ANTIBACTERIAL PROPERTIES OF SYNTHETIC PEPTIDES AND SCHIFF BASE COMPOUNDS AGAINST DIFFERENT TYPES OF CLINICAL BACTERIA

CHOW WEI CHON

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2013

ABSTRACT

The emergence of multiple drug-resistant nosocomial pathogens affects the efficacy of chemotherapeutic treatment of infectious diseases in patients. Therefore, continuous development of new synthetic antibacterial compounds such as the synthetic peptides and Schiff base complexes to complement the current antibiotic treatment is essential. The *in-vitro* antibacterial activities of six synthetic cationic peptides and twenty-nine synthetic Schiff base complexes towards selected sixteen clinical strains of multiple drug-resistant methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* (AC), *Klebsiella pneumoniae* (KB) and *Pseudomonas aeruginosa* (PA) were investigated in this study. Evaluation of the antibacterial activities were determined through disk diffusion testing, broth micro-dilution assay for minimum inhibitory concentration (MIC) determination, cell inactivation assay and bacterial killing rate (time-kill) assay. Some of the Schiff base ligands were bound to various metals including nickel (Ni), cobalt (Co), zinc (Zn), cadmium (Cd) and copper (Cu). Among the twenty-nine synthetic Schiff base complexes screened in the disk diffusion test, the complex containing cadmium, LMA Cd-N_3, was shown to be more effective as it inhibited the growth of six randomly selected bacterial strains (KB88, KB198, MRSA080925, MRSA08071, AC06127, AC08121), resulting in zones of inhibition that were comparable to the antibiotics (polymyxin B and vancomycin) used. Results from the time-kill assay showed that the LMA Cd-N_3 complex achieved complete killing of bacterial cells after exposure to the complex for 4 hours (1X MIC at 625.0 ppm), 8 hours (1X MIC at 156.3 ppm) and 12 hours (2X MIC at 625.0 ppm) for MRSA, AC and KB, respectively. On the other hand, complete killing was observed when similar strains of KB and AC were exposed to 0.5X MIC of polymyxin B (1.0 ppm) for 2 hours, while MRSA strain required 12 hours of exposure to 0.5X MIC of vancomycin (2.0 ppm).

Interestingly, KB had shown regrowth of cells within 4 hours to 24 hours of exposure to polymyxin B at 0.5X MIC, indicating that polymyxin B had lost its antibiotic effect after 4 hours of exposure. Apart from having poor antibacterial activity against all bacterial strains tested in the study, some of the cationic peptides were also shown to induce growth of bacterial cells at the range of concentration from 25.0 ppm to 375.0 ppm. However, the growth of *P. aeruginosa* strains was not affected by the cationic peptides and Schiff base complexes where no inhibition zones were observed for the strains in the disk diffusion test. Results obtained from the assays in the study showed that both of the synthetic Schiff base complexes and cationic peptides exhibited antibacterial activity against Gram-positive and Gram-negative bacteria. Schiff base cadmium complex showed comparable results to commercial antibiotics used against bacterial strains of MRSA, *A. baumannii* and *K. pneumoniae*, whereas cationic peptides (RM) exerted slight antibacterial activities towards MRSA strains. The findings implied that the cadmium-containing Schiff base complex represents a good candidate for future research in the development of novel antibacterial compounds for treatment of diseases caused by MRSA, *A. baumannii* and *K. pneumoniae*.

ABSTRAK

Kemunculan patogen nosokomia yang resistan terhadap antibiotik mempengaruhi keberkesanan rawatan kemoterapiutik penyakit berjangkit di kalangan pesakit. Oleh demikian, perkembangan berterusan sebatian antibakteria sintetik yang baru seperti peptida dan kompleks *Schiff base* untuk menambahbaik rawatan antibiotik adalah penting. Aktiviti antibakteria *in-vitro* bagi enam peptida sintetik kationik dan dua puluh sembilan kompleks sintetik *Schiff base* terhadap enam belas strain *methicillin-resistant Staphylococcus aureus* (MRSA), *Acinetobacter baumannii (AC), Klebsiella pneumoniae (KB)* dan *Pseudomonas Aeruginosa (PA)* telah dikaji. Penilaian aktiviti antibakteria bagi sebatian sintetik telah ditentukan melalui ujian resapan cakera, ujian '*broth micro-dilution*' untuk penentuan kepekatan perencatan minimum (*MIC*), ujian penyahaktifan sel dan ujian kadar pembasmian bakteria (*time-kill*). Sesetengah ligan *Schiff base* dapat mengikat dengan pelbagai logam termasuk nikel (Ni), kobalt (Co), zink (Zn), kadmium (Cd) dan kuprum (Cu). Antara dua puluh sembilan kompleks sintetik *Schiff base* yang dikaji melalui ujian resapan cakera, sebatian yang mengandungi kadmium, LMA Cd-N_3 adalah lebih berkesan kerana mampu menghalang pertumbuhan enam bakteria yang dipilih secara rawak (KB88, KB198, MRSA080925, MRSA08071, AC06127, AC08121) serta menunjukkan keputusan zon perencatan yang setanding dengan antibiotik komersial (polymyxin B dan vancomycin) yang digunakan. Keputusan daripada ujian *time-kill* menunjukkan bahawa sebatian LMA Cd-N3 mampu membasmi kesemua sel-sel bakteria selepas pendedahan kepada sebatian tersebut selama 4 jam (1X *MIC* pada 625.0 ppm), 8 jam (1X *MIC* pada 156.3 ppm) dan 12 jam (2X *MIC* pada 625.0 ppm) masing-masing bagi MRSA, AC dan KB. Manakala, pembasmian kesemua sel KB dan AC berlaku selepas 2 jam pendedahan kepada polymyxin B pada 0.5X *MIC* (1.0 ppm), sementara MRSA memerlukan 12 jam

pendedahan kepada vancomycin pada 0.5X *MIC* (2.0 ppm). Yang menariknya, KB menunjukkan pertumbuhan semula sel selepas pendedahan kepada polymyxin B pada 0.5X *MIC* dalam masa 4 jam hingga 24 jam menunjukkan bahawa polymyxin B telah kehilangan aktiviti antibiotik selepas 4 jam pendedahan. Selain daripada aktiviti antibakteria yang kurang memuaskan terhadap kesemua bakteria dalam kajian ini, sesetengah peptida kationik juga mampu menggalakkan pertumbuhan sel bakteria pada julat kepekatan 25.0 ppm hingga 375.0 ppm. Walaubagaimana pun, pertumbuhan *P. aeruginosa* tidak dipengaruhi oleh sebatian *Schiff base* dan zon perencatan tidak diperhatikan dalam ujian resapan cakera. Keputusan daripada ujian menunjukkan bahawa kedua-dua sebatian sintetik *Schiff base* dan peptida kationik menunjukkan aktiviti antibakteria terhadap bakteria Gram-positif dan Gram-negatif. Sebatian *Schiff base* kadmium mempamerkan keputusan yang standing dengan antibiotik komersial yang digunakan terhadap bakteria MRSA, *A. baumannii* dan *K. pneumoniae*, manakala peptide kationik (RM) hanya menunjukkan aktiviti antibakteria yang kurang memuaskan terhadap bakteria MRSA. Kajian ini menunjukkan bahawa sebatian *Schiff base* yang mengandungi kadmium merupakan calon yang baik dalam perkembangan sebatian antibakteria yang novel untuk rawatan penyakit yang disebabkan oleh MRSA, *A. baumannii* dan *K. pneumoniae*.

ACKNOWLEDGEMENTS

This work was carried out at the Institute of Biological Science, Microbiology Division at University Malaya, Kuala Lumpur, Malaysia.

First of all, I would like to acknowledge my co-supervisor Professor Dr. Thong Kwai Lin, Professor at the Microbiology Division at University Malaya for giving me the opportunity to work on this study and not to mention providing the funds and laboratory equipment to make this work possible.

Much appreciation is given to my main supervisor; Dr. Chai Lay Ching who had supervised the given thesis title and provide much needed guidance and advices for the completion of this dissertation. Without her assistance and continuous support, this dissertation would not be thorough.

I would also like to thank my family members; to both of my parents and particularly to my sister, Adele who provided me with supports and advices especially when things get tough in the duration of this study. Not forgetting also, a big gratitude goes to all of my friends and laboratory mates for their friendship, support, advices, jokes and those little things that cheers and kept my spirit high for me to be able to complete this dissertation.

TABLE OF CONTENTS

Title	Page
Abstract	ii
Abstrak	iv
Acknowledgements	vi
Table of contents	vii
List of figures	x
List of tables	xi
Chapter 1: Introduction	1
1.1 Objectives	2
Chapter 2: Literature review	3
2.1 Healthcare-associated infection (HAI)	3
2.1.1 *Staphylococcus aureus*	3
2.1.2 *Klebsiella pneumoniae*	4
2.1.3 *Acinetobacter baumannii* and *Pseudomonas aeruginosa*	4
2.2 Antibiotics	5
2.3 Peptides	6
2.3.1 Structure of peptides	7
2.3.2 Modes of action	8
2.3.3 Resistance to peptides	10
2.3.4 Selectivity of peptides	10
2.4 Metallic ion-bound complex	11
2.4.1 Effects of metal towards living organisms	11
2.4.2 Schiff base metal complexes	11

2.4.3 Applications of other metallic ion-bound complex 12

Chapter 3: Methodology 14

3.1 Materials 14

3.1.1 Bacterial cultures 14

3.1.2 Media 15

3.1.2.1 Lysogeny-Broth (LB) agar media composition 15

3.1.2.2 Veal infusion broth media 16

3.1.2.3 Mueller Hinton II agar (cation-adjusted) (MHII agar) 16

3.1.2.4 Mueller Hinton II (cation-adjusted) (MHII agar) 16

3.1.3 Antibiotics 17

3.1.3.1 Polymyxin B sulfate disk 17

3.1.3.2 Polymyxin B sulfate powder 17

3.1.3.3 Vancomycin disk 17

3.1.3.4 Vancomycin powder 17

3.1.4 Synthetic cationic peptides 18

3.1.5 Synthetic Schiff base complexes 19

3.2 Methods 20

3.2.1 Bacterial cell culture preparation 20

3.2.2 Antibacterial activity screening for cationic peptides 20

3.2.2.1 Peptide preparation 21

3.2.2.2 Kirby-Bauer disk diffusion antibacterial susceptibility test 22

3.2.2.3 Broth micro-dilution assay 22

3.2.2.4 Cell inactivation assay 23

3.2.3 Antibacterial activity screening for Schiff base complexes 25

3.2.3.1 Schiff base complexes preparation 25

3.2.3.2 Kirby-Bauer disk diffusion antibacterial susceptibility test 26

3.2.3.3 Broth micro-dilution assay 27

3.2.3.4 Time-kill assay 28

3.3 Data analysis 29

Chapter 4: Results 30

4.1 Antibacterial activity of cationic peptides 30

4.1.1 Kirby-Bauer disk diffusion antibacterial susceptibility test 30

4.1.2 Minimum inhibitory concentration (MIC) determination 30

4.1.3 Cell inactivation assay 31

4.2 Antibacterial activity of Schiff base complexes 35

4.2.1 Kirby-Bauer disk diffusion antibacterial susceptibility test 35

4.2.2 Minimum inhibitory concentration (MIC) determination 37

4.2.3 Time-kill assay 37

Chapter 5: Discussion 42

5.1 Antibacterial activity of cationic peptides against selected multidrug-resistant nosocomial bacteria 42

5.2 Antibacterial activity of Schiff base complexes against selected multidrug-resistant nosocomial bacteria 44

5.3 Cadmium toxicity and bacterial resistance to cadmium 47

5.4 Limitation of the study 49

Chapter 6: Conclusion 50

Bibliography 52

Appendix 61

List of Figures

Title		Page number
Figure 2.1:	Role of cationic peptides in innate immunity	8
Figure 2.2:	Activities of antimicrobial peptides	9
Figure 2.3:	Peptide interaction with cytoplasmic membrane of bacteria and four modes of action proposed	10
Figure 3.1:	General flow diagram of assays conducted for the antibacterial activity screening of cationic peptides	21
Figure 3.2:	General flow diagram of assays conducted for the antibacterial activity screening of Schiff base complexes	25
Figure 4.1:	Relative growth rate of bacterial strains tested tested in different concentrations of peptide α-RetroMAD1 (RM) at 750.0 ppm, 375.0 ppm, 187.5 ppm, 93.8 ppm and 46.9 ppm	31
Figure 4.2:	Bacterial growth inhibition of peptide α-RetroMAD1 (RM) tested at 750.0 ppm in cell inactivation assay against different strains of *Methicillin-resistant Staphylococus aureus* (MRSA), *Klebsiella pneumoniae* (KB), *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AC)	33
Figure 4.3:	Time-kill curves for *K. pneumoniae* (strain 88) against metal complex LMA Cd-N_3	39
Figure 4.4:	Time-kill curves for MRSA (strain 080925) against metal complex LMA Cd-N_3	40
Figure 4.5:	Time-kill curves for *A. baumannii* (strain 08121) against metal complex LMA Cd-N_3	41
Figure 5.1:	Chemical structure of Schiff base cadmium complex (LMA Cd-N_3)	41

List of Tables

Title		Page number
Table 3.1:	List of bacterial strains with antibiogram profiles	15
Table 3.2:	Recipe for LB agar media	16
Table 3.3:	List of synthetic cationic peptides and the concentration (ppm) of stock solution provided	18
Table 3.4:	List of synthetic Schiff base complexes tested	19
Table 4.1:	Antibacterial activities of the peptide α-RetroMAD1 (RM) tested at 750.0 ppm in cell inactivation assay	32
Table 4.2:	Growth induction effects of the α-RetroMAD1 (RM) tested at different concentrations in cell inactivation assay for MRSA08071	34
Table 4.3:	Antibacterial activities of peptides (RG, HP, CT, BC, AB and RM) tested at 25.0 ppm in cell inactivation assay	35
Table 4.4:	Zones of inhibition for Schiff base complexes	36
Table 4.5:	Minimum inhibitory concentration (MIC) of antibacterial compounds tested in broth micro-dilution assay	37

CHAPTER 1

INTRODUCTION

Antibiotics are compounds that are able to inhibit the growth of bacteria or to kill the bacteria. The halting or inhibition of bacterial growth by antibiotics is referred to as bacteriostatic while the antibiotics that kill bacteria are referred to as bactericidal (Hancock, 2005). Therefore, in the case of infectious diseases, the administration of antibiotics to patient will either kill the microorganisms responsible for the disease or weaken the microorganisms to allow the immune response system of the human body itself to eliminate them. Thus, without the advent of antibiotics, the treatment of infectious diseases would not be possible. In nature, bacteria can acquire or develop resistance to antimicrobial compounds to enhance their own survivability. However, through the utilization of antibiotics in the treatment of infectious diseases and also as additives in animal feed, pathogenic bacteria especially the medically important *Staphylococcus* spp., *Klebsiella* spp., *Acinetobacter* spp. and *Pseudomonas* spp. have adapted and developed resistance to multiple or most of the currently available antibiotics. These made clinical treatments of infectious diseases caused by these nosocomial bacteria difficult. Due to the slow development and lack of new classes of antibiotics discovered (Bax *et al*., 2000), novel compounds present an interesting opportunity to be studied for their antibacterial property against nosocomial pathogens such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Therefore, novel compounds that possess antimicrobial properties may provide an alternative to antibiotics in order to overcome challenges faced in treatment of infectious diseases. Among the compounds of interest were the synthetic compounds of cationic peptides (Hancock, 2005; Li *et al*., 2007; Hartmann *et*

al., 2010; Ross & Vederas 2010) and metal complexes (Aiyelabola *et al*., 2012; Gwaram *et al*., 2012; Kumar *et al*., 2009; Nishat *et al*., 2011; Vinuelas-Zahinos *et al*., 2011) which showed considerable potential in various applications; including anticancer, antiviral, antifungal and antibacterial properties. The study on the antibacterial effectiveness of the synthetic cationic peptides and metallic-ion bound Schiff base complexes would contribute to the crisis faced in the rapid emergence of antibiotic-resistant nosocomial pathogens in the medical sector.

1.1 OBJECTIVES

The objectives of this study were:

(1) To determine the antibacterial properties of selected synthetic cationic peptides toward methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Klebsiella pneumoniae, Acinetobacter baumanii* and *Pseudomonas aeruginosa*.

(2) To determine the antibacterial activities and kinetics of selected synthetic metal-bound Schiff base complexes toward methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Klebsiella pneumoniae, Acinetobacter baumanii* and *Pseudomonas aeruginosa*.

CHAPTER 2

LITERATURE REVIEW

2.1 Healthcare-associated infection (HAI)

Human nosocomial infections or better known as healthcare-associated infections (HAI) can be described as infections obtained from the hospitals. Several pathogenic microorganisms are the cause for these infections. In clinical settings, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are among the medically important bacteria that not only causes the nosocomial infections, but they are also highly resistant to multiple antibiotics commonly used in clinical treatments (Navon-Venezia *et al*., 2005; Casey *et al*., 2007; Sikarwar *et al*., 2011; Su *et al*., 2012).

2.1.1 *Staphylococcus aureus*

Staphylococcus aureus are Gram-positive bacteria that can be observed as spherical bacteria and appeared in grape-like cluster when viewed under microscopic view (Defres *et al*., 2009). *S. aureus* are coagulase-positive, which differentiate them from the other *Staphylococcus* spp. such as *S. epididermis*, which are coagulase-negative (Casey *et al*., 2007). *S. aureus* are among the medically important pathogenic bacteria responsible for nosocomial infections. Colonization of the bacteria is often found on the human skin and also in the nasal passageway. It is commonly associated with the skin and soft tissue infections (SSI), infections of bone and joint, endocarditis, bacteremia and also capable of producing life-threatening cytotoxins where the severity of toxicity ranges from food poisoning to serious toxic shock syndrome. Serious infections caused by *S. aureus* are treated with the glycopeptide antibiotics such as

vancomycin and teicoplanin. However, the methicillin-resistant *S. aureus* (MRSA) are known to be resistant to multiple antibiotics including the previously antibiotic of last resort for the treatment of *S. aureus* infections, vancomycin (Casey *et al*., 2007; Defres *et al*., 2009).

2.1.2 *Klebsiella pneumoniae*

The rod-shaped and Gram-negative bacteria, *Klebsiella* spp. are ubiquitous and can be found in the environment on surface water, sewage and soil. On human, it mainly colonizes the mucosal surfaces on the nasopharynx and intestinal tract. As opportunistic pathogen, *K. pneumoniae* are the medically important strain that are responsible for most of the nosocomial *Klebsiella* infections in humans. It is often associated with respiratory infections, pneumonia and urinary tract infection (UTI). Other diseases caused by the pathogen include bacteremia and septicemia (Podschun & Ullmann, 1998). Some of the multiple drug-resistant strains are capable of producing the extended-spectrum β-lactamase (ESBL) enzyme that renders all β-lactam antibiotics ineffective (Won *et al*., 2011)

2.1.3 *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

Both *Acinetobacter* spp. and *Pseudomonas* spp. are waterborne pathogens and are ubiquitous. The coccobacillus *Acinetobacter* spp. and the rod-shaped *Pseudomonas* spp. are opportunistic Gram-negative bacteria, often linked to respiratory infections and urinary tract infections. The species *A. baumannii* and *P. aeruginosa* are the medically important bacteria that are responsible for healthcare-associated infections particularly affecting patients that were admitted into the Intensive Care Units (ICUs) (Timurkaynak *et al*., 2006). They are often associated with ventilator-associated pneumonia, surgical site infections, meningitis and bacteremia. The spread of these bacteria is difficult to

control due to the ability of these bacteria to survive on most surfaces for prolonged period of time (Navon-Venezia *et al*., 2005). Traditionally, infections caused by *A. baumannii* are treated with the antibiotics imipenem and meropenem (Su *et al*., 2012). As for infections caused by the multiple drug-resistant strains, the lipopolypeptide polymyxin would be used as the antibiotic of choice for the treatment (Tomas *et al*., 2005). Both of these pathogenic bacteria are feared not only for their ability to grow and survive in unfavorable conditions, but also their highly intrinsic resistance to most of the available antibiotics (Zavascki *et al*., 2010).

