# STUDY OF KINETIC KILLING OF ANTIBACTERIAL PEPTIDE PAM-5 ON *Escherichia coli* AND *Pseudomonas aeruginosa*

By

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## ABSTRACT

### STUDY OF KINETIC KILLING OF ANTIBACTERIAL PEPTIDE PAM-5 ON *Escherichia coli* AND *Pseudomonas aeruginosa*

**NG WEI NEE**

Antibiotics are the conventional antibacterial agents used to treat bacterial infections since many decades ago. However, the effectiveness of these antibacterial compounds is slowly being compromised with the increasing incidence and prevalence of drug and multidrug-resistant bacteria. Apart from the abuse usage of antibiotics in clinical setting, the slow killing kinetic by these compound might contributes to the mutational-induced resistance among the bacteria. Hence, there is a need to develop an alternative antibacterial agent that kills the bacteria rapidly. Among the alternative antibacterial agents that have been studied, antibacterial peptides (ABPs) is regards as a potential alternative due to their rapid bactericidal effects that is able to minimize the likelihood of resistance development. In this study, the killing kinetic of a novel 15-mer synthetic antibacterial peptide, PAM-5 against *Escherichia coli* and *Pseudomonas aeruginosa* was studied using time-kill assay. In addition, the killing kinetics between PAM-5, gentamicin (aminoglycosides) and polymyxin B were also compared. In short, the minimal bactericidal concentrations (MBCs) of PAM-5, gentamicin and polymyxin B on the two bacteria were determined using microbroth dilution antibacterial assay. Subsequently, time kill assays were performed in which the bacteria were

treated with the antibacterial agents. Upon treatment, the bacteria were inoculated on Mueller-Hinton (MH) agar at each 10 minutes interval, with a total duration of 60 minutes. The findings of the assay revealed that PAM-5 was able to kill both the bacteria completely within 10 minutes. Moreover, PAM-5 demonstrated faster killing of the bacteria than gentamicin and polymyxin B. Hence, PAM-5 is more potent as compared to gentamicin and polymyxin B in terms of killing kinetic. This indicates that PAM-5 is potential to be further studied and developed into an alternative antibacterial agent against bacterial infections.

## ACKNOWLEDGEMENT

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Furthermore, I appreciate all the mental support and love provided by my family. Last but not least, I truly appreciate my lovely friends who always help me during my final year project.

## DECLARATION

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

_____

Ng Wei Nee

## APPROVAL SHEET

This project report entitled **"STUDY OF KINETIC KILLING OF ANTIBACTERIAL PEPTIDE PAM-5 ON *Escherichia coli* AND *Pseudomonas aeruginosa*"** was prepared by **NG WEI NEE** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by,

_____

Mr. Yuen Hawk Leong Date: 25th September 2018

Supervisor

Department of Biomedical Science

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**FACULTY OF SCIENCE**

**UNIVERSITI TUNKU ABDUL RAHMAN**

Date: 25th September 2018

**PERMISSION SHEET**

It is hereby certified that **NG WEI NEE** (ID Number: 15ADB07234) has completed this final year project entitled **"STUDY OF KINETIC KILLING OF ANTIBACTERIAL PEPTIDE PAM-5 ON *Escherichia coli* AND *Pseudomonas aeruginosa*"** supervised by Mr. Yuen Hawk Leong (Supervisor) from Department of Biomedical Science, Faculty of Science.

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

Yours truly,

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(NG WEI NEE)

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## LIST OF ABBREVATIONS

| | |
|---|---|
| ABPs | Antibacterial peptides |
| ATCC | American Type Culture Collection |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Standards Institute |
| Da | Dalton |
| DNA | Deoxyribonucleic acid |
| G | Glycine |
| K | Lysine |
| L | Leucine |
| LB | Luria-Bertani |
| LPS | Lipopolysaccharide |
| M | Methionine |
| MBC | Minimum bactericidal concentration |
| MIC | Minimum inhibitory concentration |
| MH | Mueller-Hinton |
| OD | Optical density |
| P | Proline |
| PBS | Phosphate buffered saline |
| R | Arginine |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| V | Valine |
| W | Tryptophan |

# CHAPTER 1

## INTRODUCTION

Conventional antibiotics have been widely used to treat bacterial infections since many decades ago. However, these compounds are losing their effectiveness towards many pathogenic bacteria due to their slow rate of antibacterial activities. (Chan et al., 2006). Slow rate of bacteriostatic or bactericidal action from antibiotics might increase the risk of acquired resistance by the fast-replicating bacteria via mutation. Along with the abuse usage of antibiotics in clinical setting and non-compliance of antibiotic consumption by the patients, this intrinsic limitation of antibiotics may become another factor contributing to the high prevalence and incidence of antibiotic resistance worldwide.

Hence, it is crucial to explore or develop novel alternative antibacterial agents that kill the bacteria rapidly before they acquire mutational resistance. Among the alternative antibacterial agents, antibacterial peptides (ABPs) are promising candidates which have been studied extensively since 1939 (Dubos, 1939). One of the great advantages of ABPs over conventional antibiotics is the ability to kill bacteria rapidly, thus minimizing the risk of bacterial resistance towards these compounds.

Previously, a novel 15-mer synthetic peptide, PAM-5, was shown to have broad spectrum of antibacterial effect against Gram-negative (Chan, 2016) (data unpublished) and drug-resistance pathogenic bacteria (Yong, 2018) (data unpublished). According to the study by Phoon (2016) (data unpublished), PAM-5 exerts its bactericidal effect through membrane disruption and permeabilization. In addition, the mode of action is further contributed by its ability to bind to bacterial DNA when it is present at high concentrations (Tan, 2018) (data unpublished). Nonetheless, the aspect of kinetic killing by this novel ABP is yet to be elucidated. Time-kill kinetics is referred as the time required by an antibacterial agent to kill the bacteria. It is used to study the efficacy of the antibacterial agent in relation to time (Peterson, 2006).

Since PAM-5 possesses membrane active mechanism and DNA binding ability on its target bacteria, it is speculated to exert faster killing effect as compared to other bactericidal antibiotics. Therefore, the objectives of this project are:

1. To study the kinetic killing of PAM-5 on *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 at its respective minimal bactericidal concentration (MBC) via plate count method.

2. To compare the kinetic killing between PAM-5 and two bactericidal antibiotics, gentamicin and polymyxin B.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Overview of Antibiotic Resistance

Antibiotics have saved millions of lives since the discovery of penicillin by Alexander Fleming in 1928 (Sengupta et al., 2013). However, the abuse usage of antibiotics in clinical setting and agriculture sector has led to the increase in incidence and prevalence of antibiotic resistance among many clinically isolated bacteria (Ventola, 2015). This medical issue has seriously limited the choice of effective therapy against many bacterial infections, especially for patients with immunodeficiency or immunocompromization due to chemotherapy. According to World Health Organization (2018), the mortality rate caused by antibiotic-resistant bacteria has been increasing worldwide. Clinical scientists have warned if the issue is not given serious attention, the predicted global mortality rate in 2050 may reach 10 million (de Kracker et al, 2016).

## 2.2 Limitations of Conventional Antibiotics

Although antibiotic-resistance is always associated with uncontrolled use of the compounds in healthcare setting and agriculture livestock, the limitation of the antibiotics also should be given considerable attention. Most of the conventional antibiotics are narrow spectrums in which they are only active against a selected group of bacteria. This limitation is always associated to the difficulty of deciding suitable initial empiric therapy against bacterial infection when the causative agent is yet to be identified (Leekha et al., 2011). Apart from that, many conventional antibiotics come with single mechanism of action, which only acts on a specific target of the bacteria (Fair and Tor, 2014). Bacteria can undergo mutation to alter the target sites of antibiotics and acquired resistance towards the antibiotics (Lambert, 2005). Moreover, antibiotics kill or inhibit the bacteria relatively slow as compared to the bacteria generation time. Generally, the generation time for majority of the culturable bacteria ranges from 15 minutes to 1 hour (Todar, 2013), while antibiotics require hours to exert their bactericidal or bacteriostatic effect (Wiuff and Andersson, 2006). In each generation time, bacteria will reproduce and evolve rapidly in response to the microenvironmental changes or stresses. The short generation time may allow the bacteria to undergo mutation and acquire resistance before they are completely killed by the slow acting antibiotics (Beatriz et al., 2015).

As the results, these antibiotic limitations are partially contributing to the emergence of multidrug-resistance bacteria (World Health Organization, 2018). Therefore, there is an urgent need to explore or develop alternative antibacterial agents that kill bacteria rapidly in order to minimize the risk of bacterial resistance. One of the well-studied alternative antibacterial agents is antibacterial peptides (ABPs).

## 2.3 Antibacterial Peptides (ABPs)

### 2.3.1 Overview

Antibacterial peptides (ABPs) are peptides that possess bacteriostatic or bactericidal effect. They were discovered as part of the innate immune effector molecules in insects, amphibians and mammals that defend them against infections by bacteria, virus and fungus (Bahar and Ren, 2013). ABPs can be naturally isolated or chemically synthesized (Diehnelt, 2013). ABPs are usually short peptides that are made up of 12 to 50 amino acid residues. Majority of the ABPs are amphipathic and cationic in nature with a net charge ranging from +2 to +9 (Midura-Nowaczek and Markowska, 2014). The amphipathicity and cationicity of ABPs play an important role in their mode of action on the bacterial membrane. Generally, cationic ABPs tend to form initial contact with the anionic bacterial membrane through electrostatic interaction. Following that, ABPs will adsorb to the surface of bacterial membrane at low peptide-to-lipid ratio. Then, the ABPs will start to insert into

the bacterial membrane once the peptide-to-lipid ratio increases and reaches a particular threshold. Hydrophobic side chain of ABPs provides lipophilic anchors to the bacterial membrane and induces membrane disruption by causing pore formation which leads to the leakage of intracellular content (Tam et al., 2002; Brown and Hancock, 2006; Cézard et al., 2011). This membrane-active mechanism represents the common action of many ABPs, which leads to bacterial death.

### 2.3.2 Advantages of ABPs

ABPs come with various advantages that made them a potential alternative antibacterial agent other than antibiotics. First of all, due to the cationic nature of ABPs, ABPs possess selective toxicity towards bacteria but not towards host mammalian cells (Epand et al., 2010). Bacterial cytoplasmic membrane is composed of high proportion of negatively-charged lipids, which plays an essential role in the selective toxicity of ABPs towards bacterial cells. As mentioned earlier, the cationic side chains of ABPs will promote electrostatic interactions between the peptides and the anionic phosphate group of lipopolysaccharide (LPS) on the membrane of Gram-negative bacteria, or lipoteichoic acids on the cell wall of Gram-positive bacteria (Jenssen et al., 2006). In contrast, ABPs bind lesser towards mammalian cell membranes which are commonly zwitterionic (Yeaman and Yount, 2003). Besides, high level of cholesterol present in mammalian cell membrane also plays a role in the selective toxicity of ABPs by rigidifying the lipid bilayer structure and

prevents the membrane disruption by the peptide (van Meer et al., 2008; Verly et al., 2008).

