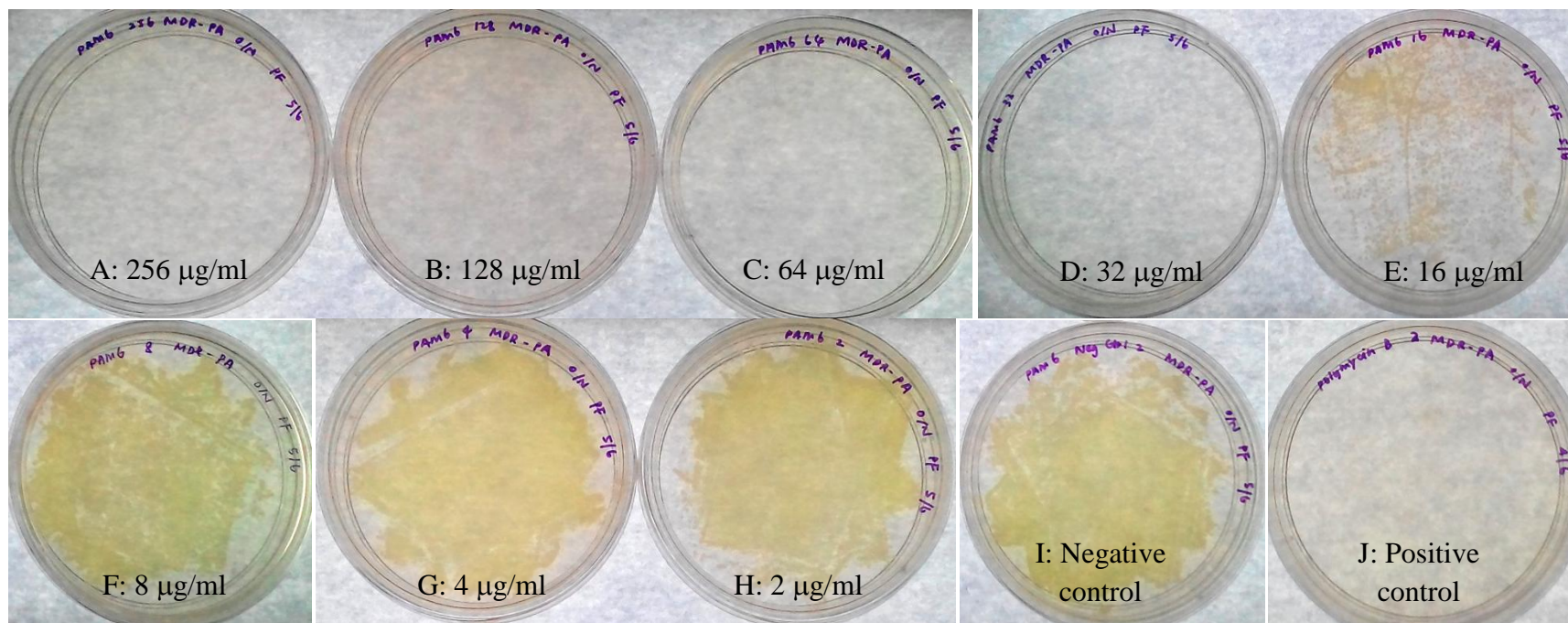
**Figure 4.4:** *in vitro* antibacterial effect of PAM-6. *P. aeruginosa* ATCC 27853 with the titer of  $10^3$  CFU/ml was treated with PAM-6 with two-fold serially diluted concentrations ranging from 2 µg/ml to 256 µg/ml. Three rounds of independence assays consistently demonstrated 4 µg/ml as the MBC. No bacterial growth was observed in positive control.

#### 4.2.2 Screening for Antibacterial Effect of PAM-6 on MDR *P. aeruginosa*

The assay as mentioned in Section 4.2.1 was conducted by using different target bacteria, which was the clinical strain of multidrug resistant (MDR) *P. aeruginosa*. Figure 4.5 (a) demonstrates the cultures of PAM-6-treated MDR *P. aeruginosa* on media plate as well as the negative and positive control. Based on the figure, it was shown that the bacteria were completely killed by PAM-6 at concentrations ranging from 32 µg/ml to 256 µg/ml (Plate A to Plate D). A reduction in the number of bacterial colony as compared to the negative control was observed when the target bacteria were treated with 16 µg/ml of PAM-6 (Plate E). Beyond this dilution (2 µg/ml to 8 µg/ml), the bacteria was able to grow to the extent that was similar to the negative control (Plate I). Based on these findings, the MBC of PAM-6 against MDR *P. aeruginosa* was determined as 32 µg/ml.