2.2 Antibiotics

In nature, antibiotics are compounds that are usually produced by microorganisms such as soil bacteria and fungi as a defense mechanism to inhibit or to kill other unwanted microorganisms in their growing environment (Walsh, 2000). Antibiotics used in the medical sector are obtained either from natural sources or through synthetic production (Ross & Vederas, 2010). Some of the known antibiotics include cephalosporin, erythromycin, fluoroquinolones, kanamycin, penicillin, streptomycin, tetracyclin. (Li & Vederas, 2009; Walsh, 2000). Three known mechanisms of antibiotic activity against microorganisms are the disruption of bacterial cell wall, inhibition of protein biosynthesis and interference of bacterial DNA biosynthesis and repair (Walsh, 2000). However, antibiotic resistance trait can be acquired by bacteria through prolonged use of antibiotics. Pathogenic microorganisms that acquired resistance to the present antibiotics, such as the methicillin-resistant *S. aureus* (MRSA) (Chomvarin *et al*., 2004), vancomycin-resistant *Enterococci* (VRE) (Bax *et al*., 2000) and the extensive drug resistance (XDR) *A. baumannii* (Liang *et al*., 2011) posed serious threat to human health. Bacteria gain the resistance traits by synthesizing modified membrane pumps (efflux pumps) to flush out the antibiotics from

bacterial cell; the deactivation of antibiotics through the production of hydrolytic enzymes such as β-lactamase before it reaches its target antigen and to modify bacterial antigen in order to lower the binding affinity of antibiotics to target antigen (Nikaido, 1998; Walsh, 2000).

2.3 Peptides

Due to the increased emergence of antibiotic-resistant pathogenic strains and the lack of long-lasting antibiotics (Frecer *et al*., 2004), an alternative compound to known antibiotics that have novel antimicrobial properties led to the studies on short polymer of amino acids such as the peptides (Nedjar-Arroume *et al*., 2008). Two early prominent discoveries of antimicrobial peptides produced by animals and insects were from amphibians and bees. The amphibian *Bombina variegata* secretes certain substances on the surfaces of its skin. The substances were then found to be a biological peptide, bombinins which had antimicrobial and haemolytic properties (Csordas & Michl, 1970). In 1972, Habermann isolated a peptide from the venom of bees, also showing antimicrobial and haemolytic properties. Other than the skin of amphibians and secretion of insects, another cationic antimicrobial peptide is the defensins which can be found in the neutrophils of mammals and it could reach to a high concentration of 10.0 mg/ml (Hancock, 2001). Gramicidin, polymyxin and colistin were some of the examples of cationic peptides that had been used as peptide antibiotics in topical applications for diseases (Hancock, 1997). Several other peptides originated from various sources with potential antimicrobial properties have been reviewed; insect cecropins (Steiner *et al*., 1981) and bee venom melittin (Habermann, 1972), amphibians magainins (Zasloff, 1987) and bombinins (Barra & Simmaco, 1995), pig protegrin I (Storici, 1993), fruit fly drosomycin (Dimarcq, 1998), plant defensins (Garcia-Olmedo,

1998), octapeptin (Rosenthal *et al*., 1977) and lantibiotics (Chatterjee *et al*., 2005; Ross & Vederas, 2010).

2.3.1 Structure of peptides

Peptide antibiotics were classified into two classes, which were the non-ribosomally synthesized peptides and the ribosomally synthesized peptides. Non-ribosomal peptides were significantly modified and were synthesized by microorganisms, mainly by bacteria and fungi. However, ribosomally synthesized peptides were produced by all organisms as the peptide plays an important role in their defenses against environmental hazards (Figure 2.1) (Hancock & Chapple, 1999). Cationic peptides are made up of 10 to 40 amino acids and are amphipathic molecules, where it possessed both hydrophobic and hydrophilic sides in its structure. The peptides have net positive charges of +2, +4, +5 or +6. The charges were contributed by the presence of amino acid residues in the hydrophilic side of the structure (Hancock, 1997).

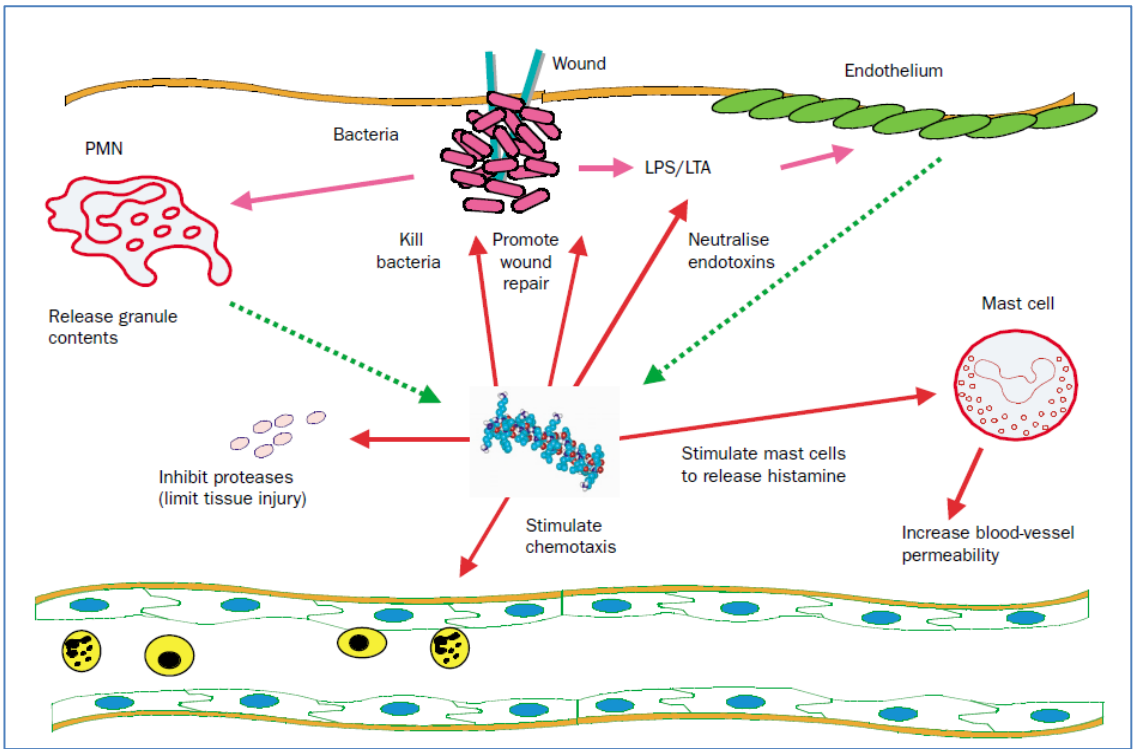


Figure 2.1 : Role of cationic peptides in innate immunity. Dotted arrows represent events that lead to increased production of extracellular cationic peptides. Solid red lines represent the actions of peptide, while solid pink lines represent the events that unfold due to invading bacteria. PMN=polymorphonuclear leococytes; LPS=lipopolysaccharide; LTA=lipoteichoic acid (Hancock, 2001)

2.3.2 Modes of action

Compared to the traditional antibiotics, some of the peptides have various mechanisms of action to inhibit or to kill the pathogens, which made it difficult for the pathogens to develop resistance traits to the peptide. The previously reported peptide, lantibiotics, possesses potent antimicrobial properties by having nano-molar minimum inhibitory concentration (MIC) activity (Chatterjee *et al.*, 2005). Some of the antimicrobial agents such as antibiotics and antifungal agents have specific or narrow-spectrum of activities whereas a number of antimicrobial peptides were reported to have broad range of antimicrobial activities against Gram-positive bacteria and Gram-negative bacteria, antifungal and antiviral activity as described in Figure 2.2.

Activities of antimicrobial peptides	Example peptides
Broad-spectrum antibacterial	Protegrin, IB-367, MSI-78, indolicidin, CEMA, gramicidin S, polyphemusin,
Anti Gram-negative bacteria	Polymyxin B
Anti Gram-positive bacteria	HNP1
Synergy with conventional antibiotics	CEMA, magainin II, MSI-78, IB-367
Antifungal	Protegrin, CEMA, indolicidin, gramicidin S, polyphemusin
Synergy with conventional antifungals	Indolicidin
Antiviral (HIV, HSV)	Indolicidin, polyphemusin, protegrin
Anticancer	CEMA, indolicidin
Synergy with conventional anticancer agents	Indolicidin
Antiparasite	Magainin II, indolicidin
Antiendotoxin	CEMA, polyphemusin variants
Wound healing	Magainins, PR39
Chemotactic	HNP-1

Figure 2.2 : Activities of antimicrobial peptides (Hancock, 2001)

Multiple hypotheses were proposed for the mechanism of action for peptides, but agreements were made that the positively-charged cationic peptides would interact with the highly anionic outer membrane of Gram negative bacteria or the thick cell wall of Gram positive bacteria. As for the actual killing mechanism of the peptide, four modes of actions have been proposed based on the model of membrane interaction (Figure 2.3); lysis of cells, damages to the internal targets of cells, the formation of channels and the breakdown of the cytoplasmic membrane (Hancock, 2001).

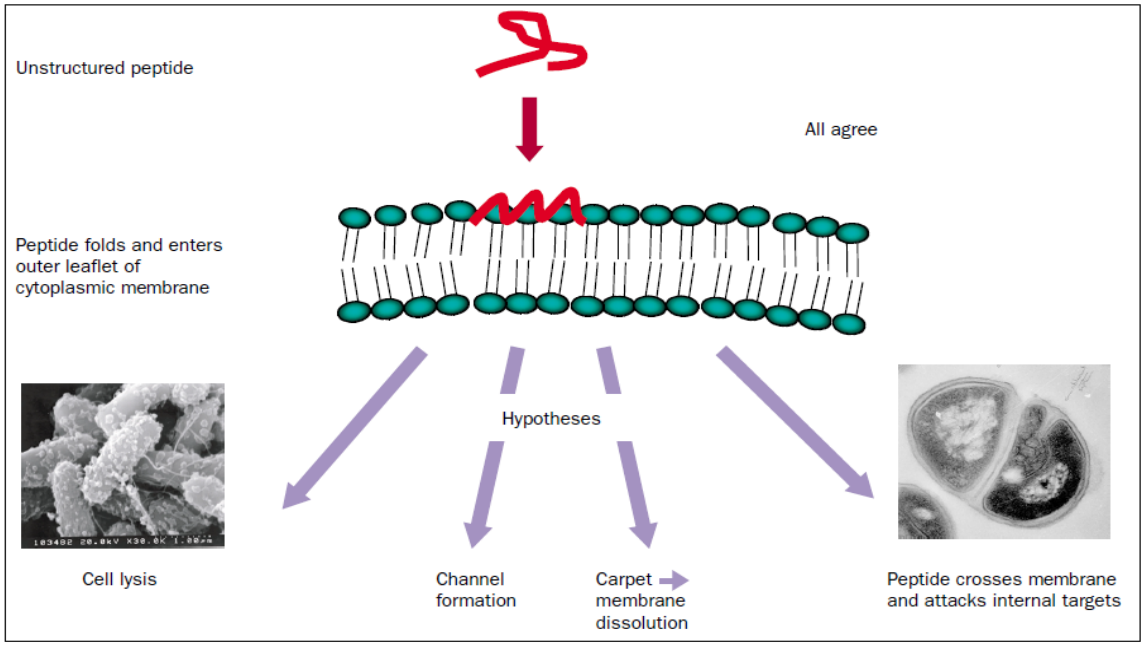


Figure 2.3 : Peptide interaction with cytoplasmic membrane of bacteria and four modes of action proposed (Hancock, 2001)

2.3.3 Resistance to peptides

The activity of antimicrobial cationic peptides was cited to be as effective against microorganisms of both the susceptible strains and the resistant strains. It was reported to be difficult for bacteria to develop resistance toward antimicrobial peptides. Multiple passages are required before the bacteria could develop increased resistance to the peptide. Though, *Burkholderia cepacia* and *Serratia* spp. were stated to be among the bacteria that are resistant to the antimicrobial effect of peptides (Hancock, 2001).

2.3.4 Selectivity of peptides

The phospholipids of the membrane in mammalian cells were comprised mainly of neutral zwitterionic phospholipids and cholesterol. However, the membranes of bacterial cells were comprised mainly of anionic lipids such as glycolipid lipopolysaccharide (LPS) and peptidoglycan. These made the cell membrane of bacteria negatively-charged. Due to the net positive charge of cationic peptides, the attraction between the opposite charges allowed the peptide to bind preferably with bacterial cells (Papo & Shai, 2003).

2.4 Metallic ion-bound complexes

Another alternative to antibiotics are synthetic compounds that contain metals in its structure. In these metallic ion-bound compounds, the ligands are first synthesized before the metallic ions are chemically bound to the ligand through various chemical reactions. These gave rise to metal complexes with antimicrobial activity as a result of the metallic ions (Yamada, 1999, Vinuelas-Zahinos *et al*., 2011, Sabik *et al*., 2012)

2.4.1 Effects of metal towards living organisms

Metal elements such as nickel, zinc, cobalt and copper are essential to living organisms at low concentration as they are involved in the production of co-factors which are vital components of some of the enzymes involved in their biological processes. In contrast to that, high concentrations of metals are toxic to most living organisms where the metals would replace or compete with other essential ions, blocking vital functional groups of biological molecules (Hassen *et al*., 1998) and affecting enzymatic activity by altering the conformation sites of the enzymes, protein denaturation and disrupting the enzyme-substrate complex (Vig *et al*., 2003). On the other hand, heavy metals such as cadmium, chromium, lead, mercury and silver have no beneficial effects to living organisms (Abou-Shanab *et al*., 2007).

2.4.2 Schiff base metal complexes

Due to the toxicity of metals toward living organisms at high concentration, much attention was placed into the antimicrobial activity of metal complexes. The condensation of primary amines with carbonyl compounds such as aldehydes and ketones resulted in the formation of Schiff bases (Sabik *et al*., 2012). Hugo Schiff was described as the first researcher to synthesize the Schiff bases and its metal complexes (Yamada, 1999). Classified by the International Union of Pure and Applied Chemistry

(IUPAC) (Moss *et al*., 1995), Schiff base were defined of a compound having the hydrocarbyl group on nitrogen atom in its structure; $R_2C=NR'$ ($R'\neq H$), where R_2 and R' denote alkyl or aryl (Kumar *et al*., 2009). Schiff bases are more often served as ligands that will be bound with other elements such as metallic ions in the synthesis of macro cyclic complexes. The presence of nitrogen (N) donor atoms in the structure of Schiff base resulted in its unique coordination behaviors with metal ions (Vinuelas-Zahinos *et al*., 2011). The chemical coordination geometry of macro cyclic complexes that are bound to metallic ions can be altered in order to change its chemical properties (Shakir *et al*., 2012). The Schiff base complexes have been the focus of researchers due to the simplicity in its synthesis and also the potential of the complex to be used as antimicrobial agents (Yamada, 1999). In the recent years, considerable number of studies was conducted on Schiff base and its metal complexes as potential antimicrobial agents, where much of the metal complexes were synthesized, characterized and tested against variety strains of bacteria and fungus (Valent *et al*., 2002; Noyce *et al*., 2006; Reiss *et al*., 2009; Sabik *et al*., 2012; Nishat *et al*., 2011; Gwaram *et al*., 2012; Gupta *et al*., 2012; Shakir *et al*., 2012; Sunitha *et al*., 2012).

2.4.3 Applications of other metallic ion-bound complexes

The study of antimicrobial metal complexes was not only limited to association with macro cyclic complexes such as Schiff bases as ligands, other novel compounds containing metals have also been studied. A natural constituent of plant, coumarin was used as a ligand bonded with silver (Ag). The silver-coumarin complex was found to be highly potent against clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA), with MIC_{80} (defined as the minimum concentration of compound required to inhibit bacterial growth to 80%) of 0.63 μM (Creaven *et al*., 2006). In 2008, Gudasi and colleagues also studied on the antibacterial activity of metal-coumarin complex. In their

studies, cadmium (Cd) and zinc (Zn) complexes were found to be more effective against the fungal strains tested, whereas nickel (Ni) and cobalt (Co) complexes have comparable antibacterial activity to the antibiotic norfloxacin used in the study as control. Kim and colleagues (2008) studied the application of silver (Ag) and copper (Cu) coating on activated carbon filters (ACFs) that were used for water filtering and purification and found that copper-plated ACFs have higher inhibitory effect on *S. aureus*. Apart from that, studies on nanomaterial exhibiting antimicrobial activity resulted in the derivative of silver, titanium and zinc metal oxide nanoparticles. Results from the study showed promising bactericidal effect of all the metal oxides synthesized, especially of the silver oxide, Ag_2O against *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Negi *et al*., 2012). The effectiveness of metals exerting antimicrobial activity was not limited only to the area of chemotherapy. In another application to study the possibility of creating novel antibacterial metals, various metals such as lead (Pb), nickel (Ni), copper (Cu), cobalt (Co), zinc (Zn), titanium (Ti) and silver (Ag) were studied for the suitability of the metals to be incorporated onto surface of steels or into the steels during its manufacturing process. As microorganisms grow on the surface of industrial metal components, the formation of bacterial biofilm would lead to the corrosion of the surface, causing material damage. Thus, antibacterial metals would prevent bacterial attachment and inhibiting its growth (Sreekumari *et al*., 2005; Yasuyuki *et al*., 2010).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Bacterial cultures

A total of sixteen bacterial strains were tested in the study. The nosocomial bacterial strains originated from clinical settings and were resistant to multiple antibiotics. The antibiogram profiles of the strains were tabulated in Table 3.1. The sixteen randomly selected strains comprised of four strains of *Acinetobacter baumannii* (AC) (AC08121, AC06127, AC07078, AC07095), four strains of *Klebsiella pneumoniae* (KB) (KB83, KB88, KB92, KB198), four strains of methicillin-resistant *Staphylococus aureus* (MRSA) (MRSA080925, MRSA080521, MRSA08061, MRSA08071) and four strains of *Pseudomonas aeruginosa* (PA) (PA30, PA4, PA102, PA104) (Table 3.1). All bacterial strains were obtained from the cultures collection of Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Kuala Lumpur, Malaysia and were used throughout the study. These pathogenic bacterial strains were used as the standard pathogenic culture for every multidrug-resistant antibacterial susceptibility tests in the laboratory. The strains were fully characterized; with their antibiogram profile included (Table 3.1).

