SCREENING OF ANTIBACTERIAL POTENCY OF ANTIBACTERIAL PEPTIDE PAM-5 AGAINST SELECTED DRUG-RESISTANT PATHOGENIC BACTERIA

By

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ABSTRACT

SCREENING OF ANTIBACTERIAL POTENCY OF ANTIBACTERIAL PEPTIDE PAM-5 AGAINST SELECTED DRUG-RESISTANT PATHOGENIC BACTERIA

Leong Yee Leng

The global threat of antibiotic resistant bacteria has prompted the search for alternative antibacterial agents. Among them, antibacterial peptides (ABPs) are possible candidates as alternative therapeutic agents against pathogenic bacteria, particularly to antibiotic-resistant bacteria. Previous studies on ABPs were focused on natural peptides isolated from different living organisms but the research attention has shifted towards synthetic peptides. In this study, a synthetic peptide, PAM-5, which was previously shown to kill several reference strains of pathogenic bacteria, was screened for its potency against several clinically isolated drug-resistant bacteria. The antibacterial potency of PAM-5 was screened against four clinically isolated pathogenic bacteria with different antibiotic resistance using microbroth dilution assay. These included extended-spectrum β-lactamases-producing (ESBL)-producing *Klebsiella pneumoniae* (*K. pneumoniae*), Cefazolin (CFZ)-resistant *Escherichia coli* (*E. coli*), Cefazolin (CFZ)-, Ceftriaxone (CRO)- and Ceftazidime (CAZ)-resistant *Acinetobacter junii* (*A. junii*), and Amoxicillin (AMX)-, Cefazolin (CFZ)-,

Cefuroxime (CXM)- and Cefoxitin (FOX)-resistant *Serratia marcescens* (*S. marcescens*). The bacteria were treated with increasing two-fold concentrations of PAM-5 ranging from 2 µg/ml to 256 µg/ml and followed by inoculation onto MH agar to determine the minimal bactericidal concentrations (MBCs) of the peptide against the bacteria. The assays were triplicated to ensure reproducibility. PAM-5 consistently demonstrated potent bactericidal effect against ESBL-producing *K. pneumoniae* at the MBC of 32 µg/ml, CFZ-resistant *E. coli* at 16 µg/ml and CFZ-, CRO- and CAZ-resistant *A. junii* at 4 µg/ml. However, PAM-5 was not potent against AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens*, as the peptide was unable to kill the bacteria at all tested concentrations. These findings suggested that PAM-5 is highly potent against several drug-resistant bacteria, with the exception of *S. marcescens*. Conclusively, PAM-5 is worth to be studied further and developed into a potential novel antibacterial agent with moderate spectrum of antibacterial activity.

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Last but not least, my heartfelt appreciation goes to my family for their constant mental support, tolerance and love. I appreciate them very much for providing me with the means to continue my education in university. Thank you for everything.

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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LEONG YEE LENG

APPROVAL SHEET

This project report entitled **"SCREENING OF ANTIBACTERIAL POTENCY OF ANTIBACTERIAL PEPTIDE PAM-5 AGAINST SELECTED DRUG-RESISTANT PATHOGENIC BACTERIA"** was prepared by LEONG YEE LENG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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(Mr. Yuen Hawk Leong) Date:……

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PERMISSION SHEET

It is hereby certified that LEONG YEE LENG (ID No: 14ADB02303) has completed this final year project entitled "SCREENING OF ANTIBACTERIAL POTENCY OF ANTIBACTERIAL PEPTIDE PAM-5 AGAINST SELECTED DRUG-RESISTANT PATHOGENIC BACTERIA" under the supervision of Mr. Yuen Hawk Leong from the Department of Biomedical Science, Faculty of Science.

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,

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(LEONG YEE LENG)

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LIST OF ABBREVIATIONS

ABPs	Antibacterial peptides
AMX	Amoxicillin
ATCC	American Type Culture Collection
CAZ	Ceftazidime
CFU	Colony forming units
CFZ	Cefazolin
CLSI	Clinical and Laboratory Standard Institutes
CRO	Ceftriaxone
CXM	Cefuroxime
Da	Dalton
ESBL	Extended-spectrum β-lactamases
FOX	Cefoxitin
G	Glycine
K	Lysine
L	Leucine
LB	Luria-Bertani
M	Methionine
MBC	Minimum bactericidal concentration
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
P	Proline
OD	Optical density
PBS	Phosphate buffered saline
R	Arginine
W	Tryptophan

CHAPTER 1

INTRODUCTION

The unwarranted use of antibiotics when it is not warranted has potentiated the incidence and prevalence of antibiotic resistance, which has been a major public health concern worldwide. In consideration to the lack of effective treatment against antibiotic-resistant bacteria, there is a need to search and develop alternative therapeutic agents. Among those, antibacterial peptides (ABPs) are extensively studied due to their great potential of antibacterial properties.

For the past few decades, scientists have been studying ABPs in hopes that they can become the alternative antibacterial agent. There have been numerous reports on the remarkable characteristics of natural and synthetic ABPs, and their potential to be a marketable therapeutic agent. However, majority of the studies were focused on their antibacterial properties against a limited spectrum of bacteria. Notably, there is a distinct lack of research on the efficacy of ABPs towards antibiotic-resistant bacteria.

Previously, a 15-mer synthetic peptide, with antibacterial effects against *Pseuodomonas aeruginosa* was designed and synthesized by Lee (2015).

Given the name PAM-5, the peptide was shown to demonstrate strong antibacterial effect against *P. aeruginosa*. In another study, the peptide was found to exert moderate spectrum of bactericidal effects towards selected reference strains of Gram negative, which include ATCC strains of *Escherichia coli*, *Acinetobacter baumanii* and *Klebsiella pneumoniae* (Chan, 2016). However, the antibacterial potencies of PAM-5 against antibiotic-resistant bacteria have not been tested. Therefore, this study was conducted with the following objective:

1. To screen for the potency of PAM-5 against selected drug-resistant pathogenic bacteria using microbroth dilution assay.
    i. To determine the minimum bactericidal concentrations (MBCs) and/or minimum inhibitory concentrations (MICs) against the selected drug-resistant pathogenic bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 The Antibiotic Resistance Crisis

The discovery of penicillin in the early 1940s had brought a significant impact on the treatment of bacterial infections and reduction of mortality rates. Following this, many other antibiotics were found and developed to fight against bacterial infections. Despite the early optimism that bacterial infectious diseases can be controlled and prevented, the emergence of antibiotic-resistant bacteria to almost every antibiotic has prompted clinicians and scientists that antibiotics are not absolutely universal. Hence, infectious diseases are still a major medical concern (Yoneyama and Katsumata, 2006). Over the years, scientists have been trying to overcome the challenge by modifying existing antibiotics and developing newer antibiotics. Combination therapy is also being used clinically in order to combat the ever-growing resistant bacteria (Bazzaz, et al., 2016). However, the serious abuse of antibiotics in the health care sector as well as in agricultural livestock has exacerbated the crisis of antibiotic resistance (World Health Organization, 2018). This is reflected by the increasing incidence and prevalence of antibiotic resistance as reported by the World Health Organization (2014), which has foreshadowed that the world is heading towards a "post-antibiotic era," where the previously effective therapeutic strategies would no longer be

relevant. Although antibiotic resistance poses a threat to public health, the development of new antibiotics is declining. This is mainly caused by factors such as the high research costs and the current focus of chronic disease treatment (Conly and Johnston, 2005). Other reasons include the United States Food and Drug Administration and European Medicines Agency need for a superior novel antibiotic rather than one that is equivalent to existing antibiotics, and the low profit of novel drugs (Eidorial Office, 2009; Projan, 2003).

Thus, with the increasing resistant bacteria and the dwindling antibiotic development, there is an urgent need to develop alternative antibacterial agents with minimal risk of bacterial resistance.

2.2 Antibacterial Peptides

2.2.1 Overview

In the efforts to search for alternative antibacterial agents, one of the potential candidates that have captured the attention of scientists is antibacterial peptide (ABP). Naturally, many ABPs are components of the innate immunity and carry immunomodulatory properties (Ganz, 2003). These peptides are usually made up of 12 to 100 amino acids, displaying an overall positive charge ranging from +2 to +9 and are amphiphilic (Jenssen, Hamill and Hancock, 2006). Under the structural classification, ABPs can be divided into α-helical,

β-sheet, or peptides with extended or random-coil structure (Nguyen, Haney and Vogel, 2011).

2.2.2 Advantages of ABPs

Many studies on ABPs have revealed the potential of ABPs as an alternative to the conventional antibiotics. Moreover, current reports indicate that there is low occurrence of resistance towards ABPs. Even though there is resistance, the resistance level is not as strong as those against antibiotics (Bahar and Ren, 2013). A possible explanation is that ABPs usually target components of the membrane that are crucial for bacterial survival and are usually conserved (Wilmes, et al., 2011). For example, a broad series of defensins target the highly conserved peptidoglycan precursor, Lipid II (Schneider and Sahl, 2010).

Secondly, the rapid killing mechanism of ABPs further reduces the likelihood of bacterial resistance towards the peptides. The capacity for the bacteria to mutate in order to develop resistance towards the peptide may be severely reduced due to the simultaneous attempt of the bacteria to repair the damage caused by the peptide (Marr, Gooderham and Hancock, 2006). In order to develop resistance, the bacteria would have to change its entire membrane structure to evade ABPs. Consequently, the rapid killing of bacteria reduces the duration of antibacterial treatment and this may reduce the likelihood of mutational-acquired peptide resistance (Zhu, et al., 2015).

The ability of ABPs to target multiple cellular targets may also lower the possibility of peptide resistance. The main action of ABPs is to interact with bacterial cell membranes (Lee and Lee, 2015). ABPs possess direct-killing ability by disrupting the membrane which results in the leakage of cell components due to cell lysis (Otvos, 2005). Several models of membrane disruption by ABPs were proposed by previous findings which are barrel-stave, toroidal and carpet models (Wimley, 2010). Despite the difference in the early steps of these models, all of them ultimately lead to bacterial lysis. Moreover, some ABPs are capable of binding to intracellular targets, thus inhibiting certain metabolic processes that are essential for bacterial survival. For instance, there is evidence that some ABPs can interact with nucleic acid and ribosomal components which lead to inhibition of protein synthesis (Jenssen, Hamill and Hancock, 2006; Guilhelmelli, et al., 2013). Unlike the action of beta-lactam antibiotics which kills only actively-dividing cells, ABPs kills the bacteria regardless of their growing phase (Sánchez-Gómez, et al., 2015).