Besides that, the microtiter plate in Figure 4.5 (b) shows the overnight PAM-6-treated bacteria before culturing onto media plate as well as the negative (N) and the positive control (P). Based on the visual inspection, bacterial growth was apparent in wells containing bacteria treated with PAM-6 at concentrations ranging from 2 µg/ml to 8 µg/ml, as indicated by the turbidity in those wells. The observation was corresponding to the bacterial culture appearance presented previously in Figure 4.5 (a), where culture media inoculated with bacteria treated with PAM-6 at concentrations from 2 µg/ml to 8 µg/ml displayed heavy growth of bacteria. No bacterial growth was observed when the target bacteria were

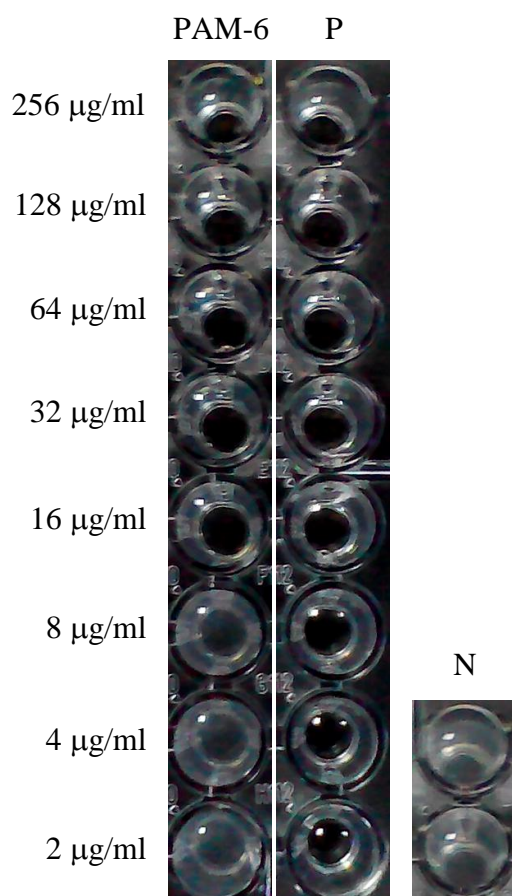
treated with PAM-6 at concentrations ranging from 16 µg/ml to 256 µg/ml. Although no sign of turbidity was observed in the well containing bacteria treated with PAM-6 at 16 µg/ml, but the media inoculated with this culture showed bacterial colonies growing at considerable amount. According to the interpretation as discussed in Figure 3.1, PAM-6 at 16 µg/ml is the MIC to the bacteria.



Bacterial inoculation titer:  $3.95 \times 10^3$  CFU/ml

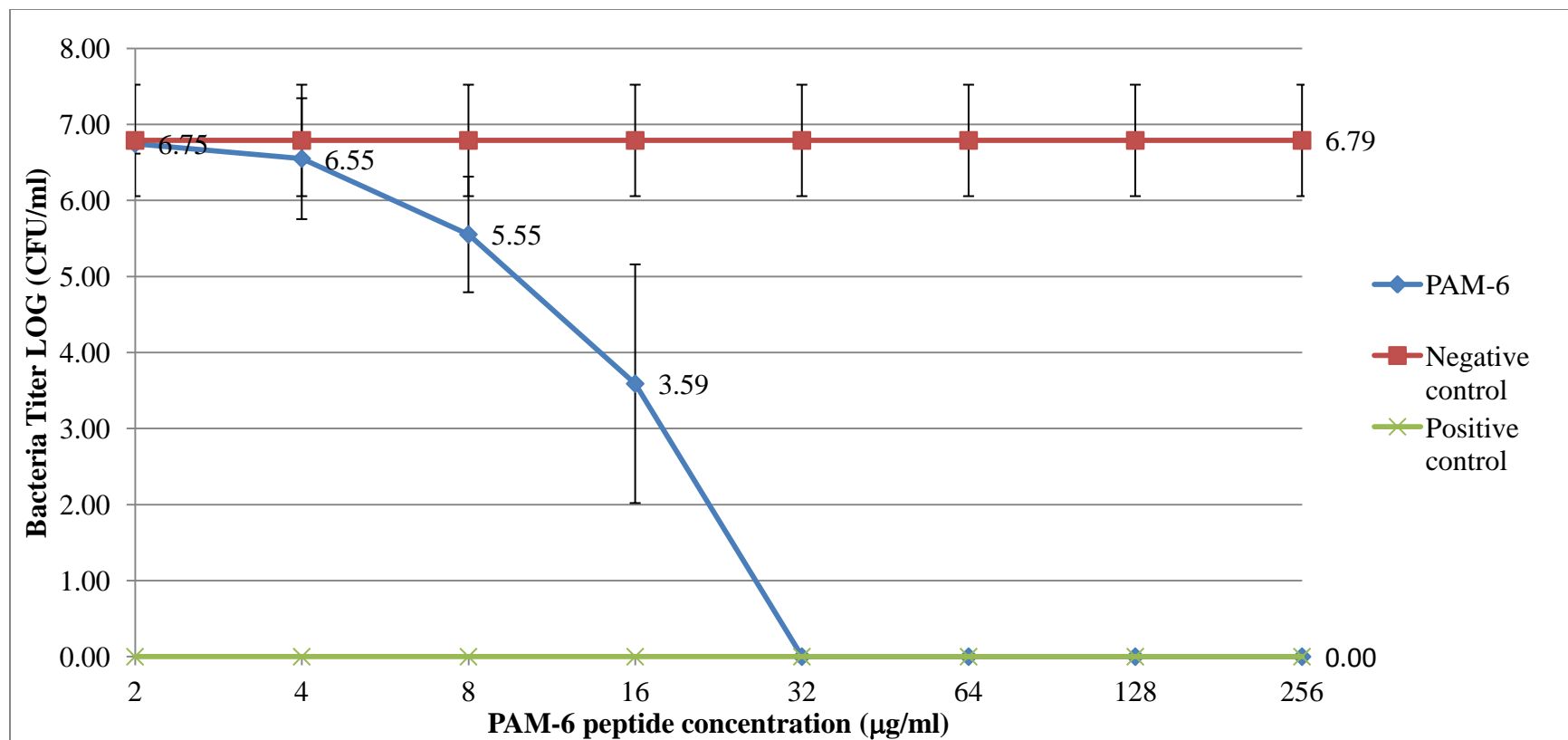
**Figure 4.5 (a): Cultures of MDR *P. aeruginosa* treated with PAM-6.** PAM-6 with concentrations ranging from 32 µg/ml to 256 µg/ml (Plate A to Plate D) showed complete bactericidal effect on the target bacteria as similar to positive control (Plate J). While PAM-6 at 16 µg/ml (Plate E) showed lesser bacterial colonies compared to negative control. No inhibition effect was shown when the bacteria were treated with PAM-6 at concentrations which ranged from 2 µg/ml to 8 µg/ml (Plate F to Plate H) compared with negative control (Plate I).