Table 3.1 : List of bacterial strains tested with antibiogram profiles

Bacterial species	Laboratory code	Origin	Resistance to antibiotics
Acinetobacter baumannii (AC)	AC08121	Clinical	CIP, CFP, CRO, CXM, AMP, MEM, IMP
	AC06127		CIP, CFP, CRO, CAZ, CXM, AMP
	AC07078		CIP, CFP, CRO, CAZ, AMP, MEM, IMP
	AC07095		CIP, CFP, CRO, CAZ, AMP, MEM, IMP
Klebsiella pneumoniae (KB)	KB83	Clinical	AMP, PIP, ATM, STR, CFP, FEP, CHL
	KB88		AMP, PIP, ATM, KAN, SXT, CIP, TET
	KB92		AMP, PIP, ATM, KAN, STR, CRO, AMK
	KB198		AMP, PIP
Methicillin-resistant *Staphylococcus aureus* (MRSA)	MRSA080925	Clinical	ERY, GEN, CIP, NET, OXA, SXT
	MRSA080521		ERY, GEN, CIP, NET, TET, OXA, SXT
	MRSA08061		ERY, GEN, CIP, NET, TET, OXA, SXT
	MRSA08071		ERY, GEN, CIP, NET, TET, OXA, SXT
Pseudomonas aeruginosa (PA)	PA30	Clinical	TET, SXT
	PA4		TET, CHL, SXT
	PA102		SXT
	PA104		TET, CTX, CRO

CIP- ciprofloxacin, CFP- cefoperazine, CRO- ceftriaxone, CXM, cefuroxime, AMP- ampicillin, MEM- meropenem, IMP- imipenem, CAZ – ceftazidime, PIP- piperacillin, ATM- aztreonam, STR- streptomycin, FEP- cefepime, CHL- chloramphenicol, KAN- kanamycin, SXT- trimethoprim-sulfomethaxazole, TET- tetracycline, AMK- amikacin, ERY- erythromycin, GEN- gentamicin, NET- netilmicin, OXA- oxacillin, CTX- cefotaxime

3.1.2 Media

3.1.2.1 Luria Bertani (LB) agar media composition

The weighed amount of media compositions were dissolved in 100.0 ml distilled water. To ensure complete dissolution, the mixture was heated with frequent agitation and followed by boiling for 1 minute. The mixture was then autoclaved at 121^{o}C for 15 minutes and was cooled down before use.

Table 3.2 : Recipe for LB agar media

Ingredient(s)	Mass (g) and Volume (ml)
Tryptone	1.0
Agar powder	1.5
Yeast extracts	0.5
Sodium chloride (NaCl)	0.5
Distilled water	100.0 ml

3.1.2.2 Veal infusion broth media (Difco, New Jersey, USA)

25.0 g of the powder was suspended in 1 L of distilled water and mixed thoroughly. To ensure complete dissolution of the powder, the mixture was heated with frequent agitation and followed by boiling for 1 minute. The mixture was then autoclaved at 121^{o}C for 15 minutes. The mixture was allowed to cool down before use.

3.1.2.3 Mueller Hinton II agar (cation-adjusted) (MHII agar) (Oxoid, Hampshire, UK)

38.0 g of the powder was suspended in 1 L of distilled water and mixed thoroughly. To ensure complete dissolution of the powder, the mixture was heated with frequent agitation and followed by boiling for 1 minute. The mixture was then autoclaved at 121^{o}C for 15 minutes and was allowed to cool down before use.

3.1.2.4 Mueller Hinton II broth (cation-adjusted) (MHII broth) (BBL, Maryland, USA)

22.0 g of the powder was suspended in 1 L of distilled water and mixed thoroughly. To ensure complete dissolution of the powder, the mixture was heated with frequent agitation and followed by boiling for 1 minute. The mixture was then autoclaved at 121^{o}C for 15 minutes and was allowed to cool down before use.

3.1.3 Antibiotics

3.1.3.1 Polymyxin B sulfate disk (Oxoid, Hampshire, UK)

Commercial antibiotics disk with concentration of 300 units of polymyxin B sulfate were used as standard control for comparison towards Gram-negative bacteria in the disk diffusion test.

3.1.3.2 Polymyxin B sulfate powder (Sigma, Missouri, USA)

Polymyxin B sulfate was used as the standard antibiotic control for comparison towards Gram-negative bacteria in the broth dilution assay. The stock solution was prepared using sterile distilled water as solvent and the stock was kept at 4^{o}C in dark until use. The potency value of polymyxin B sulfate was calculated according to the formula outlined by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 guidelines (CLSI, 2006). The stock solution prepared had a concentration of 1,600.0 ppm with the potency value of 790.0 μg/mg. The stock solution was subjected to ten-fold (10X) serial dilution with sterile distilled water to the working concentration of 16.0 ppm prior to use.

3.1.3.3 Vancomycin disk (Oxoid, Hampshire, UK)

Commercial antibiotics disk containing 30.0 μg of vancomycin (Oxoid) were used as standard antibiotic control for comparison towards Gram-positive bacteria in the disk diffusion test.

3.1.3.4 Vancomycin powder (Sigma, Missouri, USA)

Vancomycin (Sigma) was used as the standard antibiotic control for comparison towards Gram-positive bacteria in the broth dilution assay. The stock solution was prepared by using sterile distilled water as solvent and the stock was kept at 4^{o}C in dark until use. The potency value of vancomycin was calculated according to

the formula outlined by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 guidelines (CLSI, 2006). Stock solution was prepared at 1,600.0 ppm from vancomycin powder with the potency value of 1117.0 μg/mg. The stock solution prepared had a concentration of 1,600.0 ppm with the potency value of 1117.0 μg/mg. The stock solution was subjected to ten-fold (10X) serial dilution with sterile distilled water to the working concentration of 16.0 ppm prior to use.

3.1.4 Synthetic cationic peptides

A total of six cationic peptides were synthetically synthesized and provided by the Biotechnology Company, BioValence Malaysia. The peptides were labeled as follows; α-RetroMAD1 (RM) (stock solution concentration of 1,500.0 ppm and 3,500.0 ppm); RG and HP (stock solution concentration of 100.0 ppm); CT (stock solution concentration of 50.0 ppm); BC and AB (stock solution concentration of 150.0 ppm) (Table 3.3). All peptides were provided in liquid form with sterile distilled water used as solvent. The stock solution concentration refers to the highest concentration provided. The working solutions of the peptides were prepared using sterile distilled water as diluent.

Table 3.3 : List of synthetic cationic peptides and the concentration (ppm) of stock solution provided

Cationic peptides	Stock concentration (ppm)
α-RetroMAD1 (RM)	1,500.0 and 3,500.0
RG	100.0
HP	100.0
CT	50.0
BC	150.0
AB	150.0

3.1.5 Synthetic Schiff base complexes

Twenty-nine Schiff base complexes, which were labeled from S1 to S29 (stock solution concentration of 10,000.0 ppm) (Table 3.4) were synthesized and provided in crystalline solid with 99 % purity by Professor Dr. Hapipah from the Department of Chemistry, University of Malaya. The stock solution concentration refers to the highest concentration provided.

Table 3.4 : Lists of synthetic Schiff base complexes tested

Label code	Complexes
S_1	LMA Ni-N_3
S_2	LMA Co-N_3
S_3	LMA Zn-N_3
S_4	LMA Cd-N_3
S_5	2,6-DAP GH
S_6	2-AP GH
S_7	CL-AP GH
S_8	GH
S_9	Br-GH
S_{10}	CH_3-O GH
S_{11}	Ind-BZH
S_{12}	Br-NiC
S_{13}	CL-NiC
S_{14}	Ind-NiC
S_{15}	CL-BZH
S_{16}	Br-BZH
S_{17}	LHA $CuCl_2$
S_{18}	LHA $ZnCl_2$
S_{19}	LHA
S_{20}	LH-BZ
S_{21}	LHA $NiCl_2$
S_{22}	LNA $CuBr_2$
S_{23}	LNA Cu-SCN
S_{24}	LNA $ZnCl_2$
S_{25}	LNA ZnSCN
S_{26}	LMA $ZnBr_2$
S_{27}	LMA MnSCN
S_{28}	LMA ZnSCN
S_{29}	LMA CuSCN

3.2 Methods

3.2.1 Bacterial cell culture preparation

Bacterial cell cultures used in this work were kept and maintained as working stab culture and glycerol frozen culture. For short-term storage, the bacterial cultures were maintained as stab culture by stabbing the bacteria in LB agar tube before the tubes were allowed to grow overnight at 37^{o}C. The stab cultures were stored at room temperature for a storage time of no longer than one month. For long term storage of culture, overnight bacterial cultures in veal infusion broth were mixed with 10 % glycerol, vortexed and stored at -80^{o}C.

The test bacteria kept as stab cultures were inoculated onto LB agar media and were allowed to grow overnight at 37^{o}C before being used in assays. Bacterial inoculum was prepared according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 guidelines (CLSI, 2006). Bacterial suspensions density were standardized to match the 0.5 McFarland turbidity standards (approximately 10^{8} CFU/ml) using turbidity meter (Dade Behring, California, USA).

3.2.2 Antibacterial activity screening for cationic peptides

The antibacterial activity of the cationic peptides against selected nosocomial pathogens was examined using the Kirby-Bauer disk diffusion antibacterial susceptibility test, broth micro-dilution assay and cell inactivation assay (Figure 3.1).

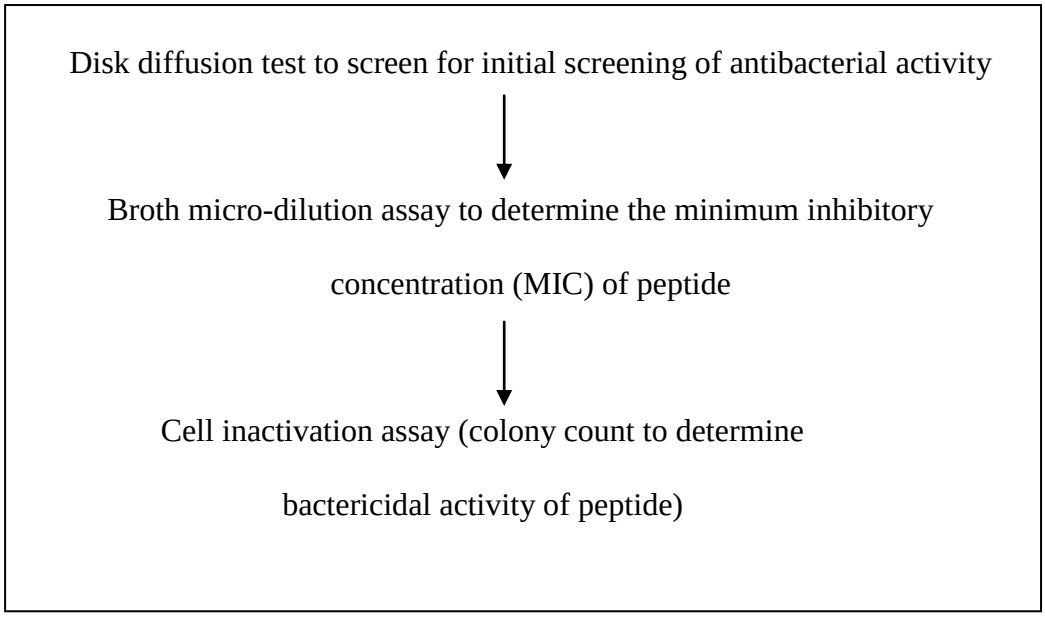


Figure 3.1 : General flow diagram of assays conducted for the antibacterial activity screening of cationic peptides

3.2.2.1 Peptide preparation

The peptides used in both the broth micro-dilution assay and cell inactivation assay were diluted using sterile distilled water as the diluent. Due to the untested antibacterial property of the compounds, the objective of the testing was to test the compounds at the highest concentration achievable. The peptide α-RetroMAD1 (RM) was used at its highest concentration from stock solution of 1,500.0 ppm and 3,500.0 ppm without further dilution in the disk diffusion test. Since all peptides were provided in liquid form, their concentrations will be diluted when mixed with bacterial suspension in the assays. Hence, a two-fold dilution was chosen as it was suitable for the assays, in terms of concentration calculation and simpler methodology. In the broth micro-dilution assay, the peptide RM was diluted two-fold from stock solution of 1,500.0 ppm to the working concentration of 750.0 ppm. In Part I of the cell inactivation assay, the peptide RM was diluted two-fold from stock solution of 1,500.0 ppm to the working concentration of 750.0 ppm. In Part II of the assay, the peptide RM was serially diluted two-fold from stock concentration of 1,500.0 ppm to 750.0 ppm, 375.0 ppm, 187.5 ppm, 93.8 ppm and 46.9 ppm. In Part III of the cell inactivation assay of peptides RM, RG, HP, CT, BC and AB, the lowest concentration of peptide provided was CT

with 50.0 ppm. Accordingly, all of the peptide stock solutions with different concentrations were diluted and standardized to working concentration of 25.0 ppm.

3.2.2.2 Kirby-Bauer disk diffusion antibacterial susceptibility test

Kirby-Bauer disk diffusion susceptibility tests were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M2-A9 guidelines (CLSI, 2006). Randomly selected cultures of AC (08121 and 06127), KB (88 and 198), MRSA (08071 and 08061) and PA (30 and 4) from stock were inoculated onto LB agar media and incubated for 16 hours to 18 hours at 37^{o}C. Using sterile swab, colonies grown on the plate were picked-up and inoculated into 0.85% NaCl (w/v) saline solution. Cell density was standardized to 0.5 McFarland turbidity standards (approximately 10^{8} CFU/ml) using turbidity meter. The standardized cell suspensions were then swabbed onto MHII agar to obtain a bacterial lawn of inoculums. Ten microliters of the α-RetroMAD1 (RM) peptide (1,500.0 ppm and 3,500.0 ppm) were transferred onto sterile paper disks using micropipette. The disks were placed firmly onto the inoculated agar surface using sterile forceps. A negative control was included in each plate by placing a paper disk with sterile distilled water. Antibiotic disk of polymyxin B (300.0 unit) and vancomycin (30.0 μg) were included as positive control in each plate. The inhibition zones around each disk were measured after 18 hours of incubation at 37^{o}C.

3.2.2.3 Broth micro-dilution assay

The broth micro-dilution assays were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 guidelines (CLSI, 2006), to determine the minimum inhibitory concentration (MIC) of the antibacterial compounds. Selected cultures of AC08121, KB88, MRSA08071 and PA30

were grown overnight on LB agar at 37^{o}C. The bacterial inoculums were prepared by suspending the fresh bacterial colonies on LB agar into 1ml of 2X MHII broth and the concentration was adjusted to 0.5 McFarland turbidity standards. Fifty microlitre of the standardized bacterial suspension were then transferred into each respective well in a 96-well microtitre plate. After that, 50.0 µl of peptide RM (1,500.0 ppm) was transferred into the first well (750.0 ppm) and then a two-fold dilution was performed for subsequent wells in the row. The content of each wells were mixed thoroughly using micropipette (the highest peptide concentration was 750.0 ppm, followed by 375.0 ppm, 187.5 ppm, 93.8 ppm and 46.9 ppm). Similar procedure was also performed using Durham glass tubes instead of 96-wells microtitre plates. The wells without test compound (with bacterial inoculants) and wells without bacterial suspensions (with test compound) were used as positive growth control and broth sterility control, respectively. The commercial antibiotics powder of polymyxin B sulfate and vancomycin were prepared beforehand and included as positive controls. The plate was incubated at 37^{o}C for 18 hours. After incubation, the turbidity of each well was visually compared to the negative control well to determine the growth end points. The MIC is the lowest concentration of the compound that completely inhibits the growth of organism in wells. After visual observation, the microtitre plate was left at room temperature for 30 minutes to equilibrate to room temperature (25^{o}C). The cell suspension in the wells was thoroughly mixed using micropipette before measuring the optical density of cell suspension using microplate reader at the wavelength of 540.0 nm.

3.2.2.4 Cell inactivation assay

The cell inactivation assay was used only to evaluate the antibacterial activity of peptide RM. This analysis was performed in three different approaches. In the first

approach, four strains from each bacterial species in test were selected randomly for the assay. The bacterial strains selected were: MRSA (080925, 080521, 08061 and 08071); KB (83, 88, 92 and 198); AC (08121, 06127, 07078 and 07095); and PA (30, 4, 102 and 104). The bacterial inoculants were prepared by suspending an approximately 10^8 of cells (0.5 McFarland turbidity standards) from freshly grown colonies on LB agar plate in 1 ml of 2X MHII broth. Then, 50.0 µl of the inoculants were transferred into microcentrifuge tubes with an equal volume (50.0 µl) of 1500.0 ppm of peptide RM. The final concentration of peptide RM in the test was 750.0 ppm. The negative control was prepared by substituting peptide RM in the microcentrifuge tube with sterile saline solution; while tubes containing no bacterial inoculant act as broth sterility control. The antibiotics polymyxin B sulfate and vancomycin were prepared and included as positive controls. The tubes were vortexed and incubated at 37^{o}C for 18 hours. After incubation, the number of cells in each tube was enumerated by plating 100.0 µl on LB agar and incubated at 37^{o}C for 24 hours. The colony-forming units (CFU) were counted after incubation. The number of bacterial cells in each tube with 750.0 ppm of peptide RM was compared to the number of cells grown in MHII broth without peptide RM (negative control).

In the second approach, MRSA strain which shown higher sensitivity to peptide RM in the first approach was tested against a series of two-fold diluted peptide RM (ranged from 46.9 ppm to 750.0 ppm). MRSA08071 was randomly selected to be used in this test. In brief, 25.0 µl of bacterial inoculants in 2X MHII broth were added to a series of durham glass tubes containing 25.0 µl of peptide RM at different concentration. In each analysis, positive controls, negative control and broth sterility control prepared as described in the previous section were included in the test. All tubes

were incubated overnight at 37^{o}C and the bacterial cells were enumerated by plating on LB agar.

In the final approach, all of the six peptides (RM, RG, HP, CT, BC and AB) were tested against bacterial strains of MRSA080925, KB88, AC08121 and PA30. The assay was conducted in Durham glass tubes following the procedure described in the first approach. The tested concentration of all of the 6 peptides was 25.0 ppm.

3.2.3 Antibacterial activity screening for Schiff base complexes

The antibacterial activity of the Schiff base complexes was examined using the Kirby-Bauer disk diffusion test, broth micro-dilution assay and time-kill assay (Figure 3.2).

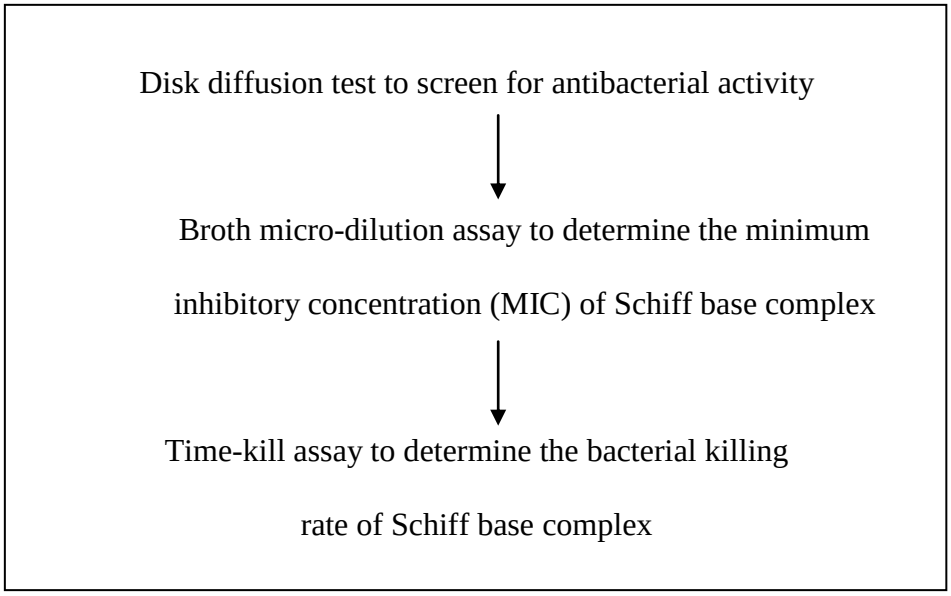


Figure 3.2 : General flow diagram of assays conducted for the antibacterial activity screening of Schiff base complexes

3.2.3.1 Schiff base complexes preparation

The entire Schiff complexes used in the disk diffusion test were dissolved in dimethyl sulfoxide (DMSO) (Seume Pharmacy, Leipzig, Germany). The Schiff base

complexes were tested at its highest concentration from stock solution of 10,000.0 ppm without further dilution in the disk diffusion test. Sterile distilled water was used as the solvent in the broth micro-dilution assay and time-kill assay. In the broth micro-dilution assay, new crystalline solids of Schiff base LMA Cd-N_3 were provided and were dissolved in sterile distilled water to 5,000.0 ppm working solution. The prepared working solution was then serially diluted two-fold to 2,500.0 ppm, 1,250.0 ppm, 625.0 ppm, 312.5 ppm, 156.3 ppm, 78.1 ppm, 39.1 ppm and 19.5 ppm. In time-kill assay, the same working solution of LMA Cd-N_3 Schiff base complexes with concentration of 5,000.0 ppm was used. The final concentration of the Schiff base complex in the experimental tube containing the mixture of the complex, broth, solvent and bacterial inoculum was prepared by diluting the working solution to the respective bacterial MICs at 1X, 2X and 4X higher MICs against the complex.

