# SCREENING OF BROAD-SPECTRUM ANTIBACTERIAL EFFECTS OF SYNTHETIC PEPTIDE PAM-5 AGAINST SELECTED PATHOGENIC BACTERIA

By

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## ABSTRACT

**SCREENING OF BROAD-SPECTRUM ANTIBACTERIAL EFFECTS OF SYNTHETIC PEPTIDE PAM-5 AGAINST SELECTED PATHOGENIC BACTERIA**

**Chan Szn Yi**

For the past two decades, antibacterial peptides (ABPs) have been extensively studied due to their great potential as alternative antibacterial agents for conventional antibiotics. However, most of the researchers prefer to study the naturally occurring peptides instead of the chemically synthesised peptides. Among the limited researches conducted on synthetic peptides, only few of them focused on the peptide broad-spectrum antibacterial activities. Thus, in this study, the broad-spectrum antibacterial activities of PAM-5, a 15-mer synthetic peptide was screened against five selected pathogenic bacteria, which included four Gram-negative bacteria namely *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), *Acinetobacter baumannii* (*A. baumannii*), *Klebsiella pneumoniae* (*K. pneumoniae*) and one Gram-positive bacterium, which was *Staphylococcus aureus* (*S. aureus*) by using microbroth dilution assay. In addition, the antibacterial potency of PAM-5 was also tested on three clinical isolates, which were *Shigella flexneri* (*S. flexneri*), multi-drug resistant *P. aeruginosa* and extended-spectrum β-lactamases producing (ESBL)

*E. coli* using the same assay. These results were further validated using PrestoBlue™ bacterial viability assay. Both assays were able to produce consistent results whereby PAM-5 was bactericidal to all tested bacteria at minimum bactericidal concentrations (MBCs) of 8 μg/ml against *P. aeruginosa*, *E. coli* and *A. baumannii*, 16 μg/ml against multidrug-resistant *P. aeruginosa*, 32 μg/ml against *K. pneumoniae* and *S. flexneri*, 64 μg/ml against ESBL-producing *E. coli* and 96 μg/ml against *S. aureus*. These findings suggest that PAM-5 is highly potent against various bacteria including the multi-drug resistant strains although the peptide efficacy was reduced for *S. aureus*. However, as the peptide was only screened on one Gram-positive bacterium, further studies of the antibacterial effects of PAM-5 against more pathogenic Gram-positive bacteria should be done. In conclusion, with the favourable antibacterial activities against various Gram-negative bacteria including the multidrug-resistant strains, PAM-5 has high potential to be developed into a novel broad-spectrum antibacterial agent.

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## DECLARATION

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

_____

CHAN SZN YI

## APPROVAL SHEET

This project report entitled "**SCREENING OF BROAD-SPECTRUM ANTIBACTERIAL EFFECTS OF SYNTHETIC PEPTIDE PAM-5 AGAINST SELECTED PATHOGENIC BACTERIA**" was prepared by CHAN SZN YI 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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**FACULTY OF SCIENCE**

**UNIVERSITI TUNKU ABDUL RAHMAN**

Date:_____

**PERMISSION SHEET**

It is hereby certified that CHAN SZN YI (ID No: 12ADB02490) has completed this final year project entitled "**SCREENING OF BROAD-SPECTRUM ANTIBACTERIAL EFFECTS OF SYNTHETIC PEPTIDE PAM-5 AGAINST SELECTED 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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(CHAN SZN YI)

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| | |
|---|---|
| ABPs | Antibacterial peptides |
| ATCC | American Type Culture Collection |
| CFU | Colony forming units |
| CLSI | Clinical and Laboratory Standard Institutes |
| Da | Dalton |
| ESBL | Extended-spectrum β-lactamases |
| G | Glycine |
| K | Lysine |
| L | Leucine |
| LB | Luria-Bertani |
| M | Methionine |
| MBC | Minimum bactericidal concentration |
| MDR | Multidrug-resistant |
| MH | Mueller-Hinton |
| MIC | Minimum inhibitory concentration |
| NADH | Reduced nicotinamide adenine dinucleotide |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| P | Proline |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| R | Arginine |
| SPATE | Serine protease autotransporters of *Enterobacteriaceae* |
| W | Tryptophan |

# CHAPTER 1

# INTRODUCTION

Antibacterial agents are substances that can kill bacteria effectively. Antibiotic is a good example of the antibacterial agents that is used widely to treat bacterial infections. However, most of the conventional antibiotics are of limited spectrum. With their limited antibacterial activities against a particular bacterial species, the etiological agent of an infection needs to be identified before deciding an antibiotic prescription, thus making these antibiotics unsuitable to be used as empirical treatments for acute infections and polyinfections. Despite the presence of antibiotics that are broad-spectrum, the high incidence and prevalence of multi-drug resistant bacteria have somehow compromised their efficacy. Thus, it is in need to search and develop alternative antibacterial agents. One of the potential alternatives is antibacterial peptides (ABPs).

In the recent years, many researchers have ventured into ABPs seeking for their potential clinical applications. However, majority of them generally target naturally occurring ABPs produced by the innate immunity. Limited studies are done on novel synthetic peptides. Even so, very few of them are exploring the spectrum of antibacterial activities by these peptides.

Previously, a 15-mer synthetic peptide, PAM-5 was designed and screened for its antibacterial effect against *Pseudomonas aeruginosa* by Lee (2014; 2015). These studies had shown remarkable findings where the peptide was highly potent against this bacterium. However, the antibacterial potencies of PAM-5 against other bacteria were still unexplored. Therefore, this project was conducted based on the following objectives:

1. To verify the antibacterial activity of PAM-5 against *P. aeruginosa* ATCC 27853.

2. To screen the spectrum of antibacterial effects of the peptide on selected pathogenic bacteria using microbroth dilution assay and PrestoBlue™ bacterial viability assay.

    i. To determine the minimum bactericidal concentrations (MBCs) and/or minimum inhibitory concentrations (MICs) of PAM-5 against these selective pathogenic bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Limitations of conventional antibiotics

The mortality and morbidity rate caused by bacterial infection has been drastically reduced ever since the discovery and development of antibiotics. However, most conventional antibiotics are of limited spectrum. With their limited antibacterial activities against a particular bacterial species, these narrow-spectrum antibiotics are unsuitable to be used in patients with polyinfections, which can be commonly seen in those who are immunocompromised. Besides, these antibiotics are also unsuitable to be used for empirical treatments as the causative agent of an infection needs to be determined before an antibiotic prescription can be decided (Leekha, Terrell and Edson, 2011). Likewise, if a wrong prescription is given, it does not only leave the infections untreated, it might also kill the normal microbiota in the patient that acts as the first line protection (Cotter, 2012). This might further worsen the condition of the patient. Indeed, there is presence of few broad-spectrum antibiotics. However, these antibiotics possess certain limitations as well. One of the limitations is the emergence of multi-drug resistant bacteria due to the excessive usage of these antibiotics (Rao, 1998). Besides, with the specific mechanism of action, the efficacy of these conventional antibiotics have also been easily compromised due to the modification by the bacteria on the drug target (Lambert, 2005).

Consequently, the development of new antibiotics remained as a challenge to those in medical science due to the rapid emergence of multi-drug resistant bacteria. This is because the duration needed for the bacteria to mutate and acquire resistance is much shorter than the duration needed for the development of a novel antibiotic (Braine, 2011).

Thus, with the decreasing effective antibacterial agents available, there is in need to search for novel therapeutic agents that have broad-spectrum antibacterial activities with minimal risk of inducing bacterial resistance so that a single agent can be used to treat multiple bacterial infections.

## 2.2 Antibacterial peptides

### 2.2.1 Overview

For the past two decades, much effort have been taken to search for alternative therapeutic agents in replacement of conventional antibiotics due to their narrow-spectrum of activities and reduced efficacy against various bacteria, particularly those multidrug-resistance strains. One of the potential compounds is antibacterial peptides (ABPs). ABPs can be naturally derived or chemically synthesised. They are usually made up of 12 to 100 amino acids, cationic with a net charge ranging from +2 to +9 and amphipatic (Hancock and Lehrer, 1998; Jenssen, Hamill and Hancock, 2006; Pasupuleti, Schmidtchen and Malmsten, 2012; Bahar and Ren, 2013).

The typical mode of action of ABPs is by causing damage to the bacterial membrane where the cationicity and amphipathicity of peptides play important roles. The cationicity allows the initial contact between the positively-charged peptide and the negatively-charged bacterial membrane through electrostatic interactions. The ABPs will start to accumulate on the bacterial membrane. The initial low peptide adsorption concentration facilitates the binding of more peptides to the bacterial membrane until a threshold concentration is reached. Thereafter, the ABPs penetrate into the phospholipid bilayer of the bacterial membrane via its hydrophobic side chain to cause permeabilisation and further downstream damages (Wieprecht et al., 1997; Diamond, 2009; Cézard et al., 2011).

Apart from the primary mode of action, recent studies had also documented the ability of ABPs to act on various intracellular components of the bacteria. For example, indolicidin was demonstrated with the ability to inhibit DNA synthesis (Hsu, 2005). In the study conducted by Kragol et al. (2001), pyrrhocoricin was found to be able to halt the actions of DnaK in *Escherichia coli* (*E. coli*) by inhibiting its ATPase activity. In the study done by Roy et al. (2015), Onc112 derived from oncocin was able to bind to the 70s ribosome of *Thermus thermophilus* that cause inhibition of its protein synthesis. Bac71-35 was also shown to inhibit protein synthesis in *E. coli* (Mardirossian et al., 2014). Apart from that, MP196, a synthetic hexapeptide was found to have the ability to interfere with cell wall synthesis (Wenzel et al., 2014).

### 2.2.2 Advantages of ABPs

ABPs possess various advantages that made them a potential alternative antibacterial agent to conventional antibiotics. Firstly, these peptides can act selectively on the target bacteria without harming the host cells. This is due to the selective electrostatic interaction between the positively-charged peptides and the negatively-charged bacterial membrane that consist of anionic phosphate group in Gram-negative bacteria and anionic glycopolymers, techoic acids in Gram-positive bacteria (Epand, Maloy and Ramamoorthy, 2010; van Meer, Voelker and Feigenson, 2008). The high level of cholesterol present in eukaryotic cell membrane also plays a role in the selective toxicity of ABP. These cholesterols are able to strengthen the phospholipid bilayer, preventing the membrane disruption by the peptides (Epand, Malay and Ramamoorthy, 2010; Verly et al., 2008).

Most importantly, ABPs are found to have broad-spectrum antibacterial activities. A number of naturally occurring peptides and their derivatives such as cathelicidin and alpha defensin were discovered and proven to have the ability to act against various bacteria (Selsted and Ouellette, 2005; Chromek et al., 2006; Hancock and Rozek, 2002). Some of these peptides could even act against fungi (Jin et al., 2010), viruses (Judd et al., 1997; Huther & Dietrich, 2007), parasites (Haines et al., 2009) and cancer cells (Hoskin and Ramamoorthy, 2008; Gaspar, Veiga and Castanho, 2013).

On top of that, unlike conventional antibiotics, where most of them usually act only against a particular biosynthetic pathway, antibacterial peptides can act on multiple targets. They are able to cause disruptions to the bacterial cell membrane by removing the divalent cations that link the neighbouring lipopolysaccharides. This leads to the leakage of the bacterial intracellular components and eventually cell death (Sawyer, Martin and Hancock, 1988). In addition to that, these peptides are also able to act against various intracellular components of the bacteria that will interfere with their vital metabolic pathways as mentioned in **Section 2.2.1**.

Furthermore, the ability of these peptide to induce rapid killing disallow the bacteria to undergo mutation or carry out cellular repair simultaneously due to the high metabolic cost. This reduces the survival rate of the bacteria. Thus, the bacteria would have minimal chances of developing resistance against these peptides (Chan, Prenner and Vogel, 2006; Marr, Gooderham and Hancock, 2006; Lv et al., 2014). With the above mentioned advantages, antibacterial peptides do have great potential to be developed as an alternative to conventional antibiotics.

### 2.2.3 Previous findings on ABPs

ABPs are widely studied over the last decade due to its advantages and promising antibacterial effects. Most of these researches usually focused on the naturally occurring peptides isolated from living organisms. However, in the recent years, the number of novel synthetic peptides being produced and

studied has been increasing due to various limitations of the natural peptides such as the labourious extraction process, low yield and unguaranteed efficacy due to their diverse primary sequences (Song et al., 2012; Jenssen, Hamill and Hancock, 2006).

A number of novel synthetic ABPs analogous to natural antibacterial peptides have been designed and synthesised throughout the years. Promising antibacterial activities were observed in most of the studies. For instance, EC5, a narrow spectrum synthetic ABP that was synthesised via phage display screening by Rao, Mohan and Atreya in 2013 was shown to be effective against *E. coli* with a minimum bactericidal concentration (MBC) of 8 μg/ml. Although it showed antibacterial activity against *Pseudomonas aeruginosa* (*P. aeruginosa*) as well, low peptide potency was observed. Besides, promising findings were obtained when the antibacterial activity of synthetic peptide S-thanatin, an analogue to thanatin synthesised by Wu et al. (2013) was screened against clinical isolates of *Klebsiella pneumoniae* (*K. pneumoniae*). In the study by Pires et al. (2015), synthetic peptide Dendrimer (G3KL) was strongly effective against both carbapenemase-producing *Acinetobacter baumannii* (*A. baumannii*) and *P. aeruginosa* at low MBCs. Meanwhile, several synthetic peptides, namely tachyplesin 3, lipopeptide, and protegrin 1 showed significant antibacterial activities against *Staphylococcus aureus* (*S. aureus*) including the multi-drug resistant strain (Baranska-Rybak et al., 2011). Besides, peptides RRIKA and RR synthesised by Mohamed and his research team in 2014 also showed high potency against various staphylococcal isolates.

However, most of these studies focused only on the antibacterial activity of antibacterial peptides against one particular type of bacteria. Limited novel ABPs with broad spectrum antibacterial activities were discovered. Thus, more studies should be done to study the broad-spectrum antibacterial activity of synthetic peptides.

### 2.2.4 Previous findings on broad-spectrum synthetic ABPs

Although limited synthetic ABPs with broad-spectrum antibacterial activities were discovered, there are indeed ABPs with this feature being successfully synthesised. For example, R-BP100 and RW-BP100 synthesised by Torcato et al. (2013) were found to be potent against both reference strains and multidrug-resistant strains of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *Streptococcus pneumoniae* (*S. pneumoniae*) and *Enterococcus faecium* (*E. faecium*). Besides, in a study by Rathinakumar, Walkenhorst and Wimley (2009), ten synthetic peptides selected from a peptide library were found to be active against *S. aureus*, *E. coli* and *P. aeruginosa* at low peptide MIC. M6 peptide isolated through phage display selection by Pini et al. (2005) was also active against various reference strains and drug-resistant clinical isolates such as *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *Enterobacter aerogenes* and *Enterobacter cloacae*. Synthetic peptide P19(9/B) was also found to be potent against *P. aeruginosa*, *Stenotrophomonas maltophilia* and *S. aueus* (Pompilio et al., 2012).

Previous studies have documented various novel synthetic peptides that possess broad-spectrum antibacterial activities. Thus, this study was also aimed to screen for the antibacterial spectrum of synthetic peptide PAM-5.

### 2.2.5 Previous findings on synthetic peptide PAM-5

Synthetic peptide PAM-5 (K-W-K-W-R-P-L-K-R-K-L-V-L-R-M) with cationicity of +7 and hydrophobicity of 46% was previously screened for its antibacterial potency against *P. aeruginosa* by Lee (2015). It was found to have significant bactericidal effect against the bacteria at MBC of 8 μg/ml. This peptide was modified from its parental synthetic peptide PAM-1 and PAM-2 by Lee in 2014 with increased cationicity. PAM-1 and PAM-2 were initially derived by Tan (2014) from PAI-20 and PA-26 that were isolated by Gwee (2011) and Lee (2012) through phage-displayed peptide library screening. As compared to PAM-5, PAM-1 and PAM-2 as well as their parental peptides, PAI-20 and PA-26 had low potencies against *P. aeruginosa*. As PAM-5 was only screened for its antibacterial effects against *P. aeruginosa*, it is worth to know whether this peptide possesses similar potency against other pathogenic bacteria. Thus, in this study, PAM-5 was tested against a variety of bacteria, including both Gram-negative and Gram-positive as well as a few clinical isolates to determine its antibacterial spectrum.

# CHAPTER 3

# MATERIALS AND METHODOLOGY

## 3.1 General experimental design

PAM-5, a 15-mer synthetic peptide was screened for its antibacterial effects against selected Gram-negative and Gram-positive pathogenic bacteria. These bacteria covered both reference strains and clinical isolated strains as well as the multidrug resistant (MDR) isolates. Using microbroth dilution assay, the bacteria were treated with different concentrations of PAM-5. Upon incubation, the treated bacteria were inoculated on growth media for colony counting in order to determine the minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) of the peptide. These results were then validated using PrestoBlue$^{TM}$ bacterial viability assay. Independent replicates were done thrice for each assay.

## 3.2 Labware and equipments

Refer to Appendix A.

## 3.3 Materials

### 3.3.1 Bacterial strains

A total of eight bacterial strains were employed in this study. There were five reference strains of bacteria which included *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC 19606), *Klebsiella pneumoniae* (ATCC 13883) and *Staphylococcus aureus* (ATCC 25923) provided by the Department of Biomedical Science, Universiti Tunku Abdul Rahman. The clinical strain of *Shigella flexneri* was kindly provided by Dr. Tan Gim Cheong from the same department. On the other hand, multidrug-resistant strains of *Pseudomonas aeruginosa* and extended-spectrum β-lactamases-producing *Escherichia coli* were isolated from patients in Gleneagles Medical Center in Penang.

*Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Shigella flexneri* were first inoculated on MacConkey agar while *Staphylococcus aureus* was inoculated on Mannitol Salt agar. The bacteria were then grown in Luria-Bertani (LB) broth, preserved in glycerol with final concentration of 20% (v/v) and stored at -80 ℃. Before performing the antibacterial assay, the bacteria were retrieved from the bacterial glycerol stock and inoculated on their respective media as a master culture plate. The

plates were then incubated overnight at 37℃ and stored at 4℃ for the maximum of seven days to ensure the freshness of the bacteria.

### 3.3.2 PAM-5 synthesis

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

### 3.3.3 PAM-5 preparation

Prior to dissolving the peptide, it was equilibrated to room temperature for about one hour. The peptide was then 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 performed using degassed, filtered-sterilised phosphate buffered saline (PBS) and the diluted peptide solution were stored in silica bottles at 4℃ for seven days at most to ensure the peptide efficacy according to the manufacturer's recommendation.

### 3.3.4 Polymyxin B preparation

Polymyxin B (Calbiochem®), made in Denmark was purchased from Merck Millipore. Similar procedures as mentioned in the preparation of PAM-5 in **Section 3.3.3** were employed in the preparation of Polymyxin B, which served as the positive control for all antibacterial assays performed.