**Figure 4.5 (b): Cultures of PAM-6-treated MDR *P. aeruginosa* before culturing onto media plates.** PAM-6 with concentrations ranging from 16 µg/ml to 256 µg/ml showed low bacterial viability or absence of bacteria as well as the positive control (P). The bacteria after treating with PAM-6 at concentrations ranging from 2 µg/ml to 8 µg/ml also showed high bacterial viability as well as the PBS-treated bacteria in negative control (N) as indicated by the turbidity in wells. From this figure, the MIC of PAM-6 against the target bacteria was determined at 16 µg/ml.

After repeating this assay for three times, the average results are shown in Figure 4.6. A gradual decreased in the bacterial titer as compared to negative control was observed as the target bacteria were treated with increasing concentrations of PAM-6 from 2 µg/ml to 32 µg/ml. This shows that the peptide was able to inhibit the growth of bacteria but not to kill the bacteria at the lower peptide concentrations. On the other hand, PAM-6 at higher concentrations ranging from 32 µg/ml to 256 µg/ml displayed complete bactericidal effect towards the bacteria where no bacteria was able to survive. The lowest concentration of PAM-6 which showed complete killing towards the MDR *P. aeruginosa* was 32 µg/ml. In another words, the MBC of PAM-6 on MDR *P. aeruginosa* is 32 µg/ml.

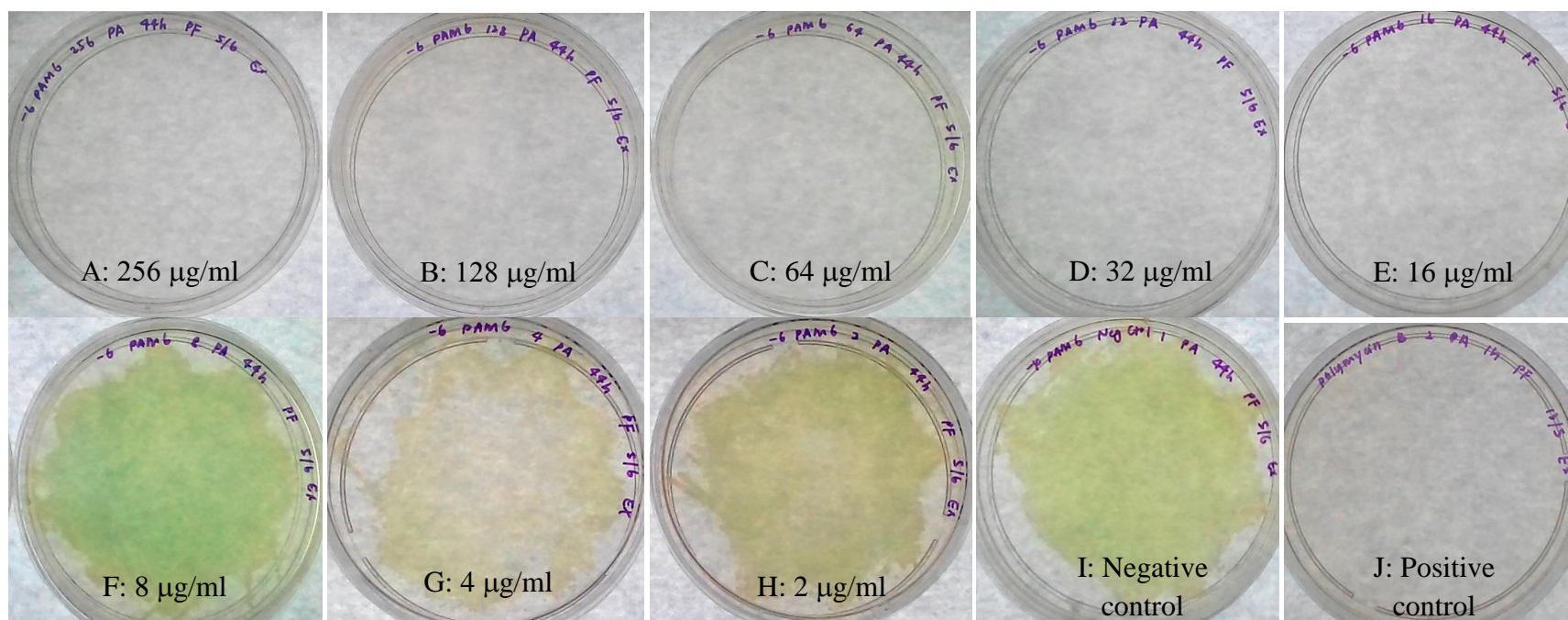


**Figure 4.6: Antibacterial activity of PAM-6 on MDR *P. aeruginosa*.** As the concentrations of PAM-6 increased, the titer of the surviving bacteria decreased as compared to negative control. Based on the graph, the MBC of PAM-6 on MDR *P. aeruginosa* was determined at 32 µg/ml. No bacterial growth was observed in positive control.