3.2.3.2 Kirby-Bauer disk diffusion antibacterial susceptibility test

Kirby-Bauer disk diffusion susceptibility tests were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M2-A9 guidelines (CLSI, 2006). Selected cultures of MRSA (080925 and 08071), KB (88 and 198), AC (06127 and 08121) and PA (30 and 4) were inoculated onto LB agar and then incubated for 16 hours to 18 hours at 37^{o}C. Using sterile swab, colonies were collected into screw-capped test tubes containing saline solution. Cell density was standardized to match those of 0.5 McFarland turbidity standards using turbidity meter. The standardized cell suspensions were then collected using swab and streaked onto MHII agar to obtain a bacterial lawn of inoculums. Twenty-four microliters of respective Schiff base complexes were transferred onto respective sterile paper disks using micropipette. The disks were placed onto agar surfaces of respective bacterial lawn of inoculums using sterile forceps. The concentration of complexes tested was 10,000.0

ppm per disk. Dimethyl Sulfoxide (DMSO) was used as solvent and was transferred onto disk as negative control. The solvent used in the preparation of the compounds was transferred onto disks as negative control. The commercial antibiotics disk of polymyxin B sulfate (300.0 units) and vancomycin (30.0 μg) were included as positive controls. Inhibition zones were observed after 18 hours of incubation of the plates at 37^{o}C.

3.2.3.3 Broth micro-dilution assay

The broth micro-dilution assays were performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 guidelines (CLSI, 2006), to determine the MIC of the complexes. Selected cultures of AC08121, KB88 and MRSA080925 were inoculated onto LB agar and were then incubated for 16 to 18 hours at 37^{o}C. In a 96-wells microtitre plate, 100.0 μl of the Schiff base complex LMA Cd-N_3 from the prepared working solution with concentration of 5,000.0 ppm was transferred into the first well before being serially diluted along the wells by two-fold using sterile distilled water. Using sterile swab, colonies were collected into screw-capped test tubes containing 1ml of 2X (two-fold greater concentration of media) MHII broth. Turbidity meter was used to standardized cell density to match the 0.5 McFarland turbidity standards. Fifty microliters of the standardized cell suspensions were transferred into respective wells. Contents of wells were mixed thoroughly using micropipette (highest peptide concentration after previous serial dilutions with sterile distilled water and cell suspension was 2,500.0 ppm, 1,250.0 ppm, 625.0 ppm, 312.5 ppm, 156.3 ppm, 78.1 ppm, 39.1 ppm and 19.5 ppm). Wells containing bacterial suspensions and wells without bacterial suspensions and compound were used as positive growth and broth sterility control, respectively. The commercial antibiotics powder of polymyxin B sulfate and vancomycin were prepared beforehand and included

as positive controls. The plate was incubated at 37^{o}C for 18 hours. After incubation, the turbidity of wells containing the compound was visually compared to the turbidity in wells without compound to determine the growth end points. The MIC is the lowest concentration of the compound that completely inhibits the growth of organism in wells.

3.2.3.4 Time-kill assay

Time-kill assay was performed according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) M26-A guidelines (CLSI 1999). The compound tested was the Schiff base metal complex; LMA Cd-N_3 against selected susceptible strains of MRSA080925, KB88 and AC08121. Cultures from stock were inoculated onto LB agar media to obtain pure culture. Plates were then incubated for 16 hours to 18 hours at 37^{o}C. Using a sterile swab, single colonies were picked into tube of fresh MHII broth before being incubated for 18 hours at 37^{o}C. Fresh 4.8 ml MHII broth was inoculated with 0.2 ml of overnight culture at the ratio of 1 to 25. The tube was incubated for 2 hours in rotary incubator at 37^{o}C to reach the exponential (log) growth phase. Turbidity meter was then used to standardize the cell suspension in MHII broth to 0.5 McFarland turbidity standards. The standardized cell suspension was added to 2X MHII broth containing the Schiff base complex at one (1X), two (2X) and four times (4X) MICs of each complex to achieve an initial cell inoculum of 5 x 10^5 CFU/ml (low inoculum). The assay was conducted in sterile polystyrene tubes. The antibiotics polymyxin B sulfate and vancomycin were prepared and tested at concentration of half (0.5X) MICs, as positive controls. Cell suspensions without compounds were included as growth control for each respective bacterial strain. The cell suspensions were incubated in rotary incubator at 37^{o}C. During incubation, aliquots of cell suspension were removed at time intervals of 0, 2, 4, 8 and 12 hours and a series of dilutions were made. The diluted cell suspension were plated onto LB agar media and incubated at

37^{o}C for 24 hours. The colony-forming units (CFU) were counted after incubation. Bacterial killing kinetic curves were plotted as the value of $\log_{10}$ CFU/ml against time of incubation (hours).

3.3 Data analysis

Bacterial cell growth populations were converted to the value of $\log_{10}$ CFU/ml. Mean of two replicates were reported and data analysis with standard deviation for time-kill kinetic was conducted using the statistical software, IBM SPSS Statistics Version 20.0.0.

CHAPTER 4

RESULTS

4.1 Antibacterial activity of cationic peptides

4.1.1 Kirby-Bauer disk diffusion antibacterial susceptibility test

From the disk diffusion susceptibility test, the clear zones around paper disks can be measured to indicate inhibition of growth by the antibacterial compounds tested. However, in the initial screening assay, no inhibition zones were observed in all sixteen strains of bacteria after incubation overnight with 1,500.0 ppm and 3,500.0 ppm of the cationic peptide α-RetroMAD1 (RM) (results were not shown).

4.1.2 Minimum inhibitory concentration (MIC) determination

In the broth micro-dilution assay, the treatments of bacterial suspensions with the peptide RM in the 96-wells microtitre plate showed the presence of cell pellets at the bottom of all wells after overnight incubation; except for the broth sterility control. No growth inhibition was observed in all bacterial strains tested. From the optical density measurements, the peptide does not show any signs of inhibition against all of the tested bacteria as compared to the positive growth control (without added peptide) (results were not shown). However, we have observed higher MRSA cell density in the tubes with peptide concentration of 46.9 ppm, 93.8 ppm and 187.5 ppm relative to the positive growth control (Figure 4.1).

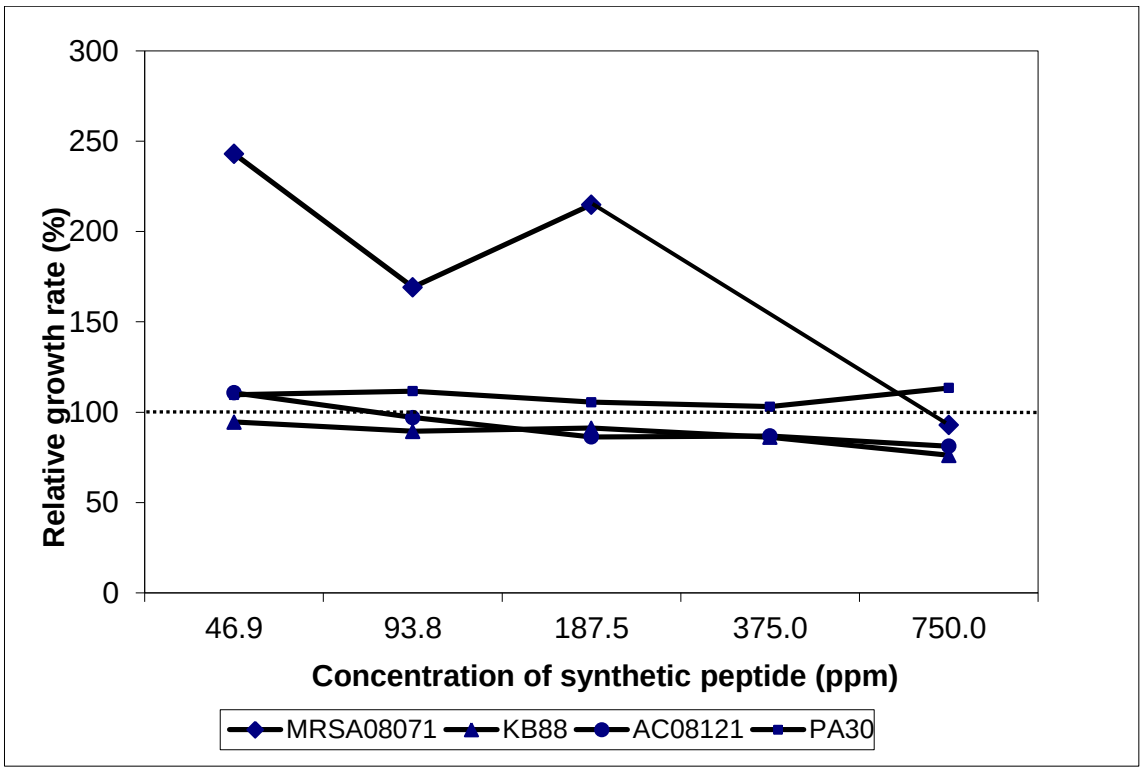


Figure 4.1 : Relative growth rate of bacterial strains tested in different concentrations of peptide α-RetroMAD1 (RM) at 750.0 ppm, 375.0 ppm, 187.5 ppm, 93.8 ppm and 46.9 ppm (The percentages of relative growth rate were measured with microplate reader at wavelength of 540 nm and calculated by taking into account of the optical density readings of cell suspensions containing peptide against those without peptide). Values above dotted lines represents increased growth rate and vice versa for values below the dotted lines for respective bacterial strains

4.1.3 Cell inactivation assay

After initial screenings with the peptide RM, no inhibition zones could be obtained from disk diffusion test and no visible changes observed in MIC determination in broth micro-dilution assay. Therefore, cell inactivation assay in liquid media was conducted with selected bacterial strains of MRSA (080925, 080521, 08061 and 08071), KB (83, 88, 92 and 198), AC (08121, 06127, 07078 and 07095) and PA (30, 4, 102 and 104) against peptide RM. In the first approach (Table 4.1 and Figure 4.2), results showed that the colony counts (CFU/ml) in the tubes with 750.0 ppm of peptide RM were lower than the tubes without peptide (positive growth control). The reduction in CFU count showed that the compound exert very little antibacterial activities towards the bacterial strains tested.

Table 4.1 : Antibacterial activities of the peptide α-RetroMAD1 (RM) tested at 750.0 ppm in cell inactivation assay

	[a] Experimental tube (with peptide) [CFU/ml]	[b] Control (without peptide) [CFU/ml]	[c] Growth reduction [CFU/ml]	[d] Experimental tube (with peptide) [log CFU/ml]	[e] Control (without peptide) [log CFU/ml]	[f] Log reduction [log CFU/ml]	**[g] Log growth inhibition (%) [log CFU/ml]**
MRSA080925	$5.5x10^7$	$1.6x10^9$	$1.5x10^9$	7.7	9.2	1.5	**15.9**
MRSA080521	$1.0x10^8$	$9.3x10^8$	$8.3x10^8$	8.0	9.0	1.0	**10.8**
MRSA08061	$6.5x10^7$	$2.9x10^9$	$2.9x10^9$	7.8	9.5	1.7	**17.5**
MRSA08071	$3.0x10^7$	$2.4x10^9$	$2.4x10^9$	7.5	9.4	1.9	**20.4**
MRSA08071 (Vancomycin)	$5.0x10^7$	$8.8x10^8$	$8.3x10^8$	7.7	8.9	1.2	**13.9**
KB83	$3.6x10^8$	$2.5x10^9$	$2.1x10^9$	8.6	9.4	0.8	**8.9**
KB88	$7.5x10^8$	$2.8x10^9$	$2.1x10^9$	8.9	9.4	0.6	**6.1**
KB92	$2.4x10^8$	$6.3x10^8$	$3.9x10^8$	8.4	8.8	0.4	**4.8**
KB198	$3.5x10^8$	$2.6x10^9$	$2.2x10^9$	8.5	9.4	0.9	**9.3**
KB83 (Polymyxin B)	$7.2x10^8$	$6.2x10^9$	$5.4x10^9$	8.9	9.8	0.9	**9.5**
AC08121	$4.4x10^8$	$1.7x10^9$	$1.3x10^9$	8.6	9.2	0.6	**6.4**
AC06127	$3.3x10^8$	$1.2x10^9$	$8.9x10^8$	8.5	9.1	0.6	**6.2**
AC07078	$1.8x10^8$	$8.7x10^9$	$6.9x10^9$	8.2	8.9	0.7	**7.8**
AC07095	$2.9x10^8$	$1.3x10^9$	$1.1x10^9$	8.5	9.1	0.7	**7.3**
AC06127 (Polymyxin B)	$1.1x10^9$	$2.2x10^9$	$1.1x10^9$	9.0	9.3	0.3	**3.3**
PA30	$3.2x10^8$	$1.8x10^9$	$1.4x10^9$	8.5	9.2	0.7	**8.0**
PA4	$2.7x10^9$	$4.1x10^9$	$1.4x10^9$	9.4	9.6	0.2	**1.9**
PA102	$5.8x10^8$	$1.1x10^9$	$5.4x10^8$	8.8	9.0	0.3	**3.2**
PA104	$4.5x10^7$	$1.8x10^8$	$1.4x10^8$	7.7	8.3	0.6	**7.3**

MRSA denotes methicillin-resistant *Staphylococcus aureus*; KB denotes *Klebsiella pneumoniae*; PA denotes *Pseudomonas aeruginosa*; AC denotes *Acinetobacter baumannii*. Vancomycin and polymyxin B were included as antibiotic control at concentration of respective bacterial MIC; 4.0 ppm for MRSA and 2.0 ppm for KB and AC

[a] Average CFU readings per ml (cell suspension with peptide)

[b] Average CFU readings per ml (control, cell suspension without peptide)

[c] Difference between [b] control and [a] experimental tubes

[d] Log_{10} ([a] experimental tube)

[e] Log_{10} ([b] control)

[f] Difference between log_{10} readings between [e] control and [d] experimental tube

[g] ([f] Log reduction/ [e] control) x 100%

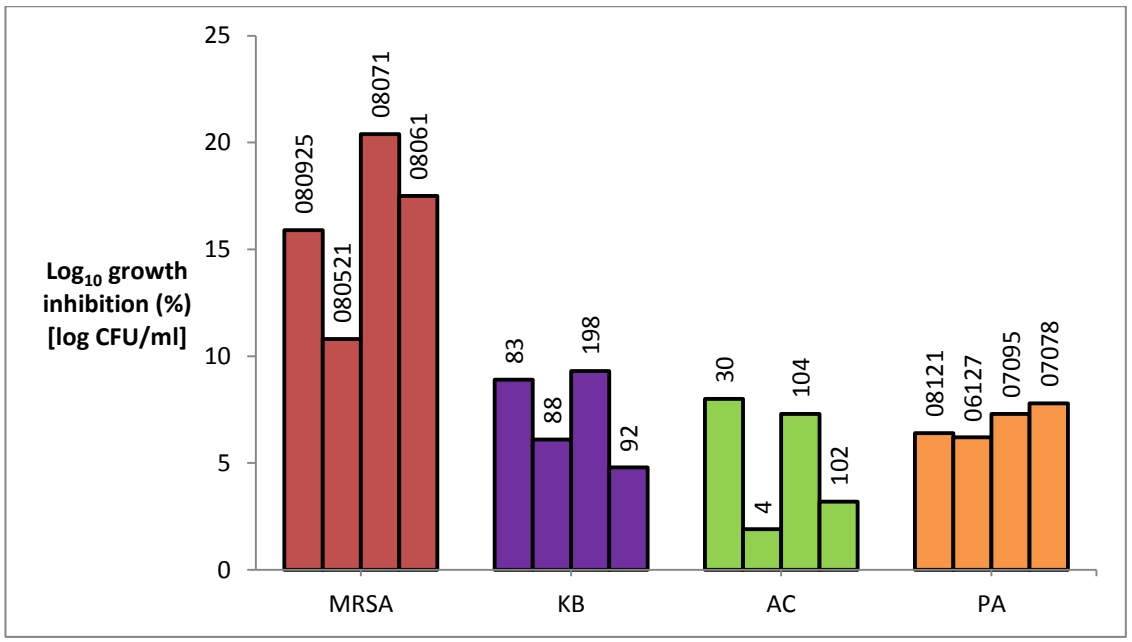


Figure 4.2 : Bacterial growth inhibition of peptide α-RetroMAD1 (RM) tested at 750.0 ppm in cell inactivation assay against different strains of *Methicillin-resistant Staphylococus aureus* (MRSA), *Klebsiella pneumoniae* (KB), *Pseudomonas aeruginosa* (PA) and *Acinetobacter baumannii* (AC)

Further analysis in the second approach revealed a surprising result that at the concentration of lower than 375.0 ppm of peptide RM; the peptide seems to support the growth of MRSA. The result showed an increase in the differences of bacterial density in the tube with peptide with the bacterial density in the tube without peptide from 0.04 to 0.28 $\log_{10}$ CFU/ml when the concentration of peptide RM dropped from 375.0 ppm to 46.9 ppm (Table 4.2). In the third approach of cell inactivation assay, the cationic peptides RG, HP, CT, BC, AB and RM at 25.0 ppm were tested against a set of selected bacterial strains. The reduction in CFU count showed that the remaining peptides also exert some antibacterial activities to a certain degree towards the bacterial strains tested. When compared among the rest of the peptides, strains KB88 and AC08121 were more susceptible to peptide CT; whereas peptide HP was shown to be more effective against strain PA30. Based on the results among all of the peptides tested, the antibacterial activity of peptide RG and RM were less effective against the tested bacterial strains, with low percentages of growth inhibition in the range of 0.3 to 13.3 % $\log_{10}$ CFU/ml.

As for peptide RM, it has poorest antibacterial activity among all of the other antibacterial peptides tested at low concentration of 25.0 ppm (Table 4.3).