### 3.3.5 Preparation of buffers and media

Refer to Appendix B.

## 3.4 Protocols

### 3.4.1 Antibacterial assay

A modified version of the Clinical and Laboratory Standard Institute (CLSI)-recommended microbroth dilution assay was applied to screen the antibacterial effects of PAM-5 against selected bacteria as mentioned in **Section 3.3.1**. Briefly, an overnight bacterial culture was prepared by inoculating two to three colonies of the target bacteria from the master culture plate into 10 ml of Mueller-Hinton (MH) broth and grown overnight at 37 ℃ in a shaking incubator (200 rpm). On the next day, a hundred-fold dilution was done by diluting 200 µl of the overnight culture into 20 ml of MH broth. The diluted culture was incubated at 37 ℃ with rotation at 200 rpm until the mid-log phase of bacterial growth was achieved, which is equivalent to $OD_{600}$ 0.500 to 0.600.

The culture was then pelleted through centrifugation at the speed of 6000 x g for 6 minutes. The pellet washed by resuspending with phosphate buffered saline (PBS, pH 7.4) followed by recentrifugation. These washing steps were repeated twice. After the last wash, the bacteria pellet was resuspended in 1 ml of PBS and serially diluted to achieve bacterial titer of $10^3$ CFU/ml.

Next, 100 µl of the bacterial suspension at $10^3$ CFU/ml was loaded into the wells of 96-well microtiter plate and 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. On the other hand, the positive controls were set up by treating the bacteria with Polymyxin B at the same series of final concentrations while the negative controls were prepared using untreated bacteria suspended in PBS. The contents of each well were summarised in **Table 3.1**. The microtiter plate was pre-incubated for 1 hour prior to the addition of 50 µl of MH broth into each well. The microtiter plate was then incubated overnight for 16 to 18 hours at 37 ℃.

After overnight incubation, the contents in the wells of the microtiter plate were subjected to visual inspection for turbidity as a sign of bacterial growth. Then, 10 µl of bacterial suspension from each well was inoculated onto MH agar to check for the presence of viable bacteria. Cultures from the wells that appeared turbid were then serially diluted with PBS and inoculated on MH agar for bacterial titering. The inoculated media were incubated overnight at 37 ℃ and the number of bacterial colonies was counted compare the titer of

PAM-5-treated bacteria with the titer of untreated bacteria. Eventually, the enumeration of minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs) of PAM-5 can be done. According to the CLSI, MIC is the lowest concentration of antibacterial agent that is able to inhibit visible bacterial growth in the growing medium (Cockerill et al., 2012), while MBC is the lowest concentration of antibacterial agent that is needed to kill at least 99.99% of the original bacterial inoculum, preventing their growth after being inoculated onto an antibiotic-free medium (Wikler et al., 2009). Determination of MBC and MIC was illustrated in **Figure 3.1**.

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

| **Contents** | **Sample 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 |

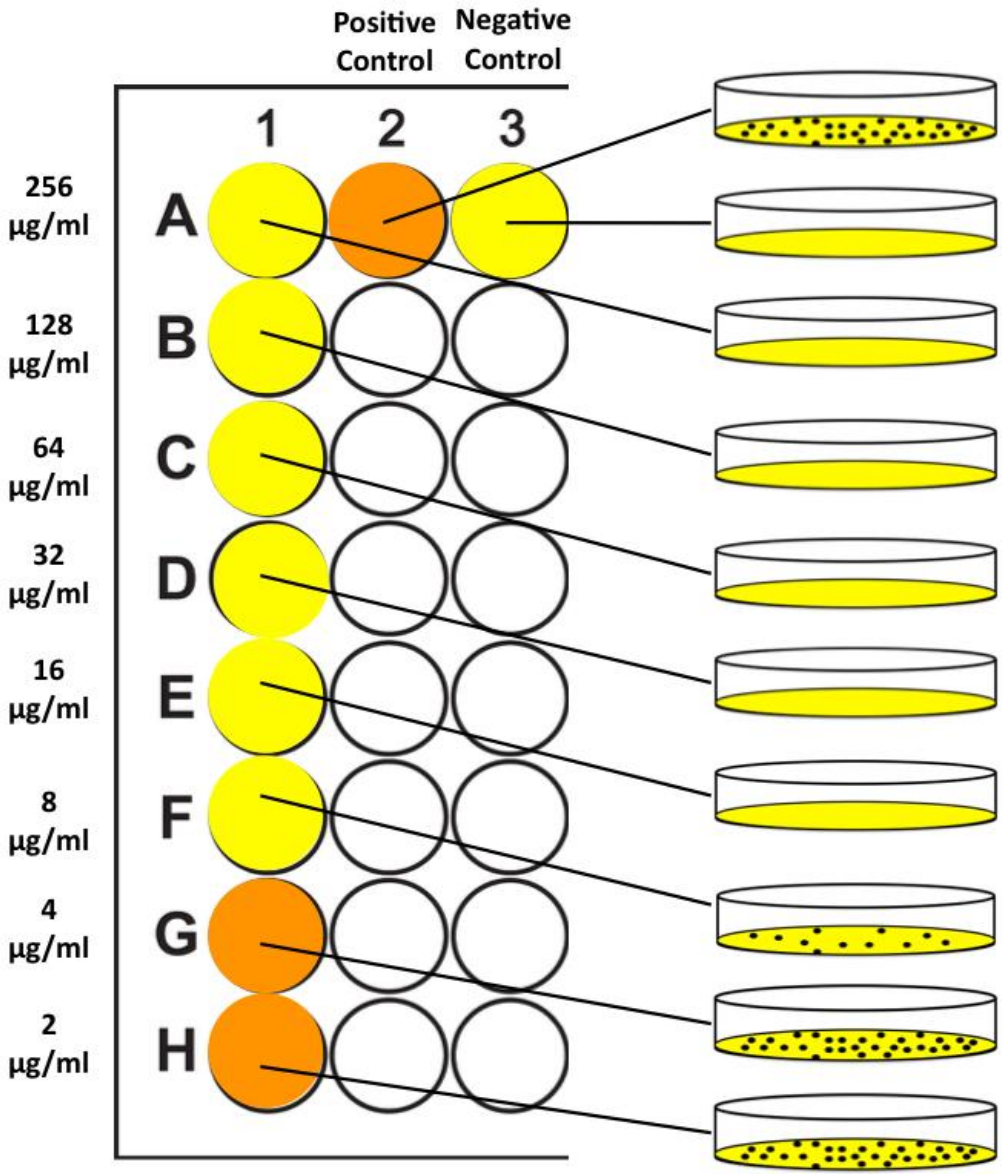


**Figure 3.1:** Determination of MIC and MBC by microbroth dilution method. Visually turbid wells were represented by orange colour: G1, H1 and A2; Visually clear wells were represented by yellow colour: A1 to F1 and A3. The well with the lowest concentration of antibacterial agent that shows no visible turbidity and reduced bacterial colonies after inoculated onto the media plate is recognised as the MIC. The well with the lowest concentration of antibacterial agent that shows no bacterial growth after being inoculated onto the media plate is recognised as the MBC. In this figure, the MIC is determined to be 8 µg/ml while the MBC is 16 µg/ml.

### 3.4.2 PrestoBlue™ bacterial viability assay

In this assay, similar procedures for the bacterial preparation were performed as described in **Section 3.4.1**. One hundred microliters of the bacterial suspension was loaded into a opaque white, 96-well microtiter plate and treated with the 100 µl of two-fold serially diluted PAM-5 at final concentrations ranging from 2 µg/ml to 256 µg/ml. Bacteria treated with Polymyxin B at the same final concentrations as the sample wells served as the positive control while untreated bacteria served as the negative control. The contents of each well were summarised in **Table 3.2**. The microtiter plate was pre-incubated at 37 ℃ for 1 hour before adding 50 µl of MH broth and 20 µl of PrestoBlue™ reagent into each well. Subsequently, the microtiter plate was incubated overnight for 16 to 18 hours at 37 ℃. The colour of each well was observed on the following day. Pink colouration was observed in wells containing viable bacteria while the PrestoBlue™ reagent remained blue in wells without viable bacteria.

**Table 3.2:** Contents of the sample, positive control and negative control wells of PrestoBlue™ bacterial viability assay.

| Contents | Sample 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 |
| PrestoBlue™ reagent | 20 µl | 20 µl | 20 µl |

# CHAPTER 4

# RESULTS

## 4.1 Evaluation of broad-spectrum antibacterial effects of PAM-5 using microbroth dilution assay

The spectrum of antibacterial activity for PAM-5 was screened against the following bacterial strains: *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *Escherichia coli* (*E. coli*) ATCC 25922, *Acinetobacter baumannii* (*A. baumannii*) ATCC 19606, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 13883 and *Staphylococcus aureus* (*S. aureus*) ATCC 25923 as well as three clinical strains which included *Shigella flexneri* (*S. flexneri*), multidrug-resistant (MDR) *P. aeruginosa* and extended-spectrum β-lactamases (ESBL)-producing *E. coli*. The minimum bactericidal concentrations (MBCs) and/or minimum inhibitory concentrations (MICs) of the peptide against these bacteria were determined according to a modified version of microbroth dilution assay recommended by the Clinical and Laboratory Standards Institute (CLSI) as described in **Section 3.4.1**.

### 4.1.1 Antibacterial effect of PAM-5 on *P. aeruginosa* ATCC 27853

Antibacterial screening using microbroth dilution assay revealed that PAM-5 was active against *P. aeruginosa* ATCC 27853. Based on **Figure 4.1 (a)**, PAM-5 at concentrations ranging from 8 μg/ml to 256 μg/ml showed complete killing of the bacteria as indicated by the absence of bacterial growth on the inoculating media (Plate I to Plate N). However, lower concentrations of the peptide (4 μg/ml and 2 μg/ml) were not able to kill or inhibit bacterial growth as shown by the bacterial lawn on the media plates (Plate O and Plate P), which was similar to the bacterial growth pattern of the negative control (Plate Q and Plate R). Polymyxin B, which served as the positive control, managed to achieve complete bactericidal effect at all concentrations as shown in Plate A to Plate H where no bacterial growth was observed. Based on **Figure 4.1 (b)** there was no MIC documented as the visually clear well containing bacteria treated with 8 μg/ml of PAM-5 (well A6) showed no growth after overnight incubation on the media plate (Plate N). However, visible bacterial growth was observed in wells containing culture treated with 4 μg/ml and 2 μg/ml of PAM-5 (well A7 and A8), which had similar turbidity as the wells of negative control (well C1 and C2) . The absence of bacterial growth on Plate I to Plate N was also corresponding to the visually clear wells with peptide concentrations at and beyond 8 μg/ml (well A1 to A6). Thus, the MBC of PAM-5 against *P. aeruginosa* ATCC 27853 was determined as 8 μg/ml with no MIC observed.

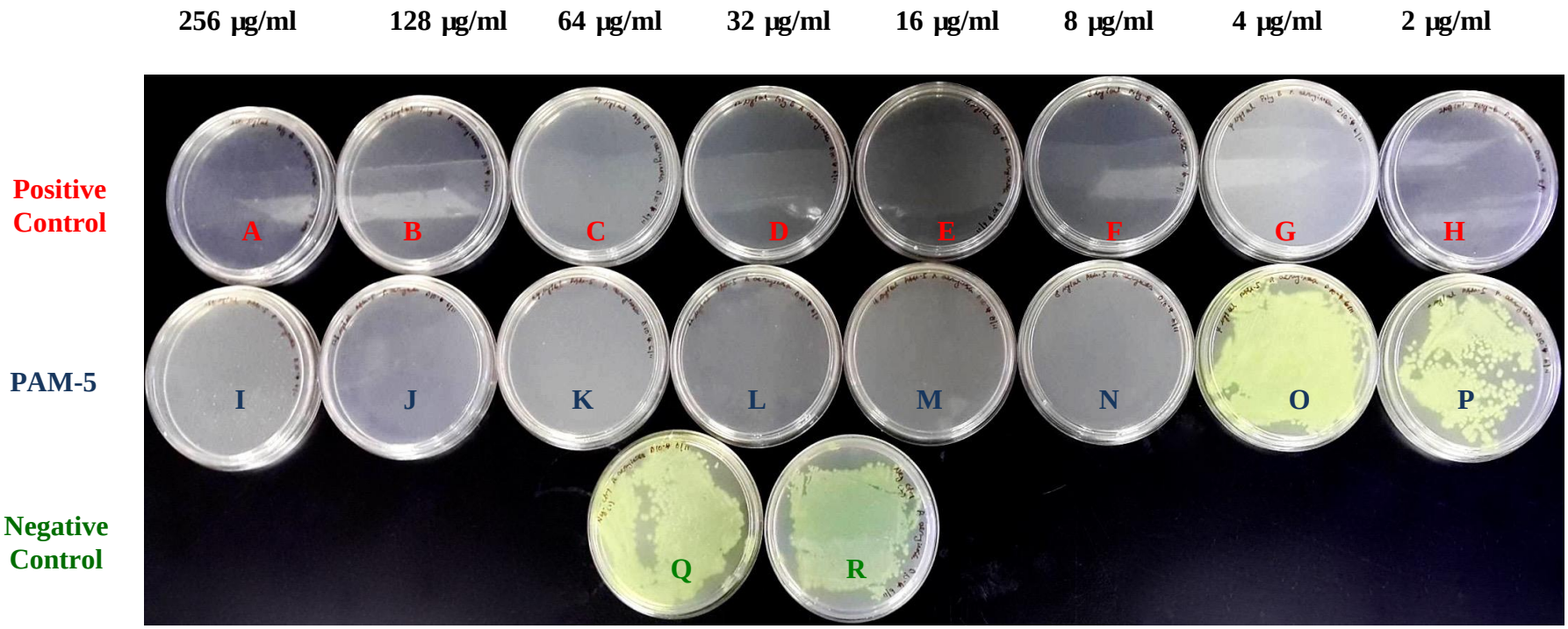


**Figure 4.1 (a):** Gross view of *P. aeruginosa* ATCC 27853 after PAM-5 treatment. Plate I to Plate P were bacterial cultures treated with PAM-5. Plate A to Plate H were the positive control plates of bacterial cultures treated with Polymyxin B while Plate Q and Plate R were the negative control plates grown with untreated bacteria. All plates were incubated overnight for 16 to 18 hours at 37 ℃. The MBC of PAM-5 against *P. aeruginosa* ATCC 27853 was determined as 8 µg/ml.

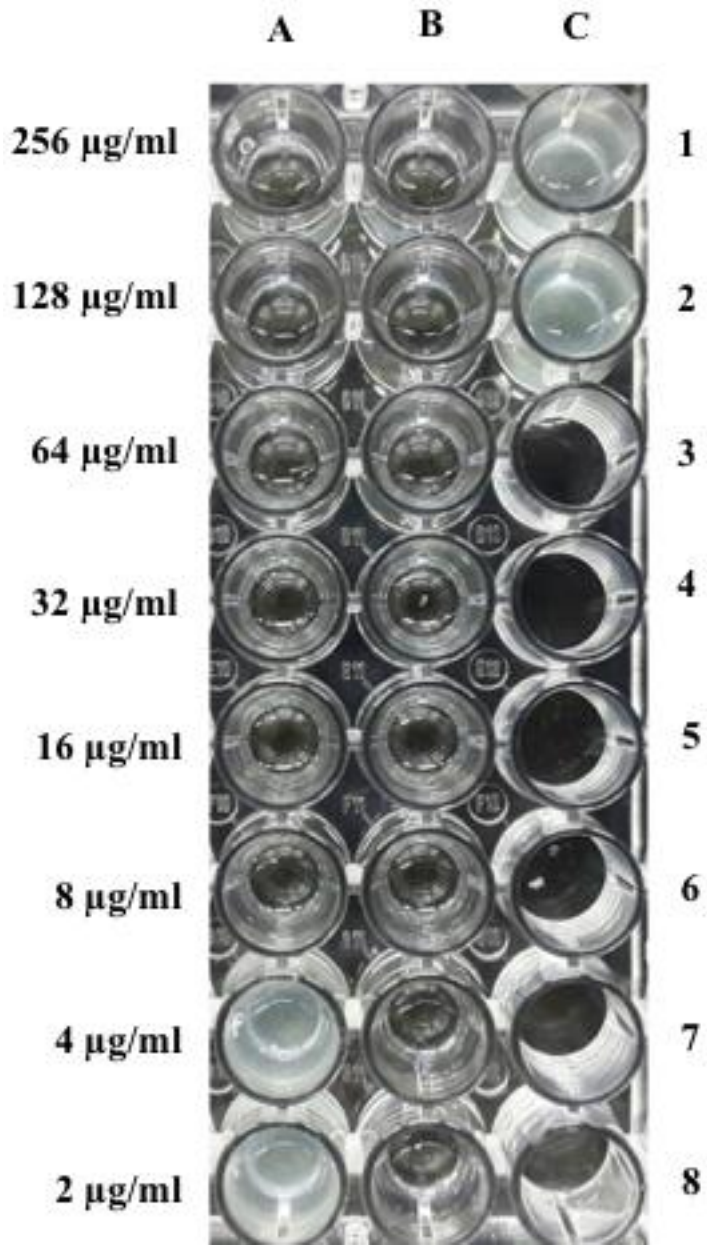


**Figure 4.1 (b):** Visual inspection of *P. aeruginosa* ATCC 27853 treated with PAM-5 in 96-well microtiter plate after overnight incubation. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.2 Antibacterial effect of PAM-5 on *E. coli* ATCC 25922

When the same assay was screened for *E. coli* ATCC 25922, it was found that the similar trend of antibacterial effect as reported for *P. aeruginosa* ATCC 27853 was observed in this *E. coli* reference strain. As demonstrated in **Figure 4.2 (a)**, heavy bacterial growth was observed for *E. coli* treated with low PAM-5 concentrations of 4 µg/ml and 2 µg/ml (Plate G and Plate H), where the growth pattern was similar to the negative controls (Plate Q and Plate R). However, when it came to peptide concentrations of 8 µg/ml and above, strong antibacterial effects were observed as reflected by the absence of bacterial growth on the media plates of A to F. Polymyxin B, which was the positive control showed complete bactericidal activity at all concentrations against the target bacteria as shown in Plate I to Plate P. These results were correspondent to the turbidity pattern of the 96-well microtiter plate shown in **Figure 4.2 (b)**, where cultures from the visually turbid wells (well A7, A8, C1 and C2) grew heavily on the inoculating MH agar while all visually clear wells (well A1 to A6 and B1 to B8) were negative for bacterial growth. Thus, the MBC of PAM-5 against the reference strain of *E. coli* was determined at 8 µg/ml while there was no MIC observed.