In addition, ABPs are found to exhibit inhibitory or killing effect to a broad spectrum of bacterial species. It has been reported that ABPs have consistent actions against both antibiotic-susceptible and antibiotic-resistant variants of bacteria (Hancock and Falla, 1996). Thus, ABPs are more effective in treating bacterial infections compared to conventional antibiotics that only act towards a limited spectrum of bacteria.

Biofilm formation is one of the main virulence factors which contribute to bacterial resistance to antibiotics and innate host defence mechanisms. Notably, there has been increasing evidence in which ABPs are able to inhibit biofilm formation (de la Fuente-Núñez, et al., 2014; Chung and Khanum, 2017). This shows that ABPs are potential anti-biofilm agents that are highly effective against biofilms as compared to conventional antibiotics.

Most importantly, ABPs selectively kill bacteria without causing significant toxicity to host cells. The molecular basis for the selectivity lies on the cationicity and amphipathicity of the peptides and the negatively-charged components of bacterial membrane such as lipopolysaccharides, phosphatidylglycerol and cardiolipin (Hammer, et al., 2010). The opposing charges between the peptides and bacterial membranes promote electrostatic interactions between these two components. On the other hand, ABPs bind less strongly to host mammalian cells in which the membranes are generally zwitterionic due to the absence of those above-mentioned anionic lipids (Epand, et al., 2010). Thus, ABPs exhibit less toxicity to mammalian cells.

2.2.3 Previous Findings on ABPs

Since the discovery in the 1920s, ABPs are under extensive research worldwide (Phoenix, Dennison and Harris, 2013). Initially, most of the studies were mainly focused on naturally occurring peptides that were isolated from microorganisms and eukaryotes such as animals, insects and plants. Recently,

the focus is diverted towards synthetic peptides due to the low yield of the tedious, time-consuming extraction of the natural ABPs (Rai, et al., 2016; Pachón-Ibáñez, et al., 2017).

Over the years, scientists are producing novel synthetic ABPs or peptides that are analogous to native ABPs. This is because modification of peptide sequences such as reducing the length, modifying the structure and replacing certain amino acids of less hydrophobicity has led to significant reduction of toxicity against host cells while maintaining or increasing their antibacterial activity (Barreto-Santamaría, et al., 2016). In a study by Chung, et al. (2017), a synthetic peptide named DRGN-1 which was modified from a peptide isolated from the Komodo dragon (*Varanus komodoensis*) was shown able to demonstrate antibacterial activity against Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) as well as the Gram-positive *Staphylococcus aureus* (*S. aureus*).

Promising results were also obtained when the antibacterial activity of peptide 35409 was screened against reference strains of *Escherichia coli* (*E. coli*), *P. aeruginosa* and *S. aureus* (Barreto-Santamaría, et al., 2016). Besides, a novel antibacterial peptide T9W synthesised by Zhu, et al. (2015), was found to be highly effective against reference strains and antibiotic-resistant strains of *P. aeruginosa* with MIC values ranging from 0.3 to 4 μg/ml.

However, the majority of these studies were focused on the antibacterial activity of ABPs against strains of bacteria that are susceptible to antibiotics. Limited studies were conducted to test the effect of ABPs against drug-resistant or multidrug-resistant bacteria. In consideration of the increasing incidence of drug-resistant bacteria, and the effectiveness of ABPs on drug-resistant bacteria which still remain unanswered, more studies should be carried out to study the antibacterial activity of synthetic peptides against drug-resistant or multidrug-resistant bacteria.

2.2.4 Novel Antibacterial Peptide PAM-5

PAM-5 is a 15-mer novel antibacterial peptide which has a peptide sequence of K-W-K-W-R-P-L-K-R-K-L-V-L-R-M. Originating from a phage-displayed peptide which was isolated during a biopanning selection process against *Pseudomonas aeruginosa* ATCC 27853, PAM-5 was produced after several modifications to the phage-displayed peptide. With the presence of several cationic and hydrophobic amino acids within the peptide, the overall cationicity and hydrophobicity of this peptide are +7 and 46%, respectively.

In a study by Lee (2015), PAM-5 was found to have bactericidal activity against *P. aeruginosa* at the MBC of 8 µg/ml. In another study by Chan (2016), PAM-5 was shown to demonstrate good antibacterial potency towards reference strains of Gram-negative bacteria including *P. aeruginosa, E. coli*, *Acinetobacter baumannii* (*A. baumannii*) and *Klebsiella pneumoniae* (*K.*

pneumoniae). As PAM-5 was mostly screened for its antibacterial effects against reference strains of bacteria, it is necessary to determine whether this peptide possesses similar potency against antibiotic-resistant pathogenic bacteria. Thus, in this study, PAM-5 was tested against selected clinical isolates of drug-resistant bacteria to determine its antibacterial potency.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 General Experimental Design

The antibacterial effect of PAM-5 was screened against selected Gram-negative pathogenic bacteria. The selected bacteria were clinical isolates of drug resistant bacteria. Using the microbroth dilution assay, the bacteria were treated with different concentrations of PAM-5. Upon incubation, the treated bacteria were inoculated onto growth media for colony counting in order to determine the minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) of the peptide. Independent assays were carried out thrice for each bacteria tested to ensure reproducibility.

3.2 Materials

3.2.1 Glassware, Consumables and Equipment

Refer to Appendix A.

3.2.2 Preparation of Buffers and Media

Refer to Appendix B.

3.2.3 Bacterial Strains

A total of four bacterial strains were used in this study. All bacterial cultures were isolated from patients in Gleneagles Medical Center, Penang. The bacteria were extended-spectrum β-lactamases (ESBL)-producing *Klebsiella pneumoniae* (1195870), cefazolin (CFZ)-resistant *Escherichia coli* (1199525), cefazolin (CFZ)-, ceftriaxone (CRO)- and ceftazidime (CAZ)-resistant *Acinetobacter junii* (1191828), and amoxicillin (AMX)-, cefazolin (CFZ)-, cefuroxime (CXM)- and cefoxitin (FOX)-resistant *Serratia marcescens* (1191741). The identities and the corresponding antibiotic-susceptibility profiles were determined by VITEK 2 automated system (bioMérieux, United States of America).

3.2.4 Bacterial Glycerol Stock and Master Culture Plate Preparation

Klebsiella pneumoniae, Escherichia coli, Acinetobacter junii and *Serratia marcescens* were first inoculated onto MacConkey agar. After ensuring their pure growth on the selective media, the bacteria was then grown in Luria-Bertani (LB) broth, preserved in glycerol with a final concentration of 25% (v/v) and stored at -80ºC. Prior to performing the antibacterial assay, the bacteria were retrieved from the bacterial glycerol stock and inoculated on Mueller-Hinton (MH) agar as a master culture plate. The plates were incubated overnight at 37ºC. The following day, the plates were stored at 4ºC for a maximum of seven days to ensure the freshness of the bacteria.

3.2.5 PAM-5 Synthesis

PAM-5 (KWKWRPLKRKLVLRM) was synthesised and purchased from Bio Basic Inc. (Canada). The peptide was received in lyophilised form. The peptide purity was 79.48% as determined by reverse-phase high performance liquid chromatography and the molecular mass of the peptide was 2038.64 Da as determined by mass spectrometry. The peptide was stored in a dry and tightly sealed vial with silica gels at -20ºC.

3.2.6 PAM-5 Preparation

Prior to dissolving the peptide, the tube was equilibrated to room temperature for approximately an hour. Next, the peptide was dissolved in degassed, filtered-sterilised distilled water to a stock concentration of 1024 µg/ml. Two-fold serial dilutions of the peptide stock solution were carried out by diluting the peptide stock from 1024 µg/ml to 4 µg/ml using degassed, filtered-sterilised phosphate buffered saline (PBS). The diluted peptide solutions were stored in silica bottles at 4ºC for a maximum period of seven days, according to the recommendation of the manufacturer to ensure the efficacy of the peptide.

3.2.7 Polymyxin B Preparation

Polymyxin B (Calbiochem®, Denmark) was purchased from Merck Milipore. Polymyxin B-treated bacteria served as the positive control for all of the antibacterial assays employed. Similar procedures as described in the

preparation of PAM-5 in **Section 3.2.6** were performed in the preparation of Polymyxin B.

3.3 Protocols

3.3.1 Preparation of Bacterial Suspension for Antibacterial Assay

The antibacterial effects of PAM-5 against the selected bacteria as mentioned in **Section 3.2.3** were determined by microbroth dilution assay as recommended by the Clinical and Laboratory Standard Institute (CLSI) with some modifications. In brief, an overnight bacterial culture suspension was prepared by inoculating two to three bacterial colonies from the master culture plate into 20 ml of Mueller-Hinton (MH) broth and grown overnight at 37ºC, in a shaking incubator at 200 rpm. The following day, 200 µl of the overnight culture was diluted into 20 ml of fresh MH broth (100-fold dilution). The diluted culture was incubated at 37ºC in a shaking incubator (200 rpm) until it reaches the mid-log phase of bacterial growth, which is equivalent to the absorbance of OD_{600} 0.500.

After reaching the mid-log phase, the bacteria culture was centrifuged at 6000 x g for 6 minutes. The pellet was washed by resuspending with phosphate buffered saline (PBS, pH 7.4), followed by recentrifugation. The washing steps were repeated twice. Following the last wash, the pellet was resuspended in 1 ml of PBS. The titre of the bacterial suspension was determined by 10-fold serial dilutions followed by inoculation onto MH agar for colony counting

on the next day. The bacterial dilution with the corresponding titre of 10^3 CFU/ml was chosen for the antibacterial assay.

3.3.2 Antibacterial Assay

Next, 100 µl of bacterial cell suspension with the bacterial titre of 10^3 CFU/ml was loaded into the wells of 96-well microplate. The loaded bacteria were treated with 100 µl of the two-fold serially diluted PAM-5 at the final concentrations ranging from 2 µg/ml to 256 µg/ml. Consequently, the positive control was set up by treating the bacteria with Polymyxin B at the same series of final concentrations while untreated bacteria suspended in PBS served as the negative control. The contents of each well were summarised in **Table 3.1** and **Figure 3.1**. The microplate was pre-incubated for 1 hour at 37ºC prior to adding 50 µl of MH broth into each well. After the addition of MH broth, the microtitre plate was then incubated overnight at 37ºC for approximately 16 to 18 hours.

Following overnight incubation, the contents of the wells of the microplate were visually inspected for turbidity as a sign of bacterial growth. Next, in order to check for the presence of viable bacteria, 10 µl of bacterial suspension from each well was inoculated onto MH agar. Bacterial suspensions from wells that appeared turbid were serially diluted using PBS and inoculated onto MH agar to determine the bacteria titre. The inoculated media were incubated overnight at 37ºC and the number of colonies formed on the agar was counted

to determine the titre of bacterial growth. Based on **Figure 3.1**, the minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) were enumerated. Independent assays were carried out thrice for each bacteria strain. According to Andrews (2001), MIC is the lowest concentration of an antimicrobial able to inhibit visible growth of a microorganism after incubating overnight in the growing medium, whereas MBC is defined as the lowest concentration of antibacterial agent showing no visible turbidity and no bacterial growth when inoculated onto MH agar.