In comparison to antibiotics, ABPs kill their target bacteria via multiple cellular targets (Teixeira et al., 2012). ABPs are well known for their non-receptor-mediated membrane-lytic activity which leads to bacteria death (Yeaman and Yount, 2003). Apart from membrane disruption, ABPs exert their bactericidal effect through intracellular targeting (Hale and Hancock, 2007). For example, Buforin II, an ABP derivative isolated from Asian toad, can penetrate bacterial membrane and inhibit cellular processes by interfering with DNA and RNA metabolism (Park et al., 1998). Another ABP that shows multiple killing mechanisms is human neutrophil peptide-1 (HNP-1). In addition to its ability to permeabilize outer and inner membrane of *Escherichia coli*, this ABP is also able to bind to DNA, RNA and interfere with protein metabolism (Lehrer et al., 1989). A hybrid peptide, DM3, was shown to exhibit broad-spectrum of bactericidal activity by affecting DNA replication and transcription, amino-acid biosynthesis pathway, ribosomal rRNA subunits and down-regulate RNA polymerase sigma factor (Le et al., 2016). Overall, the ability of ABPs to act on multiple target sites on/in the bacteria may decrease the likelihood of ABP resistance as it is metabolic costly for a bacterium to alter multiple target sites simultaneously to avoid the actions of ABPs (Marr et al., 2006).

ABPs exert broad spectrum of activity against pathogenic microorganism (Narayana and Chen, 2015). According to a study by Bharal and Sohpal (2013), bacteriocin, an antimicrobial peptide produced by *Lactobacillus acidophilus*, showed bactericidal effect against *Salmonella* Typhi, *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. In addition, it also showed bacteriostatic effect towards *Streptococcus faecalis* and *Streptococcus pyogenes*. EC5, a synthetic 12-mer ABP derived from phage displayed-peptide with high binding affinity to *Escherichia coli*, was shown bacteriostatic towards different strains of *Escherichia coli* and *Pseudomonas aeruginosa* (Sainath Rao et al., 2013). Similar findings were also found for Salusin-β, an endogenous parasympathomimetic peptide with broad spectrum of antibacterial activity against many species of Gram-positive bacteria (Kimura et al., 2014). These findings potentiate the use of ABPs in clinical setting, especially for empiric treatment before the identity of the causative bacterial agent is identified.

In contrast to the slow killing or inhibitory effects by antibiotics, the ability of ABPs to induce rapid killing of bacteria serves as another advantage that may minimize the risk of acquired resistance among the bacteria (Narayana and Chen, 2015). As mentioned earlier, the extensive membrane disruption by ABPs may serve as one of the important factors that contribute to the fast killing of bacteria. In addition, the action of ABPs on multiple cellular targets may further contribute to the rapid killing (Yan et al., 2012). As the bacteria are killed even before their doubling time, it is unlikely that the bacteria may

acquire inducible resistance in such a short period of time before they are killed.

Due to the possibility that ABPs may be able to minimize the likelihood of drug-resistance bacteria (Mohamed et al., 2016) and the above mentioned advantages, ABPs are highly recommended to be developed as an alternative antibacterial agent.

### 2.3.3 Previous Findings on the Time-kill Kinetics of ABPs

Numerous studies on ABPs have been documented since their discovery decades ago. Most of these studies are focused on their antibacterial efficacy, mechanism of action, structure-function relationship and peptide toxicity. However, very few studies are conducted on the time-kill kinetic of ABPs. Time-kill kinetic of ABPs is referred to the duration of time needed by ABPs to exert complete killing of bacteria, which provides knowledge on the antibacterial pharmacodynamics and pharmacokinetics of ABPs. This information is important to determine the suitable dosing interval at minimal inhibitory concentrations (MICs) or minimal bactericidal concentrations (MBCs) of the antibacterial agent (Levison and Levison, 2009).

As mentioned earlier, there are only few reports on the kinetic killing of ABPs. From a study conducted by Sainath Rao et al. (2013), a phage displayed-peptide EC5 with cationicity of +7 and hydrophobicity of 41%, was shown to reduce the growth of *E. coli* and *Pseudomonas aeruginosa* by 5 $\log_{10}$ reduction within 5 minutes at the MIC of 8 μg/ml. Next, a similar study conducted by Mohamed et al. (2016) showed that a novel 12-mer synthetic peptide, namely WR12, was able to exhibit complete killing towards methicillin-resistant *Staphylococcus aureus* (MRSA) within 30 minutes. Apart from that, a linear 16-mer α-helical antimicrobial peptide, T9W, demonstrated complete killing towards different strains of *P. aeruginosa* within 30 minutes (Zhu et al., 2015). These findings strongly suggested that ABPs may exert better pharmacokinetic activity as compared to conventional antibiotics, thus raising hopes for more promising candidates of alternative antibacterial agent.

### 2.3.4 Synthetic Peptide PAM-5

PAM-5 is a 15-mer synthetic peptide with the sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M. This peptide possesses a cationicity of +7 and hydrophobicity of 46% (Lee, 2015) (data unpublished), which are the common characteristics for a highly potent ABP. Originally, this peptide was derived from a phage displayed-peptide isolated from a biopanning process in which several phage clones displaying high binding affinity towards *Pseudomonas aeruginosa* were selected from a phage displayed-peptide library. The oligonucleotide that encoded the phage displayed-peptides was sequenced and

the corresponding peptide sequences were deduced. Using the online Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php), the sequence of the phage displayed-peptide was modified and elongated to 15 amino acids in length, and was eventually named as PAM-5.

In the earlier studies, PAM-5 was tested for its potency against several Gram-negative pathogenic bacteria. In the study conducted by Chan (2016) (data unpublished), PAM-5 was able to kill a range of Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Shigella flexneri* at different minimal bactericidal concentrations (MBCs). Another study conducted by Yong (2018) (data unpublished) showed that PAM-5 was able to exert bactericidal effects towards a range of drug-resistance pathogenic bacteria which include carbapenem-resistant Enterobacteriaceae *Klebsiella pneumoniae*, extended-spectrum β-lactamases producing *Escherichia coli*, cefazolin-resistant *Pseudomonas aeruginosa* and clinical isolate *Salmonella* Typhi. Subsequently, the mechanisms of action by this peptide were revealed by two separate studies, which included outer membrane disruption and inner membrane permeabilization (Phoon, 2016) (data unpublished) as well as DNA-binding (Tan, 2018) (data unpublished). By possessing more than one mechanism of antibacterial action, it is believed that PAM-5 may exert rapid killing effect towards its target bacteria. Therefore, in this study, PAM-5 was studied for its time-kill kinetics on selective Gram-negative bacteria.

# CHAPTER 3

# MATERIALS AND METHODOLOGY

## 3.1 General Overview of Experimental Design

PAM-5, a novel 15-mer synthetic peptide which was previously confirmed for its bactericidal effects against *Esherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853, was studied for its time-kill kinetics towards these bacteria in this study. The minimum bactericidal concentrations (MBCs) of PAM-5, gentamicin and polymyxin B against *Esherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were determined via microbroth dilution antibacterial assay. Next, the bacteria were treated with 2X MBC of PAM-5, gentamicin and polymyxin B. The treated bacteria were then inoculated on Mueller-Hinton (MH) agar at every 10 minutes interval. The treatment and inoculation of bacteria were carried out for a duration of one hour. The results of the inoculation were visually examined on the second day to determine the exact time point where the bacteria were completely killed. All assays were performed in 37°C and duplicated to ensure reproducibility.

## 3.2 Materials

### 3.2.1 List of Glassware, Consumables and Laboratory Equipment

Refer to Appendix A

### 3.2.2 Preparation of Buffers and Media

Refer to Appendix B

### 3.2.3 Bacteria Strains

Two reference strains of bacteria were screened in this study. The reference strain of *Esherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were provided by Dr. Sit Nam Weng from the Department of Biomedical Science, Universiti Tunku Abdul Rahman (UTAR). The bacteria were grown in Luria-Bertani (LB) broth for 4 hours. The bacteria cultures were added with 50% glycerol (v/v) to make into glycerol stock at a final concentration of 25%. The bacterial-glycerol suspension was then aliquot into individual microcentrifuge tubes and stored at -80°C. The frozen bacteria were thawed on ice before use and cultured on Mueller-Hinton (MH) agar as master culture plate. The master culture plate was incubated overnight at 37°C and stored in 4°C for a maximum of seven days to ensure freshness of the bacteria.

### 3.2.4 Synthesis and Preparation of PAM-5

PAM-5 with the peptide sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M was synthesized and purchased from Bio Basic Inc. (Canada). The molecular weight of PAM-5 is 2038.64 Dalton (Da). PAM-5 was packaged in a tightly sealed and dry plastic tube in lyophilized form, and stored at -20°C. Before use, the peptide is placed at room temperature for about 30 minutes. PAM-5 must be dissolved in sterile, degassed distilled water due to the presence of methionine residues in PAM-5 which are prone to oxidation. An amount of 1024 µg of PAM-5 was dissolved in 100 µL of degassed, filtered-sterilized distilled water. The dissolved peptide was topped up with 900 µL of sterile, degassed phosphate-buffered saline (PBS). The peptide stock solution (1024 µg/mL) was then subjected to two-fold serial dilution to yield a series of peptide concentration from 1024 µg/mL to 4 µg/mL as illustrated in **Figure 3.1**. The diluted peptide solutions were stored in silica bottles at 4 ℃ for a maximum of seven days to ensure the effectiveness of the peptide.