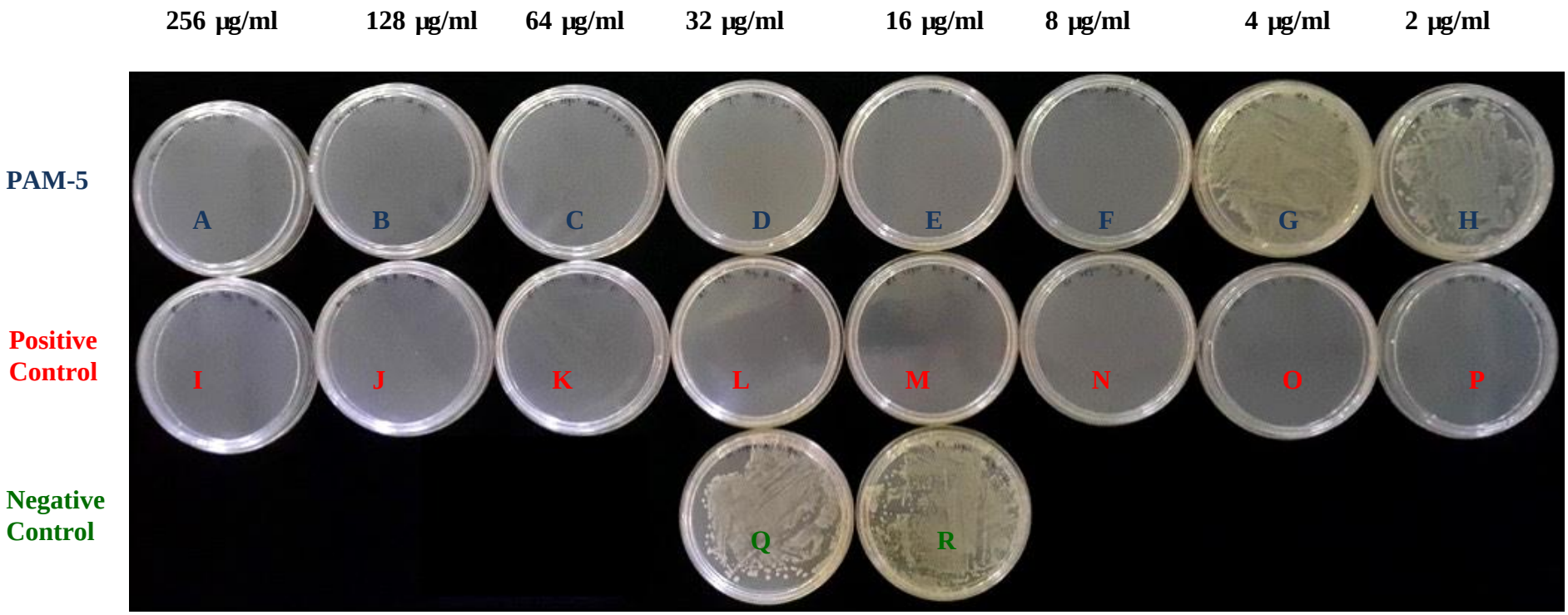


**Figure 4.2 (a):** Gross view of *E. coli* ATCC 25922 after PAM-5 treatment. The MBC of PAM-5 against *E. coli* ATCC 25922 was determined as 8 µg/ml as indicated by the absence of bacterial growth on Plate F. Plate I to Plate P served as the positive control plates while Plate Q and Plate R were the negative controls.

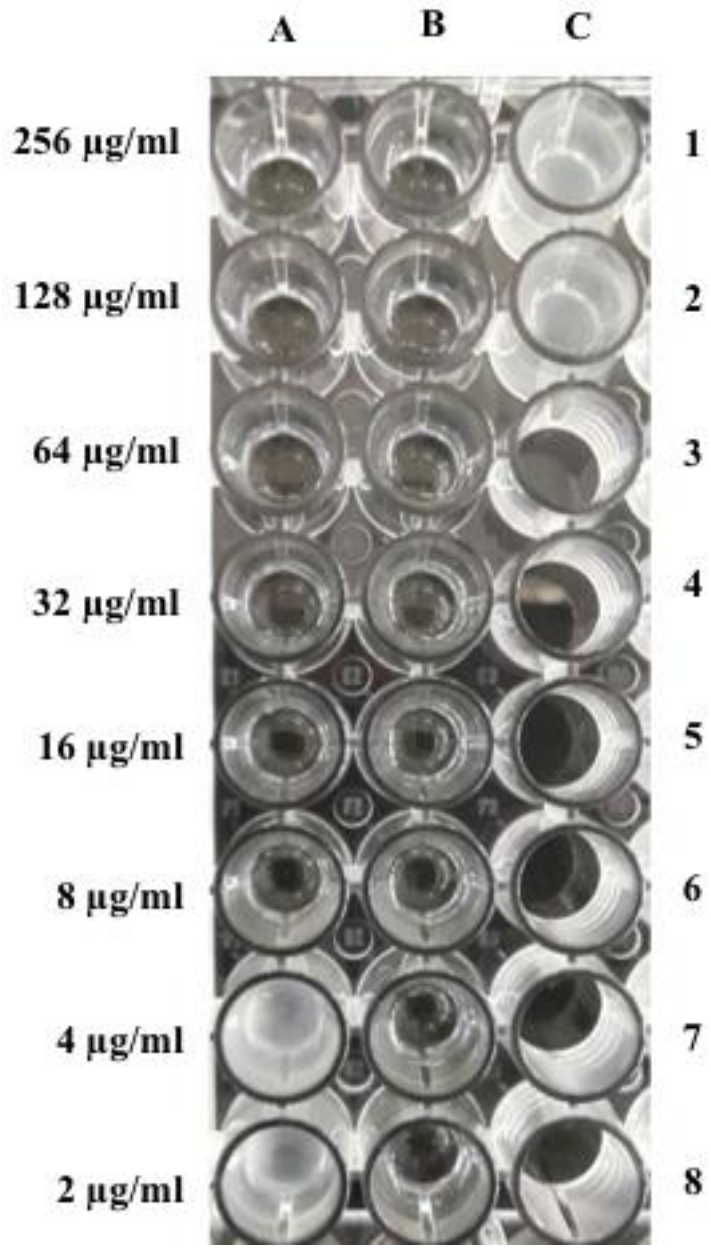


**Figure 4.2 (b):** Visual inspection of *E. coli* ATCC 25922 treated with PAM-5 after overnight incubation. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.3 Antibacterial effect of PAM-5 on *A. baumannii* ATCC 19606

As seen in **Figure 4.3 (a)**, PAM-5 had similar efficacy against *A. baumannii* ATCC 19606 as demonstrated for the reference strains of *P. aeruginosa* and *E. coli* as reported earlier. At peptide concentrations from 8 µg/ml to 256 µg/ml, the target bacteria were completely killed resulting no bacterial growth on the inoculating media (Plate A to Plate F). However, when the peptide concentrations were reduced to 4 µg/ml and below, its bactericidal effect was also compromised. This was indicated by the bacterial lawn formed on Plate G and Plate H, where the visual growth intensity was almost similar to the negative controls (Plate Q and Plate R). The positive controls showed complete killing of the target bacteria by all concentrations of Polymyxin B (Plate I to Plate P). The results of the plate inoculation demonstrated in **Figure 4.3 (a)** were corresponding to the results of the microtiter plate in **Figure 4.3 (b)**. Thus, the MBC of PAM-5 against *A. baumannii* ATCC 19606 was found to be 8 µg/ml. The peptide did not demonstrate any MIC as indicated by the absence of bacterial growth on media Plate F (**Figure 4.3 (a)**) where the media plate was inoculated with the bacterial culture treated with 8 µg/ml of PAM-5 from well A8 of the microtiter plate shown in **Figure 4.3 (b)**.

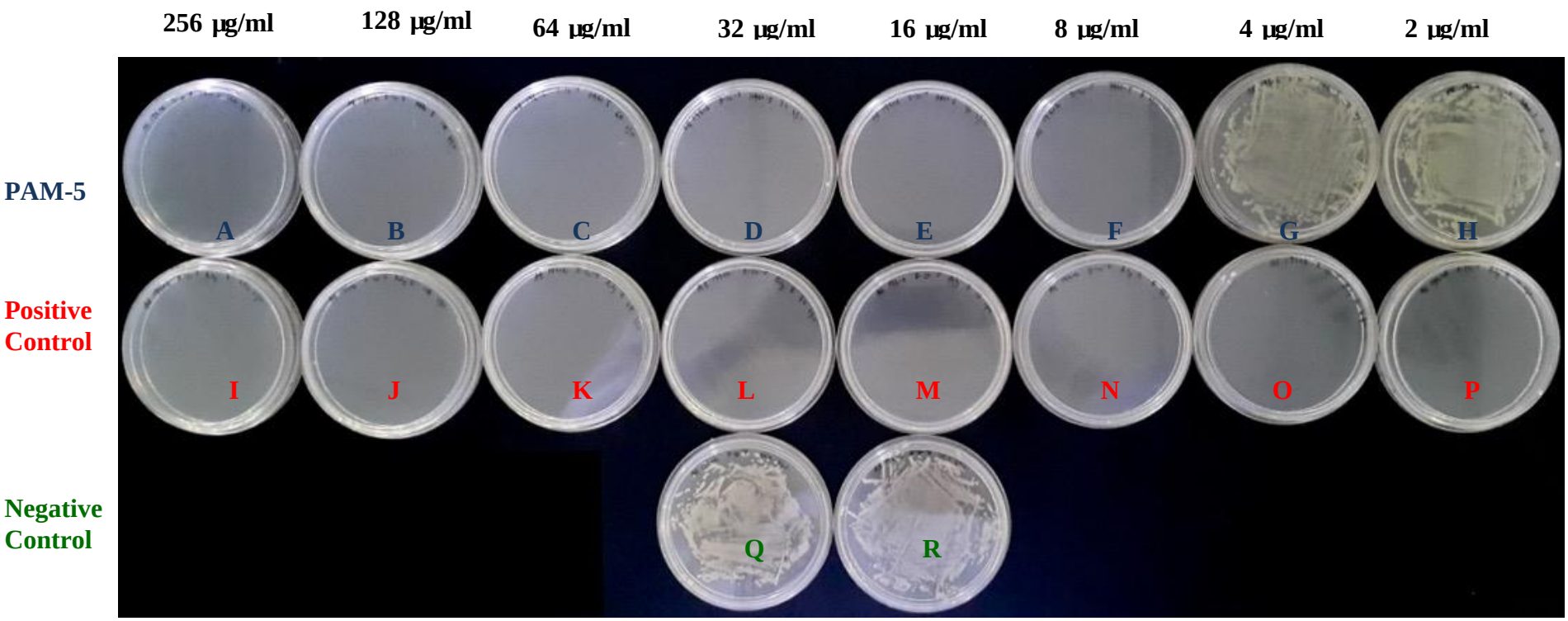


**Figure 4.3 (a):** Gross view of *A. baumannii* ATCC 19606 after PAM-5 treatment. Reference strain of *A. baumannii* was completely killed at PAM-5 concentrations of 8 µg/ml and above (Plate A to Plate F) where 8 µg/ml was determined as the peptide MBC. Plate I to Plate P served as the positive controls while Plate Q and Plate R were the negative controls.

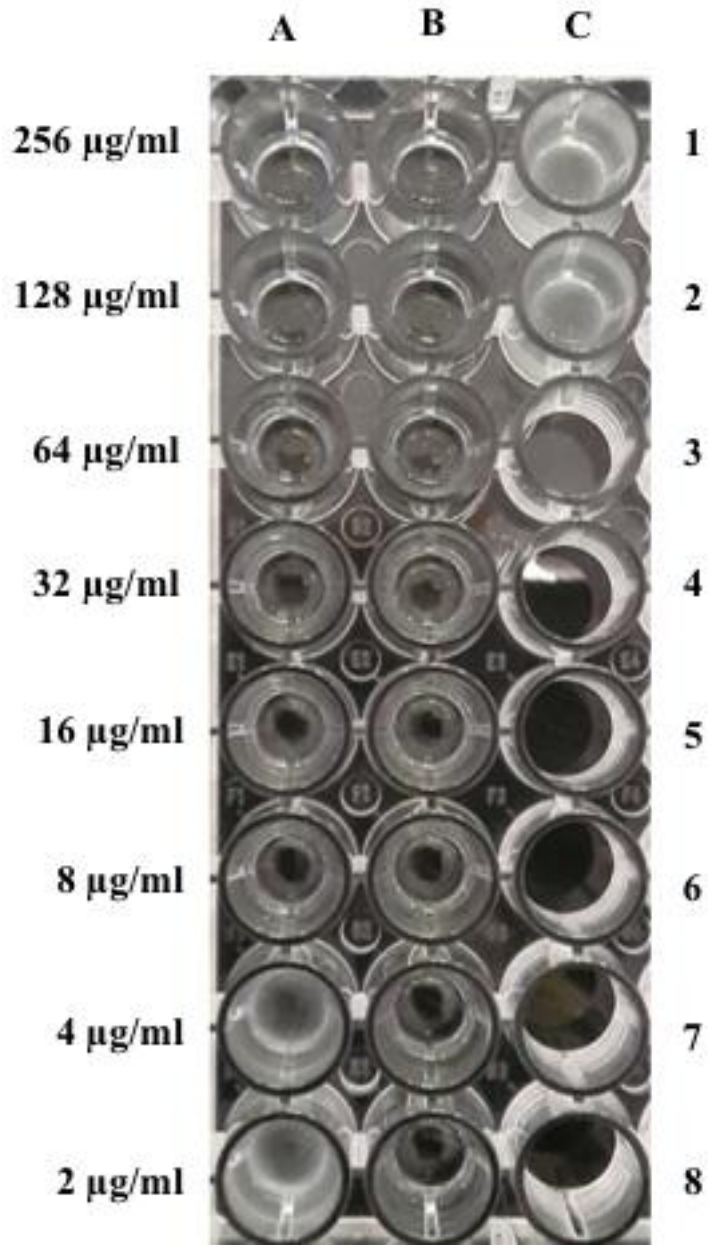


**Figure 4.3 (b):** Visual inspection of *A. baumannii* ATCC 19606 after PAM-5 treatment. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.4 Antibacterial effect of PAM-5 on *K. pneumoniae* ATCC 13883

Nevertheless, PAM-5 did not demonstrate similar potency against *K. pneumoniae* ATCC 13883 as compared to the few Gram-negative bacteria reported in the previous sections. As shown in **Figure 4.4 (a)** and **Figure 4.4 (b)**, PAM-5 was only able to kill the *K. pneumoniae* reference strain completely at 32 µg/ml. This was indicated by the heavy bacterial growth on the corresponding inoculating media plates E to H shown in **Figure 4.4 (a)** and the turbid wells in **Figure 4.4 (b)**, where the bacterial culture were treated with PAM-5 at concentrations ranged from 16 µg/ml to 2 µg/ml (well A5 to A8). Results obtained from the plate inoculation in **Figure 4.4 (a)** and the results of the microtiter plate (**Figure 4.4 (b)**) determined the MBC of PAM-5 against *K. pneumoniae* ATCC 13883 as 32 µg/ml with no MIC observed. Cultures treated with PAM-5 at 32 µg/ml (**Figure 4.4 (b)** well A4) did not grow after overnight inoculation on the media plate D (**Figure 4.4 (a)**) while cultures treated with peptide concentrations below 32 µg/ml grew to the extend similar as the negative control plates. The bacteria treated with Polymyxin B at all concentrations were completely killed.

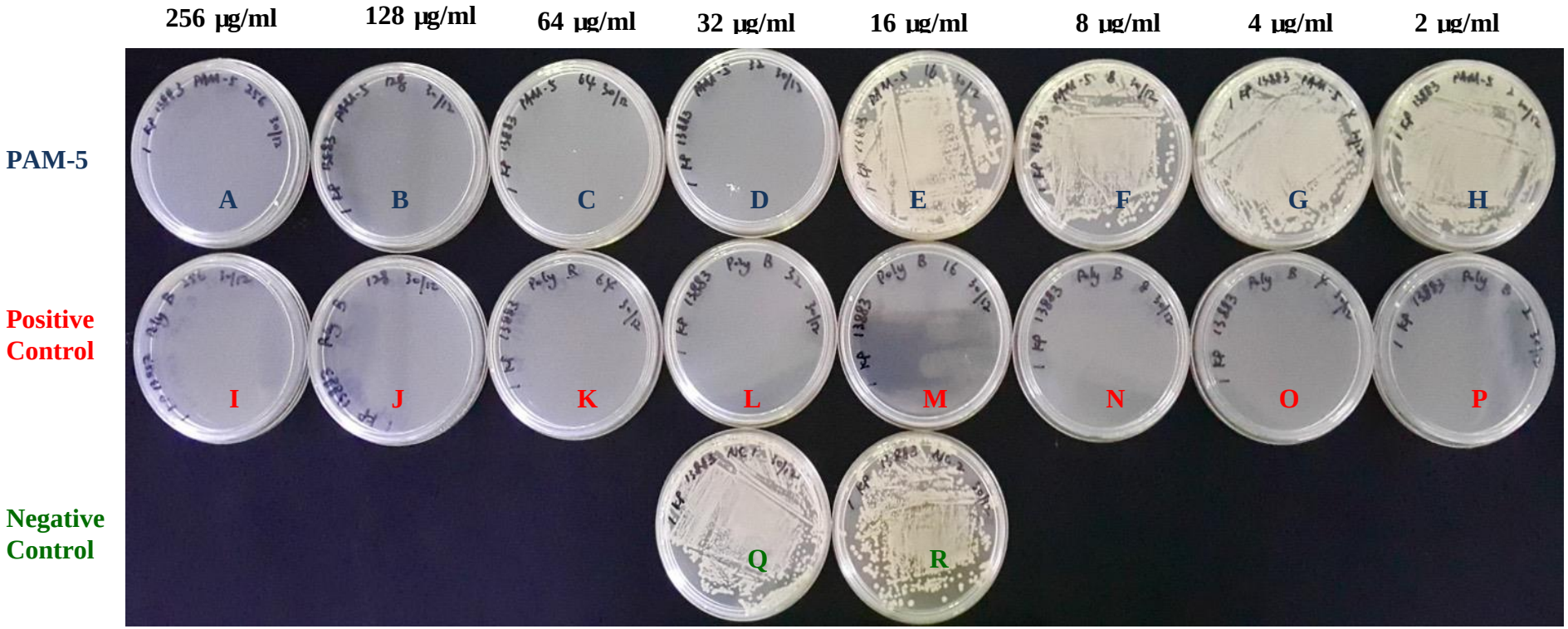


**Figure 4.4 (a):** Gross view of *K. pneumoniae* ATCC 138833 after treatment with PAM-5. Plate A to H were bacterial cultures treated with PAM-5 at concentrations ranging from 256 µg/ml to 2 µg/ml while Plate I to Plate P were culture treated with Polymyxin B of the same concentrations as PAM-5. Plate Q and Plate R were the untreated cultures that served as the negative control.