Table 3.1: Contents of the test, positive control and negative control wells of microbroth dilution assay.

Contents	**Test Well**	**Positive Control Well**	**Negative Control Well**
Bacteria suspension	100 µl	100 µl	100 µl
PAM-5	100 µl	-	-
Polymyxin B	-	100 µl	-
PBS (pH 7.4)	-	-	100 µl
MH Broth	50 µl	50 µl	50 µl

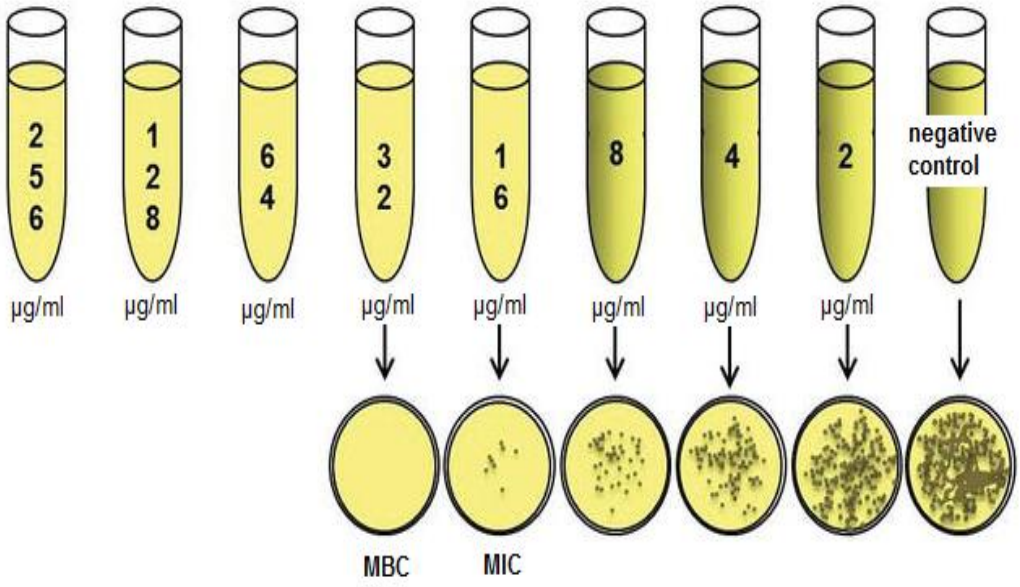

Figure 3.1: Determination of MIC and MBC by microbroth dilution method. Tubes with the concentration ranging from 2 µg/ml to 8 µg/ml were visually turbid. The bacterial suspension in the negative control tube was shown to be turbid as well. In this figure, the MIC is 16 µg/ml while the MBC is 32 µg/ml (Adapted and modified from Karaman, et al., 2017).

CHAPTER 4

RESULTS

4.1 Determination of Broad-spectrum Antibacterial Effects of PAM-5 Using Microbroth Dilution Assay

The broad-spectrum antibacterial effects of PAM-5 were screened against the following bacterial strains: extended-spectrum β-lactamases (ESBL)-producing *Klebsiella pneumoniae* (*K. pneumoniae*), cefazolin (CFZ)-resistant *Escherichia coli* (*E. coli*), cefazolin (CFZ)-, ceftriaxone (CRO)- and ceftazidime (CAZ)-resistant *Acinetobacter junii* (*A. junii*) and *Serratia marcescens* (*S. marcescens*) which is resistant to amoxicillin (AMX), cefazolin (CFZ), cefuroxime (CXM) and cefoxitin (FOX). All of the bacterial strains tested were clinical strains. The minimum bactericidal concentrations (MBCs) and/or minimum inhibitory concentrations (MICs) of the antibacterial peptide against these selected bacteria were determined in accordance to a modified version of the microbroth dilution assay as recommended by the Clinical and Laboratory Standards Institute (CLSI) as mentioned in **Section 3.3.2**. The assay was carried out in triplicate to ensure reproducibility.

4.1.1 Antibacterial Effect of PAM-5 on ESBL-producing *K. pneumoniae*

Triplicate assays consistently demonstrated that PAM-5 was able to exert antimicrobial effect against ESBL-producing *K. pneumoniae*. Based on **Figure 4.1 (a)**, visible growth was observed in the wells containing bacterial suspension treated with 2 µg/ml, 4 µg/ml and 8 µg/ml of PAM-5 (well A1, A2 and A3, respectively), in which the turbidity of the wells were comparable to the negative control (well C1 and C2). The visually clear wells containing bacteria treated with peptide concentrations from 32 µg/ml to 256 µg/ml (well A4 to A8) corresponded to the outcome of the culture on Plate E to Plate H in **Figure 4.1 (b)** where no colonies were seen. With reference to **Figure 4.1 (a)** and **Figure 4.1 (b)**, although the well containing bacteria treated with 16 µg/ml of PAM-5 (well A4) was visually clear, the MH media inoculated with the treated bacteria from the well was grown with certain number of bacterial colonies (Plate D). However, the amount of bacterial colonies on the agar (Plate D) was lesser as compared to the agar plates inoculated with bacteria treated with PAM-5 of 2 µg/ml, 4 µg/ml and 8 µg/ml (Plate A, Plate B and Plate C, respectively) as well as the negative control (Plate Q and Plate R). Beyond this, ESBL-producing *K. pneumoniae* treated with PAM-5 of higher concentrations (from 32 µg/ml to 256 µg/ml) were completely killed, as shown by Plate E to Plate H with the similar appearance as the positive control (Plate I to Plate P). According to the definition of MIC and MBC as described in **Section 3.3.2**, the MIC and MBC of PAM-5 on ESBL-producing *K. pneumoniae* are 16 µg/ml and 32 µg/ml, respectively.

By counting the colonies of the treated bacteria grown on the MH agar, the titre of the surviving bacteria after the peptide treatment can be determined. A graph of the bacterial titres in $\log_{10}$ (CFU/ml) against peptide concentrations (µg/ml) was plotted as shown in **Figure 4.1 (c).**

As seen in the graph, PAM-5 did not reduce the titre of ESBL-producing *K. pneumoniae* at peptide concentrations ranging from 2 µg/ml to 8 µg/ml. The bacterial treated with these concentrations of PAM-5 were able to grow up to titres similar to the titre of negative control. However, the bacterial culture treated with 16 µg/ml of PAM-5 showed a reduction of titre from 9.75 $\log_{10}$ CFU/ml to 3.18 $\log_{10}$ CFU/ml, indicating that the MIC of PAM-5 against ESBL-producing *K. pneumoniae* is 16 µg/ml. The potent activity of PAM-5 against the target bacteria was apparent at 32 µg/ml where the bacteria were completely killed by the peptide. Thus, the MIC and MBC of PAM-5 on ESBL-producing *K. pneumoniae* were determined to be 16 µg/ml and 32 µg/ml, respectively.

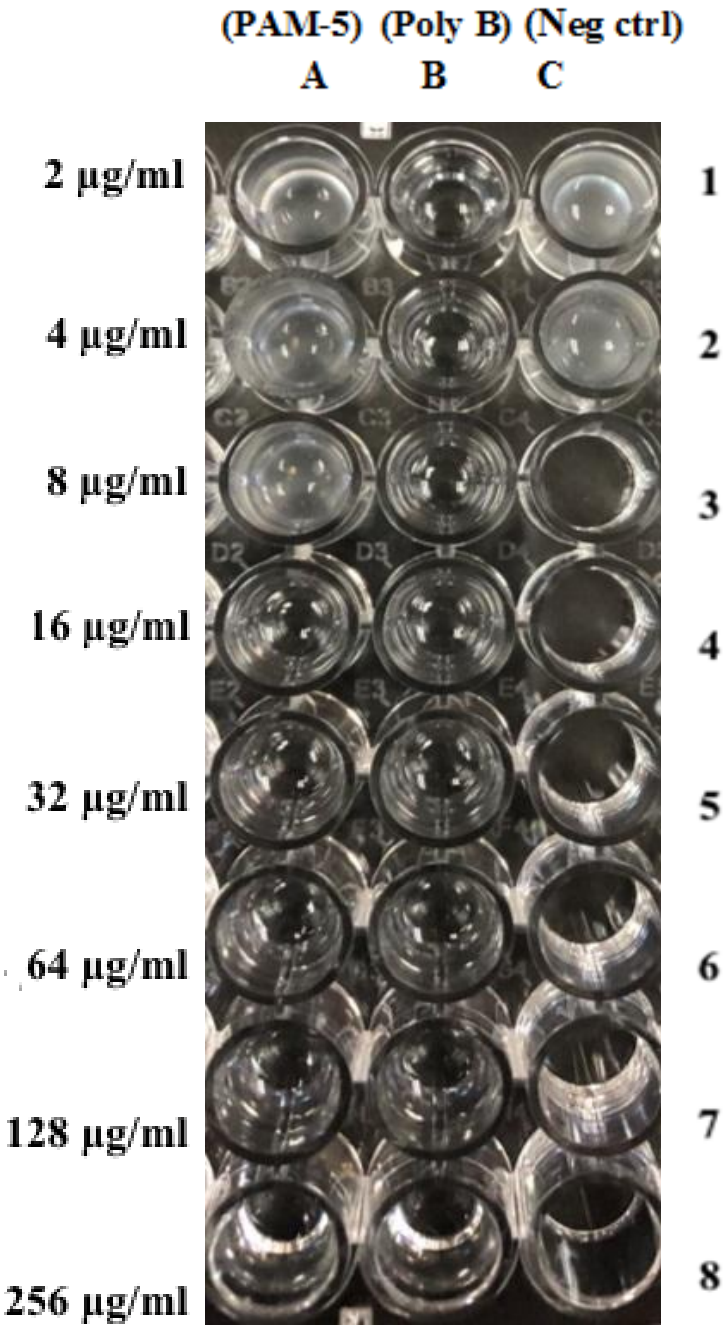

Figure 4.1 (a): Visual inspection of ESBL-producing *K. pneumoniae* treated with PAM-5 in 96-well microtitre plate after incubating overnight at 37ºC. Well A1 to well A8: bacterial suspensions treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml; well B1 to B8: bacterial suspensions treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml; wells C1 and C2: untreated bacteria suspended in PBS (negative control).