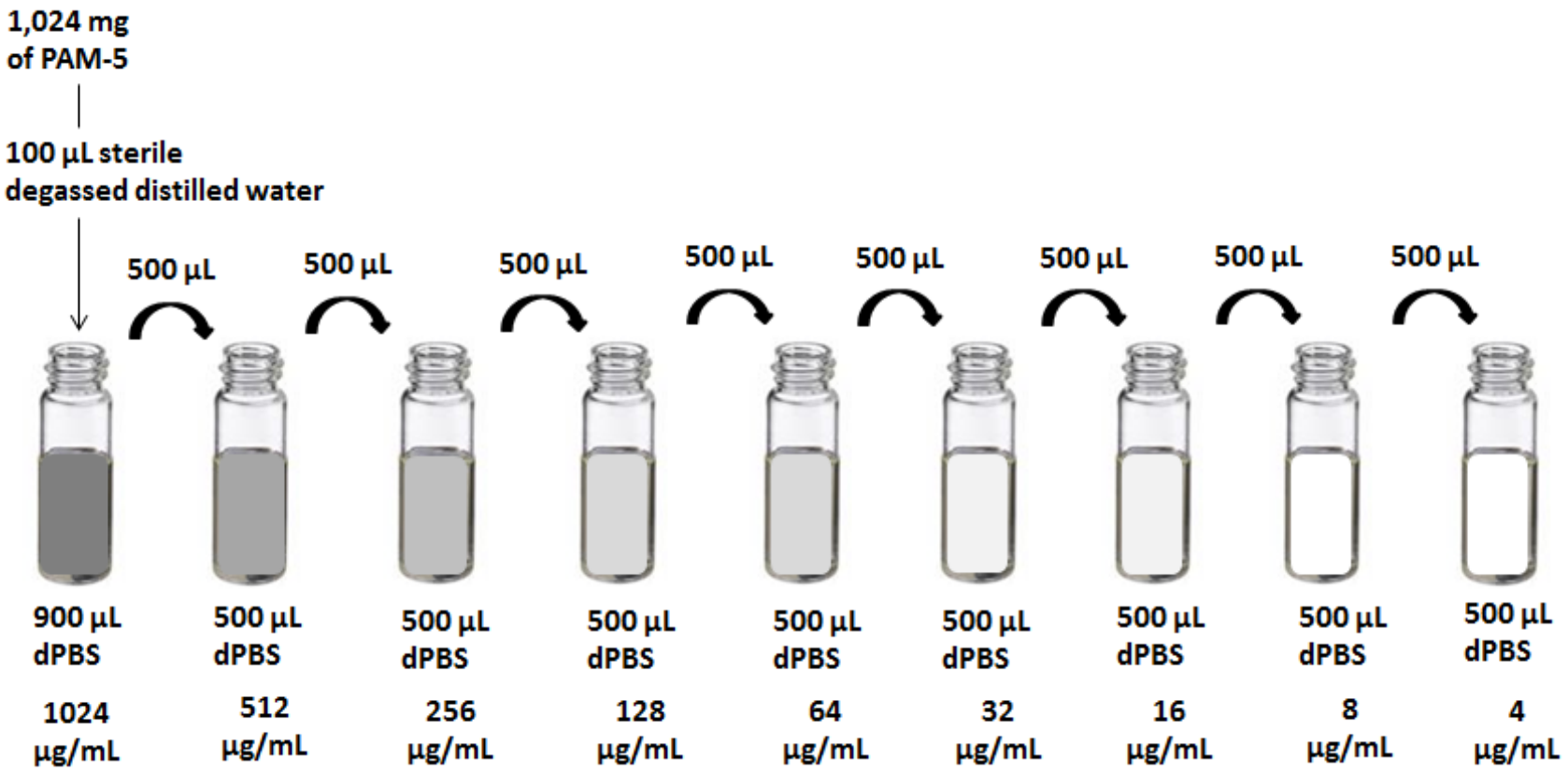


**Figure 3.1**: Illustration of serial dilution of PAM-5 into different concentrations.

### 3.2.5 Preparation of Gentamicin and Polymyxin B

Gentamicin (Calbiochem®) was purchased from EMD Chemicals, Inc (Canada) and polymyxin B (Calbiochem®, Denmark) was purchased from Merck Millipore. These antibiotics were used as comparison to PAM-5 in time kill assay and were prepared using the similar procedures as described in **Section 3.2.4**.

## 3.3 Methodology

### 3.3.1 Determination of Minimum Bactericidal Concentrations (MBCs) using Microbroth Dilution Assay

Microbroth dilution assay was used in this study to determine the MBCs of PAM-5, gentamicin and polymyxin B against *E. coli* and *P. aeruginosa*. Firstly, an overnight liquid culture of the bacteria was prepared by inoculating two to three bacterial colonies into 20 mL of Luria- Bertani (LB) broth. The bacterial culture was then grown for 16-18 hours at 37°C in a shaking incubator with a rotation speed of 200 rotations per minute (rpm). On the next day, 200 µL of the overnight culture was added into a conical flask filled with 20 mL of fresh Mueller-Hinton (MH) broth. The diluted bacteria culture was allowed to grow at 37°C with a rotation speed of 200 rpm until it reached the mid-exponential growth phase (optical density~0.5). Next, the bacteria were centrifuged at the centrifugal force of 6000 x *g* for 6 minutes at 4°C to collect the pellet. The supernatant was discarded and the bacterial pellet was washed twice by re-suspending the pellet in 2 mL of phosphate-buffered saline (PBS) (pH 7.4) followed by centrifugation at the same setting. After washing, the

bacterial pellet was re-suspended in 1 mL of degassed PBS. The bacterial suspension was then serially diluted with PBS to obtain a bacteria titer of $10^3$ CFU/mL.

In order to set up the antibacterial assay, 100 µL of the bacterial suspension with the inoculation titer of $10^3$ CFU/mL was loaded into wells of the 96-well microtiter plate. The bacteria were then treated with 100 µL of PAM-5 at the concentrations ranging from 2 µg/mL to 256 µg/mL. On the other hand, bacteria treated with 100 µL of gentamicin and polymyxin B at the final concentrations ranging from 0.25 µg/mL to 8 µg/mL were also set up. Untreated bacteria were added with 100 µL of PBS to serve as the negative control. The microtiter plate was then pre-incubated at 37 ˚C for an hour before loading 50 µL of MH broth into each well. Upon loading of MH broth, the microtiter plate was then incubated overnight at 37 ˚C. The content of each well was summarized in **Figure 3.2**.

On the following day, a volume of 10 µl of the culture suspension from each well was inoculated onto MH agar by spreading method to detect any viable bacteria. This was performed to determine the minimum bactericidal concentrations (MBCs) of PAM-5, gentamicin and polymyxin B. The MH agar plates were inspected for any bacterial growth on the following day after overnight incubation at 37 ˚C.

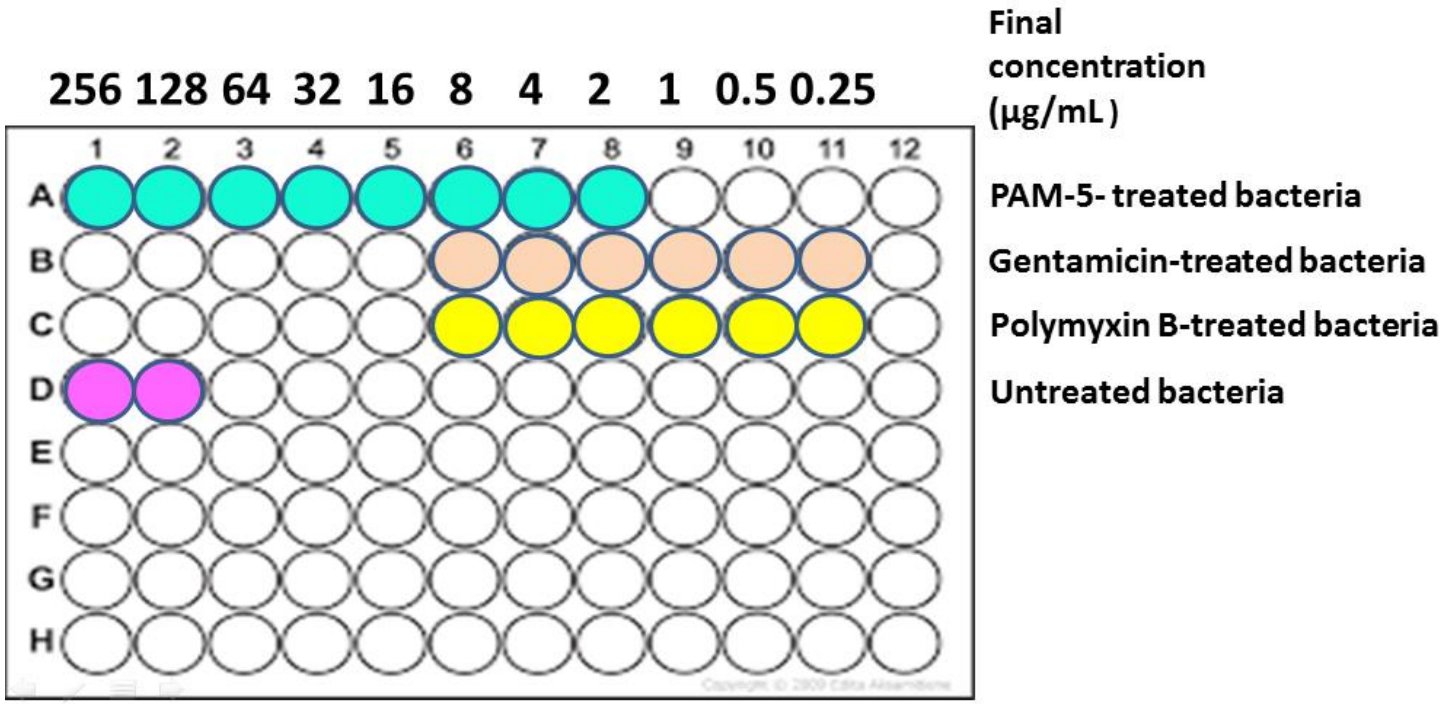


**Figure 3.2**: Illustration of culture set up for microbroth dilution antibacterial assay. The bacteria in row A, B and C were treated with PAM-5, gentamicin and polymyxin B, respectively. Untreated bacteria were located in column D to serve as the negative control.

### 3.3.2 Determination of Minimum Bactericidal Concentrations (MBCs)

The antibacterial potency of PAM-5, gentamicin and polymyxin B were determined as MBC as shown in **Figure 3.3**. MIC is defined as the lowest concentration of an antibacterial agent that is able to inhibit the visible growth of bacteria in liquid medium after overnight incubation, whereas MBC is defined as the lowest concentration of an antibacterial agent that shows 99.9% killing of bacteria in both liquid medium and solid medium (Andrews, 2001).