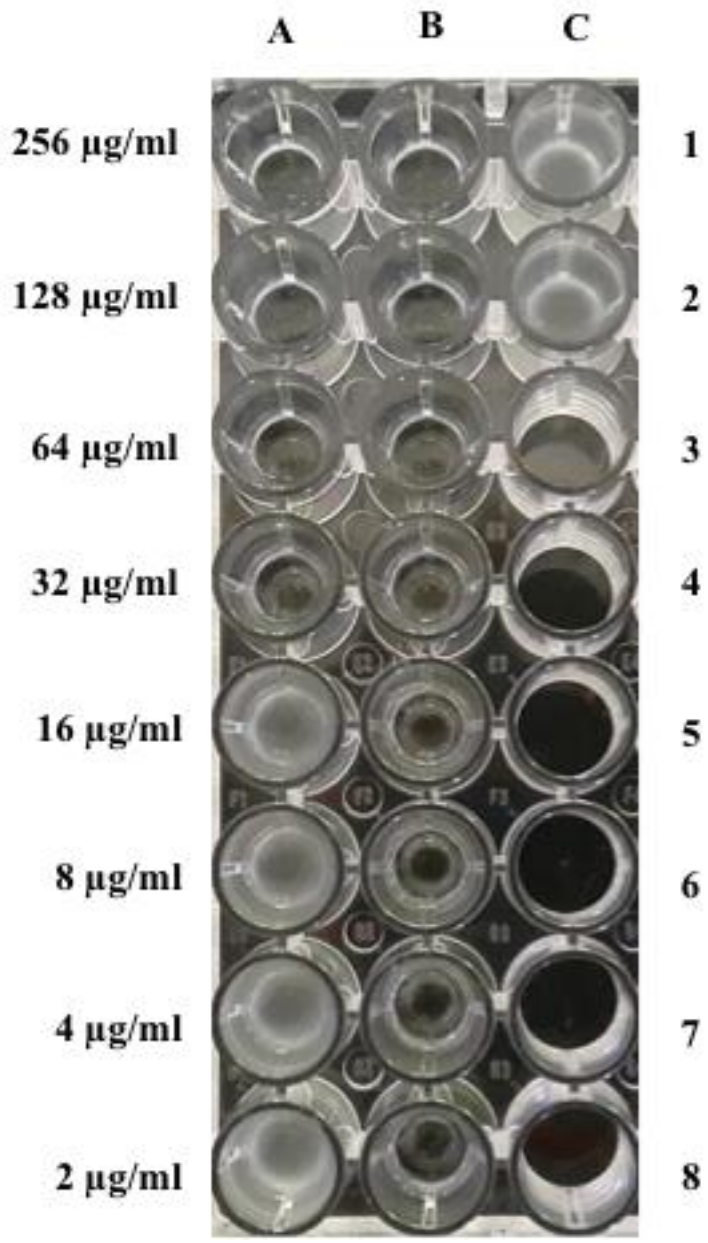


**Figure 4.4 (b):** Visual inspection of *K. pneumoniae* ATCC 13883 after overnight PAM-5 treatment. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.5 Antibacterial effect of PAM-5 on *S. aureus* ATCC 25923

Apart from the reference strains of Gram-negative bacteria reported previously, PAM-5 was also tested for its potency against a reference strain of *S. aureus* as a representative of the Gram-positive bacteria. As seen in **Figure 4.5 (a)**, the antibacterial effect of PAM-5 was only apparent at high concentrations, where both concentrations of 256 µg/ml and 128 µg/ml were bactericidal towards this bacterium (Plate A and Plate B). Further diluted concentrations (64 µg/ml to 2 µg/ml) were not potent against this bacterium, where the bacterial growth intensity on Plate C to Plate H was noted to be similar as the negative control (Plate Q and Plate R). As two different MBCs of PAM-5 against *S. aureus* ranged between 64 µg/ml and 128 µg/ml were obtained after the first and second assays, additional assays were performed to validate these findings. Out of the four independent assays performed, two assays gave the same MBC of 128 µg/ml while the other two were 64 µg/ml. Thus, an average MBC at 96 µg/ml was decided as the MBC of PAM-5 towards this bacterium. Similarly, no MIC was noted for the action of PAM-5 on this bacterium as the bacterial suspension from the visually clear well A2 filled with bacterial cultures treated with 128 µg/ml of PAM-5 in **Figure 4.5 (b)** did not grow after being inoculated on MH agar (**Figure 4.5 (a)**: Plate B). Apart from that, Polymyxin B at 16 µg/ml and above were not potent against this bacterium as well, where bacterial lawn was formed on Plate N, Plate O and Plate P. The observations of the inoculating media in **Figure 4.5 (a)** were corresponding to the results of the 96-well microtiter plate (**Figure 4.5 (b)**) where similar bacterial growth patterns were observed in cultures treated with Polymyxin B below 16 µg/ml as wells as cultures treated PAM-5 below 128 µg/ml.

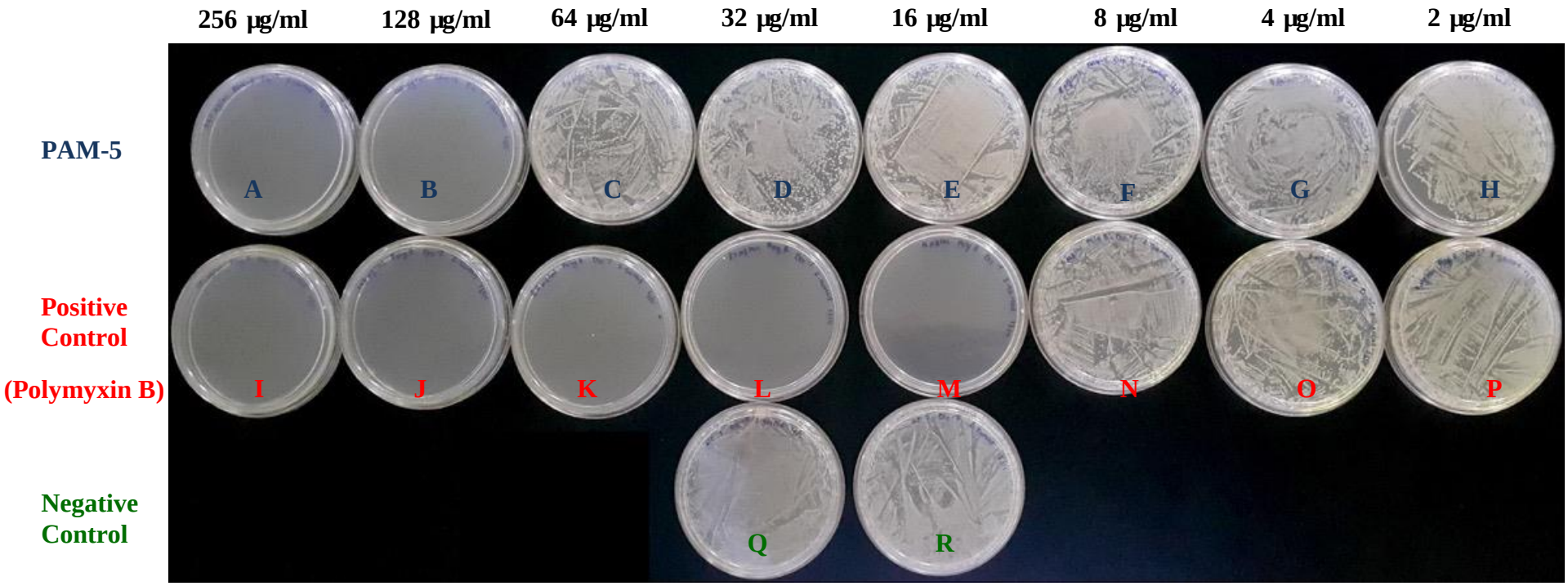


**Figure 4.5 (a):** Gross view of *S. aureus* ATCC 25923 after PAM-5 treatment (Plate A to Plate H). A high MBC of 128 µg/ml was required for PAM-5 to be completely bactericidal against the reference strain of *S. aureus*. Bacterial growth was also observed on the plates inoculated with culture treated with the lowest three concentrations of Polymyxin B (Plate N to Plate P). Plate Q and Plate R were the negative controls.

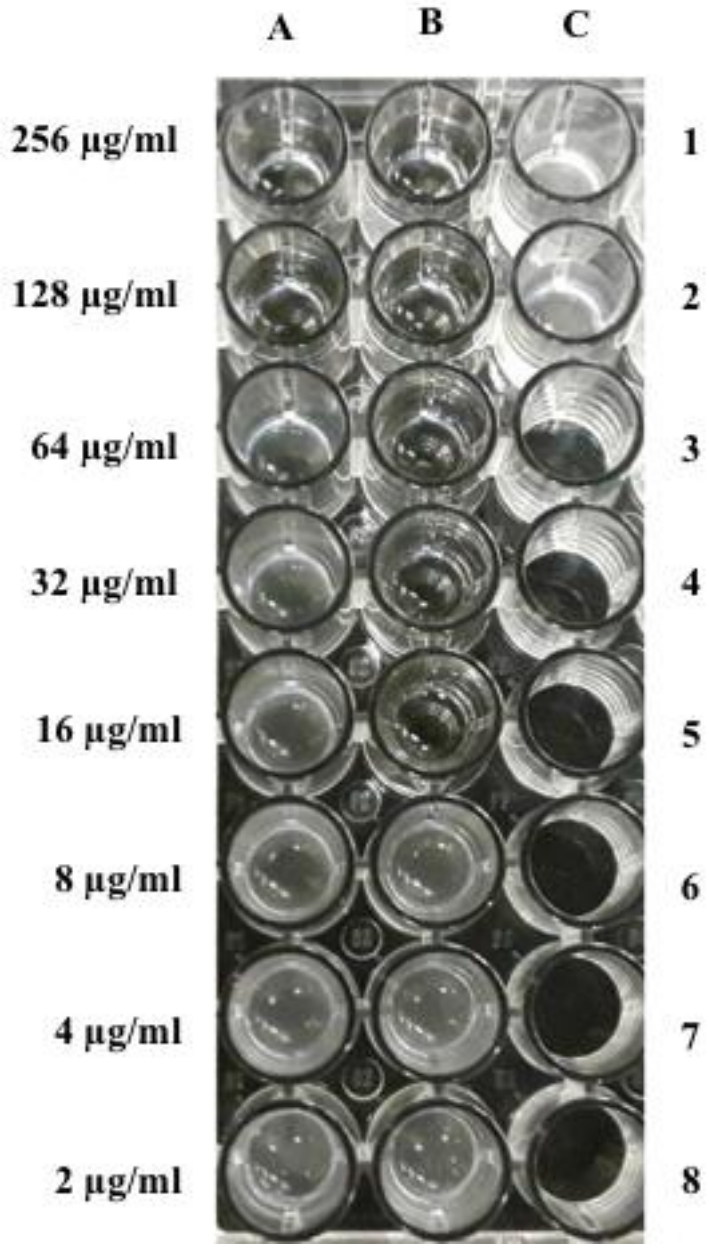


**Figure 4.5 (b):** Visual inspection of *S. aureus* ATCC 25923 after overnight PAM-5 treatment. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.6 Antibacterial effect of PAM-5 on *S. flexneri* clinical strain

Prominent antibacterial potency was observed in PAM-5 against several reference strains of bacteria, as mentioned in the previous sections. However, it is worth to know whether this peptide possesses any effect towards clinically isolated bacteria. One of the clinical strains employed in this study was *S. flexneri.* Using the same assay, the bacteria were treated with PAM-5 and their viability was accessed the next day. The results of this assay were demonstrated in **Figure 4.6 (a)** and **Figure 4.6 (b)**. The degree of bacterial growth that was similar to the negative controls on culture plates inoculated with bacteria treated with PAM-5 at concentrations from 2 µg/ml to 16 µg/ml (**Figure 4.6 (a)**: Plate E to Plate H). Conversely, starting from 32 µg/ml, bactericidal effect of PAM-5 was noted as there was no observable bacterial growth on Plate A to Plate D. Polymyxin B was able to kill the bacteria completely at all concentrations as shown in positive control plates I to P (**Figure 4.6 (a)**). These observations from the inoculating media were consistent to the visual inspection of the turbidity from wells of the microtiter plate (**Figure 4.6 (b)**). As the lowest concentration of PAM-5 that was able to achieve complete bactericidal activity was 32 µg/ml, the MBC of PAM-5 against the clinical isolate was determined as 32 µg/ml. PAM-5 did not possess any MIC against this clinical strain as indicated by the absence of bacterial growth on Plate D in **Figure 4.6 (a)**. It was because the culture treated with PAM-5 at 32 µg/ml in the visually clear well A4 did not show any bacterial growth with remarkable colony reduction when it was inoculated on the media plate D.

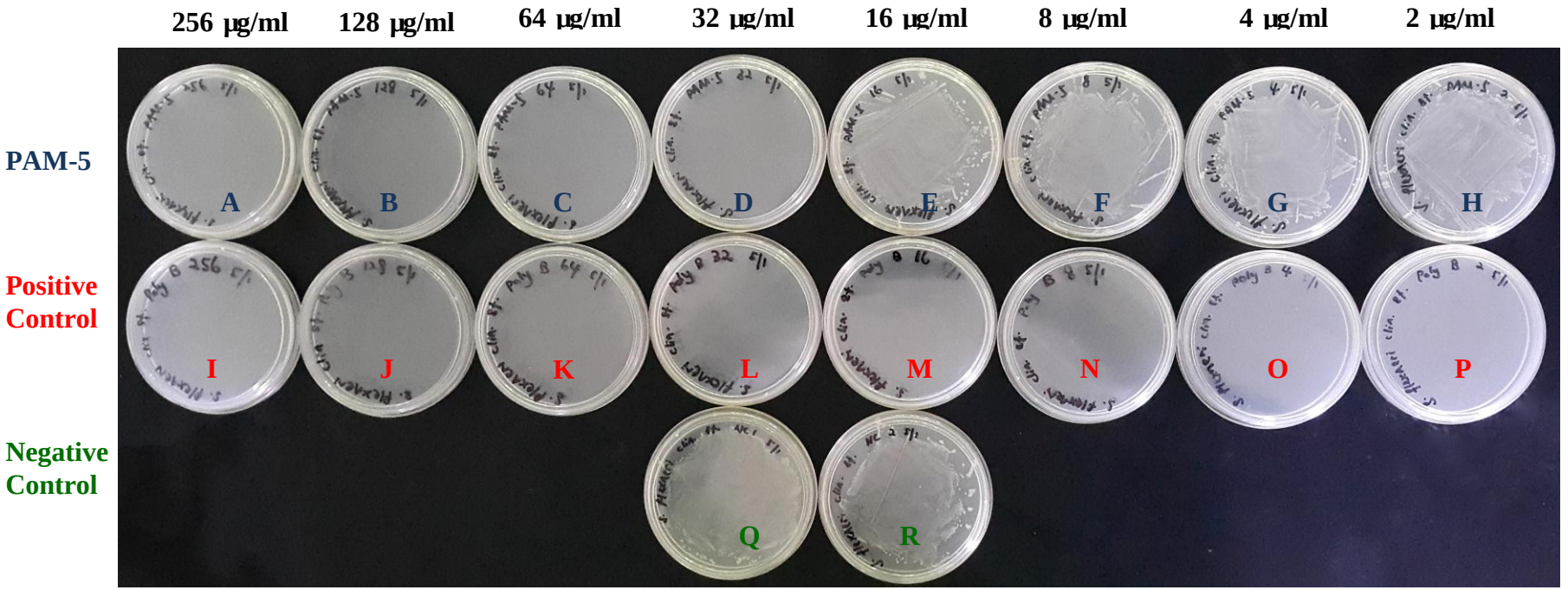


**Figure 4.6 (a):** Gross view of *S. flexneri* clinical strain after PAM-5 treatment. Plate A to Plate H were inoculated with bacterial cultures treated with different concentrations of PAM-5 while Plate I to Plate P were bacterial cultures treated with Polymyxin B (positive control). Plate Q and Plate R served as the negative control. The MBC of PAM-5 against this clinical isolate was determined as 32 µg/ml.

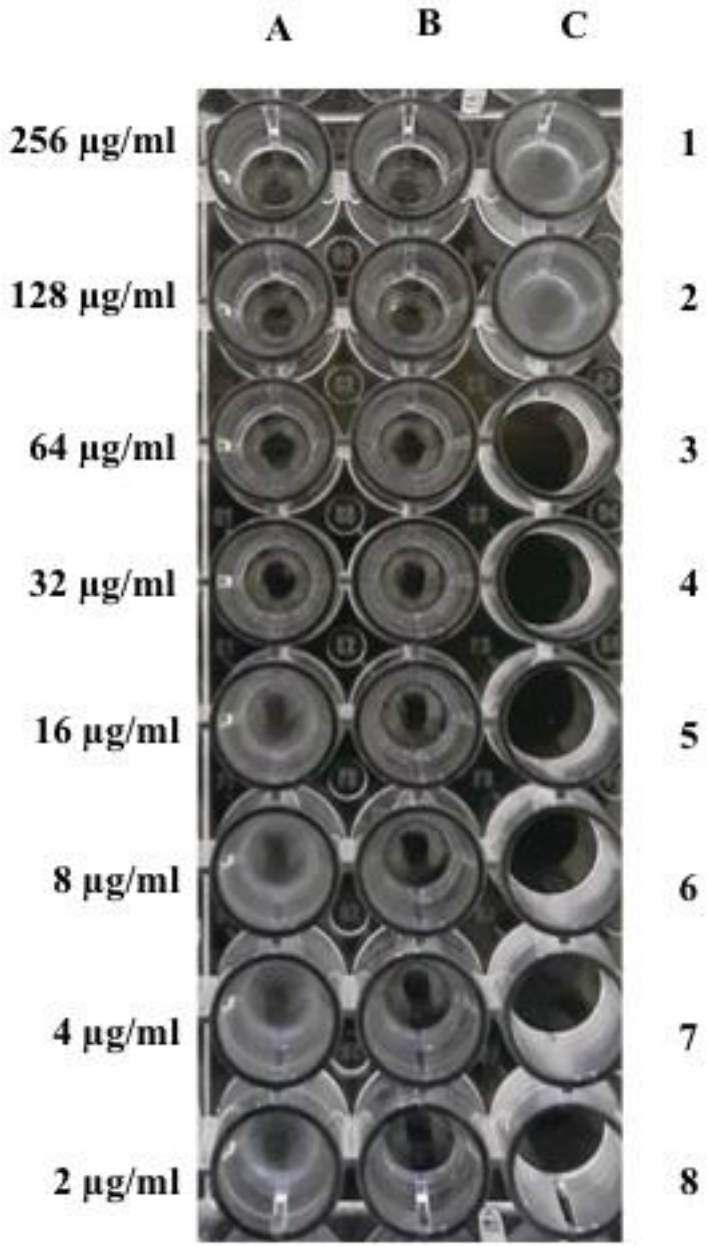


**Figure 4.6 (b):** Visual inspection of *S. flexneri* clinical strain after overnight treatment with PAM-5. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.7 Antibacterial effect of PAM-5 on *P. aeruginosa* MDR strain

The second clinical isolate to be screened was the multi-drug resistant (MDR) *P. aeruginosa.* Complete bactericidal activity was exhibited by the peptide at concentrations from 256 μg/ml down to 16 μg/ml as represented by the absence of growth on Plate A to Plate E in **Figure 4.7 (a)** and the visually clear wells A1 to A5 in **Figure 4.7 (b)**. However, PAM-5 at low concentrations from 2 μg/ml to 8 μg/ml was insufficient to kill the MDR bacterium, resulting the formation of bacterial lawn on Plate F to Plate H (**Figure 4.7 (a)**) and high turbidity in wells A6 to A8 (**Figure 4.7 (b)**), where they showed similar growth intensity as the negative controls. The present of visible bacterial growth in wells containing the cultures treated with PAM-5 concentrations below 16 μg/ml (**Figure 4.7 (b)**) represented minimal or absence of bacteriostatic effect of PAM-5 against this bacterium. Thus, based on these gross views, the MBC of PAM-5 against MDR *P. aeruginosa* was determined as 16 μg/ml with no MIC noted. The positive controls that contained bacteria treated with Polymyxin B showed no growth at all concentrations.