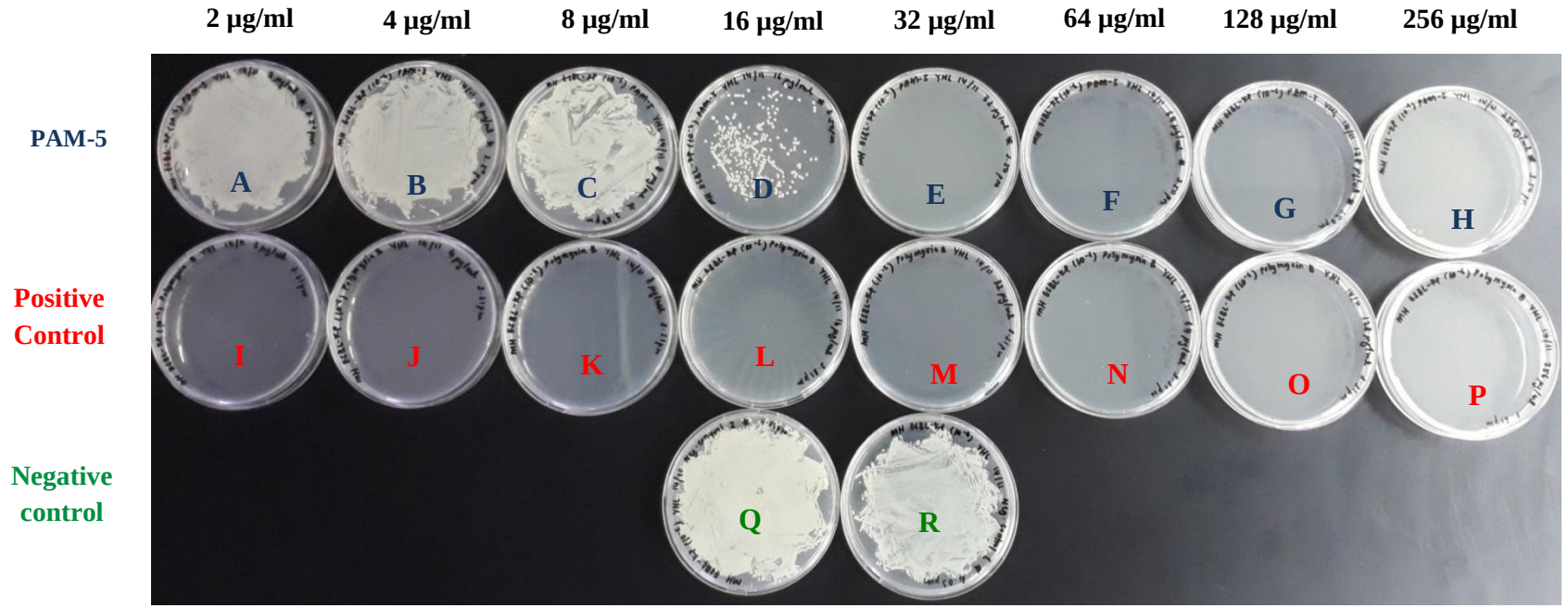

Figure 4.1 (b): Gross view of ESBL-producing *K. pneumoniae* after treatment with PAM-5. Plate A to Plate H were bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml. Plate I to Plate P were bacterial cultures treated with Polymyxin B (positive control) with concentrations from 2 µg/ml to 256 µg/ml. Plates Q and R were negative control plates grown with untreated bacteria. All plates were incubated overnight for a period of 16 to 18 hours at 37ºC. The MBC of PAM-5 against ESBL-producing *K. pneumoniae* was determined to be 32 µg/ml

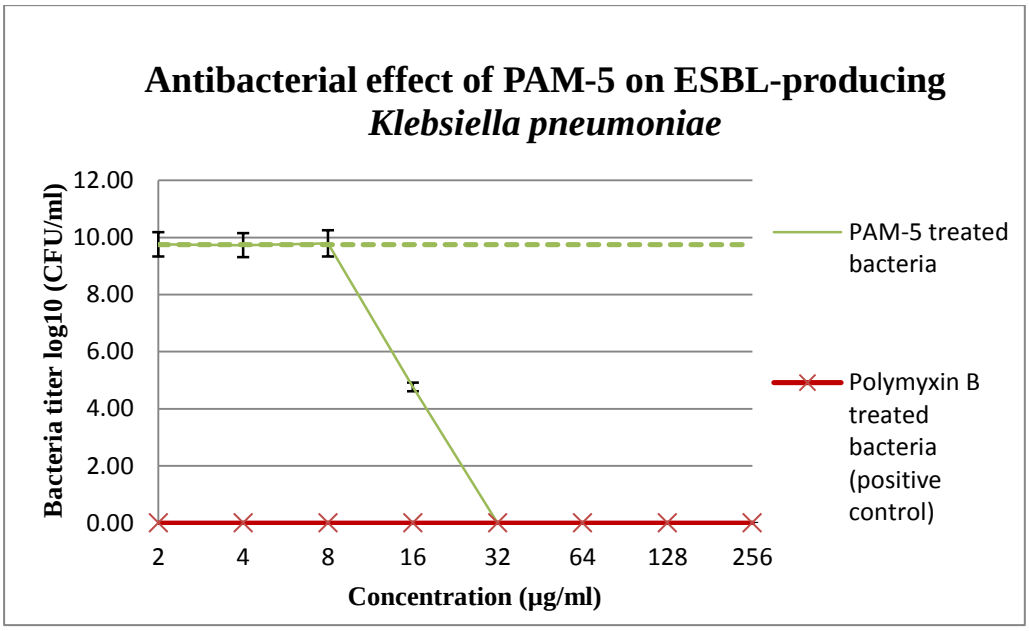

Figure 4.1 (c): Antibacterial effect of PAM-5 on ESBL-producing *Klebsiella pneumoniae*. The green solid lines (**—**) represent bacteria treated with PAM-5 while the green dashed lines (---) represent the negative controls. Polymyxin B was completely bactericidal against the tested bacteria at all concentrations.

4.1.2 Antibacterial Effect of PAM-5 on CFZ-resistant *E. coli*

As compared to ESBL-producing *K. pneumoniae*, PAM-5 was more potent against the clinical isolate of cefazolin (CFZ)-resistant *E. coli*. As shown in **Figure 4.2 (a)**, the wells filled with CFZ-resistant *E. coli* 1199525 treated with PAM-5 at concentrations 2 µg/ml, 4 µg/ml and 8 µg/ml (well A1, A2 and A3, respectively) showed turbidity which were comparable to the wells of negative control (well C1 and C2). At higher concentrations of PAM-5 (from 16 µg/ml to 256 µg/ml), no signs of bacterial growth were observed in the wells, where the contents were as clear as the positive control (well B1 to B8). These results corresponded to the plating assay as shown in **Figure 4.2 (b)**. Plates A to C were inoculated with the contents from the three turbid wells [A1 to A3 in **Figure 4.2 (a)**] and the outcome of the inoculation showed heavy growth of the bacteria. The degree of bacterial growth on these media was similar to the growth density for the negative control (Plate Q and R). On the other hand, upon treatment with PAM-5 at higher concentrations from 16 µg/ml to 256 µg/ml (Plate D to H), the bacteria was unable to grow on the media. Thus, the MBC of PAM-5 against CFZ-resistant *E. coli* 119952 was determined to be 16 µg/ml.

The trend of the antibacterial activity of PAM-5 against CFZ-resistant *E. coli* was almost similar to that of ESBL-producing *K. pneumoniae* as reported earlier. The peptide was not potent against the clinical strain of *E. coli* at low concentrations ranging from 2 µg/ml to 8 µg/ml as shown in **Figure 4.2 (c)**. The titre of the treated bacteria with these peptide concentrations did not show

much reduction as compared to the untreated bacteria. However, the bacterial titre treated with peptide concentration of 8 µg/ml showed a small decrease from 8.87 $\log_{10}$ CFU/ml (negative control) to 8.47 $\log_{10}$ CFU/ml which indicates a slight inhibitory effect towards the target bacteria. However, this could not be considered as the MIC of the peptide as the titre reduction was not apparent. At higher concentrations, which began from 16 µg/ml, the peptide was able to completely kill the bacteria. Thus, the MBC of PAM-5 against CFZ-resistant *E. coli* was evaluated to be 16 µg/ml.

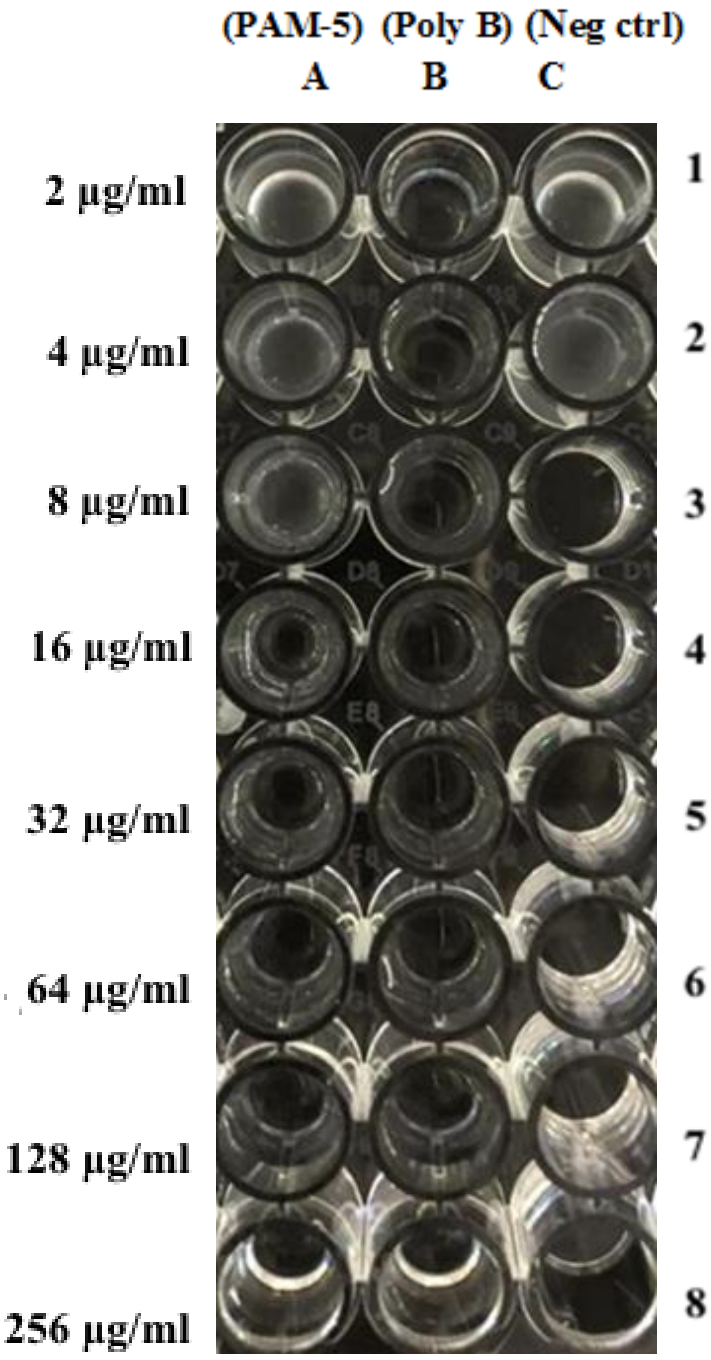

Figure 4.2 (a): Visual inspection of CFZ-resistant *E. coli* treated with PAM-5 in 96-well microtitre plate after incubating overnight at 37ºC. Wells A1 to A8: bacterial cultures treated with 2 µg/ml to 256 µg/ml of PAM-5; wells B1 to B8: bacterial cultures treated with 2 µg/ml to 256 µg/ml of Polymyxin B (positive control); wells C1 to C2: untreated bacteria suspended in PBS (negative control).