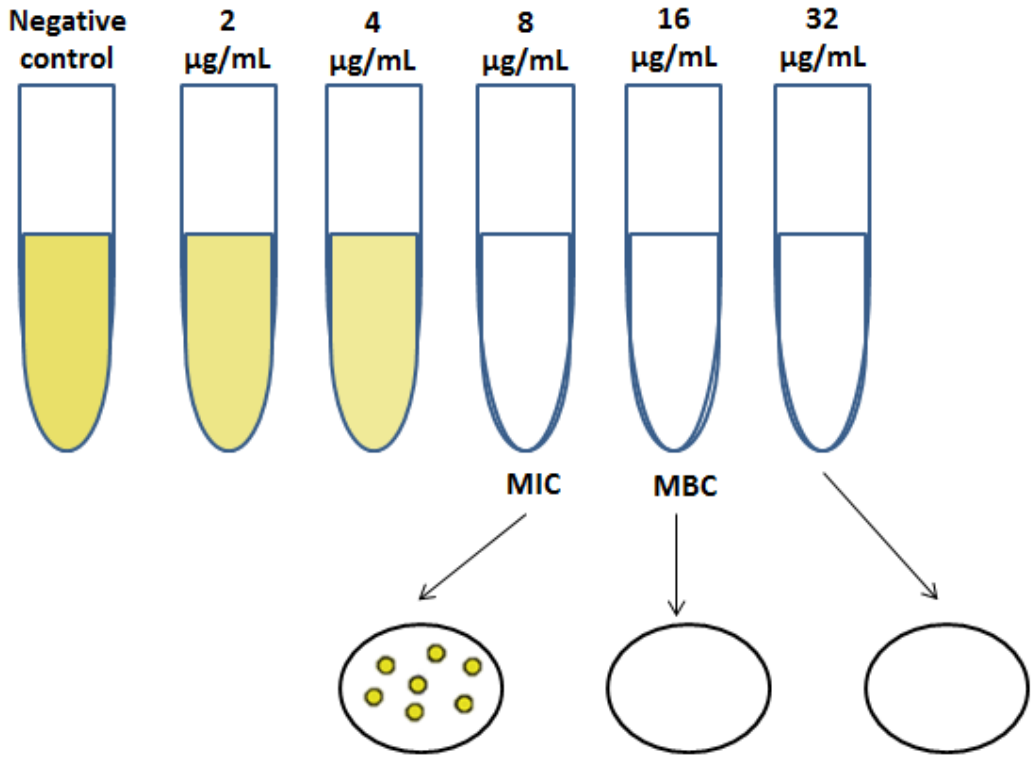


**Figure 3.3:** The illustration for determination of MIC and MBC by using microbroth dilution assay. From this example, MIC is at 8 µg/mL where the tube shows no visible bacterial growth upon visual inspection but visible colonies was observed after inoculation onto media agar plate, whereas MBC is at 16 µg/mL which shows no turbidity in the tube and no visible colonies produce after inoculating onto media agar plate.

### 3.3.3 Time-kill Assay

The kinetic killings of PAM-5 and other antibiotics on the selected bacteria as mentioned in **Section 3.2.3** were determined by using time-kill assay according to the guidelines provided by Clinical and Laboratory Standards Institute (CLSI). The overnight culture of bacteria, bacteria harvesting and bacterial inoculation titer were performed in the same way as described in **Section 3.3.1**. Serial dilution was carried out on the harvested bacteria to obtain an inoculation bacterial titer of $10^3$ CFU/mL. Subsequently, 100 µL of the bacterial suspension was added into wells of 96-well microtiter plate. The bacteria were then treated with 100 µL of PAM-5, gentamicin and polymyxin B simultaneously by using multichannel pipettor. For the negative control, 100 µL of sterile, degassed PBS (pH 7.4) was added into the bacteria suspension. The content of each well is summarized in **Table 3.1**.

**Table 3.1**: Contents and volume loaded into the wells of the 96-well microtiter plate in the time-kill assay.

| Contents | Sample Well | Antibiotic Well | Antibiotic Well | Negative Control Well |
|---|---|---|---|---|
| Bacterial suspension | 100 µL | 100 µL | 100 µL | 100 µL |
| PAM-5 | 100 µL | - | - | - |
| Gentamicin | - | 100 µL | - | - |
| Polymyxin B | - | - | 100 µL | - |
| PBS (pH 7.4) | - | - | - | 100 µL |

* The hyphen in the table indicates the absence of the particular variable.

Upon treatment with the antibacterial agents, at each 10 minute interval, a total of 60 µL of the treated bacterial suspension from each well was inoculated onto MH agar as shown in **Figure 3.4**. At the same time, 60 µL of the untreated bacteria was also inoculated onto MH agar which served as the negative control. Upper left quadrant was inoculated with PAM-5-treated bacteria, followed by upper right quadrant inoculated with gentamicin-treated bacteria, bottom right quadrant inoculated with polymyxin B-treated bacteria and bottom left quadrant inoculated with untreated bacteria to serve as the negative control. This treatment-inoculation was carried out for the duration of 60 minutes. The inoculated MH agar plates were incubated overnight at 37°C and the number of colonies was counted on the following day.

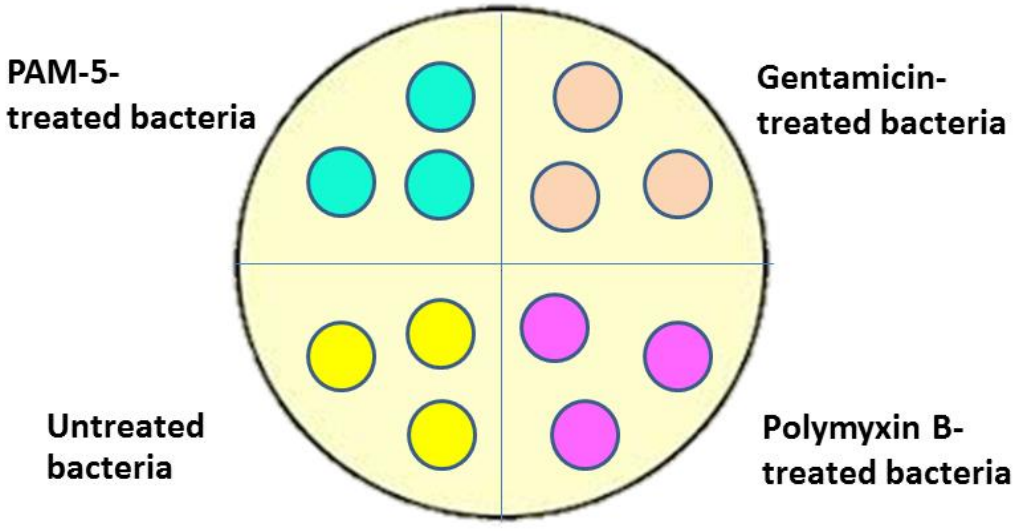


**Figure 3.4**: Illustration of quadrant setting for time-kill assay. Upper left quadrant was inoculated with PAM-5-treated bacteria, followed by upper right quadrant inoculated with gentamicin-treated bacteria, bottom right quadrant inoculated with polymyxin B-treated bacteria and bottom left quadrant inoculated with untreated bacteria to serve as the negative control.

# CHAPTER 4

# RESULTS

## 4.1 Determination of Minimum Bactericidal Concentrations (MBCs) of PAM-5, Gentamicin and Polymyxin B Towards *Escherichia coli* ATCC 35218 via Microbroth Dilution Assay

*Escherichia coli* ATCC 35218 with the inoculation titer of $10^3$ CFU/mL was treated with PAM-5, gentamicin and polymyxin B at the range of concentrations as mentioned in **Section 3.3.1.** After overnight incubation at 37˚C, the treated bacteria were inoculated onto MH agar plate after visual inspection to determine the minimal bactericidal concentrations (MBCs) of the antibacterial agents. As shown in **Figure 4.1**, plates F, G and H inoculated with bacteria treated with PAM-5 at 8 µg/mL, 4 µg/mL and 2 µg/mL, respectively, were heavily grown with bacteria, indicating that PAM-5 at these concentrations was unable to kill the bacteria. The absence of bacterial colony on the agar starting from Plate E (16 µg/mL) indicates that the MBC of PAM-5 against *Escherichia coli* ATCC 35218 is 16 µg/mL.

On the other hand, at low concentrations of gentamicin (0.25 µg/mL and 0.5 µg/mL), bacterial colonies were observed on Plate N and M. Complete killing of *E. coli* was achieved at 1 µg/mL of gentamicin (Plate L) in which no bacterial colony was present on the plate. Hence the MBC of gentamicin towards *Escherichia coli* ATCC 35218 was 1 µg/mL.

For polymyxin B, the absence of bacteria colony on the agar starting from plate S (0.5 µg/mL) indicated that the MBC of polymyxin B against *Escherichia coli* ATCC 35218 was 0.5 µg/mL.