### 4.3 Evaluation of PAM-6 Stability in Human Plasma (*ex vivo* Microbroth Dilution Assay)

The stability of PAM-6 in the presence of fresh human plasma was determined by *ex vivo* microbroth dilution assay. By comparing the antibacterial effect of PAM-6 in both *in vitro* and *ex vivo* conditions, the stability of PAM-6 could be estimated.

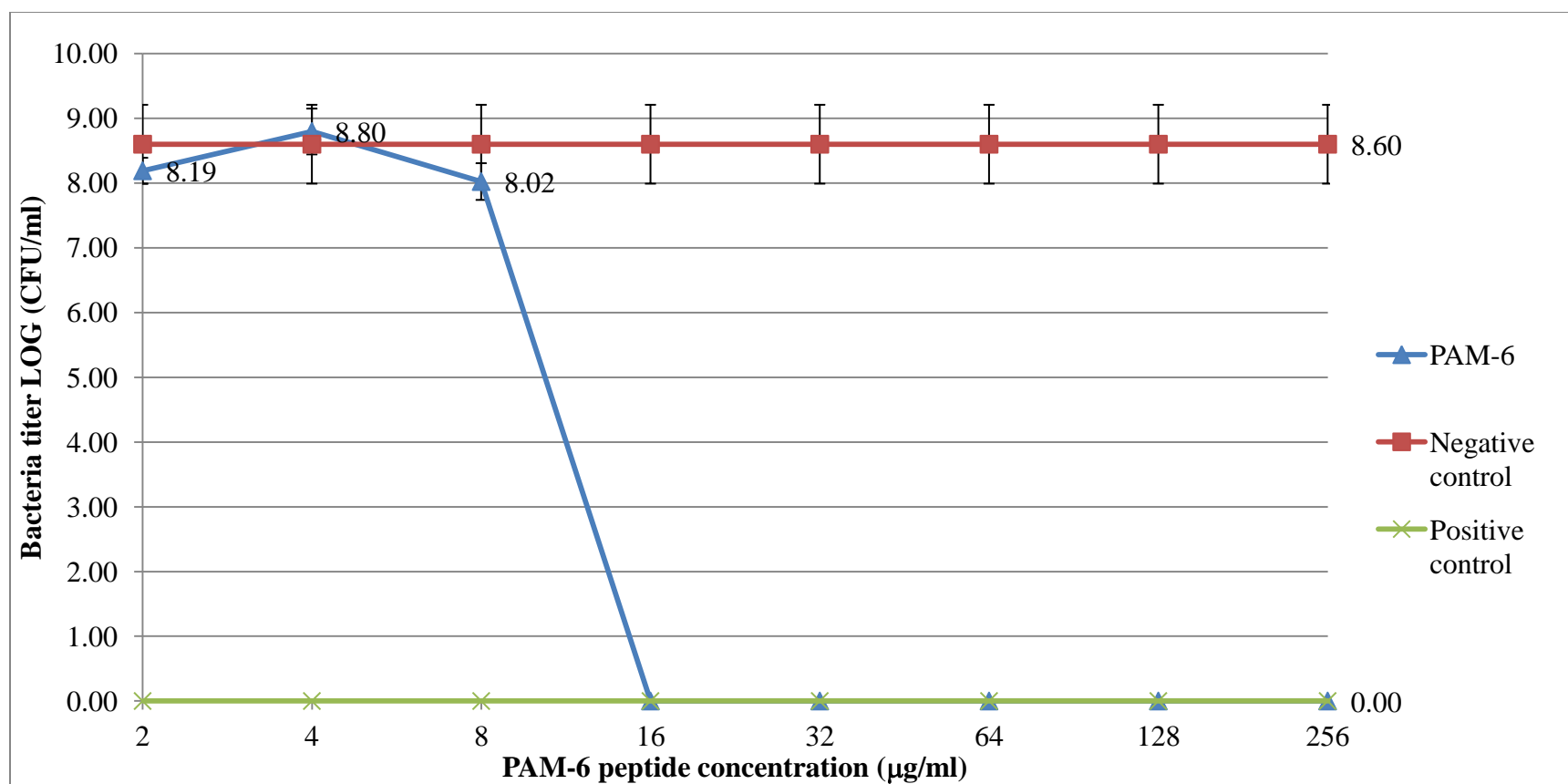
After the target bacteria in human plasma at the titer of  $1.26 \times 10^3$  CFU/ml were treated with PAM-6, the culture was inoculated onto MH media. Figure 4.7 shows the culture plating for peptide-treated bacteria in human plasma, in which PAM-6 at the concentrations ranging from 16  $\mu\text{g/ml}$  to 256  $\mu\text{g/ml}$  (Plate A to Plate E) exhibited complete bactericidal effect on the target bacteria. However, reduced or absence of antibacterial activities were observed at concentrations ranging from 2  $\mu\text{g/ml}$  to 8  $\mu\text{g/ml}$  (Plate F to Plate H) which were reflected by the similar bacteria growth as negative control (Plate I). The MBC of PAM-6 in the presence of plasma was determined at 16  $\mu\text{g/ml}$ , which was four-fold higher than the MBC in *in vitro* assay.