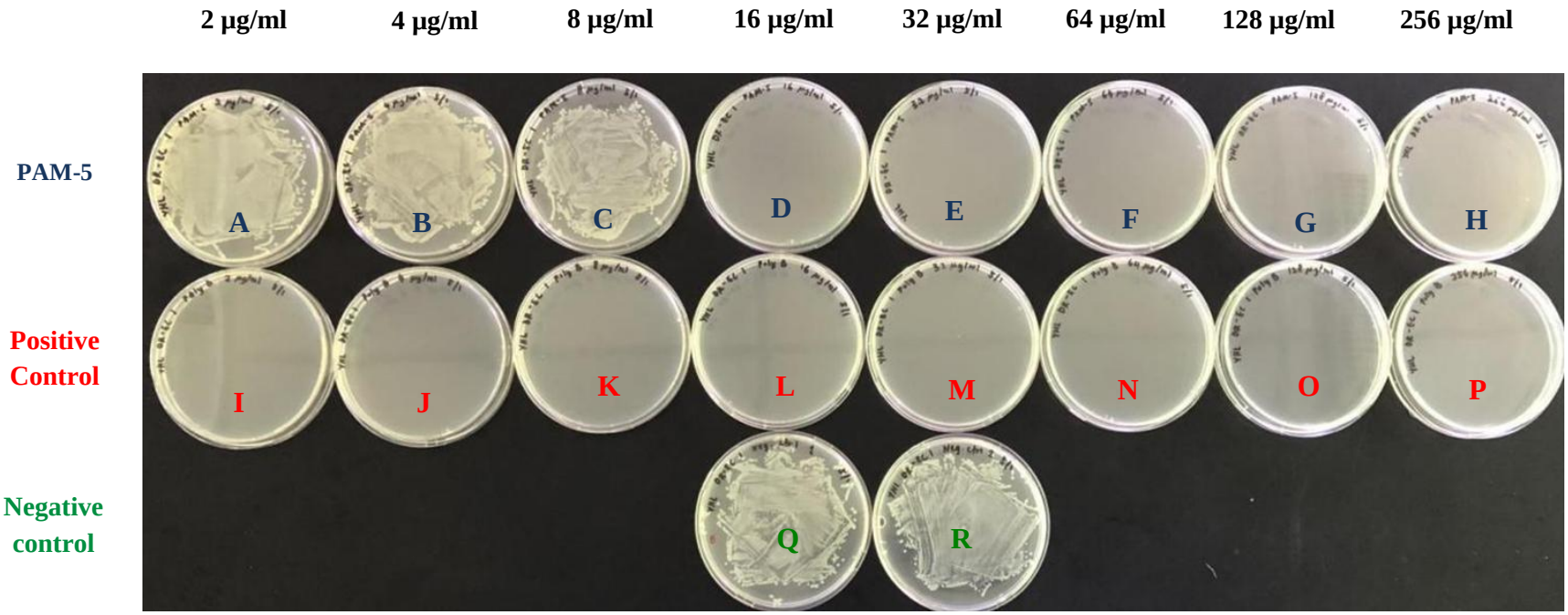

Figure 4.2 (b): Gross view of CFZ-resistant *E. coli* after treatment with PAM-5. Plate A to Plate H were bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml. Plate I to Plate P were bacterial cultures treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml. Plates Q and R were the negative control plates grown with untreated bacteria. All plates were incubated overnight for a period of 16 to 18 hours at 37 ºC. The MBC of PAM-5 against CFZ-resistant *E. coli* was determined to be 16 µg/ml.

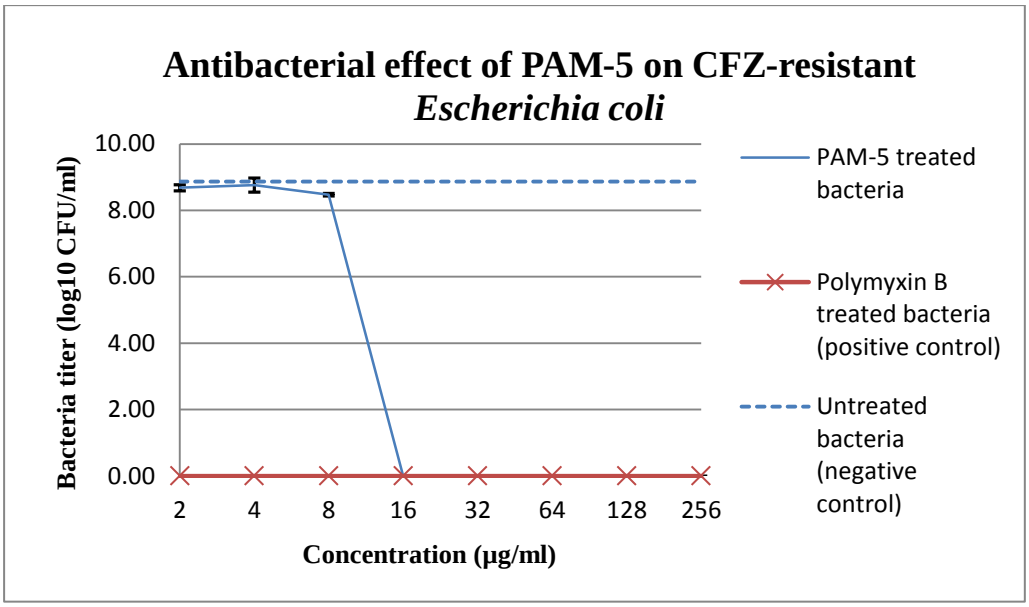

Figure 4.2 (c): Antibacterial effect of PAM-5 on CFZ-resistant *Escherichia coli*. The blue solid lines (**—)** represent bacteria treated with PAM-5 while the blue dashed lines (---) represent the negative controls. Polymyxin B was completely bactericidal against the tested bacteria at all concentrations.

4.1.3 Antibacterial Effect of PAM-5 on CFZ-, CRO- and CAZ-resistant *A. junii*

As demonstrated in **Figure 4.3 (a)** and **Figure 4.3 (b)**, PAM-5 demonstrated better potency against CFZ-, CRO- and CAZ-resistant *A. junii* 1191828 as compared to ESBL-producing *K. pneumoniae* 1195870 and CFZ-resistant *E. coli* 1199525. At all the tested concentrations of PAM-5, the antibiotic resistant bacteria only survived the peptide treatment at 2 µg/ml, before being completely killed by the peptide at higher concentrations. This resulted in the absence of bacterial growth on the inoculation media from Plate B to Plate H [**Figure 4.3 (b)**] and the corresponding absence of turbidity in well A2 to A8 [**Figure 4.3 (a)**]. Both positive and negative controls continued to provide reliable results for validation purpose. Triplicate assays consistently demonstrated that 4 µg/ml is the lowest concentration of PAM-5 that completely killed the bacteria in both the microtitre plate and agar inoculation assays, thus the MBC of PAM-5 on CFZ-, CRO- and CAZ-resistant *A. junii* is suggested to be 4 µg/ml.

As seen in **Figure 4.3 (c)**, upon treatment with PAM-5 at the concentration of xcdxd2 µg/ml, the bacterial titre showed a reduction of approximately 1 $\log_{10}$ CFU/ml when compared to the negative control. The bactericidal effect of PAM-5 was prominent at 4 µg/ml and higher concentrations where the target bacterium was killed completely. Thus, the MBC of PAM-5 against CFZ-, CRO- and CAZ-resistant *A. junii* was 4 µg/ml.

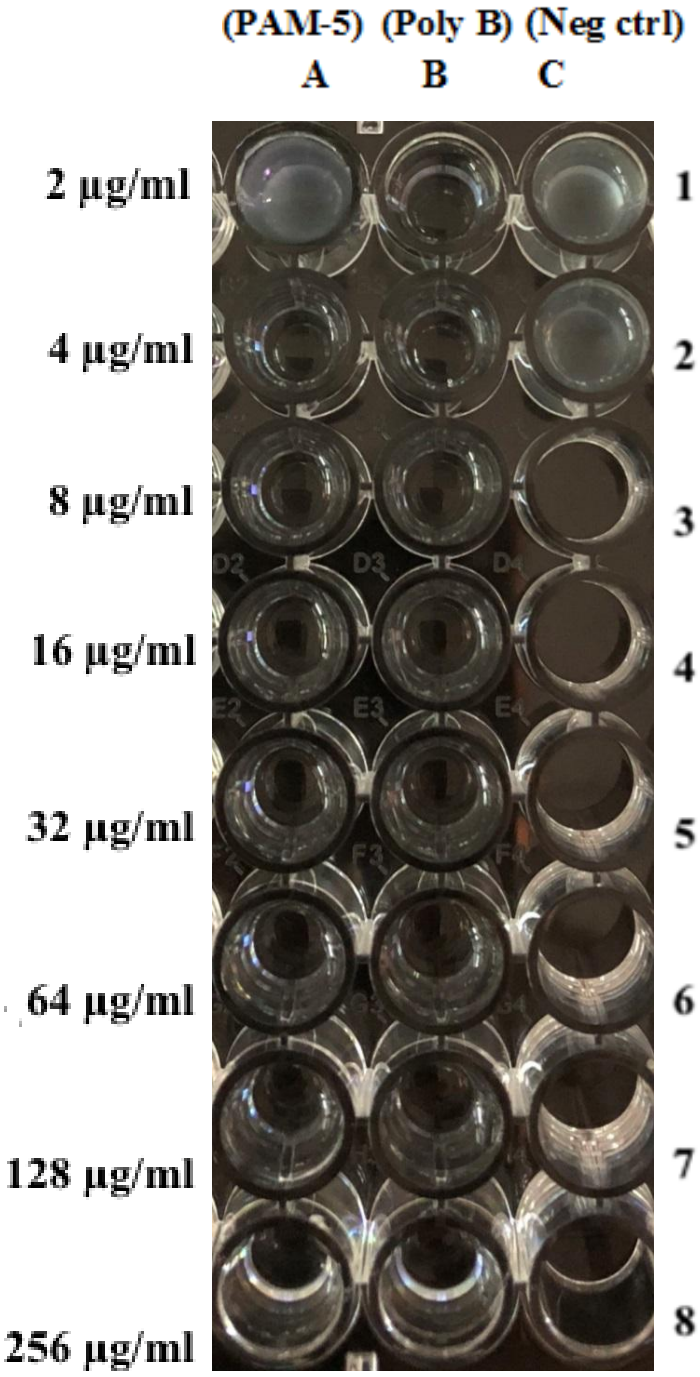

Figure 4.3 (a): Visual inspection of CFZ-, CRO- and CAZ-resistant *A. junii* treated with PAM-5 in 96-well microtitre plate after incubating overnight at 37ºC. Wells A1 to A8: bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml; wells C1 to C2: untreated bacteria suspended in PBS (negative control).