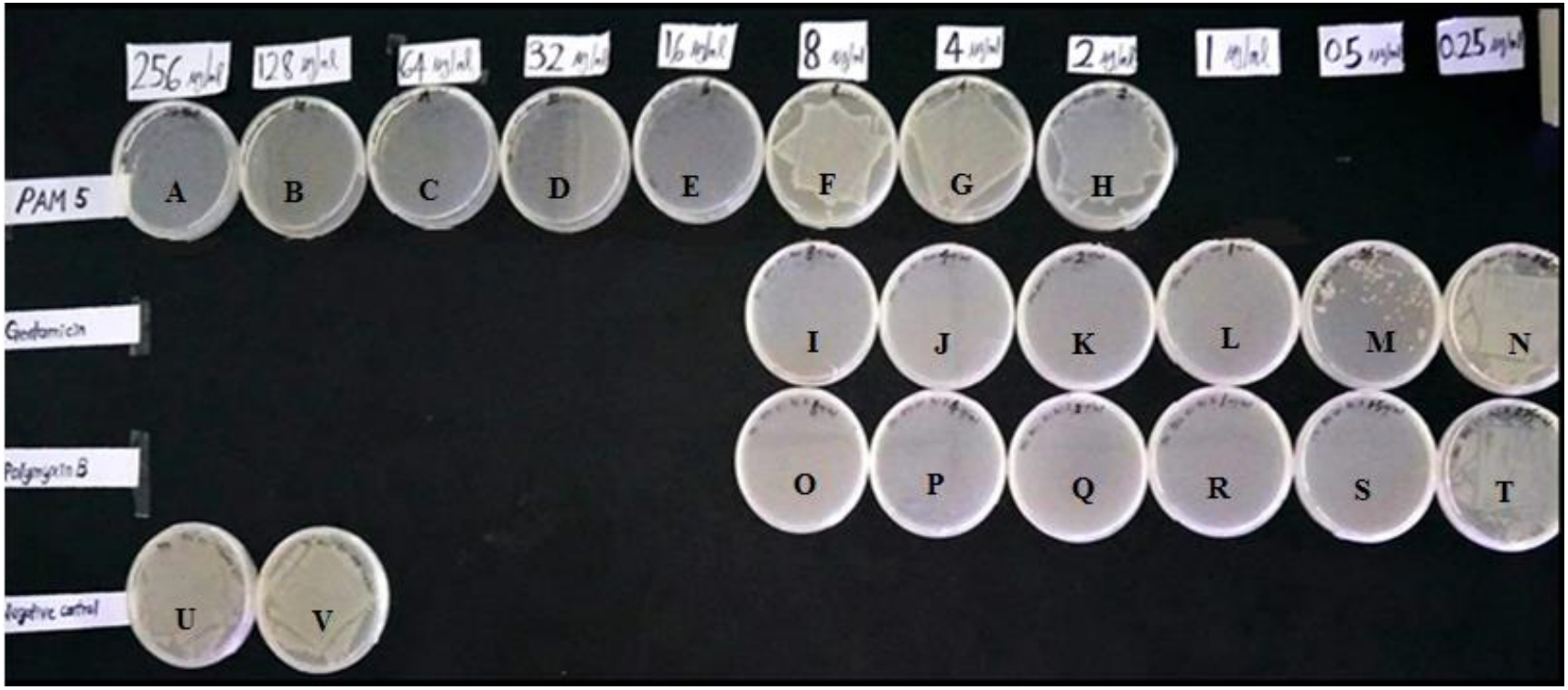


**Figure 4.1:** Gross view for culture of *Escherichia coli* ATCC 35218 after treatment with PAM-5, gentamicin and polymyxin B. Plate A to Plate H were bacteria treated with PAM-5 at concentrations ranging from 256 µg/ml to 2 µg/ml; Plate I to Plate N were bacteria treated with gentamicin at concentrations ranging from 8 µg/ml to 0.25 µg/ml; Plate O to Plate T were bacteria treated with polymyxin B with the same range of concentrations with gentamicin; Plate U and Plate V served as the negative control which consists of untreated bacterial. MBC for PAM-5 was determined as 16 µg/ml whereas MBC for gentamicin and polymyxin B were 1 µg/ml and 0.5 µg/ml, respectively.

## 4.2 Determination of Minimum Bactericidal Concentrations (MBCs) of PAM-5, Gentamicin and Polymyxin B Towards *Pseudomonas aeruginosa* ATCC 27853 via Microbroth Dilution Assay

*Pseudomonas aeruginosa* ATCC 27853 with the inoculation bacteria titer of $10^3$ CFU/mL was treated with PAM-5, gentamicin and polymyxin B at a range of concentrations as mentioned in **Section 3.3.1** at 37˚C. As demonstrated in **Figure 4.2**, PAM-5 at concentrations from 2 µg/mL to 16 µg/mL was not able to suppress the growth of *Pseudomonas aeruginosa*, as indicated by the heavy bacterial growth on Plate E to H. The degree of bacterial growth on these plates was relatively similar to the plates of negative control (Plate U and V). Beyond these concentrations, *P. aeruginosa* was completely killed by PAM-5. This was indicated by the absence of bacterial colony on Plate D to Plate A, which was inoculated with the bacteria treated with PAM-5 from 32 µg/mL to 256 µg/mL, respectively. As 32 µg/mL was the lowest concentration of PAM-5 that was able to exert complete killing towards *P. aeruginosa*, hence the MBC of PAM-5 towards this bacteria is 32 µg/mL.

As for gentamicin and polymyxin B, complete killing of *P. aeruginosa* by these two antibiotics was achieved at concentrations ranged from 0.5 µg/mL to 8 µg/mL. Hence the MBC for polymyxin B and gentamicin against *Pseudomonas aeruginosa* ATCC 27853 is 0.5 µg/mL.

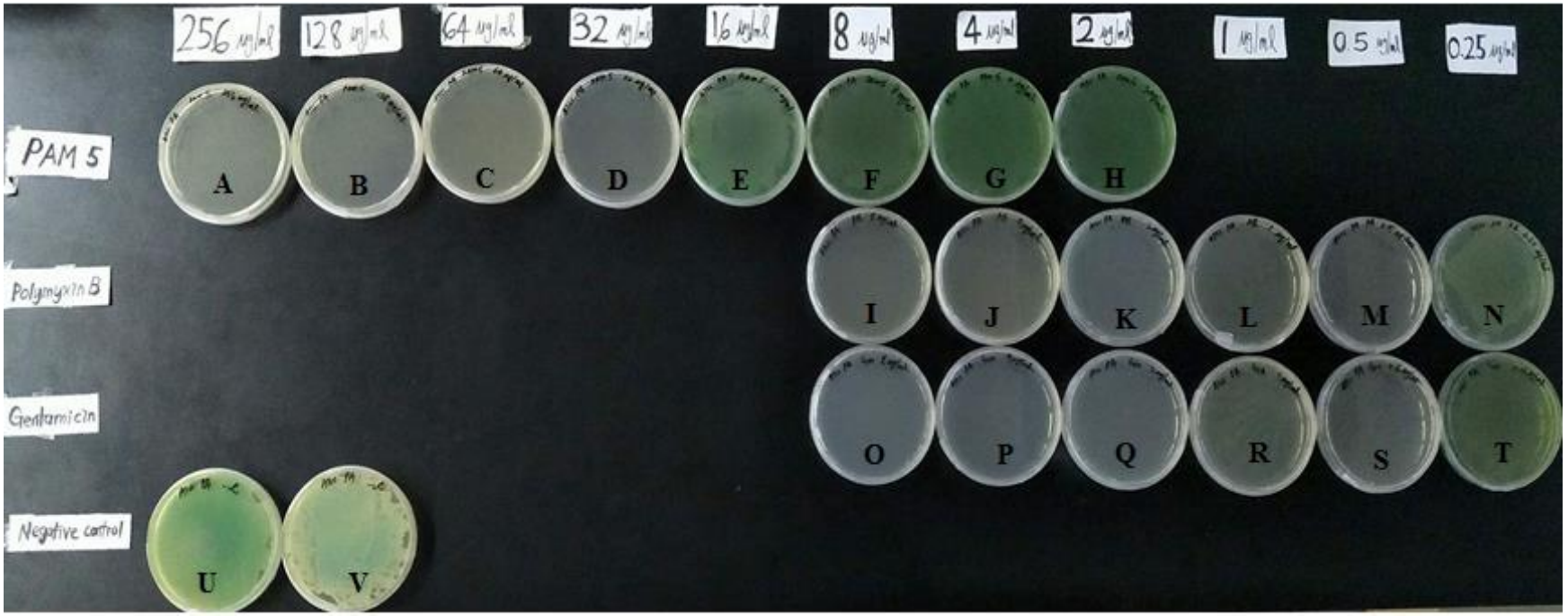


**Figure 4.2:** Gross view for culture of *Pseudomonas aeruginosa* ATCC 27853 after treatment with PAM-5, polymyxin B and gentamicin. Plate A to Plate H were bacteria treated with PAM-5 at concentrations ranging from 256 µg/ml to 2 µg/ml; Plate I to Plate N were bacteria treated with polymyxin B at concentrations ranging from 8 µg/ml to 0.25 µg/ml; Plate O to Plate T were bacteria treated with gentamicin with the same range of concentrations with polymyxin B; Plate U and Plate V served as the negative control which consists of untreated bacterial. MBC for PAM-5 was determined as 32 µg/ml whereas MBC for polymyxin B and gentamicin were both 0.5 µg/ml.

## 4.3 Time-kill Kinetic Assay for PAM-5, Gentamicin and Polymyxin B Towards *Escherichia coli* ATCC 35218

The time-kill effect of PAM-5 on *E. coli* was studied via time kill kinetic assay. As described in **Section 3.3.3**, at every 10-minute interval upon treatment, the treated bacteria were inoculated on MH media according to the designated quadrant on the media (**Figure 3.4**). As shown in Plate A of **Figure 4.3**, *E coli* was completely killed by PAM-5 at its 2X MBC within 10 minutes of the treatment (top left quadrant), as no bacterial colony was noticeable after 10 minutes of treatment by PAM-5. In comparison to the titer of untreated bacteria (negative control), which almost consistently maintained its growth titer at 3.0 $\log_{10}$ CFU/mL throughout the 60 minute-duration, PAM-5 was able to eliminate the bacteria by approximately 3 times log killing at 2X MBC in 10 minutes (**Figure 4.4**).

This rapid killing was even hardly achieved by another two tested antibacterial agents, which were gentamicin and polymyxin B. As observed in **Figure 4.3**, colonies of *E. coli* were still present at the quadrants inoculated with the bacteria treated with both the antibacterial agents even though after 30 minutes of exposure. Complete killing of *E. coli* by these two agents only can be achieved after 40 minutes (**Figure 4.3; Plate D**). Similarly, as analyzed from **Figure 4.4**, it was clearly demonstrated that both gentamicin and polymyxin B did not kill *E. coli* as drastic as PAM-5. While PAM-5 achieved a 3 $\log_{10}$ reduction of bacteria in 10 minutes, both gentamicin and polymyxin B were only able to reduce the bacterial titer by less than 0.5 $\log_{10}$, before they took approximately another 30 minutes to eliminate the bacteria.