Bacterial inoculation titer:  $1.26 \times 10^3$  CFU/ml

**Figure 4.7: Evaluation of PAM-6 stability in *ex vivo* condition.** A complete bactericidal action was observed when the target bacteria were treated with PAM-6 at concentrations ranging from 16 µg/ml to 256 µg/ml (Plate A to Plate E) whereas low or absence of antibacterial activities was shown at concentrations ranging from 2 µg/ml to 8 µg/ml (Plate F to Plate H) which were corresponding to the negative control (Plate I).

In order to determine the viability of the peptide-treated bacteria, the culture which showed visible growth of bacteria was further diluted and plated on media plates. After triplicate assays were conducted, the average data was plotted in the graph as shown in Figure 4.8. Absence or reduced in antibacterial activity was observed when the bacteria were treated with PAM-6 at concentrations ranging from 2 µg/ml to 8 µg/ml, as indicated by the closely similar bacterial titer comparatively to the negative control. On the other hand, PAM-6 with concentrations ranging from 16 µg/ml to 256 µg/ml was able to kill the target bacteria completely. The lowest concentration of PAM-6 which was able to exhibit complete inhibition towards the target bacteria is at 16 µg/ml. Therefore, 16 µg/ml was considered as the MBC of PAM-6 in the presence of human plasma.



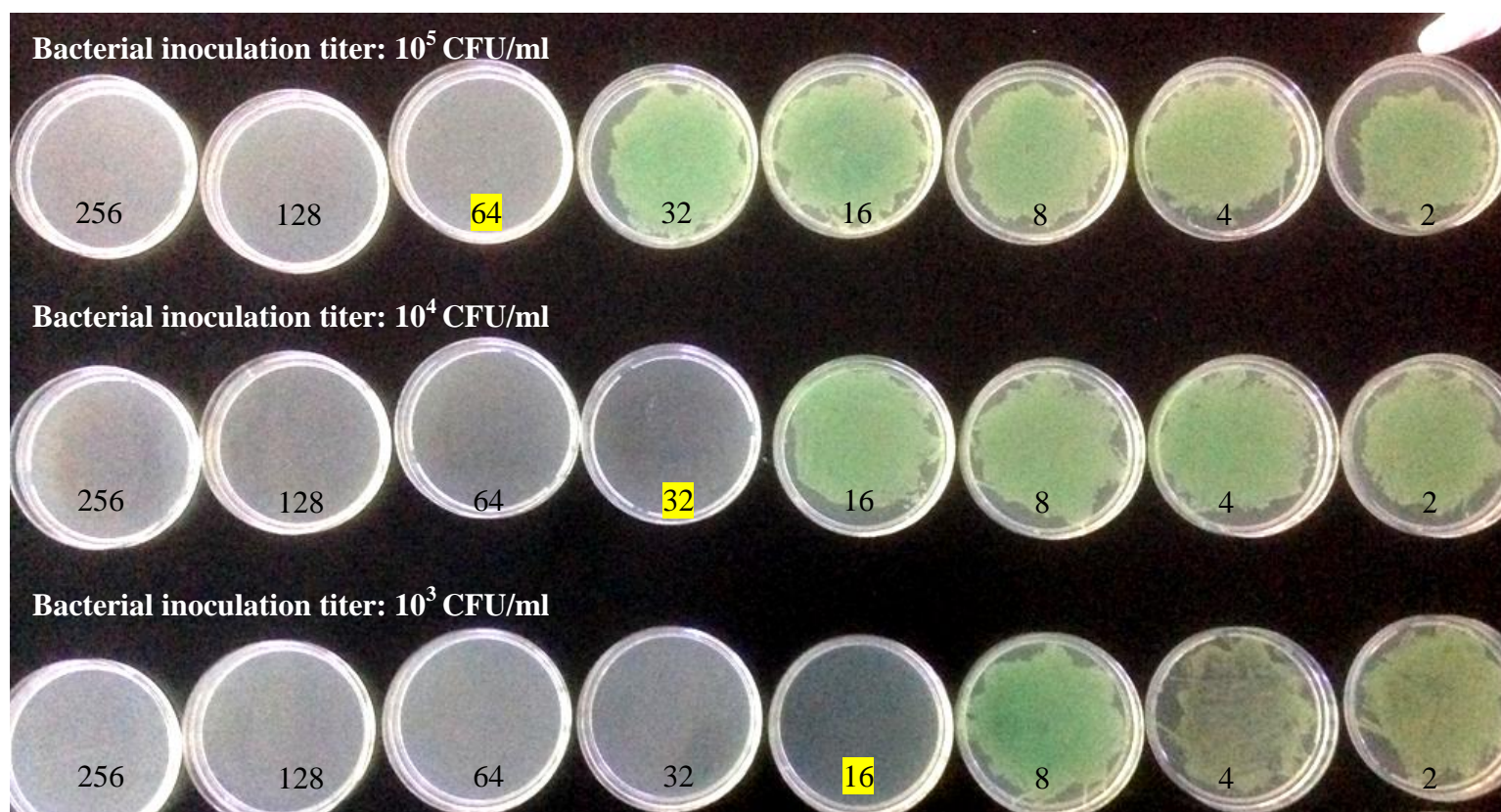
**Figure 4.8: Antibacterial effect of PAM-6 on *P. aeruginosa* ATCC 27853 in the presence of human plasma.** Based on the graph, high bacterial viability was observed while the bacteria was treated with PAM-6 at concentrations lower than 16 µg/ml whereas the peptide at concentrations higher than 16 µg/ml demonstrated complete bactericidal effect on the bacteria. Triplicate assay was performed and the MBC of PAM-6 in this condition was determined as 16 µg/ml.