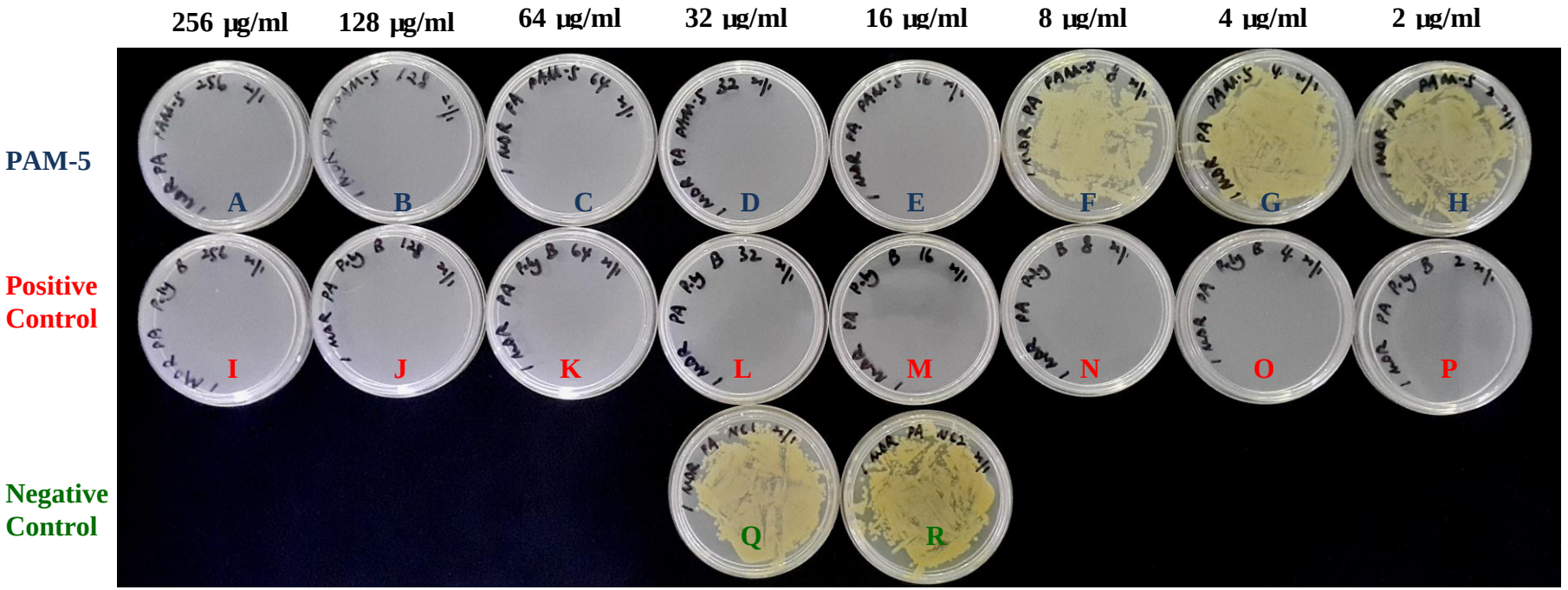


**Figure 4.7 (a):** Gross view of MDR *P. aeruginosa* after PAM-5 treatment. Plate A to Plate H were cultures treated with two-fold serially diluted PAM-5 while Plate I to Plate P were cultures treated with Polymyxin B of the same serial dilutions that served as the positive control. Plate Q and Plate R were the negative control plates. The MBC of PAM-5 against this multidrug-resistant strain was 16 µg/ml.

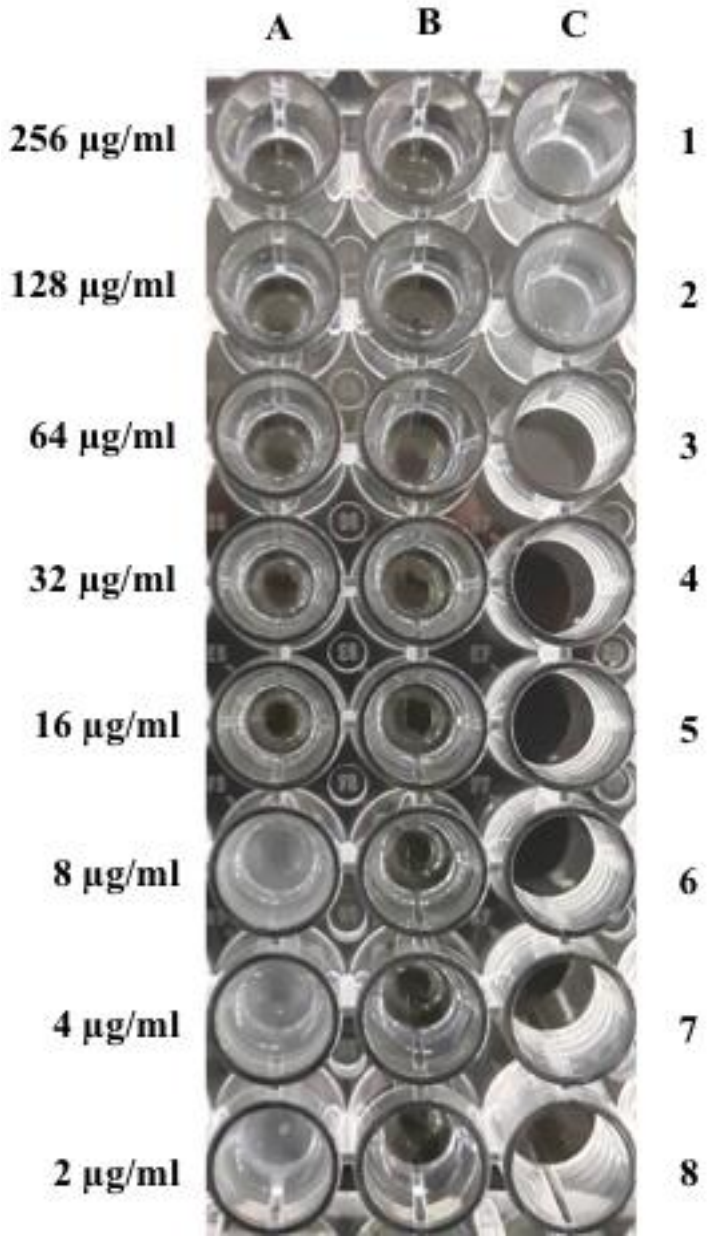


**Figure 4.7 (b):** Visual inspection of multidrug-resistance *P. aeruginosa* treated overnight with PAM-5. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

### 4.1.8 Antibacterial effect of PAM-5 on ESBL-producing *E. coli*

The last bacterial target to be tested was the clinically isolated ESBL-producing *E. coli*. As shown in **Figure 4.8 (a)** and **Figure 4.8 (b)**, PAM-5 was only able to exert complete bactericidal effect against this bacterium at 64 μg/ml and higher concentrations. This was indicated by the absence of growth on the MH agar plates A to C in **Figure 4.8 (a)** inoculated with these peptide-treated culture. Correspondingly, wells A1 to A3 in **Figure 4.8 (b)** did not show visible turbidity as well. PAM-5 at concentrations lower than 64 μg/ml was not able to kill the bacteria resulting in the apparent growth on the media Plate D to Plate H (**Figure 4.8 (a)**) as well as turbidity in wells A4 to A8 (**Figure 4.8 (b)**). These bacterial growth intensities observed were similar to the bacterial growth of the negative controls, indicated the absence of bacteriostatic effect against the ESBL-producing *E. coli*. The positive controls, which were the Polymyxin-treated bacteria, were completely killed at all concentrations. Thus, the MBC of PAM-5 against ESBL-producing *E. coli* was determined as 64 μg/ml without notable MIC.

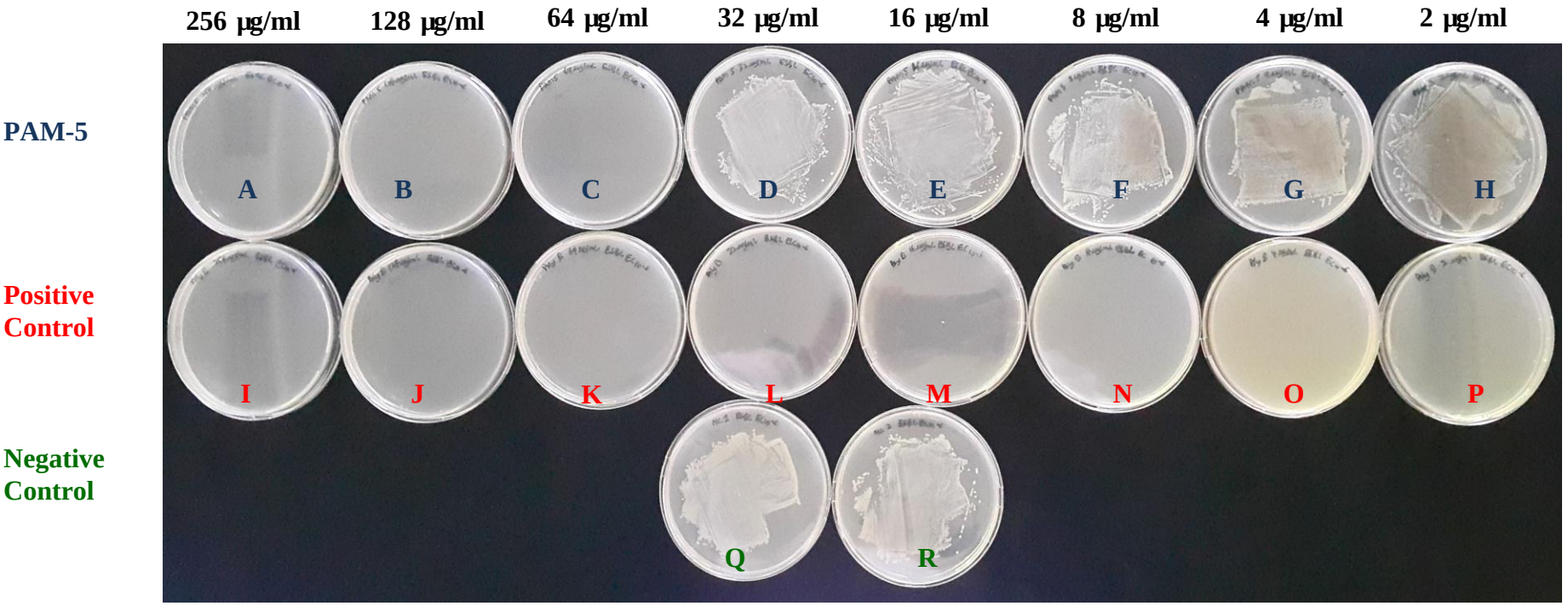


**Figure 4.8 (a):** Gross view of ESBL-producing *E. coli* after PAM-5 treatment. Plate A to Plate H were inoculated with cultures treated with PAM-5 ranging from 256 µg/ml to 2 µg/ml. Plate I to Plate P were the positive control plates while Plate Q and Plate R served as the negative controls. The MBC of PAM-5 against ESBL-producing *E. coli* was determined as 64 µg/ml.

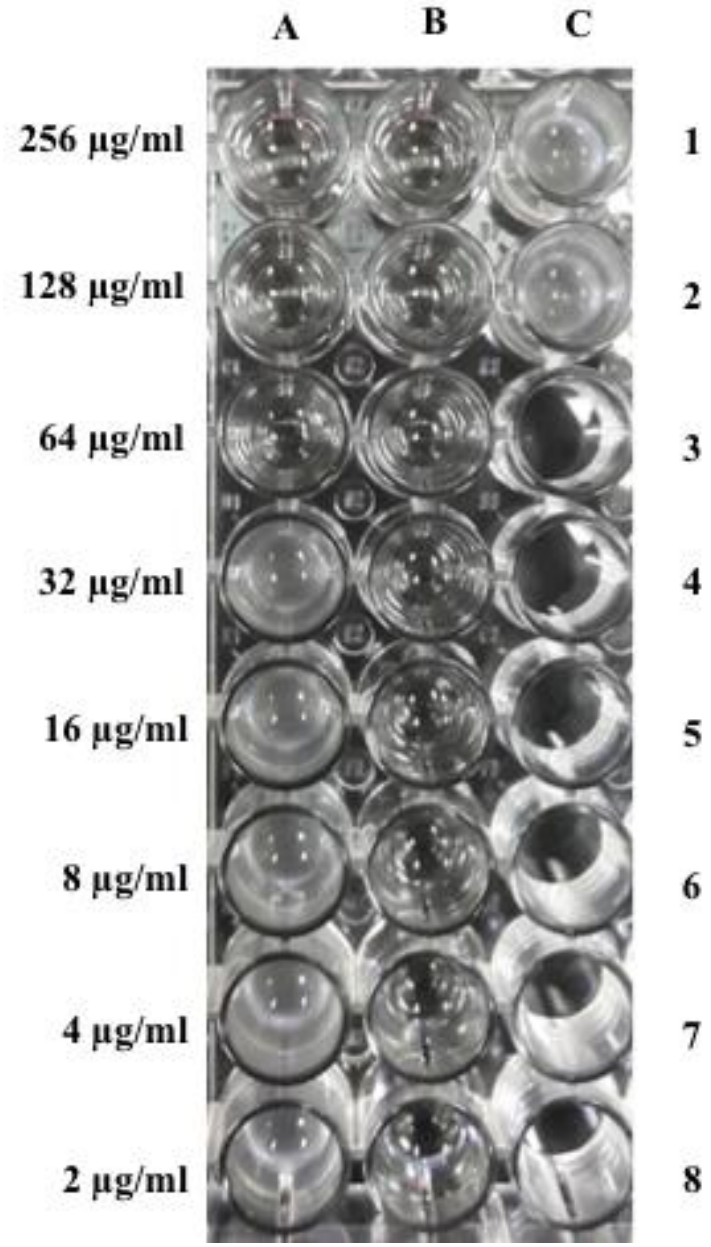


**Figure 4.8 (b):** Visual inspection of ESBL-producing *E. coli* after overnight PAM-5-treatment. Wells A1 to A8: bacterial cultures treated with PAM-5; wells B1 to B8: bacterial cultures treated with Polymyxin B (positive control); well C1 to C2: untreated bacteria suspended in PBS (negative control).

## 4.2 Analysis of antibacterial effects of PAM-5 against selected pathogenic bacteria

Gross view analysis as mentioned in the previous sections may provide visual information on the bactericidal effect of PAM-5 but may not reflect the quantitative inhibitory effect of the peptide sufficiently. Titer determination on each of the treated bacterium with observable growth is essential to disclose any minor inhibition of the peptide at a particular concentration, especially the lower concentrations. Thus, after overnight peptide treatment, the cultures from wells with visible bacterial growth were serially diluted for colony counting to determine their respective bacterial titers.

### 4.2.1 Analysis of antibacterial effects of PAM-5 against selected Gram-negative bacteria

After three independent assays, the average titers of target bacteria treated with different peptide concentrations were obtained and a graph of bacterial titers in $\log_{10}$ (CFU/ml) against peptide concentrations (μg/ml) was plotted as demonstrated in **Figure 4.9**.

As shown in the graph, PAM-5 was not potent against *P. aeruginosa* ATCC 27853 at 2 μg/ml and 4 μg/ml. The bacteria treated with PAM-5 of these low concentrations were able to grow up to the titer as almost similar to the negative control, indicating that no bacteriostatic activity was present. However, the peptide was able to kill the bacteria completely at 8 μg/ml and

above where a titer reduction of 8 $\log_{10}$ CFU/ml was achieved. Thus, the MBC of PAM-5 against the reference strain of *P. aeruginosa* was determined as 8 μg/ml with no MIC observed.

With the prominent antibacterial effects of PAM-5 against the reference strain of *P. aeruginosa*, it was also worth to know whether PAM-5 had the same efficacy against the clinically isolated MDR strain. Interestingly, PAM-5 was also potent against the MDR *P. aeruginosa* as it was able to achieve complete bactericidal effects at 16 μg/ml. As compared to its reference strain, although PAM-5 at 8 μg/ml did not manage to kill MDR *P. aeruginosa* completely, it was able to reduce the bacterial growth from 10.74 $\log_{10}$ CFU/ml (negative control) to 10.37 $\log_{10}$ CFU/ml, as shown in **Figure 4.9**. Compared to the negative control, a gradual decrease in bacterial titers was observed when the peptide concentrations were increased from 2 μg/ml to 8 μg/ml before the bacteria were completely killed at 16 μg/ml and higher concentrations. This indicated that PAM-5 possessed slight inhibitory effect towards this MDR bacterium at those low concentrations from 2 μg/ml to 8 μg/ml. However, these could not to be considered as the peptide MICs as the bacterial titers reductions were not apparent. Thus, the MBC of PAM-5 against MDR *P. aeruginosa* was identified as 16 μg/ml with no notable MIC

The trend of antibacterial effect of PAM-5 towards *E. coli* ATCC 25922 was somehow similar to that of the reference strain of *P. aeruginosa* as reported earlier. The peptide was not active against the reference strain of *E. coli* at low

peptide concentrations of 2 μg/ml and 4 μg/ml, as demonstrated by the bacterial titers that did not differ much from the negative control. However, PAM-5 at concentrations from 8 μg/ml to 256 μg/ml were bactericidal where those peptide concentrations were able to prevent the bacterial growth by decreasing the bacterial titer by approximately 8.5 $\log_{10}$ CFU/ml as compared to the negative control. Thus, the MBC of PAM-5 against *E. coli* was determined as 8 μg/ml with no MIC observed.