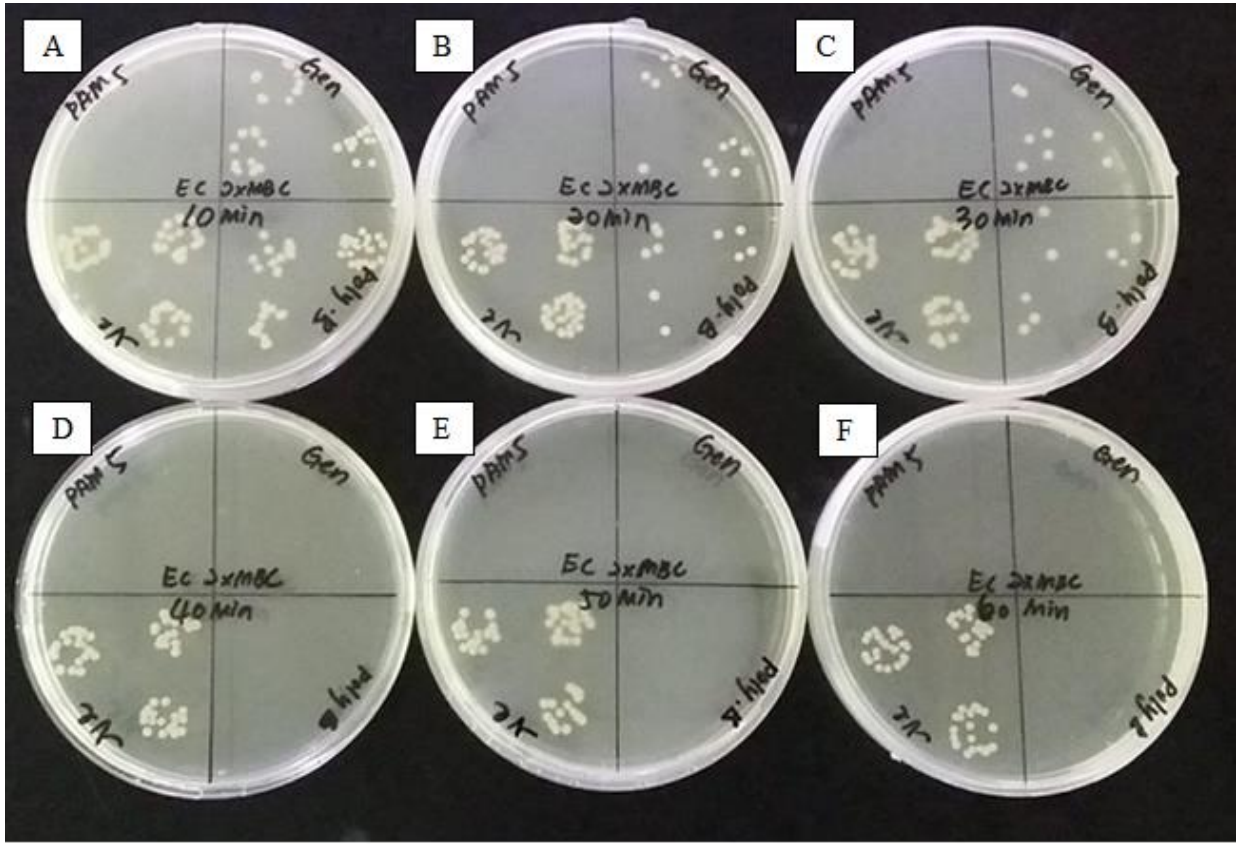


**Figure 4.3**: Result of time-kill assay of PAM-5, gentamicin and polymyxin B. Inoculation of *Escherichia coli* ATCC 35218 treated with PAM-5 (top left quadrant), gentamicin (top right quadrant), polymyxin B (bottom right quadrant) and untreated bacteria which served as the negative control (bottom left quadrant) from 10 minutes to 60 minutes (Plate A to F).

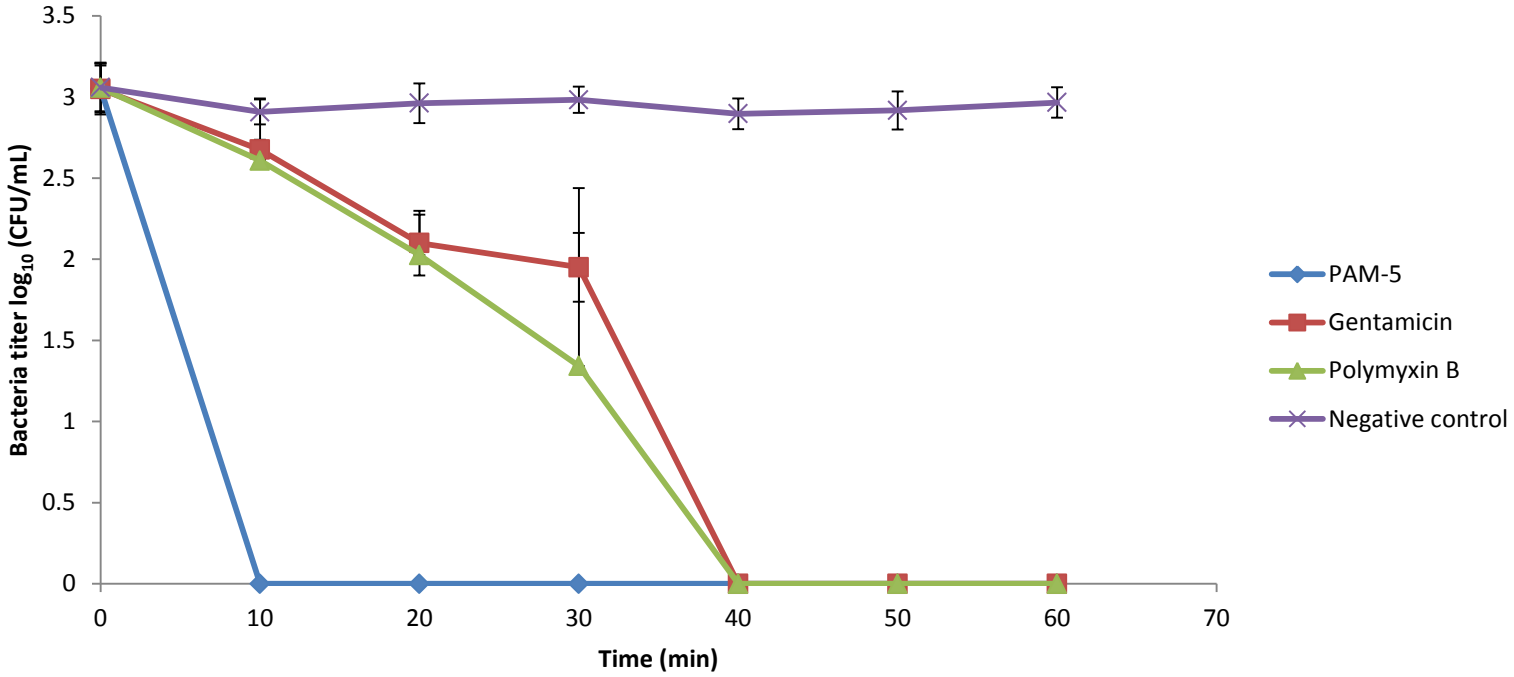


**Figure 4.4**: The graph of time-kill of PAM-5, gentamicin and polymyxin B against *Escherichia coli* ATCC 35218. The target bacteria were treated with PAM-5, gentamicin and polymyxin B for 60 minutes. The untreated bacteria suspended in PBS served as the negative control.

## 4.4 Time-kill Kinetic Assay for PAM-5, Gentamicin and Polymyxin B Towards *Pseudomonas aeruginosa* ATCC 27853

The time-kill kinetic of PAM-5 on *P. aeruginosa* was studied via time kill assay and shown in **Figure 4.5**. As mentioned in **Section 3.3.3**, the treated bacteria were inoculated on MH media according to the designated quadrant at every 10-minute interval upon treatment. As demonstrated in **Figure 4.5**, *P. aeruginosa* was completely killed by PAM-5 at its 2X MBC within 10 minutes of the treatment, which can be seen in Plate A (top left quadrant) where no bacterial colony was visible after 10 minutes of treatment by PAM-5. Throughout the 60 minutes duration, PAM-5 was able to eliminate the bacteria by approximately 3.3 times $\log_{10}$ killing at 2X MBC in 10 minutes as compared to the titer of untreated bacteria (negative control), which almost consistently maintained its growth titer at 3.3 $\log_{10}$ CFU/mL.

This rapid killing effect was not achieved by the other two tested antibacterial agents, which were gentamicin and polymyxin B. As observed in **Figure 4.5**, even though after 60 minutes of exposure to the antibacterial agents, colonies of *P. aeruginosa* were still present at the quadrants inoculated with the antibacterial agents-treated bacteria. There was no complete killing of *P. aeruginosa* by these two antibacterial agents throughout the 60 minutes of treatment (**Figure 4.5; Plate A to Plate F**). Similarly, as analyzed from **Figure 4.6**, it was clearly shown that both gentamicin and polymyxin B failed to kill *P. aeruginosa* at the similar rate as compared to PAM-5. While PAM-5 achieved a 3.3 $\log_{10}$ reduction of bacteria within 10 minutes, both gentamicin

and polymyxin B were only able to cause reduction of the bacterial titer by approximately 0.6 $\log_{10}$ CFU/mL throughout the 60 minutes of treatment.

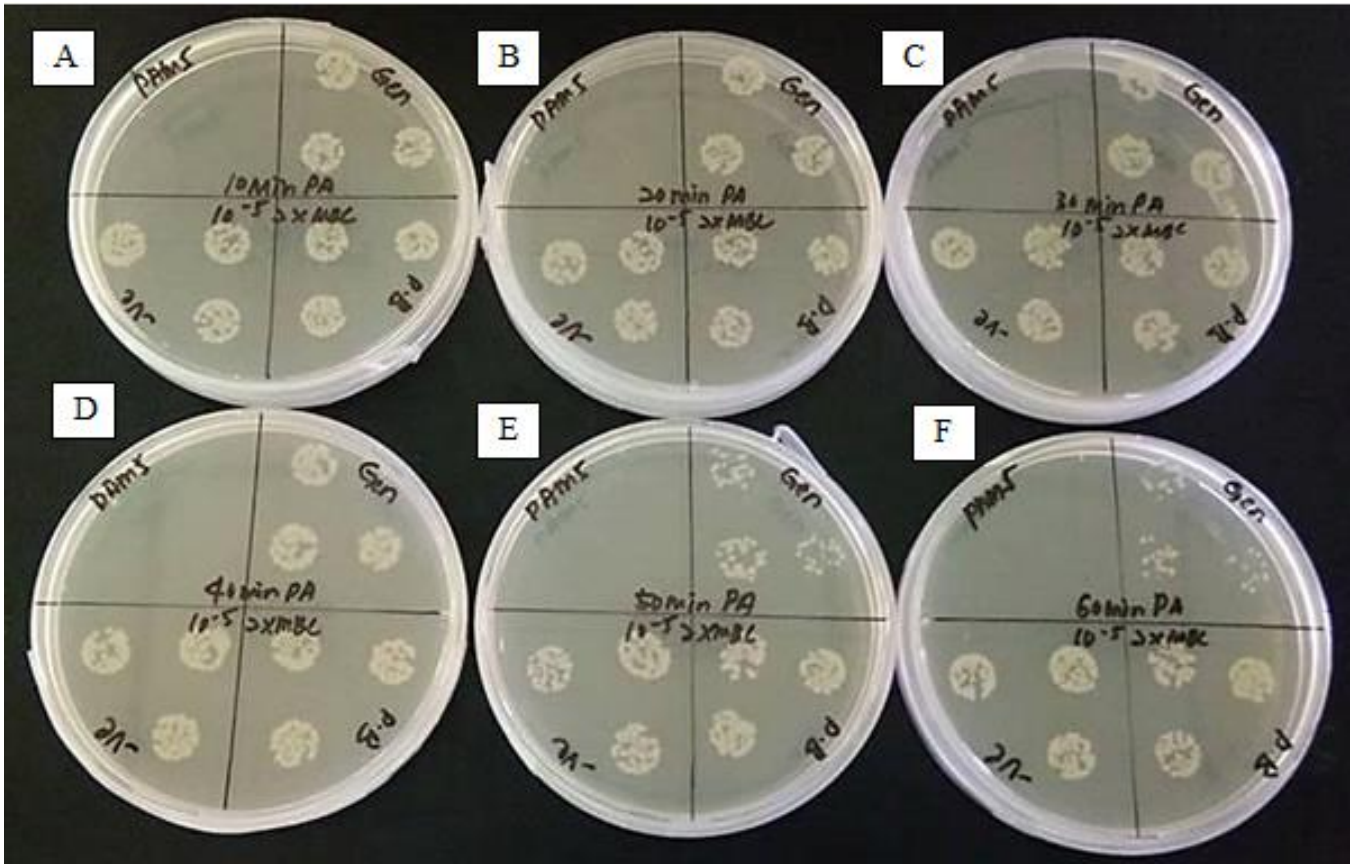


**Figure 4.5:** Result of time-kill assay of PAM-5, gentamicin and polymyxin B. Inoculation of *Pseudomonas aeruginosa* ATCC 27853 treated with PAM-5 (top left quadrant), gentamicin (top right quadrant), polymyxin B (bottom right quadrant) and untreated bacteria which served as the negative control (bottom left quadrant) from 10 minutes to 60 minutes (Plate A to F).