#### **4.4 The influence of different initial inoculation titer on MBC of PAM-6**

To investigate the relationship of the initial inoculation titer and the MBC of PAM-6, three independent antibacterial assays were carried out by using *P. aeruginosa* ATCC 27853 of different initial inoculation titer treated with the same set of PAM-6 concentrations (2 µg/ml to 256 µg/ml). The outcome of these assays is shown in Figure 4.9. When the bacterial inoculation titer was set at  $10^3$  CFU/ml, the MBC of PAM-6 was at 16 µg/ml. However, the peptide was only able to kill the bacteria completely at 32 µg/ml when the input bacteria was raised to  $10^4$  CFU/ml. On the other hand, same set of peptide concentrations were also used to treat the target bacteria at initial input titer of  $10^5$  CFU/ml. Consequently, the MBC of PAM-6 was determined at 64 µg/ml.

Therefore, the MBC of PAM-6 was proportional to the bacterial inoculation titer.





**Figure 4.9: The relationship between the bacterial initial inoculation titer and the MBC of PAM-6.** All of the numbers labeled on the bottom of the media plates indicating the concentrations of PAM-6 in  $\mu\text{g/ml}$  which was used to treat the bacteria with different input titer. The yellow highlighted numbers representing the MBC of PAM-6 for each assay. When the bacterial titer inoculated increased by ten-fold, the MBC of PAM-6 was also increased by two-fold. The MBCs of PAM-6 were at 64  $\mu\text{g/ml}$ , 32  $\mu\text{g/ml}$  and 16  $\mu\text{g/ml}$  where the bacterial titer inputs were at  $10^5$  CFU/ml,  $10^4$  CFU/ml and  $10^3$  CFU/ml, respectively.

## CHAPTER 5

### DISCUSSION

In spite of the development of potent conventional antibiotics, bacterial infectious diseases still remain as a global health problem due to the continual emergence of multidrug resistant strains of bacteria. *Pseudomonas aeruginosa* is receiving special attention because of its notorious features that could facilitate the development of antibiotic resistance. For this reason, development of novel antibacterial agent against this bacterium should be taken as precedence instead of modifying or improving the pre-existing conventional antibiotics. One of the potential candidates for the alternative antibacterial agent is antibacterial peptides (ABPs). In this study, PAM-6 was designed via modification of two previously proposed ABPs, namely PAM-1 (WPVKKWKWALRM) and PAM-2 (RPVKKWKRIILRM) by Tan (2014). In her study, PAM-1 and PAM-2 were less potent ABPs as these peptides only kill *P. aeruginosa* at very high minimum bactericidal concentration (MBC), which might be toxic to mammalian cells.

Thus, in this study, several modifications were done to PAM-1 and PAM-2 by increasing the peptide length from 12-mer to 15-mer. The resulted new peptide, namely PAM-6 (RPRGKLRWKLRLVLRM) came with higher cationicity (+7) and moderate hydrophobicity (40%) as compared to the former two. These enhanced features render PAM-6 with criteria that fulfill the requirement of an ideal

antibacterial peptide (Bahar and Ren, 2013). It is estimated that this newly synthesized peptide would demonstrate a better potency against *P. aeruginosa*.

## **5.1 Antibacterial Effect of PAM-6 *in vitro***

### **5.1.1 Antibacterial Effect of PAM-6 on *P. aeruginosa* ATCC 27853**

As PAM-6 demonstrates common characteristics of an antibacterial peptide, microbroth dilution assay was carried out to investigate whether PAM-6 could exert antibacterial activity *in vitro*. After several repeated and independent assays, it was shown that PAM-6 was able to exert bactericidal effect on *P. aeruginosa* ATCC 27853 *in vitro*, with the MBC of 4 µg/ml. This finding clearly indicates that PAM-6 was more potent than its original template peptides from which it was derived from, PAM-1 and PAM-2. The latter two were able to exert complete bactericidal action only at 512 µg/ml, a concentration which might be toxic towards mammalian cells (Tan, 2014).

The better potency of PAM-6 compared to its parental peptides can be explained by the differences in the physiochemical properties between the two generations of peptides (refer to Table 4.1). There are two main factors that may contribute to the different potency of antibacterial peptides.

The primary determinant of the peptide antibacterial potency is the peptide cationicity (Deslouches, 2005). PAM-6 was designed by increasing the peptide length with enhanced peptide cationicity. The increased cationicity was achieved by substituting and adding positively-charged amino acid residues such as arginine (R) and lysine (K) into the original peptides as shown in Table 4.1. As a result, this may promote stronger electrostatic interaction between the peptide and the anionic bacterial membrane. This finding corresponds to the study by Ueno and colleagues (2011) who found that engineered peptides which are more cationic could exert better antibacterial potency. Therefore, PAM-6 is a more potent ABP as compared to its parental peptides.