Table 4.2 : Growth induction effects of the α-RetroMAD1 (RM) tested at different concentrations in cell inactivation assay for strain MRSA08071

Concentrations of peptide (ppm)	[a] With peptide [CFU/ml]	[b] Control (without peptide) [CFU/ml]	[c] Growth induction [CFU/ml]	[d] With peptide [log CFU/ml]	[e] Control (without peptide) [log CFU/ml]	[f] Log increment [log CFU/ml]	**[g] Log growth induction (%) [log CFU/ml]**
46.9	$8.25x10^8$		$3.95x10^8$	8.9		0.28	**3.3**
93.8	$6.95x10^8$		$2.65x10^8$	8.8		0.21	**2.4**
187.5	$7.45x10^8$	$4.30x10^8$	$3.15x10^8$	8.9	8.6	0.24	**2.8**
375.0	$4.70x10^8$		$4.00x10^7$	8.7		0.04	**0.4**
750.0	$7.85x10^7$		-	-		-	**-**

Growth inductions instead of growth reductions were shown as hyphen (-)
[a] Average CFU readings per ml (cell suspension with peptide)
[b] Average CFU readings per ml (control, cell suspension without peptide)
[c] Difference between [a] experimental tubes and [b] control
[d] Log_{10} ([a] experimental tube)
[e] Log_{10} ([b] control)
[f] Difference between log_{10} readings between [e] experimental tube and [d] control
[g] ([f] Log increment / [e] control) x 100%

Table 4.3 : Antibacterial activities of peptides (RG, HP, CT, BC, AB and RM) tested at 25.0 ppm in cell inactivation assay

Bacterial strain	Compounds	[a] With peptide (CFU/ml)	[b] Control (CFU/ml)	[c] Growth reduction (CFU/ml)	[d] With peptide log_{10} (CFU/ml)	[e] Control log_{10} (CFU/ml)	[f] Log_{10} reduction (CFU/ml)	[g] **Log_{10} growth inhibition (%) (CFU/ml)**
	RG	$6.15x10^8$	$3.90x10^8$	-	8.8	8.6	-	**-**
	HP	$8.30x10^8$	$3.90x10^8$	-	8.9	8.6	-	**-**
MRSA	CT	$9.05x10^8$	$8.05x10^8$	-	9.0	8.9	-	**-**
080925	BC	$1.49x10^9$	$2.70x10^8$	-	9.2	8.4	-	**-**
	AB	$2.85x10^8$	$2.70x10^8$	-	8.5	8.4	-	**-**
	RM	$1.39x10^9$	$1.48x10^9$	$9.00x10^7$	9.1	9.2	0.03	**0.3**
	RG	$2.28x10^9$	$1.04x10^9$	-	9.4	9.0	-	**-**
	HP	$1.50x10^9$	$1.04x10^9$	-	9.2	9.0	-	**-**
	CT	$9.00x10^8$	$3.55x10^9$	$2.65x10^9$	9.0	9.6	0.6	**6.2**
KB88	BC	$1.43x10^9$	$3.04x10^9$	$1.61x10^9$	9.2	9.5	0.3	**3.5**
	AB	$1.21x10^9$	$3.04x10^9$	$1.83x10^9$	9.1	9.5	0.4	**4.2**
	RM	$1.57x10^9$	$2.73x10^9$	$1.16x10^9$	9.2	9.4	0.2	**2.5**
	RG	$5.50x10^8$	$3.58x10^9$	$3.03x10^9$	8.7	9.6	0.8	**8.5**
	HP	$3.00x10^8$	$3.58x10^9$	$3.28x10^9$	8.5	9.6	1.1	**11.3**
AC	CT	$1.80x10^8$	$3.18x10^9$	$3.00x10^9$	8.3	9.5	1.2	**13.1**
08121	BC	$3.50x10^8$	$2.96x10^9$	$2.61x10^9$	8.5	9.5	0.9	**9.8**
	AB	$3.10x10^8$	$2.96x10^9$	$2.65x10^9$	8.5	9.5	1.0	**10.3**
	RM	$3.16x10^9$	$3.62x10^9$	$4.60x10^8$	9.5	9.6	0.1	**0.6**
	RG	$3.70x10^9$	$4.00x10^9$	$3.00x10^8$	9.6	9.6	0.0	**0.4**
	HP	$2.01x10^9$	$4.00x10^9$	$1.99x10^9$	9.3	9.6	0.3	**3.1**
	CT	$1.45x10^9$	$2.00x10^9$	$5.50x10^8$	9.2	9.3	0.1	**1.5**
PA30	BC	$1.53x10^9$	$2.00x10^9$	$4.70x10^8$	9.2	9.3	0.1	**1.3**
	AB	$1.80x10^9$	$2.00x10^9$	$2.00x10^8$	9.3	9.3	0.0	**0.5**
	RM	$2.16x10^9$	$1.15x10^9$	-	9.3	9.1	-	**-**

Growth inductions instead of growth reductions were shown as hyphen (-). All cationic peptides were standardized to 25.0 ppm

[a] Average CFU readings per ml (cell suspension with peptide)

[b] Average CFU readings per ml (control, cell suspension without peptide)

[c] Difference between [b] control and [a] experimental tubes

[d] Log_{10} ([a] experimental tube)

[e] Log_{10} ([b] control)

[f] Difference between log_{10} readings between [e] control and [d] experimental tube

[g] ([f] Log reduction/ [e] control) x 100%

4.2 Antibacterial activity of Schiff base complexes

4.2.1 Kirby-Bauer disk diffusion antibacterial susceptibility test

Distinct clear zones indicating growth inhibition were observed for the LMA complexes series (S_1, S_2, S_3 and S_4) containing metal elements of nickel (Ni), cobalt (Co), zinc (Zn) and cadmium (Cd) (Table 4.4). KB, MRSA and AC were found to be susceptible to those complexes tested. No inhibition zone was observed for PA for all of the compounds tested. Based on the results, the complex LMA Cd-N_3 (S_4) was more efficient than the other compounds tested as it inhibited the growth of six bacterial

strains tested including strains KB88, KB198, MRSA080925, MRSA08071, AC06127 and AC08121, resulting in clear inhibition zones around the disc. Based on the screening result, the most active compound, S_4 was chosen for further testing of MIC determination and time-killing kinetic.

Table 4.4 : Zones of inhibition for Schiff base complexes

		Zones of inhibition (value to the nearest mm)							
		Gram-positive		**Gram-negative**					
Code	**Compound**	**MRSA 080925**	**MRSA 08071**	**KB 88**	**KB 198**	**AC 06127**	**AC 08121**	**PA 30**	**PA 4**
Ctrl	**0.85% saline**	6	6	6	6	6	6	6	6
Ctrl	**DMSO**	6	6	6	6	6	6	6	6
Ctrl	**Polymyxin B**	-	-	15	15	16	15	-	-
Ctrl	**Vancomycin**	16	17	-	-	-	-	-	-
S_1	**LMA Ni-N_3**	6	9	10	12	6	6	6	6
S_2	**LMA Co-N_3**	6	9	9	11	6	6	6	6
S_3	**LMA Zn-N_3**	6	8	8	10	6	6	6	6
S_4	**LMA Cd-N_3**	20	10	10	12	14	12	6	6
S_5	**2,6-DAP GH**	7	6	6	6	6	6	6	6
S_6	**2-AP GH**	6	6	6	6	6	6	6	6
S_7	**CL-AP GH**	11	6	6	6	6	7	6	6
S_8	**GH**	6	6	6	6	6	8	7	6
S_9	**Br-GH**	10	6	6	6	6	7	6	6
S_{10}	**CH_3-O GH**	6	6	6	6	6	7	6	6
S_{11}	**Ind-BZH**	6	6	6	6	6	6	6	6
S_{12}	**Br-NiC**	6	6	6	6	6	6	6	6
S_{13}	**CL-NiC**	6	6	6	6	6	6	6	6
S_{14}	**Ind-NiC**	6	6	6	6	6	7	6	6
S_{15}	**CL-BZH**	11	6	6	6	6	7	6	6
S_{16}	**Br-BZH**	10	6	6	6	6	7	6	6
S_{17}	**LHA $CuCl_2$**	6	6	6	6	6	6	6	6
S_{18}	**LHA $ZnCl_2$**	7	6	6	6	6	6	6	6
S_{19}	**LHA**	6	6	6	6	6	7	6	6
S_{20}	**LH-BZ**	6	6	6	6	6	8	6	6
S_{21}	**LHA $NiCl_2$**	6	6	6	6	6	6	6	6
S_{22}	**LNA $CuBr_2$**	6	6	6	6	6	6	6	6
S_{23}	**LNA Cu-SCN**	6	6	6	6	6	6	6	6
S_{24}	**LNA $ZnCl_2$**	7	6	6	6	6	6	6	6
S_{25}	**LNA ZnSCN**	6	6	6	6	6	6	6	6
S_{26}	**LMA $ZnBr_2$**	6	6	6	6	6	6	6	6
S_{27}	**LMA MnSCN**	6	6	6	6	6	6	6	6
S_{28}	**LMA ZnSCN**	6	6	6	6	6	6	6	6
S_{29}	**LMA CuSCN**	6	6	6	6	6	6	6	6

Readings of 6 mm represents disk size; no inhibition zone observed. All Schiff base complexes were tested at 10,000.0 ppm. Disc concentration of polymyxin B tested was 300.0 units; concentration of vancomycin was 30.0 µg. Polymyxin B and vancomycin were not tested on *P. aeruginosa*; represented by hyphen (-)

4.2.2 Minimum inhibitory concentration (MIC) determination

In the broth micro-dilution assay, the MICs of S_4 against strains MRSA080925, KB88 and AC08121 were shown in Table 9. The MIC of S_4 against AC08121 was the lowest among the three tested bacterial strain (156.3 ppm); while the highest MIC was recorded for strain MRSA080925 (625.0 ppm) (Table 4.5).

Table 4.5 : Minimum inhibitory concentration (MIC) of antibacterial compounds tested in broth micro-dilution assay

Bacterial strains	Minimum inhibitory concentration (MIC) (ppm)		
	Antibacterial compounds		
	LMA Cd-N_3	Vancomycin	Polymyxin B
MRSA080925	625.0	4.0	-
KB88	312.5	-	2.0
AC08121	156.3	-	2.0

Hyphen (-) represent antibiotics not tested against respective strains

4.2.3 Time-kill assay

The bacterial killing kinetic of S_4 against strains MRSA080925, KB88 and AC08121 was shown in Figures 7, 8 and 9. As defined by the Clinical and Laboratory Standards Institute (CLSI) M26-A guidelines (CLSI, 1999), a decrease of $\geq$ 3-$\log_{10}$ CFU/ml from time-kill curves indicates the 99.9% of killing rate, the compound is considered to have bactericidal activity towards the bacterial cells tested. The complex S_4 exhibited an insignificant bactericidal activity toward KB88 only after 8 hours of treatment at 1X MIC concentration (Figure 4.3). No complete killing was observed even after 24 hours of treatment with 1X MIC of complex S_4. At a higher concentration at 2X MIC and 4X MIC, bactericidal activity of the complex can be observed after 4 hours and 2 hours of treatment, respectively. For strain MRSA080925, the complex tested at concentration of 1X MIC resulted in complete killing of bacterial cells after 4 hours of treatment (Figure 4.4). At higher concentrations of 2X and 4X MIC, complete killing of cells was achieved after 2 hours of treatment. No regrowth was observed for the complex at 1X, 2X and 4X MIC when tested against strain MRSA080925. As for strain

AC08121, bactericidal activity can be observed after 4 hours of treatment with 1X MIC and after 2 hours of treatment with 2X MIC (Figure 4.5). Complete killing was observed after 2 hours, 4 hours and 8 hours of treatments for the concentrations of 4X, 2X and 1X MIC, respectively.

As for the antibiotics treatment, both the polymyxin B and vancomycin were tested in time-kill assay at 0.5X concentration of respective bacterial MIC to the antibiotics. Complete killing of cells was achieved after 2 hours of exposure to polymyxin B for both strains AC08121 and KB88, whereas the bactericidal activity of vancomycin against strain MRSA080925 was slower and bactericidal activity was observed after 4 hours of treatment. Though, complete killing of MRSA was achieved after 12 hours of treatment with vancomycin. However, in the treatment of polymyxin B at 1.0 ppm against strain KB88, regrowth of cells occurred after 4 hours of treatment and continued to grow until 24 hours (Figure 4.3).

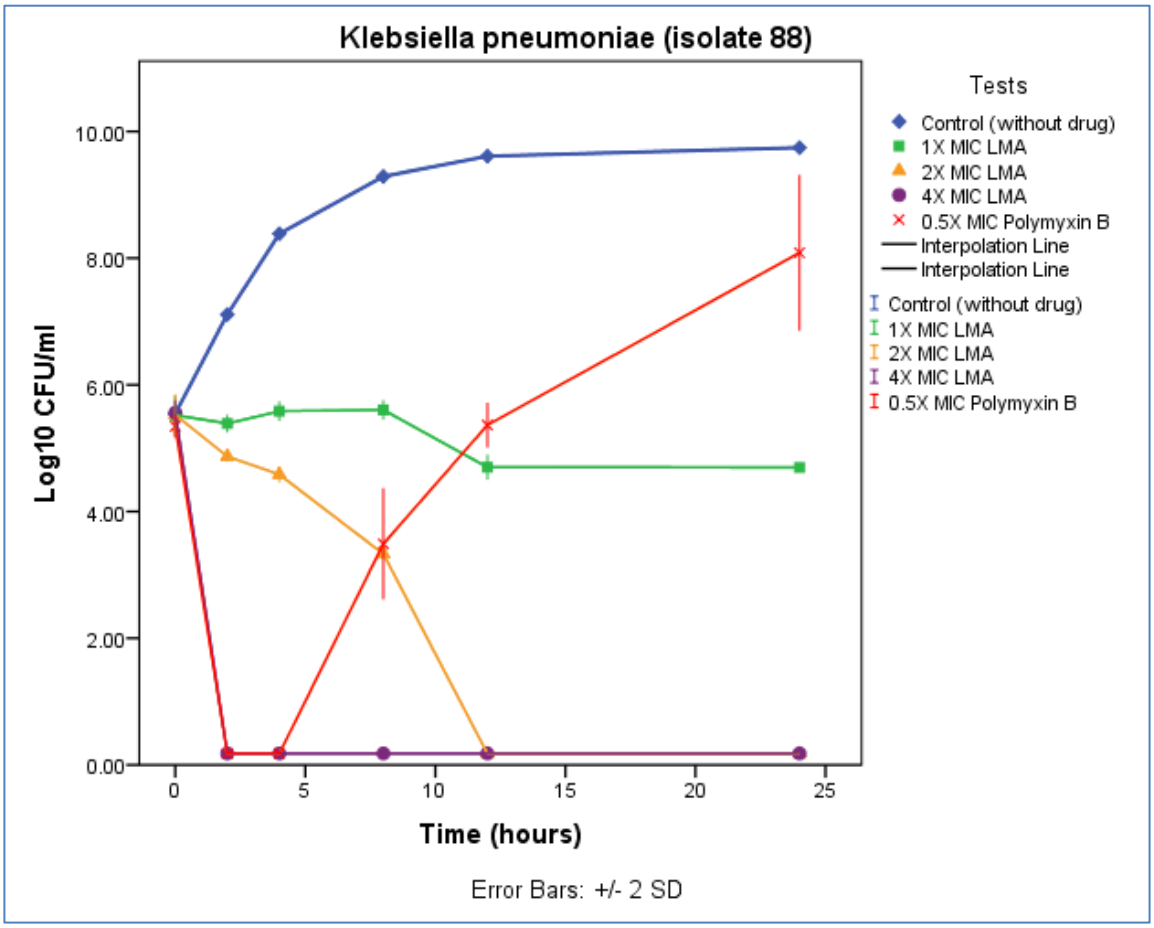


Figure 4.3 : Time-kill curves for *K. pneumoniae* (strain 88) against metal complex LMA Cd-N_3. Concentrations of compounds tested were expressed as multiples of MIC. 'LMA' represents the metal complex LMA Cd-N_3 at concentration of 1X (312.5 ppm), 2X (625.0 ppm) and 4X (1250.0 ppm). The antibiotic polymyxin B was tested at concentration of 0.5X (1.0 ppm)

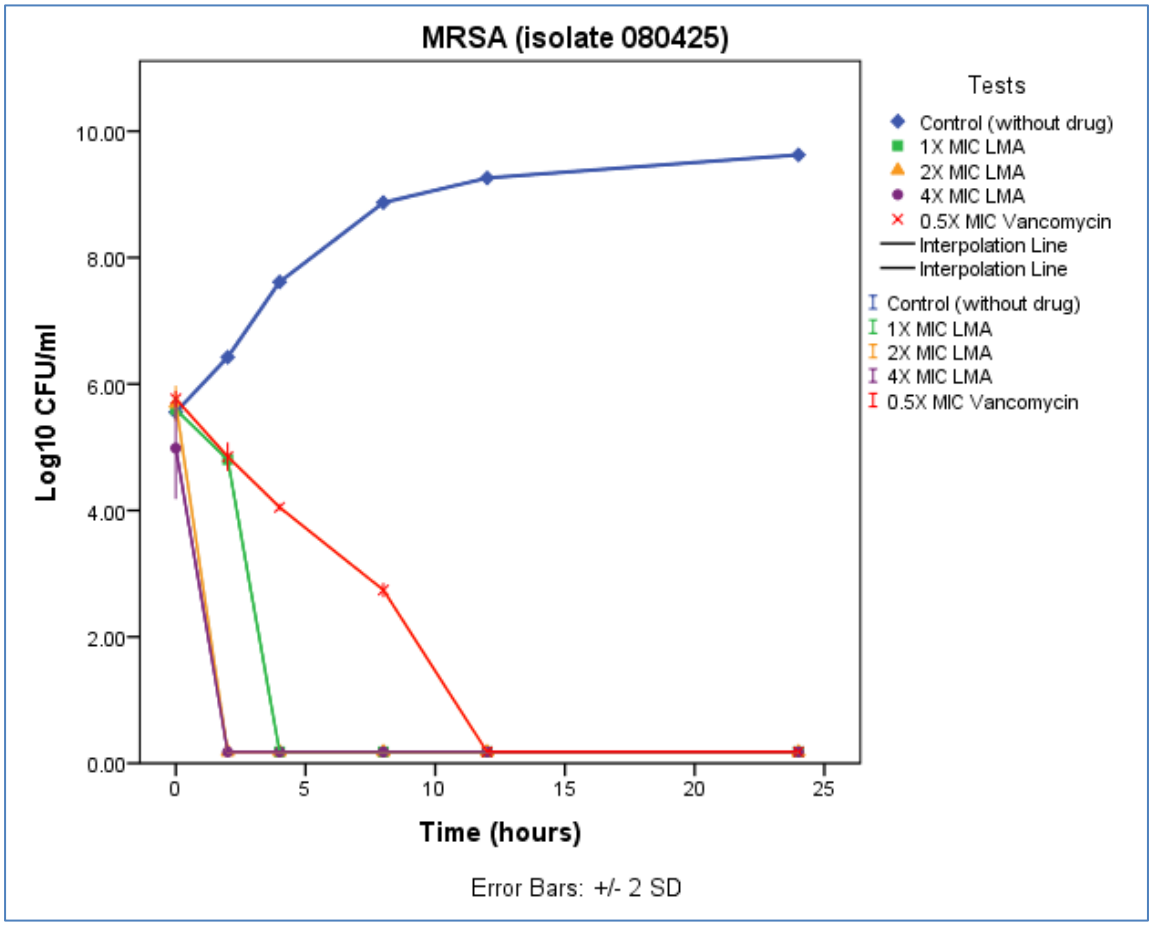


Figure 4.4 : Time-kill curves for MRSA (strain 080925) against metal complex LMA Cd-N_3. Concentrations of compounds tested were expressed as multiples of MIC. 'LMA' represents the metal complex LMA Cd-N_3 at concentration of 1X (625.0 ppm), 2X (1250.0 ppm) and 4X (2500.0 ppm). The antibiotic vancomycin was tested at concentration of 0.5X (2.0 ppm)