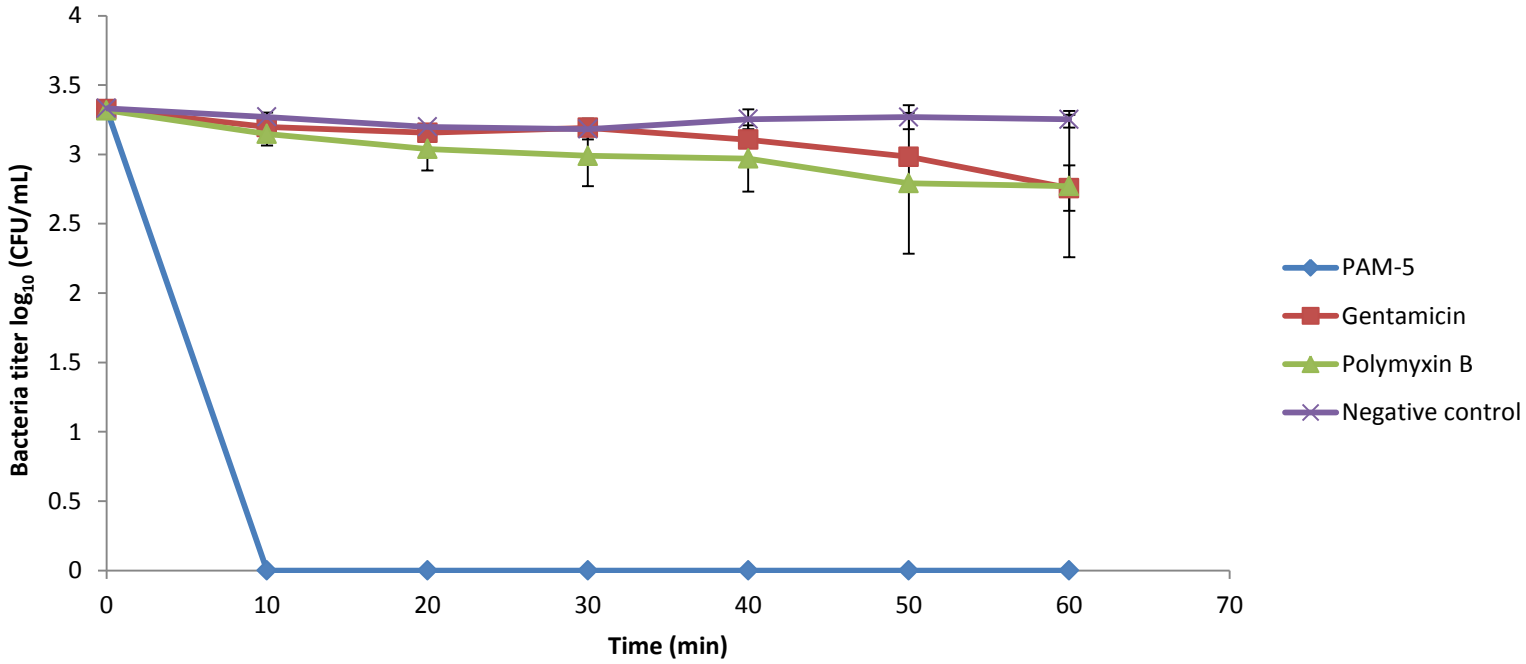


**Figure 4.6**: The graph of time-kill of PAM-5, gentamicin and polymyxin B against *Pseudomonas aeruginosa* ATCC 27853. The target bacteria were treated with PAM-5, gentamicin and polymyxin B for 60 minutes. The untreated bacteria suspended in PBS served as the negative control.

# CHAPTER 5

# DISCUSSION

The prevalence and incidence of antibiotic resistance is increasing at an alarmingly high rate. Many antibiotics which are commonly used to treat bacterial infections are becoming less effective. Consequently, high morbidity and mortality rate are resulted by the limitation of available effective antibiotics against the drug-resistant bacteria (Zaman et al., 2017). Uncontrolled antibiotic consumption such as inappropriate dosage and dosing interval are the main culprits of the antibiotic resistance. In addition, non-compliance of patients' practice in completing the entire course of prescribed antibiotics is another common reason for this medical issue. Antibiotic acts as selective pressure to bacteria which may cause them to evolve under prolonged and sub-optimal exposure if consumed irrationally and frequently (Odenholt et al, 2003).

On the other hand, the limitation of antibiotic efficacy and potency is another factor that contributes to the antibiotic resistance. Most of the antibiotics target only on single site on the bacteria to exert their antibacterial action. Hence, by altering or modifying the target site, bacteria can easily acquire resistance to the antibiotics. For example *Staphylococcus aureus* acquired resistance towards vancomycin by synthesizing additional peptidoglycan with D-Ala-D-

Ala residues that bind to vancomycin and prevent vancomycin from reaching the target site of the bacteria (Lowy, 2003). Similarly, macrolide, a class of antibiotics which inhibits protein synthesis via binding to bacterial 50S ribosomal subunit, becomes less effective when the bacteria undergo methylation of the 23 rRNA of the ribosomal subunit. This methylation impairs binding of the antibiotic to the ribosome (Munita and Arias, 2016). Based on these findings, it is clearly indicated that antibiotics that possess single mechanism of action are usually compromised by fast-mutating bacteria.

Besides, antibiotics take several hours to exert their bactericidal or bacteriostatic effect. This may allow the fast replicating bacteria with short doubling time to undergo mutation to acquire antibiotic-resistance (Hancock, 1997).

Hence there is an urgent need to explore or develop alternative antibacterial agents to fight against antibiotic-resistant bacteria. Among the alternative antibacterial agents that have been studied, antibacterial peptides (ABPs) have been given considerable research attention due to their great potential. Since their discovery many decades ago, numerous studies on these antibacterial agents have been documented. Most of these studies suggested that ABPs could work better than antibiotics by overcoming the limitations of the latter. For instance, in contrast to the conventional antibiotics, which most of them are narrow spectrum, ABPs possess strong antibacterial effects towards a broader spectrum of target bacteria, which include the drug-resistant strains

(Sainath Rao et al., 2013). This characteristic potentiates the use of ABPs in clinical setting, especially for empiric treatment before the identity of the causative bacterial agent is identified.

Next, ABPs are reported to act on multiple target sites on/in the bacteria, such as outer membrane, inner membrane, ribosomes, nucleic acids and even interfering with bacterial metabolic activities (Gottler and Ramamoorthy, 2009; Guilhelmelli et al., 2013). In contrast, most of the antibiotics used in the clinical settings are targeting only at a particular bacterial site to exert their antibacterial effect. Antibiotic-resistance can be easily acquired if the bacteria alter or modify these target sites. However, it is metabolic costly for a bacterium to change multiple target sites concurrently to avoid the actions of ABPs (Marr et al., 2006). Therefore, the issue of ABP resistance among bacteria is less likely.

PAM-5 is a 15-mer synthetic peptide which was modified from a phage displayed-peptide with strong binding affinity to *P. aeruginosa* (Gwee, 2012) (unpublished data). Like many other ABPs, PAM-5 was found to exert bactericidal effects towards several Gram-negative bacteria (Chan, 2016) (unpublished data) and drug-resistance pathogenic bacteria (Yong, 2018) (unpublished data). According to Phoon (2016) (unpublished data), the bactericidal effect of PAM-5 was attributed to its ability to cause membrane disruption and permeabilization. In addition, the mode of action is further contributed by the ability of this peptide to bind to bacterial DNA when it is

present at high concentrations (Tan, 2018) (unpublished data). However, the time-kill kinetic of this peptide is yet to be elucidated. With the issues of slow-acting antibiotics which might be associated to antibiotic-resistance, the development of a novel antibacterial agent with rapid killing should be a new strategy.

## 5.1 Time-kill Study of PAM-5 on *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853

As reported in **Section 4.1.3** and **4.1.4**, PAM-5 was able to eradicate *Escherichia coli* and *Pseudomonas aeruginosa* completely within 10 minutes, which indicates that this novel ABP possesses rapid killing effect towards the two bacteria. This rapid killing feature could be associated with its membrane-active mechanism as described in **Section 2.3**. PAM-5 is a synthetic peptide with the sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M that harbors arginine (Arg) and lysine (Lys) residues. According to Zhang et al. (2015), these two residues contribute to the peptide cationicity that enhances its antibacterial activities. The presence of these two amino acids in PAM-5 greatly enhances the peptide cationicity which may contribute to its potent bactericidal activities. Besides, PAM-5 also contains another important amino acid which is associated with antibacterial activity, which is tryptophan (Trp). Trp has uncharged side chain that makes it hydrophobic (Chan et al., 2006). Hydrophobicity of an ABP is important to promote its insertion into the hydrophobic phospholipid bilayer of bacterial membrane, which is followed

by membrane disruption. With the presence of these amino acids in an ABP, it is believed that the cationic amino acids promote its initial electrostatic interaction to the anionic bacteria membranes followed by penetration through the membrane by the hydrophobic portion of the peptide before inducing membrane disruption to the bacteria. Subsequently, the intracellular contents may leak out from the bacteria followed by cell death. Since bacterial membrane is usually the first target site of ABPs, the extensive damage of the membrane may be associated with the rapid killing of the peptides.