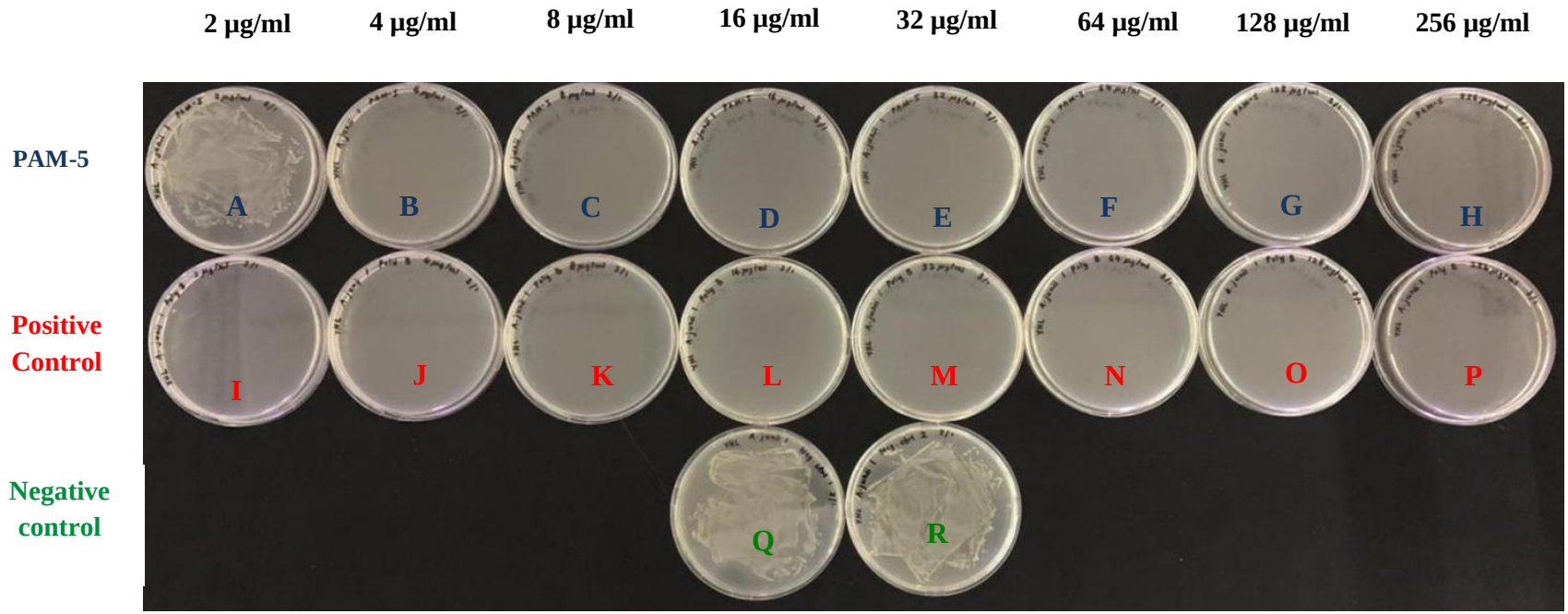

Figure 4.3 (b): Gross view of CFZ-, CRO- and CAZ-resistant *A. junii* after treatment with PAM-5. Plate A to Plate H were bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml. Plate I to Plate P were bacterial cultures treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml. Plates Q and R were negative control plates grown with untreated bacteria. All plates incubated overnight for a period of 16 to 18 hours at 37ºC. The MBC of PAM-5 against CFZ-, CRO- and CAZ-resistant *A. junii* was determined to be 4 µg/ml.

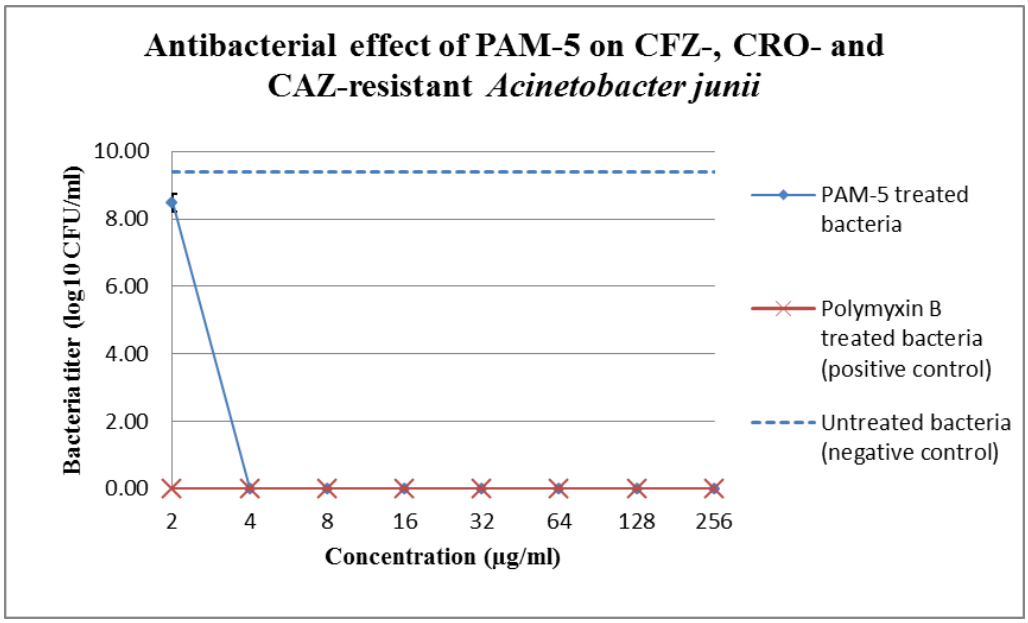

Figure 4.3 (c): Antibacterial effect of PAM-5 on CFZ-, CRO- and CAZ-resistant *Acinetobacter junii*. The blue solid lines (**—**) represent bacteria treated with PAM-5 while the blue dashed lines (---) represent the negative controls. Polymyxin B was completely bactericidal against the tested bacteria at all concentrations.

4.1.4 Antibacterial effect of PAM-5 on AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens*

Nevertheless, PAM-5 did not exhibit similar efficacy against AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens* as compared to the previous bacteria. With reference to **Figure 4.4 (a)**, the wells containing bacteria treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml (well A1 to A8) showed turbidity which was comparable to the wells of negative control (well C1 and C2). These results corresponded to the plating assay as shown in **Figure 4.4 (b)**. Despite the strong action on the previous three bacteria, Polymyxin B was unable to inhibit the growth of *S. marcescens* at concentrations ranging from 2 µg/ml to 32 µg/ml (Plate I to Plate M) as seen in **Figure 4.4 (b).** Similar visual observations were obtained from wells B1 to B5 which contained bacteria treated with Polymyxin B at theses concentrations [**Figure 4.4 (a)**]. However, Polymyxin B was able to completely kill the target bacteria at concentrations of 64 µg/ml and above as indicated by the visually clear wells (well B6 to well B8) on the microtitre plate in **Figure 4.4 (a)** and the absence of bacterial colonies on the media plate (Plate N to P) in **Figure 4.4 (b)**. Thus, PAM-5 is unable to kill AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens* 1191741 at 2 µg/ml to 256 µg/ml.

As shown in **Figure 4.4 (c),** the bacteria treated with PAM-5 at concentrations of 2 µg/ml to 256 µg/ml were able to grow up to the similar titre of the untreated bacteria, indicating the absent or insufficient antibacterial effect. Polymyxin B was unable to kill the bacteria at concentrations ranging from 2

µg/ml to 32 µg/ml as reflected by the similar titre of the treated bacteria to the titre of the negative control. Polymyxin B completely killed the target bacteria at high concentrations ranging from 64 µg/ml to 256 µg/ml. Since *S. marcescens* grew heavily on the MH media despite the treatment by PAM-5 at all the treated concentrations, no MIC and MBC can be determined by this range of concentrations.

Based on the overall analysis, PAM-5 is able to exert moderate bactericidal effect against the clinical strain of *K. pneumoniae.* The peptide has a strong efficacy towards the clinical strains of *A. junii* and *E. coli*. In contrast, PAM-5 was unable to demonstrate bactericidal activity when tested against the clinical strain of *S. marcescens* at PAM-5 concentrations from 2 µg/ml to 256 µg/ml. The MBCs and MICs of PAM-5 against all the tested bacteria were compiled and summarised in **Table 4.1**.