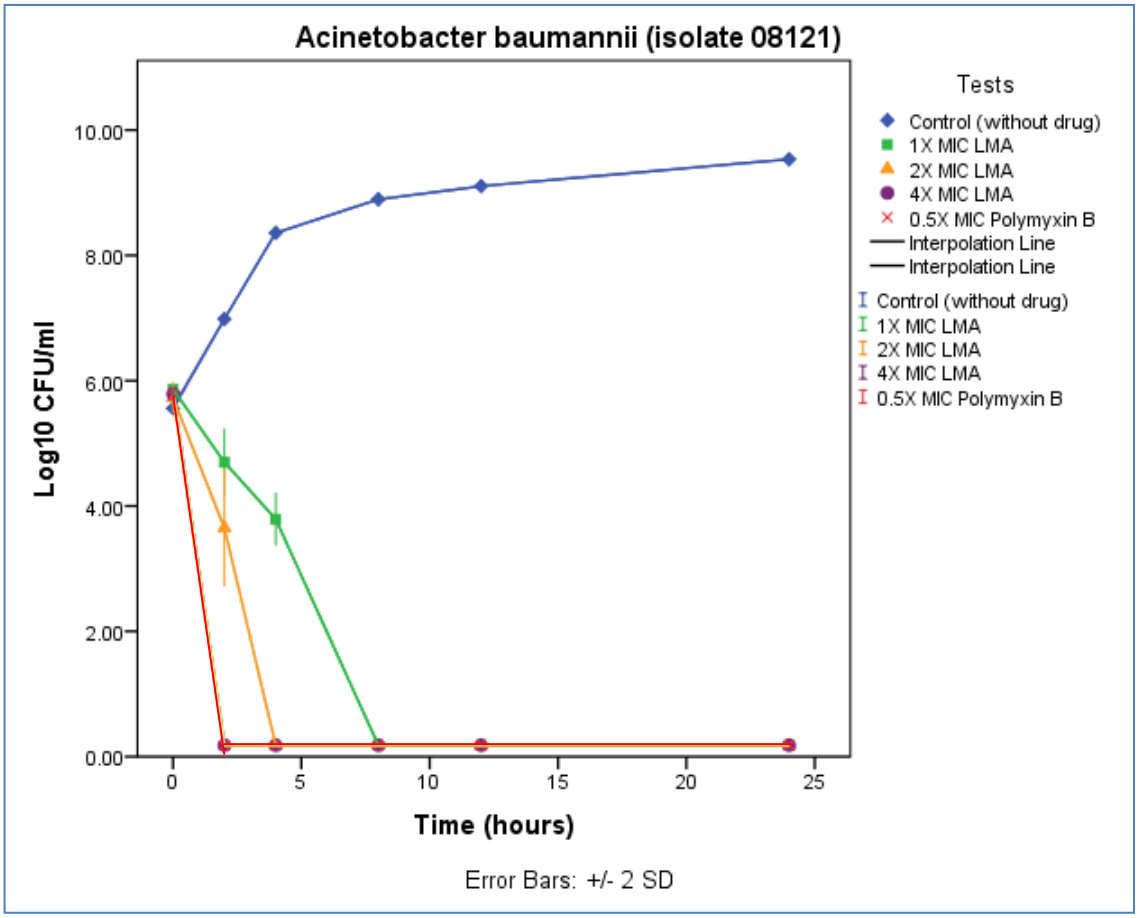


Figure 4.5 : Time-kill curves for *A. baumannii* (strain 08121) against metal complex LMA Cd-N_3. Concentrations of compounds tested were expressed as multiples of MIC. 'LMA' represents the metal complex LMA Cd-N_3 at concentration of 1X (156.3 ppm), 2X (312.5 ppm) and 4X (625.0 ppm). The antibiotic polymyxin B was tested at concentration of 0.5X (1.0 ppm)

CHAPTER 5

DISCUSSION

5.1 Antibacterial activity of cationic peptides against selected multidrug-resistant nosocomial bacteria

Discovery of antimicrobial cationic peptides, such as polymyxin B has churned up many interests worldwide on continuous search for new antimicrobial cationic peptides. To date, more than 600 cationic peptides have been discovered in virtually all organisms from microorganisms to man. These cationic peptides have a broad spectrum of antimicrobial activity including activity against bacteria, eukaryotic parasites, viruses, and fungi (Hancock, 2001). In this study, six potential antimicrobial cationic peptides were designed and synthesized by a local biotechnology company. The structure and composition of these cationic peptides provided by the company were unknown, as well as the antimicrobial activity and antimicrobial mechanism. Due to the limitation in the volume of the cationic peptide provided, only one of the peptide, α-RetroMAD1 (RM), was selected for methodology optimization. Initially, Kirby-Bauer disk diffusion test was used to screen for the antibacterial activity. However, no activity could be detected with this method. No clear inhibition zone could be detected around the disc impregnated with peptide RM. This could be due to (i) low or no antibacterial activity of the peptide; or (ii) the peptide was unable to diffuse through the agar media. The agar media used in the study was prepared according to Clinical and Laboratory Standards Institute (CLSI) M2-A9 guidelines (CLSI, 2006). However, the peptides were unable to produce any inhibition zones even when tested against all bacterial strains at a high concentration of 3,500.0 ppm (results were not shown). Hence, based on the results obtained from the screening, it was safe to make assumption that the size of peptide

molecules could be larger than the matrix size of agar used. Therefore, to confirm the result, broth micro-dilution method was then adopted. Based on most of the studies on antibacterial activity of cationic peptides published (Hancock and Lehrer, 1998; Friedrich *et al*., 2000; Hancock and Rozek, 2002), broth micro-dilution method was proved to be efficient and suitable for the determination of the antibacterial property of cationic peptides. Nonetheless, even at the highest concentration (750.0 ppm) tested, the peptide RM did not show any substantial capability to inhibit the growth of the multidrug-resistant bacterial pathogens tested (Table 4.1). However, the cell inactivation assay employed in this work captured some antibacterial activity of peptides RG, HP, CT, BC and AB when tested at 25.0 ppm toward the multidrug-resistant *Acinetobacter baumanii* (strain AC08121) (Table 4.3) and peptide RM against MRSA and multidrug-resistant *Klebsiella pneumoniae* tested (Table 4.1, Figure 4.2). The log reduction of strain AC08121 with 25.0 ppm of the peptides ranged from 0.8 to 1.2 $\log_{10}$ CFU/ml (Table 4.3). It is believed that if the bacterial strains were to be challenged with higher concentrations of the peptides, the antibacterial activity of these peptides will be more significant.

According to the work of Hancock and Rozek (2002), virtually all cationic peptides cause severe membrane perturbations in bacteria if high enough concentrations are administered. Complete and rapid disruption of membrane potential and all macromolecular synthesis occur at concentrations of 10-fold higher than the minimum bactericidal concentration (MBC); at the MBC concentration, only macromolecular synthesis is inhibited (Hancock and Rozek, 2002). At the concentration of 750.0 ppm, the inhibitory effect of peptide RM against MRSA ($\log_{10}$ reduction of 1.0 to 1.9 $\log_{10}$ CFU/ml); multidrug-resistant *Acinetobacter baumannii* ($\log_{10}$ reduction of 0.6 to 0.7 $\log_{10}$ CFU/ml) and multidrug-resistant *Klebsiella pneumoniae* ($\log_{10}$ reduction 0.4 to 0.9

$\log_{10}$ CFU/ml) were comparable to the antibacterial effect of Vancomycin (1.2 $\log_{10}$ reduction), Polymyxin B (0.3 $\log_{10}$ reduction), and Polymyxin B (0.9 $\log_{10}$ reduction), respectively (Table 4.1). However, when the peptide was tested at a low concentration of 46.9 ppm, the peptide RM induce higher bacterial growth in MRSA where it showed an increase of CFU count of bacterial suspension with peptide when compared to bacterial suspension without peptide (Table 4.2). Based on the results, it would appear that at lower concentration, the peptide RM tested in the study were able to be utilized by the bacteria as an additional or alternative source of nutrient that support an increase in biomass. However, further studies need to be conducted in order to verify the assumptions made.

As for the mechanism of action of the cationic peptides, considerable studies had been carried out to validate the attraction of positively-charged peptide towards the negatively-charged bacterial cell membrane, where most of the morphology of bacterial cells viewed under scanning electron microscope (SEM)/transmission electron microscope (TEM) (Shimoda *et al*., 1995; Friedrich *et al*., 2000; Hartmann *et al*., 2010) and atomic force microscope (AFM) (Li *et al*., 2007) revealed the damaging effect of the peptides toward the cell membranes of bacteria.

5.2 Antibacterial activity of Schiff base complexes against selected multidrug-resistant nosocomial bacteria

Up to date, published data on bacterial killing rate (time-kill assay) of Schiff base metal complexes toward multidrug-resistant bacteria is limited. In this work, the antibacterial activity of 29 synthetic Schiff base compounds were screened using disk diffusion and broth micro-dilution assays; while the bacterial killing kinetic was determined with time-kill assay. From the time-kill assay (Figure 4.3, Figure 4.4 and

Figure 4.5), the bactericidal activity of cadmium-containing Schiff base complex was shown to be more effective against MRSA, followed by multidrug-resistant *A. baumannii* and *K. pneumoniae*. The order of susceptibility to the complex was also supported by the results of disk diffusion test (Table 4.4). The findings indicated that the bactericidal activity of Schiff base cadmium complex (LMA Cd-N_3) was more effective against Gram-positive bacteria. Gram-positive bacteria were described to be more sensitive to metals when compared to Gram-negative bacteria (Wang *et al*., 2010). The enhanced antimicrobial activity of metal complexes compared to unbound metallic ions and ligands was extensively studied (Mohamed *et al*., 2011; Vinuelas-Zahinos *et al*., 2011; Sabik *et al*., 2012; Aiyelabola *et al*., 2012; Sunitha *et al*., 2012). Based on chelation theory, the enhancement is due to the increased lipophilic nature of the metal complex, achieved by the overlapping of ligand orbital with metal orbital in the complex, which causes partial sharing of the positive charge of metals with the donor groups on ligands. This coordination chemistry reduces the polarity of metal and thus increasing the lipophilic nature of the metal to the lipid layer of bacterial cell membrane (Figure 5.1) (Nishat *et al*., 2011; Sabik *et al*., 2012; Sunitha *et al*., 2012).

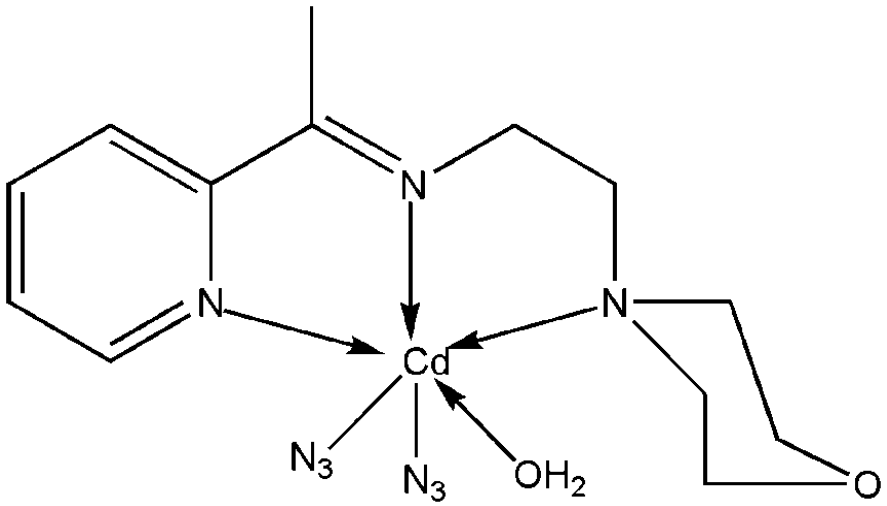


Figure 5.1: Chemical structure of Schiff base cadmium complex (LMA Cd-N_3); Aqua{2-morpholino-N-[1-(2-pyridyl)-ethylidene] ethanamine-k^3N,N',N"}-bis(azido-k^N) cadmium (II)

In agreement with studies done by Vinuelas-Zahinos and colleagues (2011), among the complexes of various metals, cadmium compounds were found to be the most active compound against *Bacillus subtilis* and *Staphylococcus epidermidis*. The heavy metal cadmium, was described to disrupt normal cellular processes of living organisms by binding to different cellular target sites; by displacing zinc in some of the zinc-containing essential enzymes and inhibits activity of the enzymes (Wang *et al*., 2010) and the damaging effect towards membrane structure when cadmium binds to phosphate ligands present on the membrane (Vig *et al*., 2003). Due to the highly negative-charged LPS on the cell walls of Gram-negative bacteria and the opposite charges of both the cationic metal ions and cationic peptides, metal ions will be adsorbed to bacterial cell surfaces through passive biosorption (Chakravarty *et al*., 2012). Likewise, the adsorption of positively-charged cadmium to the cell wall of Gram-positive bacteria, *Bacillus subtilis* was also reported (Boyanov *et al*., 2003).

Utilizing the antibiotic polymyxin B in time-kill assay, regrowth of *K. pneumoniae* strains after 4 hours signifies the loss of bactericidal activity of the antibiotics beyond 4 hours of treatment. In agreement with similar time-kill studies done by Pournaras and colleagues (2001), the treatments of clinical strains of *K. pneumoniae* with polymyxin E (colistin) also showed regrowth after 8 hours of exposure to the antibiotics. Polymyxins were a class of decapeptide antibiotics that have antimicrobial spectrum against Gram-negative bacteria such as *K. pneumoniae* and *A. baumannii*. Out of the five polymyxins available (A, B, C, D and E), only polymyxin B and polymyxin E were administered in treatments of infectious diseases in clinical settings (Falagas & Michalopoulos, 2006). The incidences of cellular regrowth following exposure to various antibiotics were also common among the studies of bacterial killing rate in time-

kill assays. Some of the regrowth were observed in the studies of clinical strains bacteria from using the antibiotics piperacillin and cefepime against *K. pneumoniae* (Burgess & Hall, 2004); amikacin and ceftobiprole against *P. aeruginosa* (Kresken *et al*., 2011) and daptomycin against vancomycin-resistant *Enterococcus faecium* (Sakoulas *et al*., 2012).

In the time-kill assay, the effect of antibiotic carryover was controlled by dilutions of broth aliquots before plating. Besides, as described in CLSI M26-A guidelines (CLSI, 1999), the effect of antibiotic carryover occurs mainly at higher concentrations (>16X MIC) of the antibacterial compounds tested. Due to the ineffectiveness of most of the antibacterial compounds tested against the strains of *P. aeruginosa*, the bacteria were excluded from the time-kill assay. Likewise, other novel thiosemicarbazone-derived Schiff base metal complexes of cobalt (Co), nickel (Ni), zinc (Zn) and cadmium (Cd) were also found to have no antimicrobial activity against *P. aeruginosa* in studies done by Vinuelas-Zahinos and colleagues (2011).

5.3 Cadmium toxicity and bacterial resistance to cadmium

The element cadmium can be found in the environment. Human exposure to cadmium generally occurs through food sources such as vegetables and cereals that were grown in polluted soils, or seafood from polluted water, inhalation of polluted air from either industrial processes or tobacco smoking; and polluted drinking-water (Satarug *et al*., 2000). Accumulations of cadmium in human body take place mainly in the kidney. High amounts of accumulated cadmium lead to renal dysfuntion, which resulted in increased excretion of biologically important proteins of low molecular weight such as β_2-microglobulin and retinol-binding protein in the urine (Buchet *et al*., 1990). Renal dysfunction is more prevalent among those with diabetics; deficiency in

vitamin C, Ca, P and Zn; and women with reduced body Fe. Other diseases include the formation of kidney stones and disruption of calcium metabolism (Satarug *et al*., 2000). The Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Expert Committee on Food Additives (JECFA) had established a Provisional Tolerable Monthly Intake (PTMI) for cadmium with 25 μg/kg of human body weight, 3 μg/L of drinking-water and an annual average of 5 ng/m^3 of air inhalation exposure (WHO, 2010).

Resistance to heavy metal ions such as arsenic, mercury and cadmium had been reported in the hospital isolates of *Staphylococcus aureus* (Chopra, 1975; Witte *et al*., 1986). The resistance to cadmium is due to the presence of two genes on the resistance (R) plasmid; cadA and cadB genes. The cadmium resistance ability is primarily governed by the cadA gene which offers high level of resistance while the cadB gene provides resistance to a lesser extend (Weiss *et al*., 1978). The cadA gene is located on mostly plasmid that encodes for penicillinase production (Chopra, 1975). Up to date, there are limited published data regarding the resistance to antibacterial Schiff base metal complexes. Despite the bacterial innate or acquired resistance to heavy metals, the Schiff base Cd complex showed comparable results to the commercial antibiotics used for the treatments of diseases caused by MRSA and *K. pneumoniae* (Table 4.4, Figure 4.3 and Figure 4.4). Besides, due to the relatively new compounds (Gwaram *et al*., 2012; Gupta *et al*., 2012; Shakir *et al*., 2012; Sunitha *et al*., 2012), the resistance of microorganisms towards Schiff base metal complexes remains to be studied in future researches. Furthermore, the cytotoxicity studies of Schiff base complexes tested in this study were conducted by the research team of Professor Dr. Hapipah for the development of potential anticancer drugs (Gwaram, personal communication, 2012).

5.4 Limitation of the study

Unfortunately, there were some limitations to the study; namely the availability and the effectiveness of the compounds which causes minor restriction to the study. From the beginning and towards the end of the study, the synthetic compounds were synthesized and provided by different individuals. The cationic peptide, α-RetroMAD1 (RM) was the first peptide provided at the concentration of 1,500.0 ppm. A higher concentration at 3,500.0 ppm of the said peptide arrived afterwards. The rest of the peptides; RG, HP, CT, BC and AB were obtained at different concentrations shortly after. The different concentrations of compounds obtained at different time periods resulted in inconsistent working concentrations of the compounds. In the case of the peptides RG, HP, CT, BC and AB; the compound labeled CT was provided at the lower concentration of 50.0 ppm when compared to the rest of the peptides. Due to that, the working concentration of the rest of the peptides (RG, HP, BC and AB) would have to be standardized to 25.0 ppm for the assays as the solution would be diluted by two-fold. Furthermore, only small volumes of the peptides were provided and this limits the amount of assays that can be conducted in this study. Besides that, some of the peptides were shown to increase growth of bacterial cells at low concentration. Due to the low availability and low bactericidal property of the peptides, another new synthetic compound would have to be obtained and the focus of the work was shifted to the Schiff base complexes in the later part of the study. However, both of the synthetic peptides and Schiff base complexes were part of bio-prospective projects and proprietary for respective individuals. Therefore, only the compound abbreviations code and concentrations were revealed.

CHAPTER 6

CONCLUSION

The overall objective of the dissertation was to determine the antibacterial properties of synthetic compounds comprising of cationic peptides and Schiff base complexes. The study was conducted in the hopes of identifying potential antibacterial agents as alternatives to existing antibiotics. All of the compounds obtained were screened for its antibacterial properties against selected nosocomial bacterial strains of methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The synthetic cationic peptides have poor bactericidal activity against all bacterial strains even when tested at a high concentration of 750.0 ppm in disk diffusion screening, broth micro-dilution assay and cell inactivation assay. Slight percentages of growth inhibition of 10 % to 20 % of $\log_{10}$ CFU/ml were observed when the peptide (RM) was tested at 750.0 ppm against strains of MRSA. Some of the peptides were even found to enhance the bacterial growth of MRSA, *K. pneumoniae* and *P. aeruginosa* tested at a lower concentration of 25.0 ppm. Furthermore, the strains of *P. aeruginosa* were not affected by all of the peptides tested in the assays. Consequently, the cationic peptides tested in this study may not be suitable to be used as antibacterial agents.