In addition, PAM-5 possesses ability to bind to bacterial nucleic acids (Tan, 2018) (unpublished data). The action of PAM-5 on bacterial DNA could be attributed to the increased membrane permeability induced by PAM-5, hence allowing the rest of the peptides to translocate into the intracellular compartment of the bacteria. The cationic PAM-5 can bind to the anionic phosphate group on DNA backbone through electrostatic interaction. Binding of PAM-5 to the DNA may interferes with DNA replication or synthesis and subsequently inhibit expression of proteins needed for cellular processes, thus lead to bacterial death. This statement is supported by a study of van Eijk et al. (2017) that inhibition of DNA replication could be achieved by disrupting the formation of replisome complex. This complex involves the binding of primase, helicase, DNA polymerase and single-stranded DNA-binding (SSB) proteins to the DNA before initiating DNA replication. The interference with this complex formation will inhibit DNA replication which results bacterial death.

Therefore, it is believed that the disruption of the bacteria membrane and inhibition of bacterial DNA metabolism contributed to the rapid killing of the target bacteria by PAM-5. This is supported by a study from Zhu et al. (2015) that a linear 16-mer α-helical antimicrobial peptide named T9W demonstrated complete killing towards different strains of *P. aeruginosa* within 30 minutes by inducing bacterial membrane damage. WR12, a 12-residue peptide mainly composed of arginine and tryptophan, exhibits rapid bactericidal activity towards methicillin-resistant *Staphylococcus aureus* (MRSA) within 30 minutes by disrupting bacterial membrane and leakage of intracellular contents (Mohamed et al., 2016). Since PAM-5 also possesses membrane-active mechanisms similar to the above-mentioned ABPs, this explains the rapid killing of the peptide.

## 5.2 Comparison of Kinetic Killing of PAM-5 and Gentamicin on *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853

Although the microbroth dilution antibacterial assay used in this study could reflect the antibacterial potency of PAM-5, gentamicin and polymyxin B, but the killing kinetics by these antibacterial agents might be obscured in this assay as the antibacterial potency was determined by the overnight effect. Interestingly, in the time-kill kinetic assay, PAM-5 demonstrated faster bacterial killing than gentamicin and polymyxin B, even though the latter two exert better bactericidal potency towards *E. coli* and *P. aeruginosa* at much lower MBC than PAM-5. These findings indicate that an antibacterial agent

which kills its target bacteria effectively at low MBC does not necessary mean it can achieve that rapidly.

The slower killing kinetic of gentamicin might be associated with its slower mode of action which is time consuming. Gentamicin is an aminoglycoside that only inhibits protein synthesis in susceptible bacteria (Hahn and Sarre, 1969). In order to exert its antibacterial effect, gentamicin first needs to bind to the lipopolysaccharide on the bacterial membrane before infusing through the outer and inner membrane to reach the bacterial cytoplasm. Once in the cytoplasm, gentamicin binds irreversibly to the 30S subunit of ribosome, which disrupts the normal protein synthesis and produced mistranslated polypeptides that damage the bacteria membrane (Poole, 2005). As the entire process of bactericidal action by gentamicin (aminoglycoside) involves several steps which are time-consuming, and inhibition of protein synthesis is the sole action of gentamicin, these could explain why gentamicin needs a longer duration to eliminate the two bacteria as compared to PAM-5. The findings in this study also correspond to a previous study which also demonstrated the slow bactericidal action of aminoglycosides as compared to ABPs. A study by Mohamed et al. (2014) showed that six synthetic short peptides, namely RRIKA, RR, $(KFF)_3K$, IK8, WR-12 and Penetratin were able to achieve rapid killing towards their target bacteria within 60 minutes as compared to amikacin (aminoglycosides) which only achieved that after 12 hours.

## 5.3 Comparison of Kinetic Killing of PAM-5 and Polymyxin B on *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853

Similarly, polymyxin B also exerted complete killing of the two tested bacteria at a relatively longer duration as compared to PAM-5. Polymyxin B is a cyclic lipo-decapeptide antibiotic with cationicity of +5 (Vaara, 2009). The slow killing of polymyxin B on these bacteria might be due to the differences in cationicity and peptide length between PAM-5 and polymyxin B. As mentioned previously, PAM-5 is a 15-mer synthetic peptide with cationicity of +7. According to Jiang et al. (2008), higher cationicity of an ABP is associated with better potency of the peptide due to the stronger electrostatic interaction formed between cationic peptide and anionic bacterial membrane. This could be further supported by a study by Dathe et al. (2001), which showed that increasing the cationicity of magainin 2 from +4 to +5 would increase its antimicrobial activity. Similar findings were demonstrated by Jiang et al. (2008), in which better antibacterial potency can be achieved by increasing the net positive charge of an ABP named V13K from +4 to +8. Hence, the slower killing of polymyxin B could be explained by the lower cationicity as compared to PAM-5 which required longer time to reach the threshold for a peptide to induce membrane disruption.

According to Liu et al. (2007), peptide with longer chains is more effective in killing bacteria. This is supported by a study by Benincasa et al. (2003) who demonstrated that a 15-residue synthetic peptide which is derived from the C-terminus of melittin, a linear peptide with 26 amino acid residues, has five to

seven times less antimicrobial activity as compared to the latter. This could be explained by the increasing peptide length that provides more surface area for the peptide adsorption to the anionic bacterial membrane, followed by pore formation and leakage of intracellular content (Ringstad et al., 2006). Hence, the slower killing of polymyxin B might be explained by the shorter length of the peptide as compared to PAM-5 that required longer time to fully adsorb on the anionic membrane in order to induce membrane disruption.

## 5.4 Implications of Studies

The rapid killing of *E. coli* and *P. aeruginosa* by PAM-5 as found in this study implicates that the peptide is a potent antibacterial agent against these bacteria. The doubling time for *E. coli* and *P. aeruginosa* are 20 minutes and 30 minutes, respectively (Gibson et al., 2018), which means that both the bacteria might be killed completely by PAM-5 before achieving their replication. Therefore, the likelihood for the bacteria to acquired resistance towards PAM-5 might be very low.

On the other hand, PAM-5 demonstrates better kinetic killing as compared to gentamicin and polymyxin B. This finding indicates that PAM-5 is a more potent antibacterial agent than the latter two, and might also imply that PAM-5 could be used to treat infections by gentamicin-resistant bacteria.

## 5.5 Limitations of Current Study and Proposed Future study

Time-kill study of PAM-5 against *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 had indicated that PAM-5 is a fast acting ABP. However, this study only focused on reference strains bacteria from American Type Culture Collection (ATCC), which might not reflect the similar action on clinically isolated bacteria. Therefore, the antibacterial potency of PAM-5 can be tested on wider range of clinical strains of Gram-negative bacteria, including antibiotic-resistant bacteria.

Secondly, only one bacteria inoculation titer ($10^3$ CFU/mL) was used in this study, which might not provide the information about the inoculum effect on the kinetic killing of PAM-5. Hence, different bacterial titers can be used in the future to study the inoculum effect on killing effect of PAM-5.

# CHAPTER 6

# CONCLUSION

In conclusion, PAM-5 exhibits rapid killing effects against *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853, in which it kills both the bacteria completely within 10 minutes. Moreover, PAM-5 demonstrates faster killing of the bacteria than gentamicin and polymyxin B. Hence, PAM-5 is more potent than gentamicin and polymyxin B in terms of kinetic killing. This indicates that PAM-5 is potential to be further studied and developed into an alternative antibacterial agent against bacterial infections.

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## APPENDIX A

## LIST OF GLASSWARE AND LABORATORY EQUIPMENT

| Lab ware/Equipments | Manufacturers |
|---|---|
| 15 ml centrifuge tube | Greiner, Germany |
| 50 ml centrifuge tube | Axvgen® Scientific, USA; NEST, China |
| 250 ml conical flask | DURAN®, Germany |
| 96-well microplate, transparent, flat-bottomed | NEST Biotechnology, China |
| Biosafety Cabinet Level-2 | TELSTAR, Philippines |
| Bunsen burner | Campingaz, France |
| Centrifuge machine | Sigma Laboratory Centrifuges, Germany |
| Incubator | Memmert, Germany |
| Shaking incubator | Yihder Technology, Taiwan |
| Microcentrifuge tube | Greiner Bio-One, Austria |
| Micropipette set | Eppendorf, Germany |
| Micropipette tip | Axvgen® Scientific, USA; NEST, China |
| Multichannel pipettor | Pipetman®, USA |
| PCR tube | Axygen® Scientific, United States |
| Petri dish | NEST Biotechnology, China |
| Schott bottle | DURAN®, Germany |
| Spectrophotometer | Biochrom Libra S22, UK |
| Syringe (10 ml) | Terumo, Japan |
| Syringe filter (0.2μm) | Pall corporation, USA |
| Vortex mixer | Gemmy Industrial Corporation, Taiwan |

# APPENDIX B

## PREPARATION OF BUFFERS AND MEDIA

### Preparation of Luria-Bertani (LB) broth

About 8 g of LB broth powder (Merck Millipore) was dissolved in 400 mL of distilled water and autoclaved at 121ºC for 15 minutes.

### Preparation of Mueller-Hinton (MH) broth

About 8.4 g of MH broth powder (Liofilchem) was dissolved in 400 mL of distilled water and autoclaved at 121ºC for 15 minutes.

### Preparation of Mueller-Hinton (MH) agar

MH agar was prepared by dissolving 20.4 g (Merck Millipore) or 22.8 g (HiMedia) of MH agar powder in 600 mL of distilled water and autoclaved at 121ºC for 15 minutes. Then, the medium was poured into petri dishes and stored at 4ºC after the agar has solidified.

### Preparation of MacConkey agar

MacConkey agar was prepared by dissolving 25 g of MacConkey agar powder (Merck Millipore) in 500 mL of distilled water and autoclaved at 121ºC for 15 minutes. The medium was then poured into petri dishes and stored at 4ºC after the agar has solidified.

**Preparation of phosphate buffered saline (PBS)**

A stock solution of 10X PBS in 100 mL was prepared by adding 8.00 g of sodium chloride (NaCl) (Merck Millipore), 0.20 g of potassium chloride (KCl) (Systerm), 1.44 g of potassium dihydrogen phosphate ($KH_2PO_4$) (QRec$^{TM}$) and 0.24 g of disodium hydrogen phosphate ($Na_2HPO_4$) (Systerm) in about 80 mL of distilled water. The mixture was thoroughly mixed and the pH of the solution was adjusted to a pH of 7.4 by using 1M sodium hydroxide (NaOH). After the pH was adjusted, the mixture was topped up with distilled water to reach 100 mL. The solution was then autoclaved at 121ºC for 15 minutes. This stock solution can be diluted into 1X working solution following a ratio at 1:10 by the addition of distilled water.