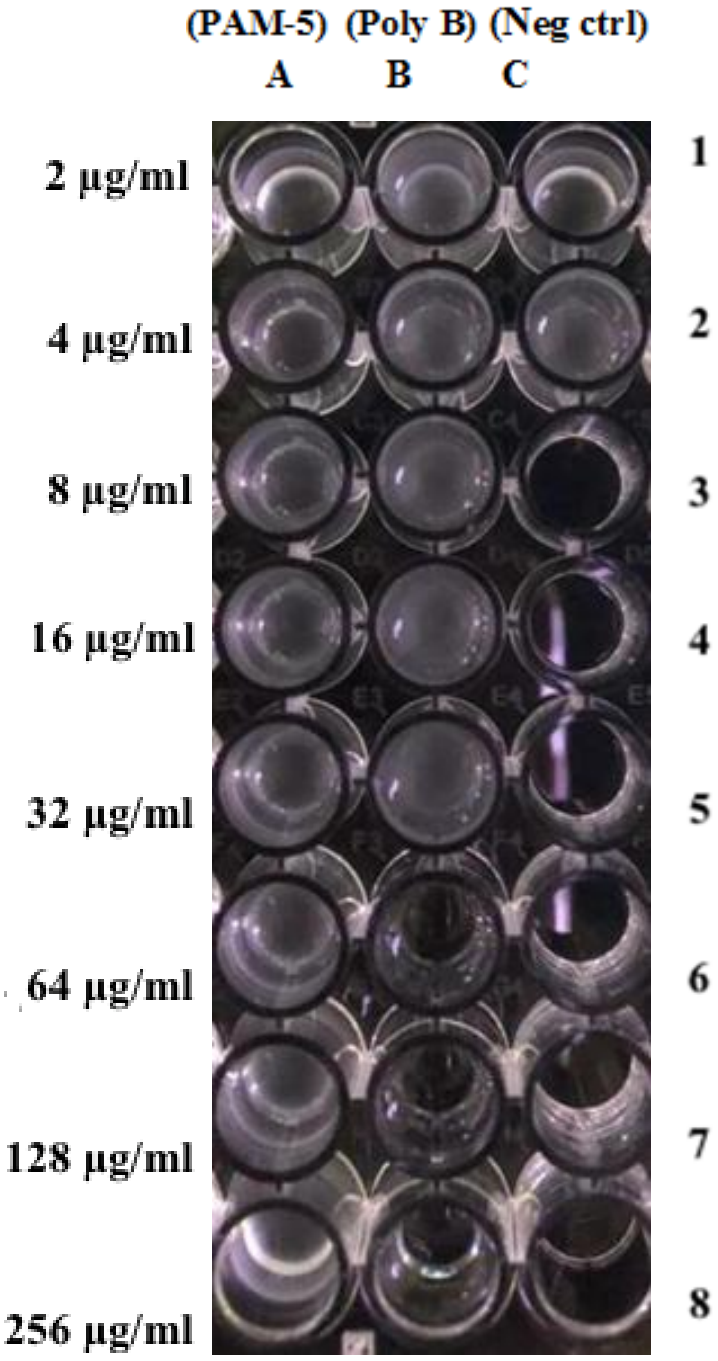

Figure 4.4 (a): Visual inspection of AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens* treated with PAM-5 in 96-well microtitre plate after incubating overnight at 37ºC. Wells A1 to A8: bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml; wells C1 to C2: untreated bacteria suspended in PBS (negative control).

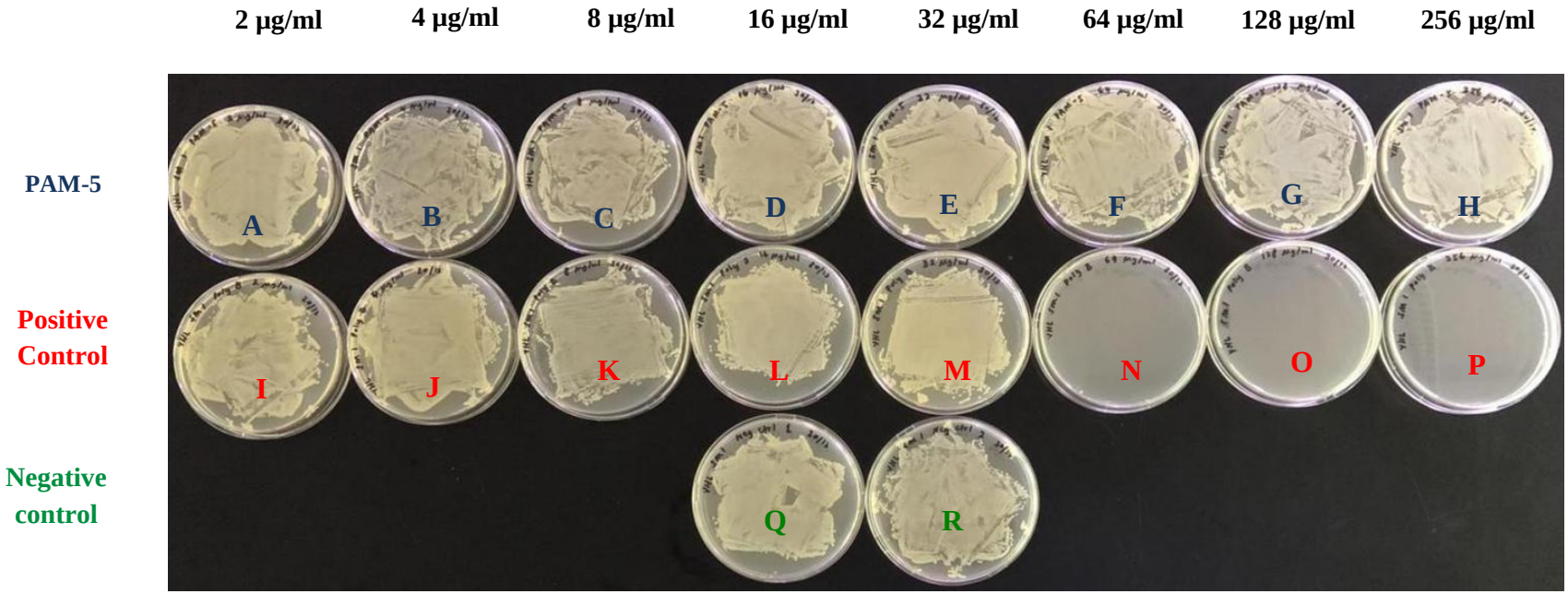

Figure 4.4 (b): Gross view of AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens* after treatment with PAM-5. Plate A to Plate H were bacterial cultures treated with PAM-5 at concentrations from 2 µg/ml to 256 µg/ml. Plate I to Plate P were the bacterial cultures treated with Polymyxin B (positive control) at concentrations from 2 µg/ml to 256 µg/ml. Plates Q and R were the negative control plates grown with untreated bacteria. All plates incubated overnight for a period of 16 to 18 hours at 37ºC.

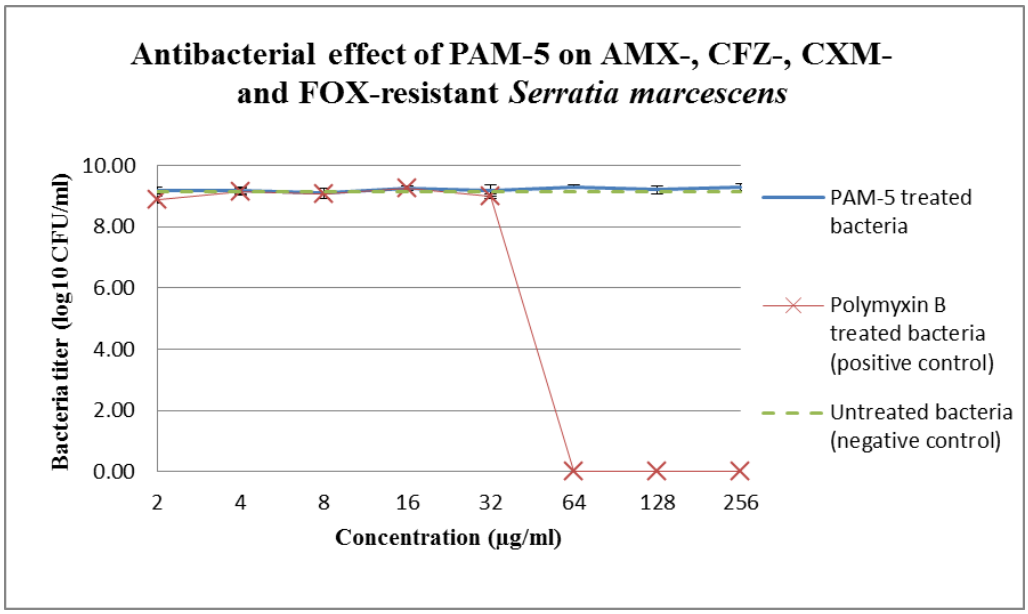

Figure 4.4 (c): Antibacterial effect of PAM-5 on AMX-, CFZ-, CXM- and FOX-resistant *Serratia marcescens.* The blue solid lines (**—**) represent bacteria treated with PAM-5 while the green dashed lines (---) represent the negative controls. Polymyxin B was completely bactericidal against the tested bacteria at 64 µg/ml, 128 µg/ml and 256 µg/ml.

Table 4.1: Summary of antibacterial effects of PAM-5 against selected clinical strains of Gram-negative bacteria.

Bacterial Species and Strain	Relevant Feature	MIC (µg/ml)	MBC (µg/ml)
Klebsiella pneumoniae	Extended-spectrum β-lactamases-producing	16	32
Escherichia coli	CFZ-resistant	-	16
Acinetobacter junii	CFZ-, CRO- and CAZ-resistant	-	4
Serratia marcescens	AMX-, CFZ-, CXM- and FOX-resistant	-	-

CHAPTER 5

DISCUSSION

Antibiotic resistance among pathogenic bacteria remains a serious threat globally causing increased morbidity and mortality rate (World Health Organization, 2018). The misuse and overuse of antibiotics in clinical and agricultural sectors, as well as non-adherence to antibiotic prescription among patients have resulted in the alarming increase of incidence and prevalence of antibiotic-resistance bacteria which is compromising the efficacy of almost all available antibiotics (Centers for Disease Control and Protection, 2017). In particular, health care centres serve as reservoirs for antibiotic-resistant bacteria due to heavy consumption or inappropriate use of antibiotics in management of bacterial infections (Khan, Baig and Mehboob, 2017). The ESKAPE pathogens which represent *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter* species (*Acinetobacter* spp.), *Pseudomonas aeruginosa* (*P. aeruginosa*), *and Enterobacter* species (*Enterobacter* spp.) are responsible for the majority of nosocomial infections throughout the world (El-Mahallawy, et al., 2016). These bacteria can easily acquire resistance to antibiotics via mutation or resistance genes transfer as a result of pressure by excessive antibiotic usage (Exner, et al., 2017). Meanwhile, bacteria such as *Escherichia coli* (*E. coli*) and *Serratia marcescens* (*S. marcescens*) are members of the family Enterobacteriaceae that are also commonly associated with antibiotic

resistance in clinical settings (Yadav, et al., 2015). Therefore, in this study, *K. pneumoniae, A. junii*, *E. coli* and *S. marcescens* with different profiles of antibiotic resistance were chosen as target bacteria for the screening of antibacterial effect of PAM-5.

5.1 Evaluation of Antibacterial Potency of PAM-5 Against Selected Drug-resistant Bacteria

An ideal ABP is usually bactericidal towards bacteria including antibiotic resistant strains with a minimum inhibitory concentration (MIC) ranging from 1 to 8 µg/ml (Hancock, 1997). PAM-5 was highly potent against the clinical strain of CFZ-, CRO- and CAZ-resistant *A. junii* with the MBC of 4 µg/ml. Similarly, PAM-5 was found to have high potency against clinical isolates of CFZ-resistant *E. coli* with the MBC of 16 µg/ml. On the other hand, PAM-5 exhibited moderate potency against the clinical strain of ESBL-producing *K. pneumoniae* where the MIC and MBC were 16 µg/ml and 32 µg/ml, respectively. In constrast, the peptide was unable to exhibit bactericidal effect towards clinical isolate of AMX-. CFZ-, CXM- and FOX-resistant *S. marcescens* at all tested concentrations ranging from 2 to 256 µg/ml. Thus, PAM-5 is assumed to have low potency against *S. marcescens*.