Besides peptide cationicity, the peptide hydrophobicity also plays an important role in determining the antibacterial potency of an ABP (Chan, Prenner and Vogel, 2006). The hydrophobicity of an ABP has to be maintained at optimum range, and beyond this range, the antibacterial activity would be affected (Bahar and Ren, 2013). As mentioned earlier, PAM-6 conferred hydrophobicity at moderate level which is relatively lower than its parental peptides, PAM-1 and PAM-2. ABPs with higher hydrophobicity tends to associate with each other before binding to the bacterial membrane and hence reduces the effective concentration of the peptide that is required to disrupt the bacterial membrane. Thus, this leads to the reduced antibacterial activity of the peptide in bacteria (Yin, et al., 2012). In this study, PAM-6 with relatively lower hydrophobicity could exert greater antibacterial effect as compared to its parental peptides.

### 5.1.2 Antibacterial Effect of PAM-6 on MDR *P. aeruginosa*

PAM-6 was also able to exert bactericidal effect on clinical strain of MDR *P. aeruginosa*. This peptide was able to kill the bacteria completely at MBC of 32 µg/ml but concurrently exerting bacteriostatic effect as well on the pathogen at the MIC of 16 µg/ml. In particular, PAM-6 was less potent in killing MDR *P. aeruginosa* than the wild type of *P. aeruginosa* (ATCC 27853) which was reflected by a higher MBC as compared to the latter.

The lower potency of PAM-6 in killing MDR *P. aeruginosa* can be explained by a possible factor. Several studies on different clinical isolates of MDR *P. aeruginosa* demonstrated a similar resistant mechanism towards cationic antibacterial peptides (McPhee, Lewenza and Hancock, 2003; Moskowitz, Ernst and Miller, 2003; Moskowitz, et al., 2011). These clinical isolates of *P. aeruginosa* were found to have mutated *pmrB* gene which may cause constitutive expression of aminoarabinose that is positively-charged. Subsequently, the mutated gene leads to the addition of aminoarabinose to the anionic bacterial membrane which may cause the bacterial membrane to become less negatively-charged and hence decrease the binding tendency of cationic ABPs to bacterial membrane (Moskowitz, et al., 2011; Yu, et al., 2014). Therefore, higher concentration of PAM-6 was needed to compromise the bacterial membrane of MDR *P. aeruginosa* and thus, resulting in higher MBC of PAM-6.

## 5.2 Stability of PAM-6 in Human Plasma

While PAM-6 possesses promising bactericidal effect *in vitro*, the *in vivo* potency of this peptide is also a main concern in this study. As human blood may contain factors such as protein or nucleic acid, the activity of ABPs might be compromised (Sainath Rao, et al., 2013). Besides that, degradative enzymes such as protease in the blood may degrade or deactivate ABPs as well (Kim, et al., 2013). Therefore, all these blood factors might reduce the potency of PAM-6 *in vivo*. Hence, the *in vivo* condition was simulated by using *ex vivo* assay in order to evaluate the antibacterial activity of PAM-6 in blood matrices.

Upon treating *P. aeruginosa* with PAM-6 in the presence of human plasma, it was found that the lowest concentration of PAM-6 that was able to kill the bacteria completely was 16 µg/ml. This concentration was four folds higher than the MBC of PAM-6 in the *in vitro* microbroth dilution assay, thus suggesting that the peptide might be unstable in plasma. As the plasma was derived from fresh blood collected prior to the assay, the interfering factors mentioned above might still present in considerable amount. It is possible that PAM-6 is compromised by these inhibitors, thus requiring higher concentration of the peptide to exert the similar bactericidal effect.

Nonetheless, the lower potency of PAM-6 in plasma can be explained by another variable factor implicated in the *ex vivo* assay, which is salinity of Alsever's

solution. Alsever's solution is anticoagulant which contains sodium ions that built up the salinity in the culture environment of PAM-6. The presence of these ions might interfere with the antibacterial effect of the peptide. According to the findings by Kandasamy and Larson (2006), increased salinity may lead to decreased in available space for peptide binding in the phospholipid bilayer as sodium ions are able to bind to the lipid head group. The positive charge of sodium ion masks the anionic membrane of bacteria and thus, weakens the binding strength between the peptide and the bacterial membrane. Therefore, greater amount of antibacterial peptides is required to kill the same amount of bacteria in high salt culture medium. This might explain the higher MBC of PAM-6 (16 µg/ml) required to kill the bacteria completely in the *ex vivo* assay.

### **5.3 Relationship of Initial Inoculating Bacterial Titer and MBC of PAM-6**

In order to study the influence of bacterial input titer on the MBC of PAM-6, the antibacterial assay was repeated by using initial bacterial input titer at different orders of magnitude ( $10^3$ ,  $10^4$  and  $10^5$  CFU/ml). Clearly demonstrated in Section 4.4, different bacterial input titer at different order of magnitude did influence the MBC of PAM-6. The decreased efficacy of PAM-6 was indicated by higher MBC when the bacterial titer input was set at higher order of magnitude. In another words, greater amount of PAM-6 was required to kill or inhibit the bacteria with higher inoculation titer. This observation suggested that the efficacy of PAM-6 is subjected to inoculum effect. The inoculum effect is described as the decreasing

efficacy of an antibacterial agent that caused by increasing initial bacterial inoculating titer (Tan, et al., 2012). In particular, the inoculum effect is always associated with those antibiotics that targeting the bacterial membrane such as beta-lactams (Chin, et al., 2007). Therefore, this finding may be a reflection of mechanism of action by PAM-6.