Apart from the *E. coli* reference strain, PAM-5 was also tested against an ESBL-producing strain. As compared to the antibacterial effect of PAM-5 against its reference strain, PAM-5 had lower potency against this ESBL-producing *E. coli*. The peptide was unable to kill the bacteria at concentrations lower than 64 μg/ml. However, reductions of bacterial titers were observed at 2 μg/ml (8.29 $\log_{10}$ CFU/ml), 4 μg/ml (8.64 $\log_{10}$ CFU/ml), 8 μg/ml (8.86 $\log_{10}$ CFU/ml), 16 μg/ml (8.9 $\log_{10}$ CFU/ml) and 32 μg/ml (8.45 $\log_{10}$ CFU/ml) as compared to the negative control (8.94 $\log_{10}$ CFU/ml). This indicated that the peptide had slight bacteriostatic effect at these low concentrations although the effect was not too strong. PAM-5 was only able to kill the bacteria completely at 64 μg/ml and above. Thus, the MBC of PAM-5 against ESBL-producing *E. coli* was 64 μg/ml with no MIC seen.

As compared to the efficacy of PAM-5 against the previous two Gram-negative reference strains (*P. aeruginosa* and *E. coli*), PAM-5 was shown to have better antibacterial potency against *A. baumannii* ATCC 19606 at those low peptide

concentrations that were unable to inhibit the former two bacteria. At 2 μg/ml and 4 μg/ml, PAM-5 was able to cause approximately 1 $\log_{10}$ CFU/ml reduction of bacterial growth as compared to the negative control. These reductions were consistent throughout the three independent assays, indicating that PAM-5 had slight bacteriostatic effects against *A. baumannii*. In the contrary, PAM-5 became bactericidal beyond 4 μg/ml where it was able to completely kill this bacterium from 8 μg/ml to 256 μg/ml. Therefore, the MBC of PAM-5 against *A. baumannii* was determined as 8 μg/ml with no MIC observed.

On the other hand, PAM-5 was found to have reduced potency against *K. pneumoniae* ATCC 13883 as it was only able to exert complete killing of the bacterium at 32 μg/ml and above. *K. pneumoniae* that was treated with 2 μg/ml of PAM-5 was able to grow to the titer as almost similar to the untreated bacteria. However, reductions in bacterial titers were observed from bacteria treated with PAM-5 from 4 μg/ml to 16 μg/ml but the reductions were not remarkably great. These indicated the presence of slight bacteriostatic effects in PAM-5 on this bacterium although these peptide concentrations were not considered as MICs. Thus, the MBC of PAM-5 against *K. pneumoniae* was determined as 32 μg/ml with no MIC observed.

The last Gram-negative bacteria screened was the clinical strain of *S. flexneri* and it was found that a gradual decrease in bacterial titers was observed from bacteria treated with PAM-5 at concentrations from 4 μg/ml to 16 μg/ml while

the bacteria from the negative control grew up to the titer of 9.72 $\log_{10}$ CFU/ml, their counterpart which were treated with PAM-5 at concentrations of 4 µg/ml, 8 µg/ml and 16 µg/ml showed a reduction of titer to 9.53 $\log_{10}$ CFU/ml, 8.56 $\log_{10}$ CFU/ml and 8.1 $\log_{10}$ CFU/ml, respectively. Above these concentrations (32 µg/ml to 256 µg/ml), PAM-5 appeared to be bactericidal towards *S. flexneri*. Thus, the MBC of PAM-5 against this clinical isolate of *S. flexneri* was determined as 32 µg/ml. The peptide did not possess any MIC although PAM-5 demonstrated slight bacteriostatic effect against this bacterium as indicated by the reduction of bacterial titers in *S. flexneri* cultures treated with peptide concentrations from 4 µg/ml to 16 µg/ml.

In short, PAM-5 was able to exert slight bacteriostatic effects on all tested bacteria at concentrations below their respective MBCs. However, these could not be considered as the peptide MICs as the titer reductions were not apparent and no bacterial growth was observed when the cultures from wells with no visible growth were inoculated onto the media plates as mentioned in the previous sections. Bacterial growth in the positive control was completely hampered at all concentrations of Polymyxin B. The MBCs and MICs of PAM-5 against all tested bacteria were summarised in **Table 4.1**.

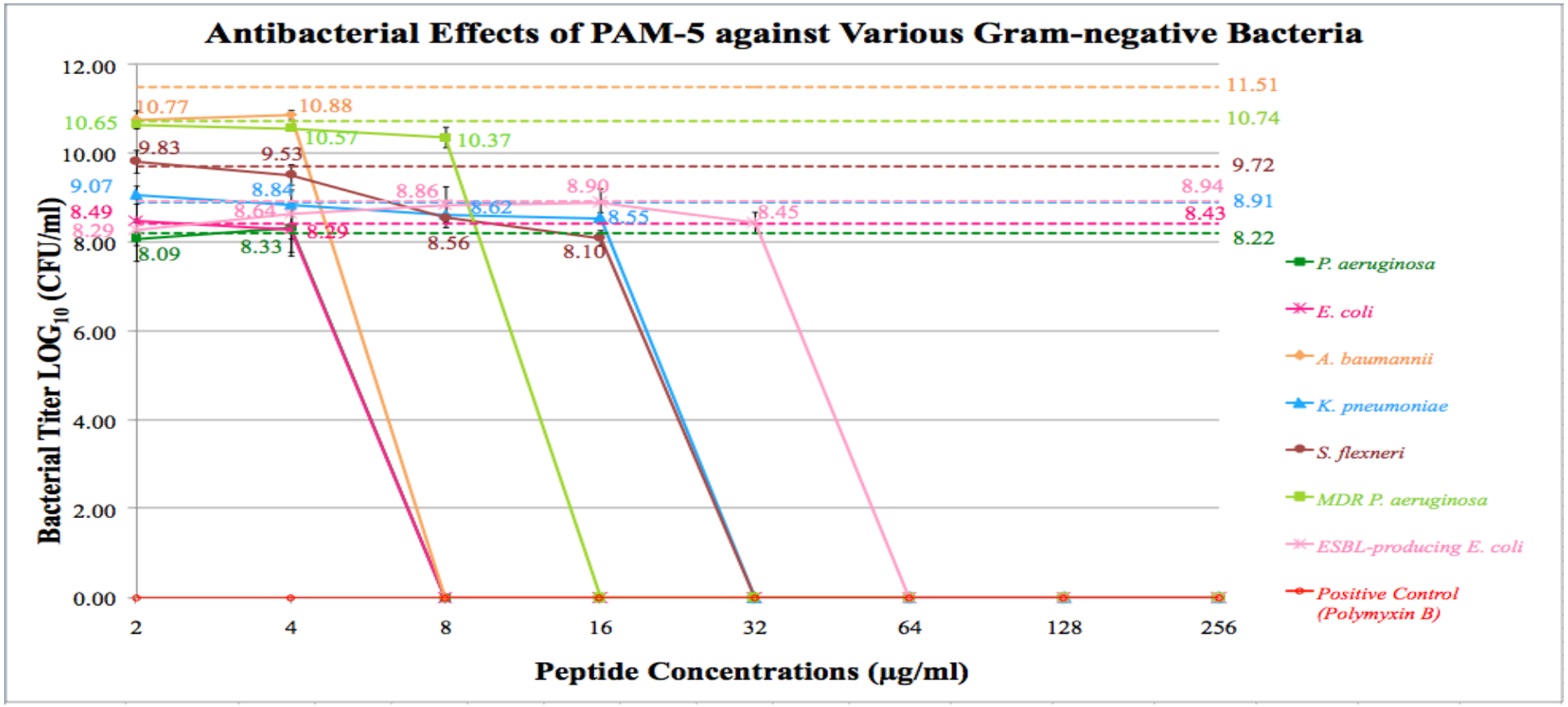


**Figure 4.9:** Antibacterial effects of PAM-5 against selected Gram-negative bacteria. The solid lines (—) represented the bacteria treated with PAM-5 while the dashed lines (---) were their respective negative controls. Polymyxin B was completely bactericidal against all tested bacteria at all concentrations.

### 4.2.2 Analysis of antibacterial effects of PAM-5 against selected Gram-positive bacteria

Apart from screening the antibacterial effects of PAM-5 against various Gram-negative bacteria, PAM-5 was also screened against *S. aureus* ATCC 25923. The average bacterial titer of each well with visual turbidity as shown in **Figure 4.5 (b)** was calculated after four independent assays and presented in **Figure 4.10**. Based on the graph, it was noticed that PAM-5 demonstrated lower antibacterial potency against this Gram-positive bacterium as compared to the few Gram-negative bacteria that were tested in the previous sections. Out of the four rounds of assays, two rounds yielded a MBC of 64 μg/ml while the other two were 128 μg/ml. Thus, an average MBC of 96 μg/ml was decided to be the MBC of PAM-5 against this bacterium. Bacteria treated with all other lower concentrations of PAM-5 were not suppressed but able to grow to the extent similar to the negative control, indicating the presence of negligible bacteriostatic effect. Hence, there was no MIC observed in PAM-5 against *S. aureus* ATCC 25923. The positive control, where the bacterium was treated with Polymyxin B also showed bacterial growth from concentrations of 2 μg/ml to 8 μg/ml, indicating that it was not an ideal candidate of a positive control against *S. aureus*. The MBCs and MICs of PAM-5 against all tested bacteria were summarised in **Table 4.1**.

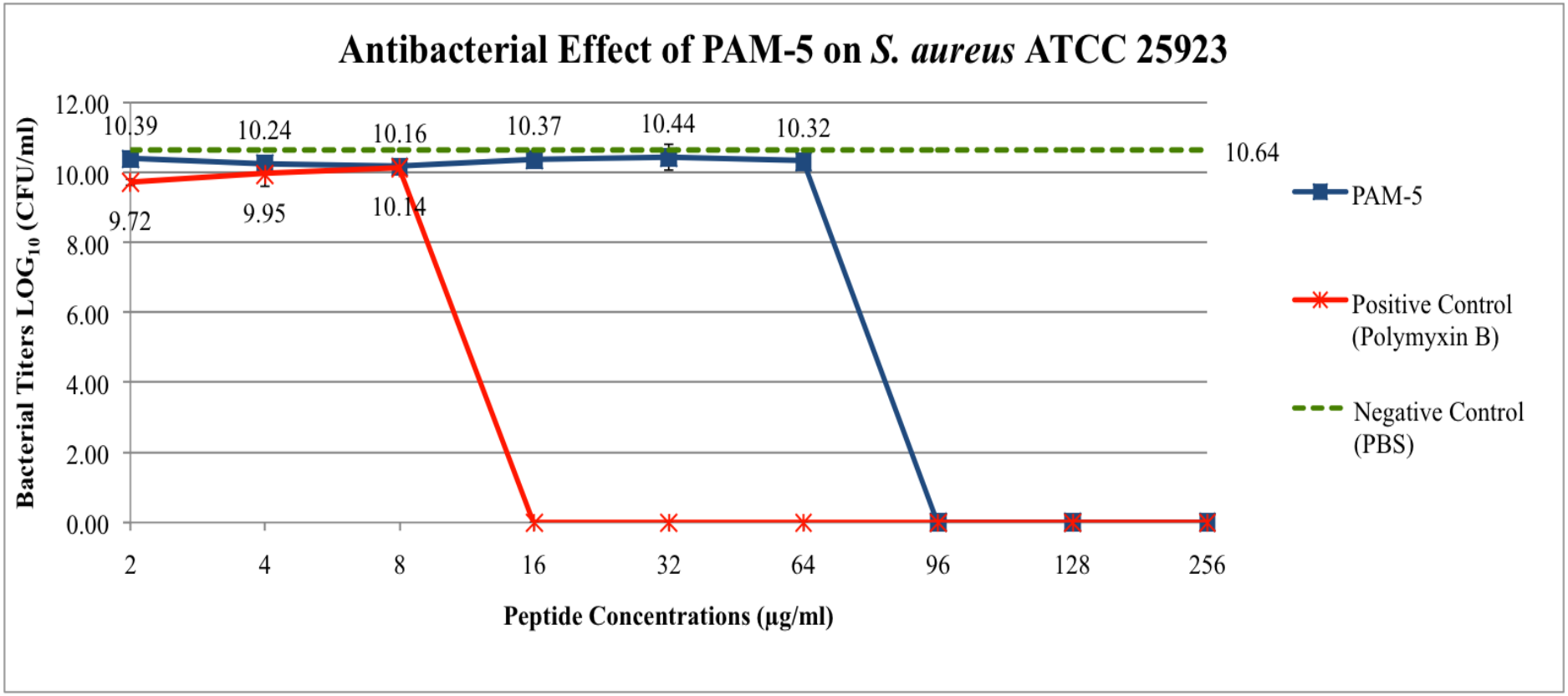


**Figure 4.10:** Antibacterial effect of PAM-5 on *S. aureus* ATCC 25923. After four independent assays, the average MBC of PAM-5 against this bacterium was decided as 96 µg/ml. Polymyxin B, as the positive control showed no bacterial growth at concentrations at 16 µg/ml and beyond.

**Table 4.1:** Summary of antibacterial effects of PAM-5 against various Gram-negative and Gram-positive bacteria.

| Bacterial Species and Strain | Relevant Feature | MIC (μg/ml) | MBC (μg/ml) |
|---|---|---|---|
| *Pseudomonas aeruginosa* ATCC 27853 | Reference strain | - | 8 |
| *Pseudomonas aeruginosa* | Multidrug-resistant | - | 16 |
| *Escherichia coli* ATCC 25922 | Reference strain | - | 8 |
| *Escherichia coli* | Extended-spectrum β-lactamases-producing | - | 64 |
| *Acinetobacter baumannii* ATCC 19606 | Reference strain | - | 8 |
| *Klebsiella pneumoniae* ATCC 13883 | Reference strain | - | 32 |
| *Shigella flexneri* | Clinical strain | - | 32 |
| *Staphylococcus aureus* ATCC 25923 | Reference strain | - | 96 |

## 4.3 Results validation using PrestoBlue™ bacterial viability assay

PrestoBlue™ bacterial viability assay was conducted to qualitatively validate the results obtained by microbroth dilution assay. Colour change from blue to pink in the wells loaded with treated or untreated bacteria indicate the presence of viable bacteria while the wells with no viable bacteria remain blue. This is due to the ability of viable bacteria to reduce the blue resazurin to pink fluorescent resorufin (Lall et al., 2013). Hence, in this study, formation of pink content in the wells indicates negative inhibitory effect of PAM-5.

### 4.3.1 Validation of the antibacterial effect of PAM-5 against *P. aeruginosa* ATCC 27853

Based on the visual observation in **Figure 4.11**, blue colouration was developed in the wells of positive control in which the bacteria were treated with Polymyxin B at concentrations ranged from 2 μg/ml to 256 μg/ml. This indicated complete killing of the bacteria by Polymyxin B of all the tested concentrations. On the other hand, the two wells of negative control containing untreated bacteria turned into pink colour upon incubation, indicating the presence of actively growing bacteria. PAM-5 at concentrations from 8 μg/ml to 256 μg/ml were potent in killing *P. aeruginosa* as indicated by the blue colour in the wells with bacteria treated with PAM-5 of these concentrations. However, at lower peptide concentrations of 2 μg/ml and 4 μg/ml, the peptide was not sufficient to prevent the reduction of resazurin to resorufin by the viable bacteria. This shows that the MBC which was obtained in this assay is consistent to the results in **Section 4.1.1**.

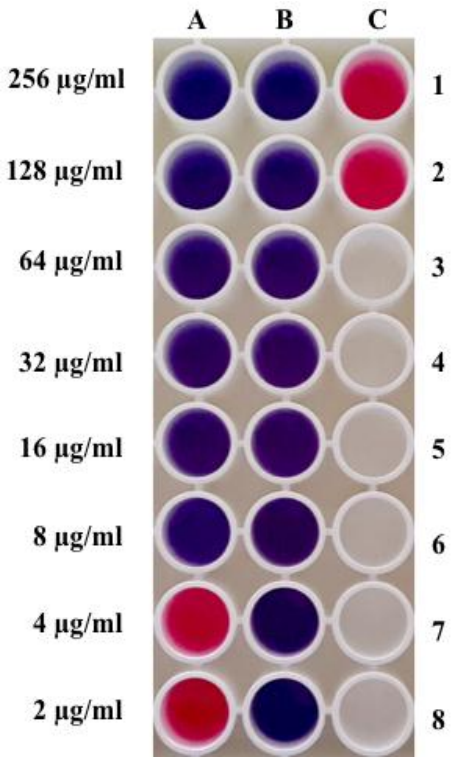


**Figure 4.11:** Qualitative determination of antibacterial effect of PAM-5 on *P. aeruginosa* ATCC 27853 using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Viable bacteria were indicated by the pink colour formation while non-viable bacteria were represented by the blue colouration. The MBC of PAM-5 was determined as 8 µg/ml against *P. aeruginosa* ATCC 27853.

### 4.3.2 Validation of the antibacterial effect of PAM-5 against *E. coli* ATCC 25922

Similar pattern of validation was obtained for *E. coli* ATCC 25922 treated with the same set of PAM-5 concentrations. As shown in **Figure 4.12**, reduced PAM-5 was not able to kill the bacteria at 2 µg/ml (well A8) and 4 µg/ml (well A7), where the blue resazurin was reduced to pink resorufin by sufficient amount of viable bacteria. However, bacteria treated with PAM-5 at 8 µg/ml (well A6) and other higher concentrations (A1 to A5) lost their metabolic capability to reduce the resazurin as they might have been completely killed by

these peptide concentrations. These data were consistent to the data from the microbroth dilution assay as reported in **Section 4.1.2**.

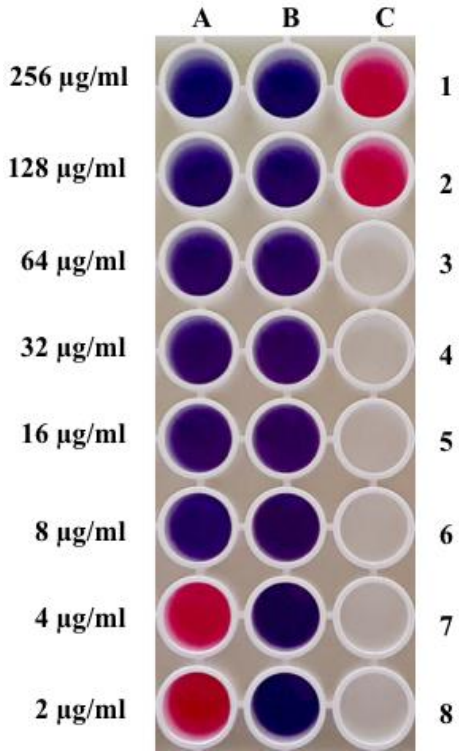


**Figure 4.12:** Qualitative determination of antibacterial effect of PAM-5 on *E. coli* ATCC 25922 using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Pink coloured content indicated presence of viable bacteria while blue coloured content indicated presence of non-viable bacteria. The MBC of PAM-5 against *E. coli* ATCC 25922 was determined as 8 µg/ml.