5.2 Antibacterial Potency of PAM-5 Against Selected Clinical Strains of Gram-negative Bacteria

5.2.1 Antibacterial Potency of PAM-5 Against CFZ-, CRO- and CAZ-resistant *A. junii*

Despite the presence of resistance towards ceftazidime, ceftriaxone and cefazolin, PAM-5 is still able to kill the resistant phenotype of *A. junii* at a relatively low concentration. This indicates that the resistant mechanisms possessed by the bacteria do not affect the efficacy of PAM-5. Ceftazidime, ceftriaxone and cefazolin are members of cephalosporins, a class of antibiotics which inhibit bacteria via interfering with the transpeptidation reactions that build the bacterial cell wall. Certain bacteria may acquire resistance towards these antibiotics by producing an enzyme named cephalosporinase that hydrolyses the antibiotics. *Acinetobacter* spp. is associated with this resistance mechanism as represented by a few studies. For example, *A. baumannii* inherently produces an AmpC-type cephalosporinase which is also known as *Acinetobacte*r-derived cephalosporinases (Mak, et al., 2008). The complete killing of cephalosporin-resistant *Acinetobacter junii* as found in this study indicates that the peptide kills the bacteria via other mechanism(s) that is/are not compromised by cephalosporinase.

5.2.2 Antibacterial Potency of PAM-5 Against CFZ-resistant *E. coli*

Similarly, the peptide was able to exert high potency against the clinical strain of CFZ-resistant *E. coli* where complete bactericidal effect was observed at peptide concentration of 16 µg/ml. Based on the antibiotic susceptibility profile,

cefazolin was only able to kill this strain of *E. coli* at concentrations of 64 µg/ml. Cefazolin is a first generation cephalosporin and possesses the ability to bind and inactivate penicillin-binding proteins located at the inner membrane of bacterial cell wall. This causes weakening of the cell wall, resulting in cell lysis (National Cancer Institute, 2018). PAM-5 is able to exhibit better potency than conventional antibiotics. This may be due to the multimodal mechanism of action of PAM-5, unlike cefazolin which only has a single mode of action.

5.2.3 Antibacterial Potency of PAM-5 Against ESBL-producing *K. pneumoniae*

The peptide was able to exert moderate potency against the clinical strain of ESBL-producing *K. pneumoniae* where complete killing was accomplished at the concentration of 32 µg/ml. Although, PAM-5 exerts similar mechanism of action to different bacteria, and we might presume a very close or same MBC value in the killing of *Klebsiella pneumoniae* as compared to the previous two bacteria, but the MBC for *K. pneumoniae* is two-fold higher than the MBC for *E. coli*. One of the possible explanations for this is the ability of *K. pneumoniae* to produce capsular polysaccharides as a protective shield to limit the interaction of ABPs with the surface. These capsular polysaccharides may serve as decoys that bind to antibacterial compounds, thus reducing the accumulation of ABPs on the bacterial membrane. Consequently, more ABPs are required in order to achieve the threshold for membrane disruption. Moreover, the capsular polysaccharides of *K. pneumoniae* are able to alter the surface charge. As a result, the anionicity of the bacterial membrane is reduced

and, this would affect the electrostatic interaction between the peptide and bacterial membrane (Campos, et al., 2004).

5.2.4 Antibacterial Potency of PAM-5 Against AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens*

In contrast, PAM-5 was unable to demonstrate any inhibiting or killing effect towards the clinical isolate of AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens*. The low potency of PAM-5 against *S. marcescens* may be caused by the bacterial intrinsic resistance. One of these intrinsic resistance is the production of extracellular enzymes. Among the extracellular enzymes secreted by *S. marcescens*, there are at least two distinct proteases produced which include a minor serine protease and a metalloprotease (Hines, et al., 1988). These enzymes may confer cross-resistance to the action of antibacterial peptide. Notable examples include trypsin-like serine proteases that are able to hydrolyse amide bonds at positively-charged arginine (R) and lysine (K) residues, whereas chymotrypsin-like serine proteases are able to cleave polypeptides at the hydrophobic phenylalanine (F), tyrosine (Y) and tryptophan (W) residues (Di Cera, 2009; Horn, et al., 2014). PAM-5 (**K**-**W**-**K**-**W**-**R**-P-L-**K**-**R**-**K**-L-V-L-**R**-M) consists of amino acids which include arginine (R), lysine (K) and tryptophan (W), thus indicating the possibility that PAM-5 might be subjected to a certain degree of degradation by these enzymes. The cleavage of the peptide will lead to the loss of cationicity and hydrophobicity that may reduce the efficacy of PAM-5. Hence, the ability of *S. marcescens* to

secrete extracellular enzymes is the possible reason that contributes to the resistance of the bacterium to PAM-5.

5.3 Implications and Limitations of Study

As mentioned in Chapter 4, PAM-5 possessed good efficacy against the various clinical strains of drug-resistant Gram-negative bacteria with the exception of *S. marcescens*. Thus, PAM-5 is potentially able to be developed as an alternative antibacterial agent against drug- and multidrug-resistant bacteria except for *S. marcescens*. However, in this study, the antibacterial potency of PAM-5 was only screened for four clinical strains of antibiotic-resistant Gram-negative bacteria. Moreover, the antibacterial effect of PAM-5 has only been tested *in vitro* and the efficacy of the peptide *in vivo* has not been tested.

5.4 Future Studies

Due to the difference in resistance profile of clinical strains, it is difficult to determine the efficacy of PAM-5 in every case of nosocomial infections. Thus, the antibacterial effects of PAM-5 against more clinical strains of drug- and multidrug-resistant bacteria can be studied in future research. Lastly, after conducting *multiple in vitro* studies of the antibacterial effect of PAM-5, it is also worth it to find out the efficacy of the peptide when applied *in vivo*. With the findings of the antibacterial spectrum of PAM-5, testing the efficacy of the peptide in an animal model is a worthy pursuit.

CHAPTER 6

CONCLUSION

In conclusion, the spectrum of antibacterial activity of synthetic peptide PAM-5 was screened and was found to have relatively different potencies against selected clinical isolates. The peptide demonstrated high potency against clinical strains of CFZ-, CRO- and CAZ-resistant *A. junii and* CFZ-resistant *E. coli* at the MBC of 4 µg/ml and 16 µg/ml, respectively. Moderate potency of PAM-5 was observed for the clinical strains of ESBL-producing *K. pneumoniae* where the MBC was determined to be 32 µg/ml. In contrast, PAM-5 was unable to exhibit antibacterial effects against AMX-, CFZ-, CXM- and FOX-resistant *S. marcescens* at all tested peptide concentrations ranging from 2 µg/ml to 256 µg/ml. It cannot be deduced that the synthetic peptide, PAM-5 is able to exert antibacterial effects towards all clinical isolates that are drug-resistant as the peptide was only potent against a few clinical bacterial strains.

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APPENDIX A

LIST OF AND EQUIPMENTS

Labware/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, China
Biosafety Cabinet Level-2	TELSTAR, Philippines
Bunsen burner	Campingaz, France
Centrifuge machine	SIGMA, Germany
Incubator	Memmert, Germany
Shaking incubator	Yihder, Taiwan
Microcentrifuge tube	Greiner Bio-One, Austria
Micropipette set	Eppendorf Research® plus, Germany
Micropipette tip	Axvgen® Scientific, USA; NEST, China
Petri dish	NEST, 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

MH broth was prepared by dissolving 4.2 g of MH broth powder (Liofilchem) in 200 ml of distilled water. The medium was 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 were stored at 4ºC after the agar has solidified.

Preparation of MacConkey agar

Approximately 25.0 g of MacConkey agar powder (Merck Millipore) was dissolved in 500 ml of distilled water and sterilised by autoclaving at 121ºC for 15 minutes. The medium was allowed to cool slightly before being poured into the petri dishes and stored at 4ºC.

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 approximately 80 ml of distilled water. The mixture was thoroughly mixed and the pH of the solution was adjusted to a pH of 7.4 using 1M sodium hydroxide (NaOH). After the pH was adjusted, the mixture was topped up with distilled water to reach the desired volume of 100 ml. The solution was then sterilised by autoclave at 121ºC for 15 min. This stock solution can be diluted into a 1X working solution following a ratio of 1:10 by the addition of distilled water.

APPENDIX C

BACTERIAL STRAINS WITH RESISTANCE PROFILES

ESBL- producing *Klebsiella pneumoniae* 1195870

Resistant to:

Antibiotic	Class	MIC
Amoxicillin	Penicillin	≥32
Ampicillin	Penicillin	≥32
Cefazolin	Cephalosporin(1st gen)	≥64
Cefuroxime	Cephalosporin(2nd gen)	≥64
Cefotaxime	Cephalosporin(3rd gen)	≥64
Ceftazidime	Cephalosporin(3rd gen)	16
Ceftizoxime	Cephalosporin(3rd gen)	16
Ceftriaxone	Cephalosporin(3rd gen)	≥64
Cefepime	Cephalosporin(4th gen)	32
Aztreonam	Monobactam	≥64
Gentamicin	Aminoglycoside	≥16
Nitrofurantoin	Nitrofuran	128
Trimethoprim	Sulfonamides	≥320

Cefazolin (CFZ)-resistant *Escherichia coli* 1199525

Resistant to:

Antibiotic	Class	MIC
Cefazolin	Cephalosporin (First generation)	≥64

Cefazolin (CFZ)-, ceftriaxone (CRO)- and ceftazidime (CAZ)-resistant *Acinetobacter junii* 1191828

Resistant to:

Antibiotic	Class	MIC
Cefazolin	Cephalosporin(1^{st} gen)	≥64
Ceftriaxone	Cephalosporin(3^{rd} gen)	≥64
Ceftazidime	Cephalosporin(3^{rd} gen)	32

Amoxicillin (AMX)-, cefazolin (CFZ)-, cefuroxime (CXM)- and cefoxitin (FOX)-resistant *Serratia marcescens* 1191741

Resistant to:

Antibiotic	Class	MIC
Amoxicillin	Penicillin	≥32
Cefazolin	Cephalosporin(1^{st} gen)	≥64
Cefuroxime	Cephalosporin(2^{nd} gen)	≥64
Cefoxitin	Cephalosporin(2^{nd} gen)	32