Among the twenty-nine Schiff base complexes, the cadmium-containing complex (LMA Cd-N_3) was found to have the highest antibacterial activity against both the Gram-positive bacteria MRSA and the Gram-negative bacteria *K. pneumoniae* and *A. baumannii* in the disk diffusion screening. However, the strains of *P. aeruginosa* were also not affected by the entire Schiff base complexes tested in the disk diffusion

screening and were omitted from the time-kill assay. Interesting to note that, the exposure of *K. pneumoniae* (strain KB88) to the antibiotic polymyxin B in time-kill assay showed regrowth of bacterial cells after exposure to the antibiotic for 4 hours. Whereas the exposure of similar bacterial strain to the complex LMA Cd-N_3 showed no regrowth even after 24 hours of incubation. As for MRSA (strain 080925), complete killing of bacterial cells were achieved after 4 hours of exposure to LMA Cd-N_3, 8 hours earlier than the exposure to the antibiotic vancomycin. The findings suggest that the Schiff base cadmium complex had considerable antibacterial activity towards both multiple drug-resistant nosocomial Gram-positive bacteria and Gram-negative bacteria, particularly MRSA, *A. baumannii* and *K. pneumoniae*. These results warrant further studies on the application of the Schiff base metal complexes in the treatments of infectious diseases caused by these pathogenic bacteria. These could overcome or alleviate the problems faced in the antibiotic-resistant trait of pathogenic microorganisms.

BIBLIOGRAPHY

Abou-Shanab R.A.I., Berkum P.V. and Angle J.S. (2007). Heavy metal resistance and genotypic analysis of metal resistance genes in Gram-positive and Gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of Alyssum murale. *Chemosphere*, 68, 360-367.

Aiyelabola T.O., Ojo I.A., Adebajo A.C., Ogunlusi G.O., Oyetunji O., Akinkunmi E.O. and Adeoye A.O. (2012). Synthesis, characterization and antimicrobial activities of some metal (II) amino acids' complexes. *Advances in Biological Chemistry*, 2, 268-273.

Barra D. and Simmaco M. (1995). Amphibian skin: A promising resource for antimicrobial peptides. *Trends in Biotechnology*, 13, 205-209.

Bax R., Mullan N. and Verhoef J. (2000). The millennium bugs-the need for and development of new antibacterials. *International Journal of Antimicrobial Agents*, 16, 51-59.

Boyanov M.I., Kelly S.D., Kemner K.M., Bunker B.A., Fein J.B. and Fowle D.A. (2003). Adsorption of cadmium to *Bacillus subtilis* bacterial cell walls: A pH-dependent X-ray absorption fine structure spectroscopy study. *Geochimica et Cosmochimica Acta*, 67(18), 3299-3311.

Buchet J.P., Lauwerys R., Roels H., Bernard A., Bruaux P., Claeys F., Ducoffre., De Plaen P., Staessen J., Amery A., Lijnen P., Thijs L., Rondia D., Sartor F., Saint Remy A. and Nick L. (1990). Renal effects of cadmium body burden of the general population. *The Lancet*, 336, 699-702.

Burgess D.S. and Hall R.G., II. (2004). *In vitro* activity of parenteral β-lactams, levofloxacin and tobramycin alone or in combination against extended-spectrum β-lactamase producing *Klebsiella pneumoniae*. *International Journal of Antimicrobial Agents*, 24, 48-52.

Casey A.L., Lambert P.A. and Elliott T.S.J. (2007). Staphylococci. *International Journal of Antimicrobial Agents*, 29, S23-S32.

Chakravarty R. and Banerjee P.C. (2012). Mechanism of cadmium binding on the cell wall of an acidophilic bacterium. *Bioresource Technology*, 108, 176-183.

Chatterjee C., Paul M., Xie L.L. and Donk W.A.V.D. (2005). Biosynthesis and mode of action of lantibiotics. *Chemicals Review*, 105, 633-683.

Chomvarin C., Siripornmongcolchai T., Chaicumpar K., Limpaiboon T., Wongkham C. and Yutanawiboonchai W. (2004). Evaluation of polymerase chain reaction, conventional and MRSA screen latex agglutination methods for detecion of methicillin-resistant, -borderline and –susceptible *Staphylococcus aureus*. *Southeast Asian Journal of Tropical Medicine and Public Health*, 35(4), 879-885.

Chopra I. (1975). Mechanism of plasmid-mediated resistance to cadmium in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 7(1), 8-14.

Clinical and laboratory standards institute (CLSI) document M26-A. (1999). Methods for determining bactericidal activity of antimicrobial agents: Approved guideline. *Clinical and Laboratory Standards Institute, Wayne, PA, USA.*

Clinical and laboratory standards institute (CLSI) document M2-A9. (2006). Performance standards for antimicrobial disk susceptibility tests: Approved standard-ninth edition. *Clinical and Laboratory Standards Institute, Wayne, PA, USA.*

Clinical and laboratory standards institute (CLSI) document M7-A7. (2006). Methods for dilution susceptibility tests for bacteria that grow aerobically: Approved standard-seventh edition. *Clinical and Laboratory Standards Institute, Wayne, PA, USA.*

Creaven B.S., Egan D.A., Kavanagh K., McCann M., Noble A., Thati B. and Walsh M. (2006). Synthesis, characterization and antimicrobial activity of a series of substituted coumarin-3-carboxylatosilver (I) complexes. *Inorganica Chimica Acta*, 359, 3976-3984.

Csordas A. and Michl H. (1970). Isolation and structure of a haemolytic polypeptide from the defensive secretion of European Bombina species. *Monatshefte fur Chemie,* 101, 182–189.

Defres S., Marwick C. and Nathwani D. (2009). MRSA as a cause of lung infection including airway infection, community-acquired pneumonia and hospital-acquired pneumonia. *European Respiratory Journal,* 34(6), 1470-1476.

Dimarcq J.L., Bulet P., Hetru C. and Hoffmann J. (1998). Cysteine-rich antimicrobial peptides in invertebrates. *Biopolymers,* 47, 465-477.

Falagas M.E. and Michalopoulos A. (2006). Polymyxins: Old antibiotics are back. *The Lancet*, 367, 633-634.

Frecer V., Ho B. and Ding J.L. (2004). De novo design of potent antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, 48(9), 3349-3357.

Friedrich C.L., Moyles D., Beveridge T.J. and Hancock R.E.W. (2000). Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. *Antimicrobial Agents and Chemotherapy*, 44(8), 2086-2092.

Garcia-Olmedo F., Molina A., Alamillo J.M. and Rodriguez-Palenzuela P. (1998). Plant defense peptides. *Biopolymers*, 47, 479-491.

Gudasi K.B., Patil M.S. and Vadavi R.S. (2008). Synthesis, characterization of copper (II), cobalt (II), nickel (II), zinc (II) and cadmium (II) complexes of [7-hydroxy-4-methyl-8-coumarinyl] glycine and a comparitive study of their microbial activities. *European Journal of Medicinal Chemistry*, 43, 2436-2441.

Gupta Y.K., Agarwal S.C., Madnawat S.P. and Narain R. (2012). Synthesis, Characterization and antimicrobial studies of some transition metal complexes of Schiff bases. *Research Journal of Chemical Sciences*, 2(4), 68-71.

Gwaram N.S., Ali H.M., Khaledi H., Abdulla M.A., Hadi A.H.A., Thong K.L., Chai L.C. and Cher L.O. (2012). Antibacterial evaluation of some Schiff bases derived from 2-acetylpyridine and their metal complexes. *Molecules*, 17, 5952-5971.

Habermann E. (1972). Bee and wasp venom. *Science*, 177, 314-322.

Hancock R.E.W. and Chapple D.S. (1999). Peptide antibiotics: mini review. *Antimicrobial Agents and Chemotherapy*, 43(6), 1317-1323.

Hancock R.E.W. and Lehrer R. (1998). Cationic peptides: a new source of antibiotics. *Trends in Biotechnology*, 16, 82-88.

Hancock R.E.W. and Rozek A. (2002). Role of membranes in the activities of antimicrobial cationic peptides. *Federation of European Microbiological Societies Microbiology Letters*, 206, 143-149.

Hancock R.E.W. (1997). Peptide antibiotics: Review. *Lancet Infectious Diseases*, 349, 418-422.

Hancock R.E.W. (2001). Cationic peptides: Effectors in innate immunity and novel antimicrobials: Review. *Lancet Infectious Diseases,* 1, 156-164.

Hancock R.E.W. (2005). Mechanisms of action of newer antibiotics for Gram-positive pathogens. *Lancet Infectious Diseases,* 5, 209–218.

Hartmann M., Berditsch M., Hawecker J., Ardakani M.F., Gerthsen D. and Ulrich A.S. (2010). Damage of the bacterial cell envelope by antimicrobial peptides Gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy,* 54(8), 3132-3142.

Hassen A., Saidi, N., Cherif M. and Boudabous A. (1998). Resistance of environmental bacteria to heavy metals. *Bioresource Technology,* 64, 7-15.

Kim B.J. and Park S.J. (2008). Antibacterial behavior of transition-metals-decorated activated carbon fibers. *Journal of Colloid and Interface Science,* 325, 297-299.

Kresken M., Korber-Irrgang B., Lauffer J., Decker-Burgard S. and Davies T. (2011). *In vitro* activities of ceftobiprole combined with amikacin or levofloxacin against *Pseudomonas aeruginosa*: Evidence of a synergistic effect using time-kill methodology. *International Journal of Antimicrobial Agents,* 38, 70-75.

Kumar S., Dhar D.N. and Saxena P.N. (2009). Applications of metal complexes of Schiff bases- A review. *Journal of Scientific and Industrial Research,* 68, 181-187.

Liang W., Liu X.F., Huang J., Zhu D.M., Li J. and Zhang J. (2011). Activities of colistin- and minocycline-based combinations against extensive drug resistant Acinetobacter baumannii isolates from intensive care unit patients. *BioMed Central Infectious Disease,* 11, 1-7.

Li A., Lee P.Y., Ho B., Ding J.L. and Lim C.T. (2007). Atomic force microscopic study of the antimicrobial action of sushi peptides on Gram negative bacteria. *Biochimica et Biophysica Acta,* 1768, 411-418.

Li J.W.H. and Vederas J.C. (2009). Drug discovery and natural products: end of an era or an endless frontier? *Science,* 325, 161-165.

Mohamed G.G., El-Halim H.F.A., El-Dessouky M.M.I. and Mahmoud W.H. (2011). Synthesis and characterization of mixed ligand complexes of lomefloxacin drug and glycine with transition metals. Antibacterial, antifungal and cytotoxicity studies. *Journal of Molecular Structure,* 999, 29-38.

Moss G.P., Smith P.A.S. and Taverner D. (1995). Glossary of class names of organic compounds and reactive intermediates based on structure. *Pure and Applied Chemistry*, 67, 1307-1375.

Navon-Venezia S., Ben-Ami R. and Carmeli Y. (2005). Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Current Opinion in Infectious Diseases*, 18, 306-313.

Nedjar-Arroume N., Delval V.D., Adje E.Y., Traisnal J., Krier F., Mary P., Kouach M., Briand G. and Guillochon D. (2008). Bovine hemoglobin: An attractive source of antibacterial peptides. *Peptides*, 29, 969-977.

Negi H., Agarwal., Zaidi M.G.H. and Goel R. (2012). Comparative antibacterial efficacy of metal oxide nanoparticles against Gram negative bacteria. *Annals of Microbiology*, 62, 765-772.

Nikaido H. (1998). Multiple antibiotic resistance and efflux. *Current Opinion in Microbiology*, 1, 516-523.

Nishat N., Hasnain S., Ahmad T. and Parveen A. (2011). Synthesis, characterization, and biological evaluation of new polyester containing Schiff base metal complexes. *Journal of Thermal Analysis and Calorimetry*, 105, 969-979.

Noyce J.O., Michels H. and Keevil C.W. (2006). Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant *Staphylococcus aureus* in the healthcare environment. *Journal of Hospital Infection*, 63, 289-297.

Podschun R. and Ullmann U. (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomoy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews*, 11(4), 589-603.

Papo N. and Shai Y. (2003). Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides*, 24, 1693-1703.

Pournaras S., Vrioni G., Neou E., Dendrinos J., Dimitroulia E., Poulou A. and Tsakris A. (2011). Activity of tigecycline alone and in combination with colistin and meropenem against *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae strains by time-kill assay. *International Journal of Antimicrobial Agents*, 37, 244-247.

Reiss A., Florea S., Caproiu T. and Stanica N. (2009). Synthesis, characterization, and antibacterial activity of some transition metals with the Schiff base N-(2-furanylmethylene)-3-aminodibenzofuran. *Turkish Journal of Chemistry*, 33, 775-783.

Rosenthal K.S., Ferguson R.A. and Storm D.R. (1977). Mechanisms of action of EM 49, membrane-active peptide antibiotic. *Antimicrobial Agents and Chemotherapy*, 12(6), 665-672.

Ross A.V. and Vederas J.C. (2010). Fundamental functionality: recent developments in understanding the structure-activity relationships of lantibiotic peptides. *The Journal of Antibiotics*, 64, 27-34.

Sabik A.E., Karabork M., Ceyhan G., Tumer M. and Digrak M. (2012). Polydentate Schiff base ligands and their La(iii) complexes: Synthesis, characterization, antibacterial, thermal, and electrochemical properties. *International Journal of Inorganic Chemistry*, 1-11.

Sakoulas G., Bayer A.S., Pogliano J., Tsuji B.T., Yang S.J., Mishra N.N., Nizet V., Yeaman M.R. and Moise P.A. (2012). Ampicillin enhances daptomycin- and cationic host defense peptide-mediated killing of ampicillin- and vancomycin-resistant E*nterococcus faecium*. *Antimicrobial Agents and Chemotherapy*, 56(2), 838-844.

Satarug S., Haswell-Elkins M.R. and Moore M.R. (2000). Safe levels of cadmium intake to prevent renal toxicity in human subjects. *British Journal of Nutrition*, 84, 791-802.

Shakir M., Khanam S., Firdaus F., Latif A., Aatif M. and Al-Resayes S.I. (2012). Synthesis, spectroscopic characterization, DNA interaction and antibacterial study of metal complexes of tetraazamacrocyclic Schiff base. *Spectrochimica Acta Part A: Molecular Spectroscopy*, 93, 354-362.

Shimoda M., Ohki K., Shimamoto Y. and Kohashi O. (1995). Morphology of defensing-treated Staphylococcus *aureus*. *Infection and Immunity*, 63(8), 2886-2891.

Sikarwar A.S. and Batra H.V. (2011). Challenge to healthcare: Multidrug resistance in *Klebsiella pneumoniae*. *International Conference on Food Engineering and Biotechnology*, 9, 130-134.

Sreekumari K.R., Sato Y. and Kikuchi Y. (2005). Antibacterial metals- A viable solution for bacterial attachment and microbiologically influenced corrosion. *Materials Transactions*, 46(7), 1636-1645.

Steiner H., Hultmark D., Engstrom A., Bennich H. and Boman H.G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, 292, 246-248.

Storici P. and Zanetti M. (1993). A novel cDNA sequence encoding a pig leukocyte antimicrobial peptide with a cathelin-like pro-sequence. *Biochemical and Biophysical Research Communications*, 196, 1363-1368.

Sunitha M., Jogi P., Ushaiah B. and Kumari C.G. (2012). Synthesis, characterization and antimicrobial activity of transition metal complexes of Schiff base ligand derived from 3-ethoxy salicylaldehyde and 2-(2-aminophenyl) 1-*H*-benzimidazole. *Journal of Chemistry*, 9(4), 2516-2523.

Su C.H., Wang J.T., Hsiung C.A., Chien L.J., Chi C.L., Yu H.T., Chang F.Y. and Chang S.C. (2012). Increase of Carbapenem-Resistant *Acinetobacter baumannii* Infection in Acute Care Hospitals in Taiwan: Association with Hospital Antimicrobial Usage. *PLoS ONE*, 7(5), 1-6.

Timurkaynak F., Can F., Azap O.K., Demirbilek M., Arslan H. and Karaman S.O. (2006). In vitro activities of non-traditional antimicrobials alone or in combination against multidrug-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from intensive care units. *International Journal of Antimicrobial Agents*, 27, 224-228.

Tomas M.D.M., Cartelle M., Pertega S., Beceiro A., Llinares P., Canle D., Molina F., Villanueva R., Cisneros J.M. and Bou G. (2005). Hospital outbreak caused by a carbapenem-resistant strain of *Acinetobacter baumannii*: patient prognosis and risk-factors for colonisation and infection. *Clinical Microbiology and Infectious Diseases*, 11(7)**,** 540-546.

Valent A., Melnik M., Hudecova D., Dudova B., Kivekas R. and Sundberg M.R. (2002). Copper(II) salicylideneglycinate complexes as potential antimicrobial agents. *Inorganica Chimica Acta*, 340, 15-20.

Vig K., Megharaj M., Sethunathan N. and Naidu R. (2003). Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: A review. *Advances in Environmental Research*, 8, 121-135.

Vinuelas-Zahinos E., Luna-Giles F., Torres-Garcia P. and Fernandez-Calderon M.C. (2011). Co(III), Ni(II), Zn(II) and Cd(II) complexes with 2-acetyl-2-thiazoline thiosemicarbazone: Synthesis, characterization, X-ray structures and antibacterial activity. *European Journal of Medicinal Chemistry*, 46, 150-159.

Walsh C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, 406, 775-781.

Wang F., Yao J., Si Y., Chen H., Russel M., Chen K., Qian Y., Zaray G. and Bramanti E. (2010). Short-time effect of heavy metals upon microbial community activity. *Journal of Hazardous Materials*, 173, 510-516.

Weiss A.A., Silver S. and Kinschrf T.G. (1978). Cation transport alteration associated with plasmid-determined resistance to cadmium in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 14(6), 856-865.

Witte W., Green L., Misra T.K., and Silver S. (1986). Resistance to mercury and cadmium in chromosomally resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 29(4), 663-669.

Won S.Y., Munoz-Price S., Lolans K., Hota B., Weinstein R.A. and Hayden M.K. (2011). Emergence and rapid regional spread of *Klebsiella pneumoniae* carbapenemase-producing Enterobacteriaceae. *Clinical Infectious Diseases*, 53(6), 532-540**.**

World Health Organization (2010). Exposure to cadmium: a major public health concern. *WHO, Geneva.*

Yamada S. (1999). Advancement in stereochemical aspects of Schiff base metal complexes. *Coordination Chemistry Reviews*, 190-192, 537-555.

Yasuyuki M., Kunihiro K., Kurissery S., Kanavillil N., Sato Y. and Kikuchi Y. (2010). Antibacterial properties of nine pure metals: A laboratory study using *Staphylococcus aureus* and *Escherichia coli*. *Biofouling*, 26(7), 851-858.