### 4.3.3 Validation of the antibacterial effect of PAM-5 against *A. baumannii* ATCC 19606

Similar observation was also noted in the assay for *A. baumannii* ATCC 19606 as compared to the former two. PAM-5 was able to prevent the bacterial metabolic activity at concentrations ranged from 8 µg/ml (well A6) to 256

µg/ml (well A8). However, at concentrations lower than 8 µg/ml, viable bacteria were detected as indicated by the pink colouration formed in wells A7 to A8 as presented in **Figure 4.13**. The consistent outcome for both the positive and negative controls signified the validity of these results. Comparatively, the data obtained via this assay and the previously reported microbroth dilution assay confirmed the MBC of this peptide on *A. baumannii*, which is 8 µg/ml.

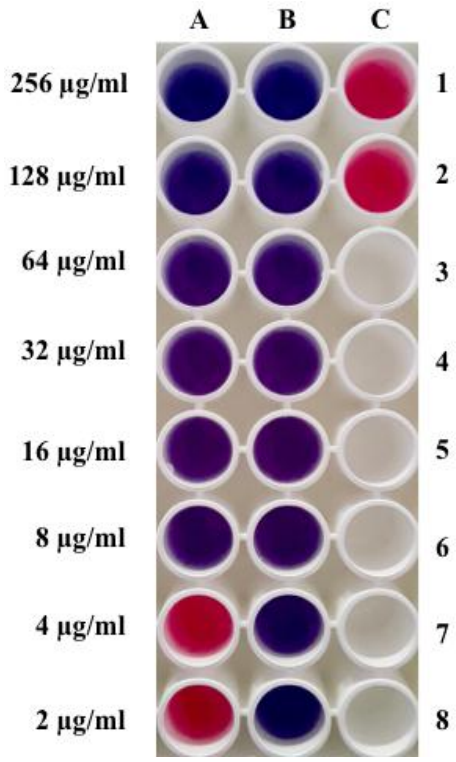


**Figure 4.13:** Qualitative determination of antibacterial effect of PAM-5 on *A. baumannii* ATCC 19606 using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Presence of viable cells was indicated by the formation of pink resorufin while absence of viable cells was indicated by the blue resazurin in the wells. The MBC of PAM-5 against this bacterium was determined as 8 µg/ml.

### 4.3.4 Validation of the antibacterial effect of PAM-5 against *K. pneumoniae* ATCC 13883

By using PrestoBlue$^{TM}$ bacterial viability assay, inconsistent findings were seen with the findings in microbroth dilution assay where a peptide MBC of 32 μg/ml was obtained. At the similar bacterial input titer of ~$10^3$ CFU/ml for this assay, different MBCs were obtained. As shown in **Figure 4.14 (a)**, viable bacteria were observed in cultures treated with PAM-5 at 2 μg/ml (well A8) to 64 μg/ml (well A3), as indicated by the pink coloured-resorufin produced by the viable bacteria. On the other hand, based on **Figure 4.14 (b)**, viable cells were observed in cultures treated with PAM-5 from 2 μg/ml to 128 μg/ml (wells A8 to A2). Thus, the MBCs of PAM-5 against *K. pneumoniae* ATCC 13883 in **Figure 4.14 (a)** and **Figure 4.14 (b)** were determined as 128 μg/ml and 256 μg/ml respectively.

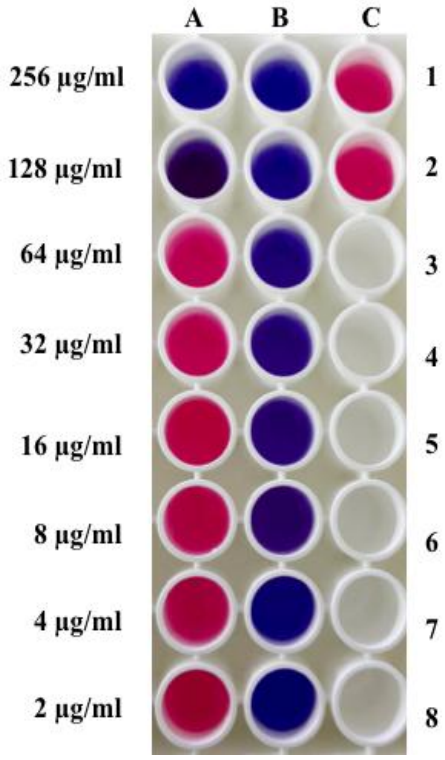


**Figure 4.14 (a):** Qualitative determination of antibacterial effect of PAM-5 on *K. pneumoniae* ATCC 13883 using PrestoBlue™ bacterial viability assay at bacterial input titer of 8 X $10^3$ CFU/ml. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Presence of viable cells was indicated by the formation of pink colouration while absence of viable cells was indicated by the blue colouration in the wells. The MBC of the peptide against this bacterium was determined as 128 µg/ml.

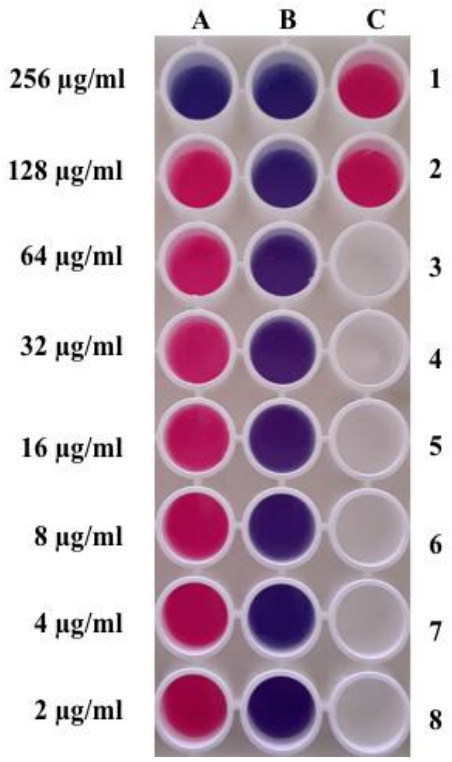


**Figure 4.14 (b):** Qualitative determination of antibacterial effect of PAM-5 on *K. pneumoniae* ATCC 13883 using PrestoBlue™ bacterial viability assay at bacterial input titer of 5 X $10^3$ CFU/ml. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Pink colouration formed in the wells indicated presence of viable bacteria while blue colouration formed in the wells indicated the presence of non-viable bacteria. The MBC of PAM-5 was determined as 256 µg/ml.

### 4.3.5 Validation of the antibacterial effect of PAM-5 against *S. aureus* ATCC 25923

PAM-5 was shown to have low potency against *S. aureus* ATCC 25923 in microbroth dilution assay, and this finding was confirmed again in PrestoBlue™ assay. As shown in **Figure 4.15**, viable bacteria were grown actively despite treatment with PAM-5 from 2 µg/ml to 64 µg/ml, which resulted in the production of pink coloured-resorufin. Polymyxin B that was served as the positive control in this assay was only able to exert complete

killing starting from concentrations of 16 µg/ml to 256 µg/ml. Thus, it was not an ideal candidate for positive control.

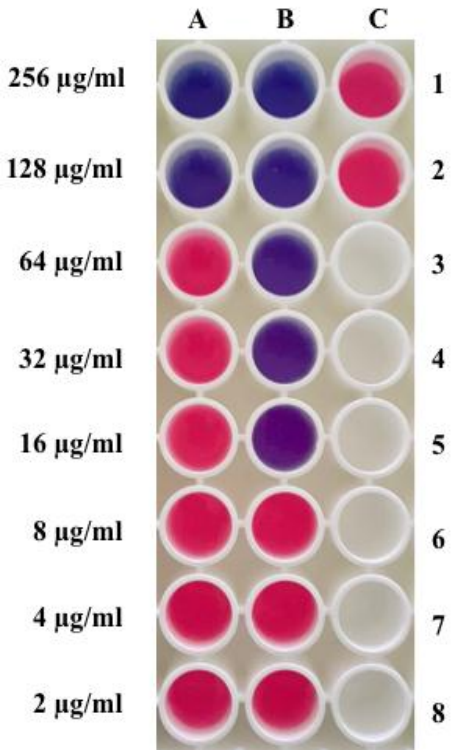


**Figure 4.15:** Qualitative determination of antibacterial effect of PAM-5 on *S. aureus* ATCC 25923 using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Pink colouration content indicated presence of viable bacteria while blue colouration content indicated the presence of non-viable bacteria. The MBC of PAM-5 against this bacterium was determined as 128 µg/ml.

### 4.3.6 Validation of the antibacterial effect of PAM-5 against clinical strain of *S. flexneri*

The outcome of PrestoBlue™ bacterial viability assay on the effect of PAM-5 towards *S. flexneri* was consistent to the finding by microbroth dilution assay. As seen in **Figure 4.16**, only moderate to high concentrations of PAM-5 (32

μg/ml to 256 μg/ml) were able to suppress the viability and metabolic activity of *S. flexneri*, in which the wells containing these treated bacterial cultures remained blue. At peptide concentrations of 2 μg/ml to 16 μg/ml, viable bacteria were present, indicated by the pink colour of the content in the wells.

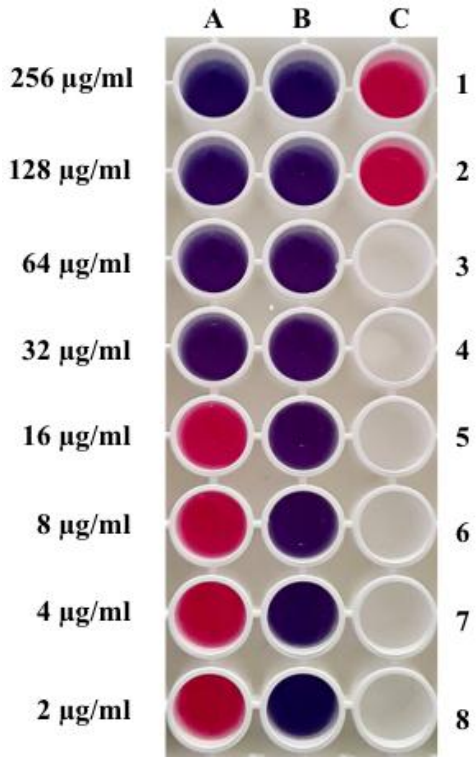


**Figure 4.16:** Qualitative determination of antibacterial effect of PAM-5 on *S. flexneri* clinical strain using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). The formation of pink colouration indicated presence of viable bacteria while blue colouration content indicated the presence of non-viable bacteria. The MBC of PAM-5 against this clinical strain was determined as 32 μg/ml.

### 4.3.7 Validation of the antibacterial effect of PAM-5 against MDR strain of *P. aeruginosa*

As reported in **Section 4.1.7**, higher concentration of PAM-5 (16 μg/ml) was needed to completely kill the multidrug resistant strain of *P. aeruginosa* as compared to its reference strain. The finding in PrestoBlue™ bacteria viability assay was consistent to the result of microbroth dilution assay, where the MBC of PAM-5 against MDR *P. aeruginosa* remained as 16 μg/ml. In **Figure 4.17**, viable bacteria were observed in wells filled with cultures treated with PAM-5 at concentrations ranging from 2 μg/ml to 8 μg/ml, as shown by pink colour formation in wells A8 to A6. Starting from 16 μg/ml to 256 μg/ml (A5 to A8), bacterial viability was suppressed as the content in the wells remained blue.

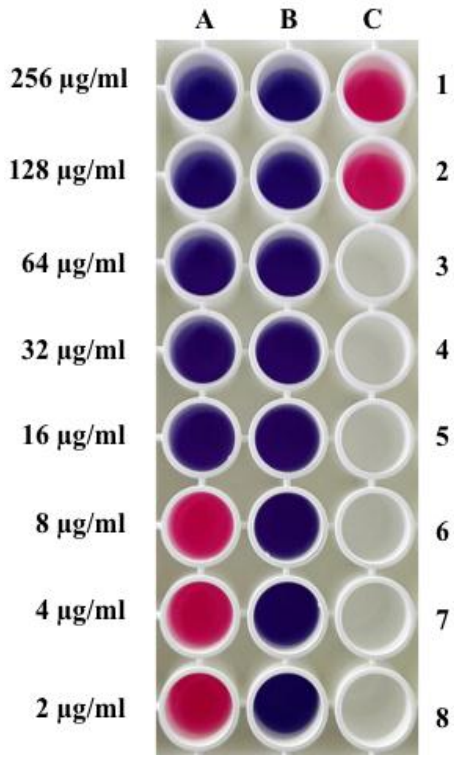


**Figure 4.17:** Qualitative determination of antibacterial effect of PAM-5 on MDR *P. aeruginosa* using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). Viable bacteria were indicated by the pink colouration formed while non-viable bacteria were indicated by the blue colouration formed. The MBC of the peptide against the multidrug-resistant strain of *P. aeruginosa* was determined as 16 µg/ml.

### 4.3.8 Validation of the antibacterial effect of PAM-5 against ESBL-producing *E. coli*

Similarly, reduced potency of PAM-5 on ESBL-producing *E. coli* in this assay was also observed. Both microbroth dilution assay and PrestoBlue™ assay revealed the same MBC of the peptide on this ESBL-producing bacterial strain, which is 64 µg/ml. As presented in **Figure 4.18**, bacterial growth was halted after treatment with PAM-5 concentrations at 64 µg/ml to 256 µg/ml as shown by the presence of resazurin that remained blue. On the other hand, viable

bacteria were detected in wells containing cultures treated with peptide concentrations lower than 64 µg/ml where the initial blue resazurin had been reduced to pink resorufin.

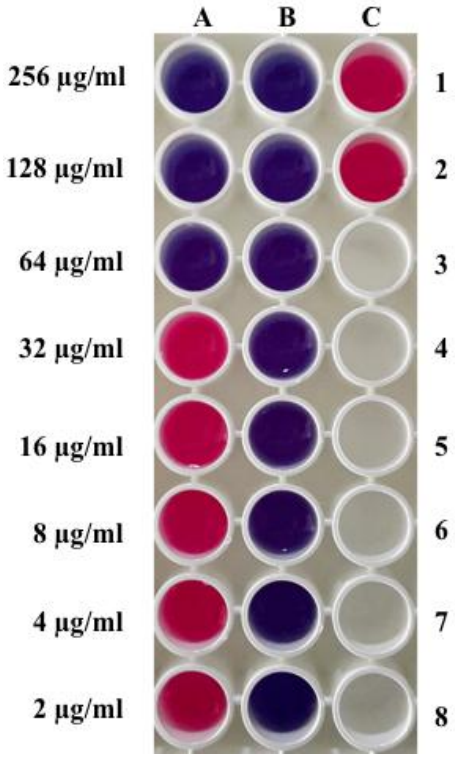


**Figure 4.18:** Qualitative determination of antibacterial effect of PAM-5 on ESBL-producing *E. coli* using PrestoBlue™ bacterial viability assay. Wells A1 to A8: Bacteria treated with PAM-5; wells B1 to B8: bacteria treated with Polymyxin B (positive control); wells C1 to C2: untreated bacteria (negative control). The presence of viable bacteria was indicated by the pink resorufin formed while the presence of non-viable bacteria was indicated by the blue resazurin. The MBC of PAM-5 against the ESBL-producing bacterium was determined as 64 µg/ml.

# CHAPTER 5

# DISCUSSION

Conventional antibiotics with narrow-spectrum antibacterial activity are less effective in treating bacterial infections, especially polyinfections which are commonly occurring in patients with compromised immunity. Moreover, this limitation always imposes difficulty to clinician to decide the best empirical therapy for a patient who is suffering from bacterial infection before identification of the actual infection is obtained. Under these circumstances, combinations of antibiotics are usually needed for successful elimination of the bacterial pathogens. However, excessive usage of these antibiotics is always associated with increased emergence of multidrug resistant bacteria (World Health Organization, 2015). Thus, an ideal antibacterial agent is expected to possess broad-spectrum antibacterial activity so that a single agent is sufficient to battle against multiple bacterial infections. This triggers the interest of various researchers to search for broad-spectrum antibacterial agents that have minimal risk in inducing resistance among bacteria. Antibacterial peptides (ABPs) are one of the potential alternatives that are extensively studied over the last decade. However, most of these researches focused on the naturally occurring peptides and very few of them studied on the synthetic peptides. Although there are recent studies on the feasibility of synthetic ABPs but limited novel ABPs with broad-spectrum antibacterial activities were discovered. Therefore, in this study, a 15-mer synthetic peptide PAM-5 which was previously found to be potent against *P. aeruginosa* by Lee (2014) was

screened for its potency against various pathogenic bacteria, including two multi-drug resistant strains using microbroth dilution assay. The results obtained from this assay were subsequently verified by using PrestoBlue™ bacterial viability assay.

## 5.1 Validation of PAM-5 potency on *P. aeruginosa* from previous finding

The antibacterial potency of PAM-5 against *P. aeruginosa* ATCC 27853 was confirmed using microbroth dilution assay and PrestoBlue™ bacterial viability assay. The minimum bactericidal concentration (MBC) of PAM-5 against this bacterium was determined as 8 μg/ml without notable minimum inhibitory concentration (MIC), which is corresponding to the results obtained by Lee in 2015. Collectively, these two consecutive works have demonstrated that PAM-5 is an ideal bactericidal agent towards *P. aeruginosa.* These findings are notably significant as *P. aeruginosa* is notorious for its multidrug-resistance that easily compromises the efficacy of many bacteriostatic antibiotics. As PAM-5 is bactericidal instead of bacteriostatic towards *P. aeruginosa*, it is anticipated to eliminate the bacteria effectively with minimal risk of developing ABP-induced resistance from the bacteria.

## 5.2 Evaluation of antibacterial spectrum of PAM-5

PAM-5 displayed highly potent antibacterial effects against several reference strains of bacteria such as *P. aeruginosa*, *E. coli* and *A. baumannii* where it

was bactericidal towards these bacteria at a MBC as low as 8 μg/ml. Although PAM-5 was less potent against reference strains of *K. pneumoniae* and clinical strain of *S. flexneri*, bactericidal effects on these two bacteria were still observed at peptide concentrations of 32 μg/ml. This indicates that PAM-5 is able to exert ideal range of antibacterial effects towards the selected Gram-negative bacteria, which represent the most common community-acquired and hospital-acquired infections. However, high PAM-5 concentration (96 μg/ml) was needed for complete killing of *S. aureus*, which was the only tested Gram-positive bacterium. This indicates its low potency against the Gram-positive bacterium.

As mentioned in Chapter 2, PAM-5 is a cationic peptide with a positive net charge of +7 with moderate hydrophobicity of 46%. The cationicity of the peptide may allow its binding to the anionic bacterial membrane surface via electrostatic interaction while the hydrophobic portion of the peptide may promote its anchoring onto the phospholipid bilayer of the bacterial membrane. Consequently, these interactions may cause disruption to the bacterial membrane followed by various downstream damages such as membrane pore formations that lead to cellular leakage and ultimately cell death. Thus, the primary determinant of an ABP potency is usually its cationicity (Guilhelmelli et al., 2013). In this study, the high potency of the synthetic peptide PAM-5 might be attributed to its ideal cationicity. As reported by the studies of Matsuzaki (2009), Yeaman (2003) and Powers and Hancock (2003), cationiticy of an ABP is crucial for the initial contact with the bacterial cells. Thus, with a positive net charge of +7, PAM-5 is believed to be able to initiate early contact

with the bacteria that subsequently accounted for further downstream damages to the bacteria.