#### **5.4 Future Studies**

As PAM-6 possesses potent antibacterial effect towards both reference strain and multidrug-resistant strain of *P. aeruginosa*, it is worth knowing that this peptide is able to exert antibacterial activity on other pathogenic bacteria such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus* and other. Therefore, the study of broad spectrum antibacterial activity of PAM-6 should be considered. It is an important finding if PAM-6 is able to kill pathogenic bacteria from both gram-negative and gram-positive category, a feature which is yet to be achieved by many conventional antibiotics.

In this study, Alsever's solution was used as an anticoagulant for the blood in *ex vivo* assay. However, the sodium ions in this solution may reduce the antibacterial effect of PAM-6. Thus, in future, the *ex vivo* assay should be carried out by replacing the Alsever's solution with EDTA anticoagulant.



Besides that, pharmacodynamic properties of PAM-6 such as time-kill kinetics could be carried out. This study enables the investigation of the rate of bacterial killing by PAM-6 at particular concentration. A peptide that exhibits rapid killing towards bacteria may reduce the possibility in developing bacterial resistance towards the peptide.

Moreover, the protease resistance of PAM-6 can be evaluated. These pharmacokinetic properties play a vital role in determining the bioavailability and the efficacy of PAM-6. This assay can be carried out by incubating the peptide and the target bacteria in the presence of purified trypsin, chymotrypsin or aureolysin. By understanding the susceptibility of PAM-6 to these substances, the peptide can be further modified to enhance its stability.

In spite of its good potency in bactericidal effect, the toxicity of PAM-6 should be taken into consideration to ensure the safety of PAM-6 in clinical application. An ideal peptide should possess selective toxicity against bacteria without targeting to the host cells. Therefore, cell viability assay on selected mammalian cell lines which utilizing MTT and Prestoblue<sup>TM</sup> reagent should be carried out.

Lastly, it is worth to understand the killing mechanisms that possessed by PAM-6 in order to predict the target of the peptide on bacteria. Several mechanisms of

action such as membrane depolarization, membrane lysis, ATP synthesis inhibition or disruption of other intracellular target or metabolic activity in the bacteria can be investigated.

## CHAPTER 6

### CONCLUSION

In summary, PAM-6 (RPRGKLRWKLRVLRM) had been successfully designed with enhanced cationicity and moderate hydrophobicity from its parental peptides PAM-1 and PAM-2. PAM-6 exhibits better potency in killing reference strain of *P. aeruginosa* ATCC 27853 than PAM-1 and PAM-2 as indicated by a much lower MBC at 4 µg/ml. Apart from that, PAM-6 is also potent in killing clinical strain of MDR *P. aeruginosa* at MBC of 32 µg/ml. This significant finding suggests that PAM-6 is a potential alternative to conventional antibiotics in treating infections by *P. aeruginosa*. However, the stability of PAM-6 in human plasma is yet to be determined as Alsever's solution that containing substantial sodium ions may cause reduced antibacterial activity of PAM-6. Finally, PAM-6 is influenced by the inoculum effect which was demonstrated by the decreased of potency and efficacy of its antibacterial activity with increased initial bacterial titer inoculated.

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## **APPENDIX A**

### **PREPARATION OF BUFFERS AND REAGENTS**

#### **Mueller-Hinton (MH) broth**

The MH broth powder from HiMedia Laboratories Pvt. Ltd. was weighed at 8.4 g and dissolved in 400 ml of distilled water. Then, the medium was autoclaved at 121°C and 15 psi for 20 min.

#### **Mueller-Hinton (MH) agar**

A total of 30.4 g of Difco™ MH agar powder was weighed by using an electronic balance and then dissolved in 800 ml of distilled water. The medium was then autoclaved at 121°C and 15 psi for 20 min before the medium was poured into petri dishes. Once the agar was solidified, the agar plate will be dried in the laminar flow for 45 min before stored in 4°C.

**Phosphate Buffered Saline (PBS) solution (pH 7.4)**

Four tablets of PBS tablet manufactured by MP Medicals, LLC which were free from magnesium and calcium were dissolved in 400 ml of distilled water. The solution was then autoclaved at 121°C and 15 psi for 20 min.

**Alsever's solution**

Two grams of D-glucose (System), 0.8 g of sodium citrate (System), 0.5 g of citric acid (Fisher Scientific UK limited) and 0.42 g of sodium chloride (System) was mixed and dissolved in 100 ml of distilled water. The solution was autoclaved at 121°C and 15 psi for 20 min before filter-sterilized by using 0.22 µm of nylon syringe filter.

## APPENDIX B

### LIST OF LAB WARES AND EQUIPMENT USED WITH THE RESPECTIVE MANUFACTURERS

Lab wares/ Equipments	Manufacturers
2 <sup>nd</sup> level Biosafety Cabinet	TELSTAR, Philippines
Spectrophotometer	Genesys, US
Avanti J-E Centrifuge	Beckman Coulter, US
Incubator	Memmert, Germany
Vortex mixer	VELP <sup>®</sup> Scientific, Europe
Microplate reader	BMG Labtech, Australia
96-well plate, flat-bottomed	Becton Dickson, USA
Beaker	GQ, Malaysia
Bunsen burner	Campingaz, UK
Falcon <sup>™</sup> tube	BD Bioscience, USA
Measuring cylinder	GQ, Malaysia
Micropipette set	Biohot, USA
Micropipette tip	Axygen <sup>®</sup> Scientific, USA
Petri dish	Labmart, Malaysia
Schott bottle	DURAN <sup>®</sup> , Germany