Apart from its primary mode of action on the bacterial membrane, several studies had also documented the interactions of ABPs with various bacterial cellular targets, such as the bacterial cell wall, nucleic acid and protein synthesis (Brogden, 2005; Straus and Hancock, 2006; Jenssen, Hamill and Hancock, 2006). Also, with the multiple targets of actions, the same peptide can act concurrently on various cellular targets, resulting in the high antibacterial activities (Guilhelmelli et al., 2013). Although it is not clear that PAM-5 is able to act on multiple targets of the same bacteria, but its potent bactericidal effects on these bacteria suggested the possibility of this potential which is worth to be studied in the future.

All the tested Gram-negative bacteria were classified under the class of *Gammaproteobacteria* (Williams et al., 2010). Thus, they might possess similar characteristics that allow the peptide to interact in a similar manner. This could explain the ability of PAM-5 to act with the same potency against *P. aeruginosa* (*Pseudomonadaceae* family), *E. coli* (*Enterobacteriaceae* family) and *A. baumannii* (*Moraxellaceae* family) even though these bacteria are of different families.

### 5.2.1 Reduced potency of PAM-5 against selected test bacteria

As mentioned briefly in **Section 5.2**, PAM-5 was slightly less potent against *K. pneumoniae* as well as *S. flexneri* and poorly active against *S. aureus.* For these bacteria, relatively higher MBCs of PAM-5 were required to kill them completely. This indicates that PAM-5 might not be an ideal antibacterial agent towards *K. pneumoniae*, *S. aureus* and *S. flexneri*. As these three bacteria were never subjected to prior exposure to PAM-5, it is unlikely to deduce that induced-resistance towards PAM-5 was being developed in these bacteria upon the assay in this study. The possible explanations to the reduced potency of PAM-5 towards *K. pneumoniae*, *S. aureus* and *S. flexneri* might be due to the intrinsic or natural resistance of these bacteria.

Similar to *E. coli*, *K. pneumoniae* and *S. flexneri* are under the family of *Enterobacteriaceae*. Based on this consideration, one would anticipate that PAM-5 should theoretically have the same potency against these three Gram-negative bacteria. However, the findings in this study clearly ruled out this assumption. One of the possible explanations is the various protective mechanisms that are present naturally in these bacteria. In particular, *K. pneumoniae* is able to produce capsular polysaccharides that contribute to its mucoid appearance on the media. These matrices can be released from the bacteria and may bind to many antibacterial agents, thus reducing the effective killing or inhibitory concentrations of these agents or blocking them from interacting directly to the bacterial membrane. In relation to ABPs, the presence of these anionic exopolysaccharides may bind to the cationic ABPs

through electrostatic interactions, thus reducing the availability of free peptides that can access to the bacterial membrane. Consequently, the peptide may not reach the sufficient concentration threshold to disrupt the bacterial membrane or initiate any downstream damages (Hale and Hancock, 2007). As the result, higher peptide concentrations are required to kill these bacteria as more peptides are needed to achieve the threshold for membrane disruption (Llobet, Tomas and Bengoechea, 2008; Willis and Whitfield, 2013). In a study done by Campos et al. in 2004, increased resistance of *K. pneumoniae* to Polymyxin was reported in strains with positive capsule production ability. Correspondingly, this may explain the higher MBC of PAM-5 that was needed to kill *K. pneumoniae* in this study.

On the other hand, the reduced potency of PAM-5 against *S. flexneri* may be due to the ability of this bacterium to secrete extracellular proteases. SepA and Pic are the two major proteases secreted by *S. flexneri*. In a study conducted by Chen et al. (2014), SepA and Pic were discovered in the outer membrane vesicles produced by *S. flexneri*. They are part of the serine protease autotransporters of *Enterobacteriaceae* (SPATEs) family, which consist similar active sites as the serine proteases that are able to hydrolyse various proteins (Henderson et al., 1999; Dautin, 2010). Thus, it could be assumed that these proteases may also able to cross-react with PAM-5 by degrading the peptide which in turn leads to the reduction of antibacterial activities. Meanwhile, Pic was shown to have proteolytic activity towards mucin by Henderson et al. (1999) and Dutta et al. (2002), while SepA was found to have the ability to cleave several peptides in a study by Benjelloun-Touimi et al.

(1998). Therefore, we postulated that similar proteolytic activities may also happen to PAM-5 when the peptide was engaged with *S. flexneri* during the assay. In addition to that, there may be presence of drug-efflux pump that might be able to exclude the peptide out from the bacterial cells. As demonstrated in the research by Kim et al. (2008), expression of genes that are encoding for efflux pumps in *S. flexneri* was greatly enhanced following the exposure of the bacterium to fluoroquinolonones. As this is a clinical isolate, it is rational to assume that *S. flexneri* may have been previously exposed to antibiotic treatments. Thus, the efflux pumps might be actively expressed present in this clinical isolate that caused the exclusion of PAM-5 from the bacterial cells.

In contrast, the reduced potency of PAM-5 on *S. aureus* may not be due to the intrinsic resistance as discussed earlier, but instead more to the structural difference between Gram-positive and Gram-negative bacteria. Gram-positive bacteria, for instance, *S. aureus* possess a layer of thick cell wall formed by peptidoglycan. As most of the ABPs are interacting directly with the bacterial membrane instead of cell wall, the thick cell wall of *S. aureus* provides protection to the bacteria from the membrane damaging antibacterial agents (Silhavy, Kahne and Walker, 2010). As the result, more ABPs are needed to penetrate through the cell wall in order to interact with the bacterial cell membrane. In a review study by Wimley in 2010, ABPs must penetrate through the thick peptidoglycan of Gram-positive bacteria in order to interact with the bacterial membrane. This could explain the high concentrations of PAM-5 as well as Polymyxin B that were needed to achieve complete

bactericidal effects towards *S. aureus* in this study. Apart from that, studies have shown that Gram-positive bacteria usually resist to ABPs through the production of peptide-degrading enzymes that prevent the peptides from contacting the bacterial cells (Nawrocki, Crispell and McBride, 2014). According to the studies by Sabat et al., (2000) and Sieprawska-Lupa et al. (2004), *S. aureus* is able to produce an extracellular protease, namely aureolysin that degrades a human ABP, LL-37. Besides, many Gram-positive bacteria are also said to be able to secrete other peptide-degrading enzymes such as metalloproteases and cysteine proteases (Wu and Chen, 2011; Del Papa et al., 2007). It is anticipated that PAM-5 may have been cleaved by some of these peptide-degrading enzymes produced by *S. aurues.* Thus, more peptides are needed to overcome the resistant mechanisms of the bacterium.

## 5.3 Antibacterial potency of PAM-5 against multidrug-resistant bacteria

### 5.3.1 Antibacterial potency of PAM-5 against MDR *P. aeruginosa*

As mentioned in **Section 4.1.7**, PAM-5 was also able to exert bactericidal effect against clinical isolate of MDR *P. aeruginosa*. Nevertheless, at the MBC of 16 μg/ml, the peptide was slightly less potent against this multi-drug resistant strain as compared to its wild-type counterpart where a lower MBC of 8 μg/ml was reported.

It was known that the MDR strain of *P. aeruginosa* used in this study was a clinical strain isolated from a patient who was under prolonged antibiotic treatment. Therefore, it is noteworthy to assume that the reduced potency of PAM-5 to this bacterium could be due to cross-resistance mechanism induced by the previous antibiotics exposure. According to many studies, one of the common induced-resistance is the alginate overexpression (Shigeta et al., 1997; Chan, Burrows and Deber, 2004; 2005; Aoki and Ueda, 2013). Alginate is an exopolysaccharide produced by the bacterium in response to stress. It serves as a protection for the bacteria from the harsh environmental conditions as well as to enhance bacterial adhesion to solid surfaces, which is normally seen in biofilm formation. According to Boyd and Chakrabarty (1995), increased alginate production is usually associated with the transcription of activated alginate biosynthetic genes upon anchoring of the bacteria to a solid surface. As this bacterial strain was a clinical strain, the transcription of the alginate regulatory genes may have been activated during its colonisation in the patient. In addition to that, it also can be assumed that a high rate of alginate synthesis was present in the bacterium as it was previously exposed to various antibiotics because alginate is an antibiotic-induced mucoid exopolysaccharide (Bayer et al., 1991; Balasubramanian et al., 2010).

Besides, the anionic alginate that is present as a protective layer on the bacterial cells may prevent the ABPs to act on the bacterial membrane. Without the initial electrostatic interactions between the peptides and the bacterial membrane, it would not be possible for the downstream actions to be produced. This anionic alginate layer would also trap the cationic peptides by

interacting electrostatically with them, thus decreasing the availability of free peptides that can act on the bacterial cells (Llobet, Tomas and Bengoechea, 2008). Hence, more peptides are needed to saturate the alginate before binding to the bacterial membrane in order to reach the sufficient threshold concentration for membrane disruption (Hale and Hancock, 2007).

### 5.3.2 Antibacterial potency of PAM-5 against ESBL-producing *E. coli*

The potency of PAM-5 was even lower against ESBL-producing *E. coli* where it was only able to kill the bacterium completely at 64 μg/ml, as compared to the concentration needed for its wild-type. As an extended spectrum β-lactamases-producing bacterium, there might be possible cross-resistance to other antibacterial agents due to the presence of other peptide-degrading enzymes that was being produced conjointly with the β-lactamases. These enzymes might be able to cleave the amide bonds of ABPs, thus compromising their antibacterial potency. In view of this study, β-lactamases that were produced by the bacterium might be able to cross-react with PAM-5. According to documented findings, there are different classes of β-lactamases and few of these classes are named Class A, Class C and Class D. These enzymes are able to act in such way like serine-active proteases (Mayers et al., 2009; Drawz and Bonomo, 2010; Thenmozhi et al., 2014). Serine proteases can be further classified into trypsin-like group, chymotrypsin-like group and elastase-like group according to their substrate selectivity. Trypsin-like group is able to hydrolyse the amide bonds of arginine (R) and lysine (K); chymotrypsin-like group is able to cleave non-polar amino acids such as

tryptophan (W) and leucine (L); while the elastase-like group can break down small non-polar amino acids such as glycine (G) and valine (V) (Ovaere, Lippens, Vandenabeele and Declercq, 2009). As these amino acids are also present in PAM-5 (**K**-**W**-**K**-**W**-**R**-P-**L**-**K**-**R**-**K**-**L**-**V**-**L**-**R**-M), it could be suspected that serine proteases produced by this ESBL-producing bacterium might cleave the peptide. This may lead to the lost of some positively-charged amino acids as well as the non-polar amino acids, which in turn reduces the cationicity and hydrophobicity of PAM-5, hence the lost of antibacterial effects of the peptide. Thus, more peptides are needed to completely kill the ESBL-producing *E. coli* as the activities of β-lactamases have to be blocked by saturating their active sites, so that the remaining available functional peptides can interact with the bacterial membrane. This could explain the high MBC of this peptide towards the ESBL-producing *E. coli.*

## 5.4 Results validation using PrestoBlue™ bacterial viability assay

PrestoBlue™ reagent is a resazurin-based reagent that contains a blue non-fluorescent membrane permeable compound resazurin in its oxidised form. However, upon reduction by the NADH dehydrogenase or NADPH dehydrogenase produced by viable cells, the non-fluorescent blue resazurin will change irreversibly into a red or pink fluorescent resorufin. Thus, by visual inspection of the colour change, the presence of viable bacteria can be determined, allowing the determination of MBC similar as microbroth dilution assay (Lall et al., 2013).

Based on the results presented in Chapter 4, the findings of PrestoBlue™ bacterial viability assays were consistent with the findings of microbroth dilution assays except for *K. pneumoniae* ATCC 13883.

Inconsistent results were obtained for the PrestoBlue™ assay when PAM-5 was screened against *K. pneumoniae* ATCC 13883. The MBC of PAM-5 obtained through this assay (**Section 4.3.4**) did not match with the results of its microbroth dilution assay as mentioned in **Section 4.1.4**. This inconsistency may be due to the high sensitivity of the PrestoBlue™ reagent. As PrestoBlue™ reagent is known as a growth indicator, it is very sensitive in the detection of viable bacterial cells (Lall et al., 2013). In this study, there might be negligible amount of viable bacteria that have escaped the peptide treatment due to the protection of its capsular layer. These viable cells could be detected by the PrestoBlue™ assay but not through microbroth dilution assay. The amount of viable bacteria present may be insufficient to grow on the media plates. It had been shown in the study by Funes-Huacca et al. (2012) that PrestoBlue™ reagent can detect *E. coli* titer as low as 100 CFU per 100 µl. Thus, it may allow the detection of bacterial growth that could not be detected using microbroth dilution assay.

## 5.5 Implications and limitations of study

As described in Chapter 4, PAM-5 possessed favourable antibacterial effects against various Gram-negative bacteria including the multidrug-resistant bacteria. Therefore, PAM-5 has great potential in being developed as an

empirical treatment for acute infections and treating polyinfections. With these advantages, the shortage of effective infection treatments, especially for infections caused by multi-drug resistant bacteria might be overcome. However, in this study, PAM-5 was only screened against one type of Gram-positive bacteria. Thus, the antibacterial spectrum against more Gram-positive bacteria could be studied in the future.

## 5.6 Future studies

The antibacterial spectrum of PAM-5 had been screened in this study and it was found out that this peptide had promising antibacterial effects against various Gram-negative bacteria as well as the multi-drug resistant strains with relatively different potencies. The peptide was also tested on a Gram-positive bacterium represented by *S. aureus* but yielded a poor potency. However, as the peptide was only tested against one Gram-positive bacterium, it could not be concluded that PAM-5 is not potent against all other Gram-positive bacteria. Thus, the antibacterial effects of PAM-5 against more Gram-positive bacteria such as *Streptococcus pneumoniae*, *Streptococcus pyrogenes*, *Listeria monocytogenes*, *Enterococcus faecalis* and *Corynebacterium diphtheriae* could be studied.

Despite of the moderate bactericidal activity of the peptide against *K. pneumoniae,* inconsistency was still observed in this study. Thus, further investigation can be done to validate the antibacterial activity of PAM-5 against this bacterium of different strains.

Lastly, with the findings on antibacterial potency of PAM-5 against the MDR *P. aeruginosa* and ESBL-producing *E. coli*, it is also worth to find out whether the spectrum of antibacterial effects could also apply to other MDR bacterial strains such as the MDR *A. baumannii*, ESBL-producing *K. pneumoniae* and the methicillin-resistant *S. aureus*.

# CHAPTER 6

# CONCLUSION

In conclusion, the spectrum of antibacterial effects of synthetic peptide PAM-5 was screened and found to have relatively different potencies against the selected test bacteria. The antibacterial effect of PAM-5 against *P. aeruginosa* ATCC 27853 was consistent with the previous finding where a MBC of 8 μg/ml was obtained. The peptide was also revealed to be highly potent against reference strains of *E. coli* and *A. baumannii* with the same MBC as *P. aeruginosa*. Moderate potency was observed in reference strain of *K. pneumoniae* and *S. flexneri* clinical strain where the MBC of PAM-5 was determined at 32 μg/ml. However, the antibacterial effect of the peptide against *K. pneumoniae* was yet to be verified. On the other hand, high peptide concentration of 96 μg/ml was needed to kill *S. aureus* ATCC 25923 completely. PAM-5 was also found to exhibit promising antibacterial effects against MDR *P. aeruginosa* and ESBL-producing *E. coli* at MBC of 16 μg/ml and 64 μg/ml, respectively. It could not be deduced that synthetic peptide PAM-5 is able to exert broad-spectrum antibacterial effects against both Gram-negative and Gram-positive bacteria as only one type of Gram-positive bacterium was tested with low potency obtained. However, PAM-5 might be broad-spectrum against Gram-negative bacteria to a certain extend as indicated by the satisfactory bactericidal effects observed in the few tested Gram-negative bacteria. PAM-5 was also found to be bactericidal against all bacteria tested.

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## APPENDIX A

## LIST OF LABWARE AND EQUIPMENTS

| Labware/Equipments | Manufacturers |
|---|---|
| 15 ml centrifuge tube | Greiner, Germany |
| 50 ml centrifuge tube | Axvgen® Scientific, USA |
| 96-well microplate, transparent, flat-bottomed | Greiner CELLSTAR®, Germany |
| 96-well microplate, white opaque, flat-bottomed | Greiner CELLSTAR®, Germany |
| Biosafety Cabinet Level-2 | TELSTAR, Philippines |
| Bunsen burner | Campingaz, France |
| Centrifuge machine | Eppendorf 5430 R, Germany |
| Incubator | Memmert, Germany |
| Measuring cylinder | GQ, Malaysia |
| Microcentrifuge tube | Axvgen® Scientific, USA |
| Microplate reader | BMG Labtech FLUOstar Omega, Australia |
| Micropipette set | Eppendorf Research® plus, Germany |
| Micropipette tip | Axvgen® Scientific, USA |
| Petri dish | BIOAN, Malaysia |
| Schott bottle | DURAN®, Germany |
| Spectrophotometer | Thermo Scientific Genesys 20, Malaysia |
| Vortex mixer | VELP® Scientific, Europe |

# APPENDIX B

## PREPARATION OF BUFFERS AND MEDIA

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

A total volume of 8 g of LB broth powder (Merck Millipore) was dissolved in 400 ml of distilled water and autoclaved at 121 ℃ for 15 minutes.

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

MH broth was prepared by dissolving 8.4 g of MH broth poweder (Difco$^{TM}$) in 400 ml of distilled water. The medium was then autoclaved at 121 ℃ for 15 minutes.

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

MH agar was prepared by dissolving 23.8 g (Merck Millipore) of MH agar powder in 700 ml of distilled water and autoclaved at 121 ℃ for 15 minutes. Then, the medium was poured into the petri dishes after being cooled down. The solidified agar plates were stored at 4 ℃.

**Preparation of MacConkey agar**

A total volume of 36.4 g of MacConkey agar powder (Oxoid) was dissolved in 700 ml of distilled water and sterilised by autoclaving at 121 ℃ for 15 minutes. Then, the cooled medium was dispensed into the petri dishes and the solidified agar plates were stored at 4 ℃.

**Preparation of Mannitol Salt agar**

Mannitol salt agar was prepared by dissolving 33.3 g of Mannitol Salt agar powder (Laboratorios CONDA) in 300 ml of distilled water and sterilised through autoclave at 121 ℃ for 15 minutes. The medium was cooled prior being dispensed into the petri dishes. The solidified agar plates were stored at 4 ℃.

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

Seven PBS tablets (Takara Bio, Inc) which contained no magnesium and calcium were dissolved in 700 ml of distilled water. The solution was then sterelised by autoclave at 121 ℃ for 15 min.