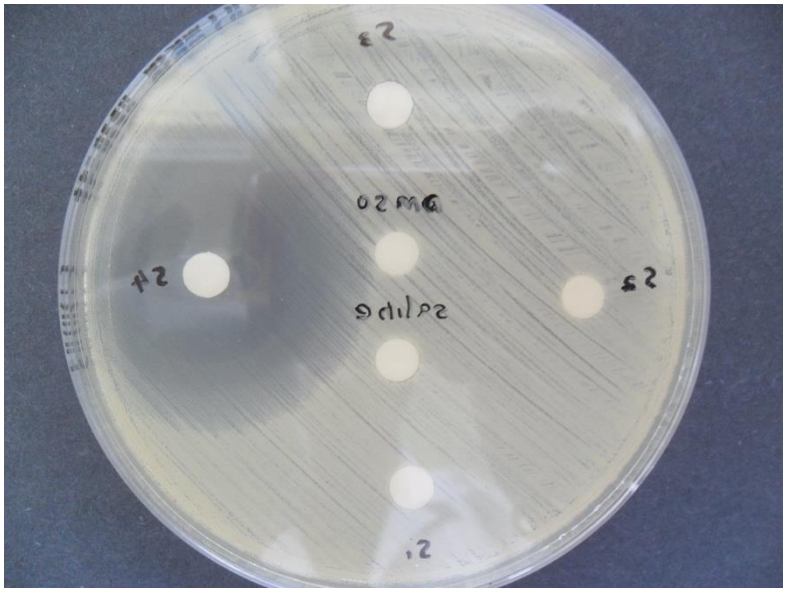
Zasloff M. (1987). Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America*, 84(15), 5449-5453.

Zavascki A.P., Carvalhaes C.G., Picao R.C. and Gales A.C. (2010). Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. *Expert Review of Anti-Infective Therapy*, 8(1), 71-93.

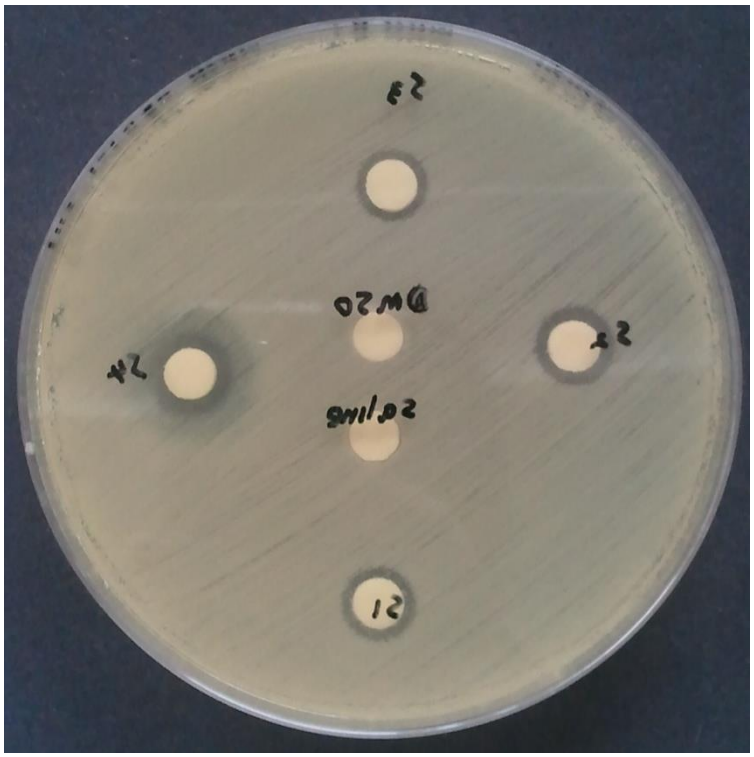
APPENDIX

Kirby-Bauer disk diffusion antibacterial susceptibility test

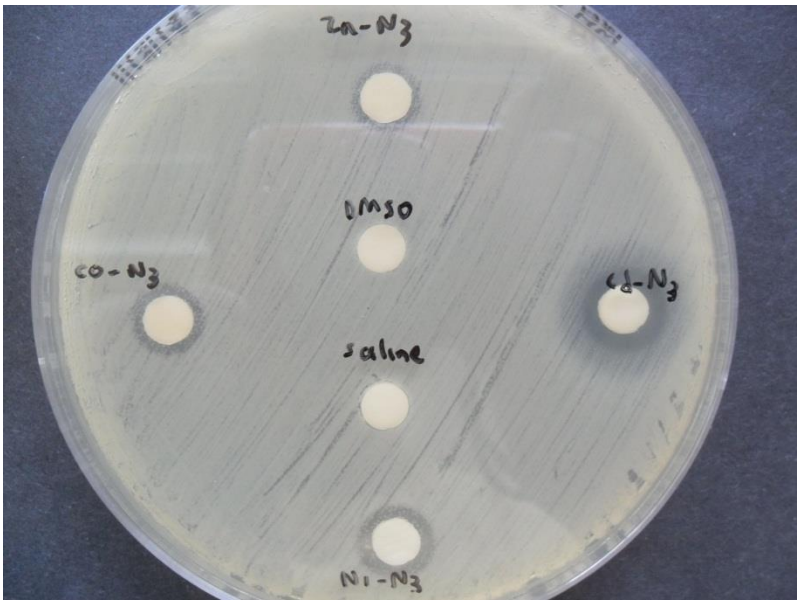
Schiff base metal complexes S_1, S_2, S_3 and S_4 of LMA series of 10,000.0 ppm; containing nickel (Ni) {S_1}, cobalt (Co) {S_2}, zinc (Zn) {S_3} and cadmium (Cd) {S_4}



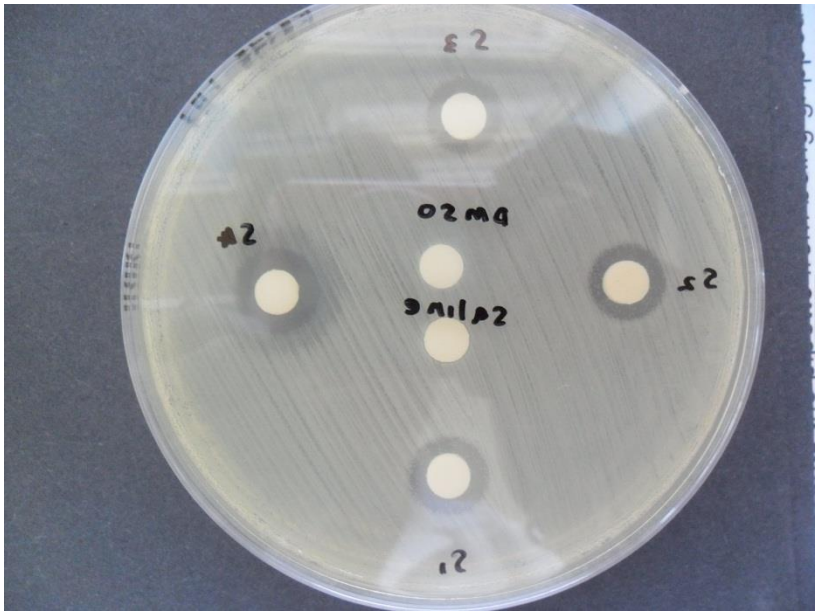
Bacterial lawn of MRSA080425

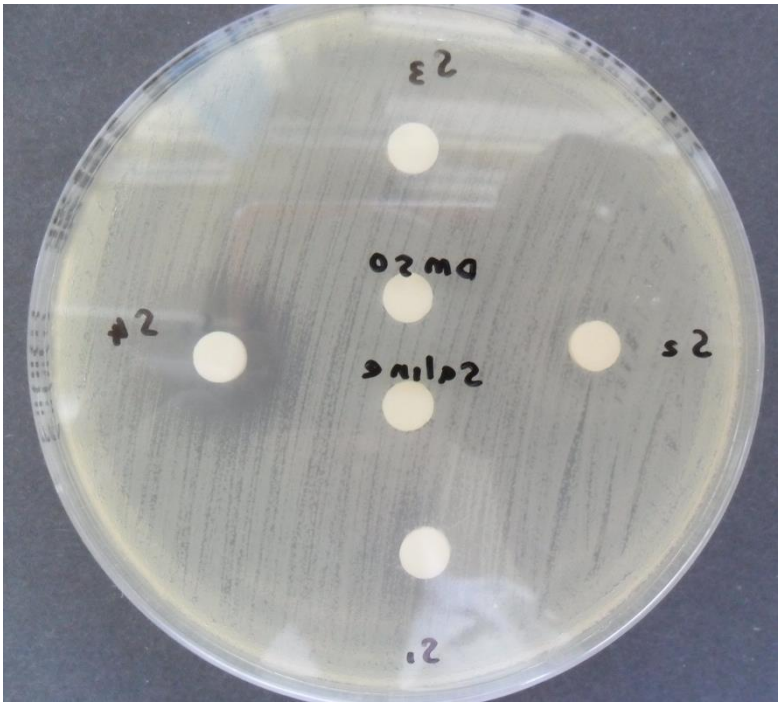


Bacterial lawn of MRSA08071

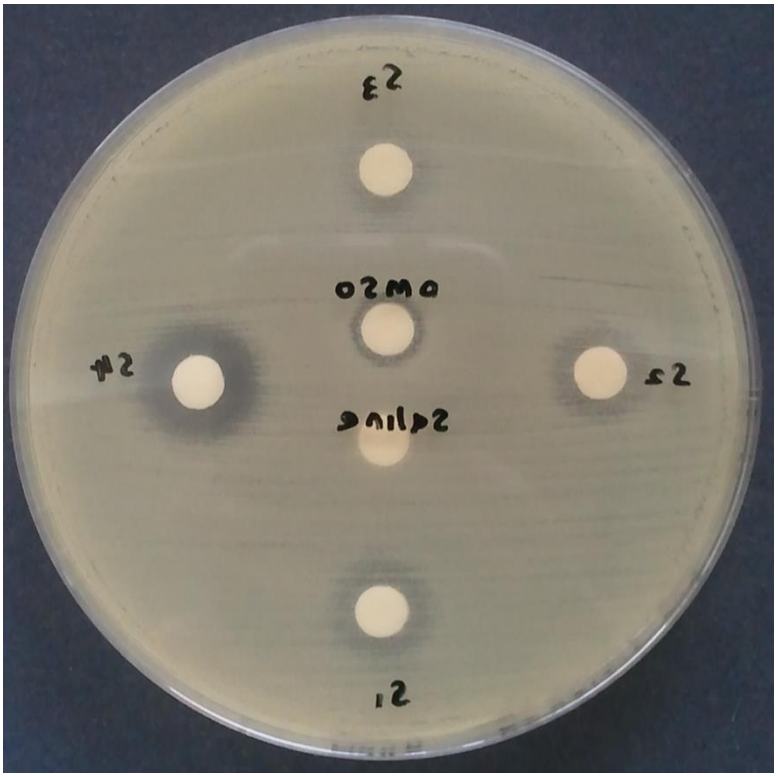


Bacterial lawn of KB88


Bacterial lawn of KB198

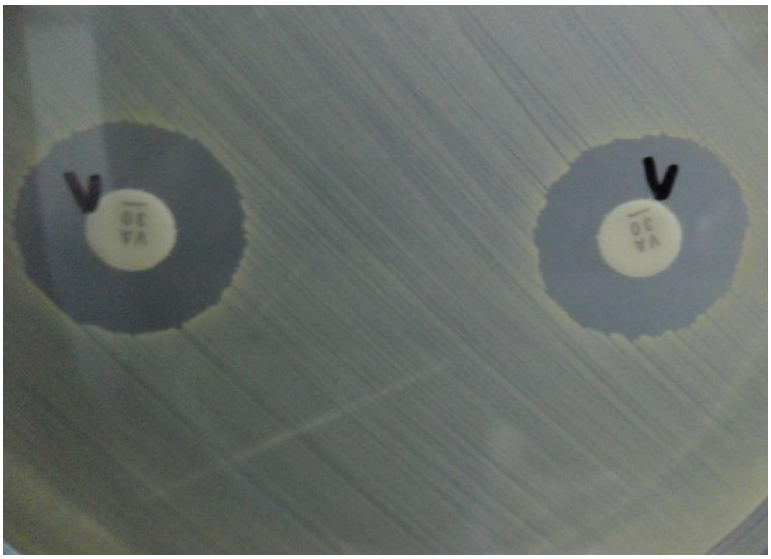


Bacterial lawn of AC06127

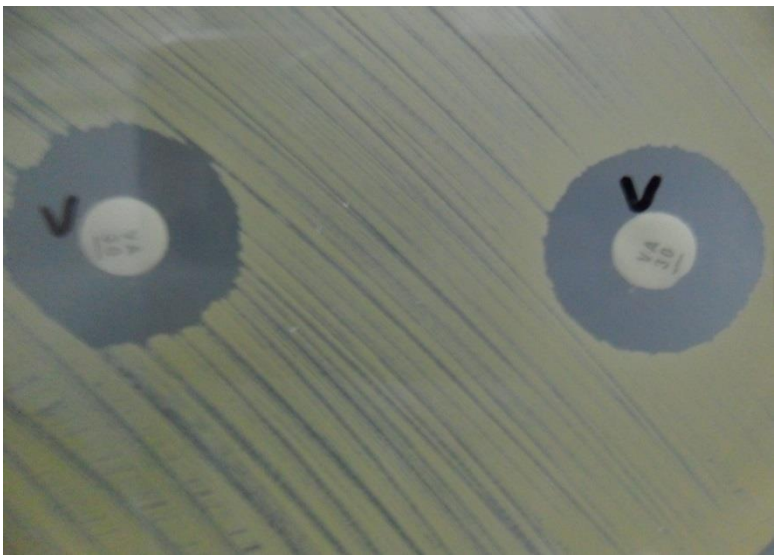


Bacterial lawn of AC08121

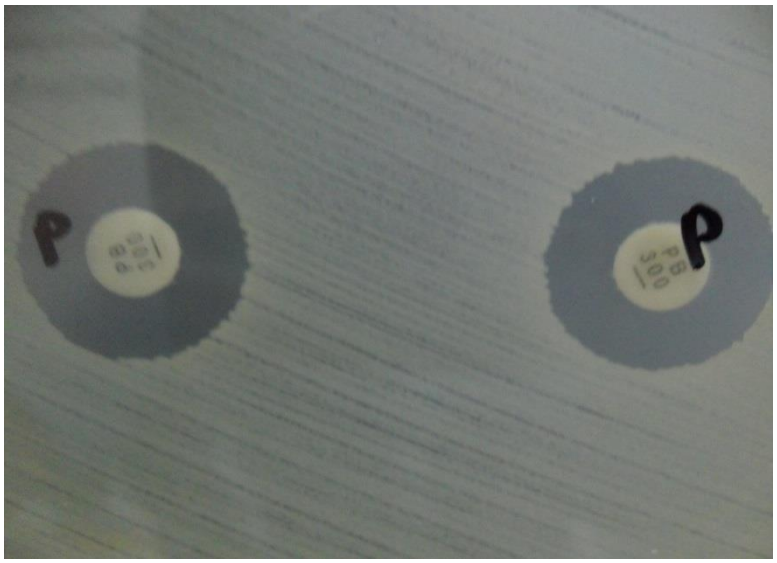
Antibiotic disks (300.0 units polymyxin B and 30.0 µg vancomycin)



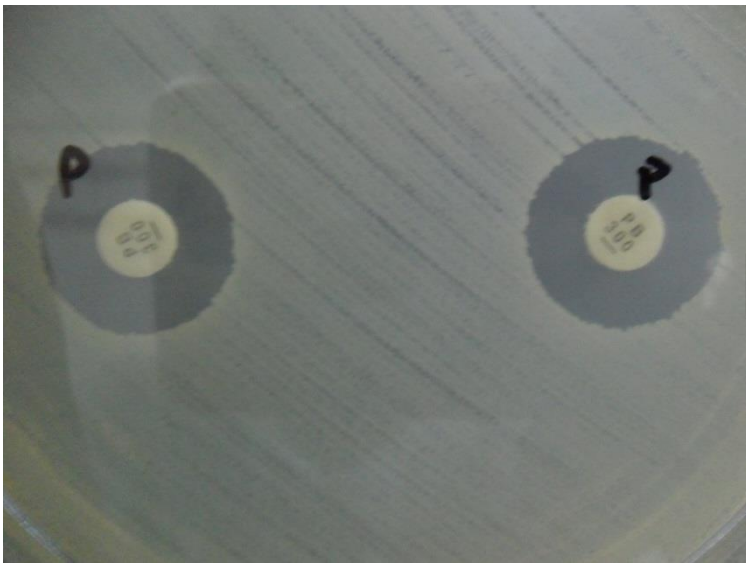
Bacterial lawn of MRSA080425, 30.0 µg vancomycin



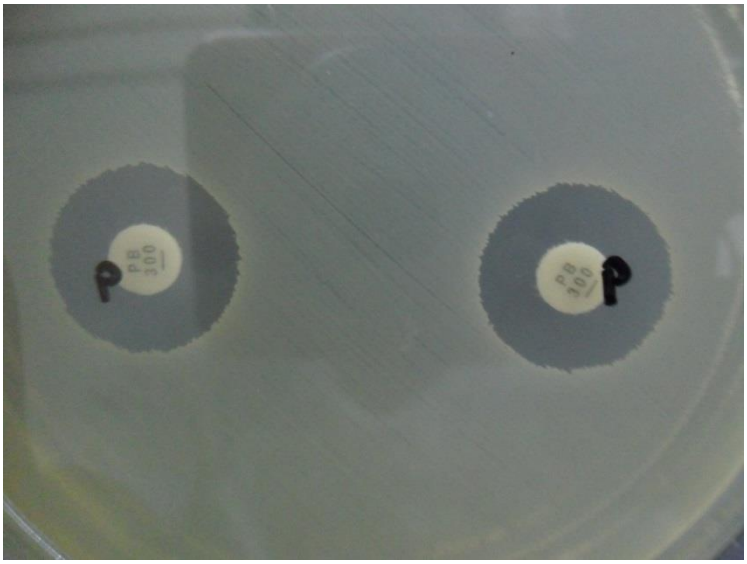
Bacterial lawn of MRSA08071, 30.0 µg vancomycin



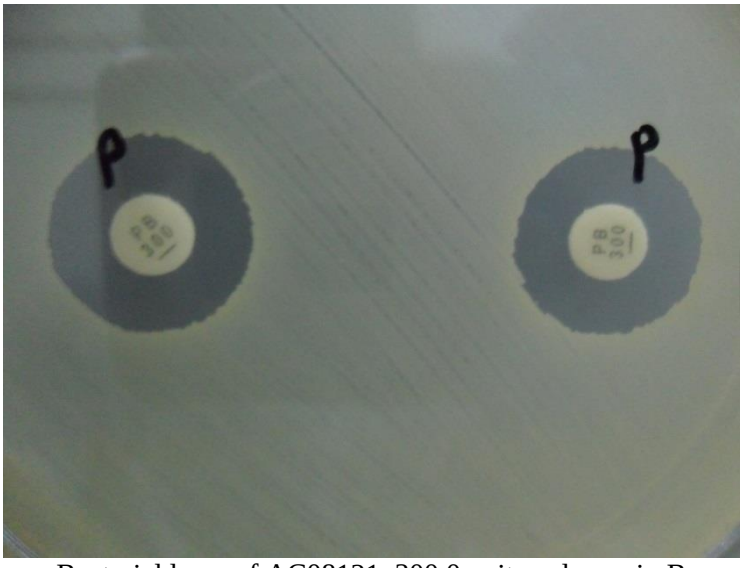
Bacterial lawn of KB88, 300.0 units polymyxin B



Bacterial lawn of KB198, 300.0 units polymyxin B



Bacterial lawn of AC06127, 300.0 units polymyxin B



Bacterial lawn of AC08121, 300.0 units polymyxin B

Time-kill assay

Raw data of bactericidal activity of compounds towards *K. pneumoniae* (strain 88) in time-kill assay

	KB88	LMA Cd-N_3					Polymyxin B	
Time (hours)	control	1X	Log reduction	2X	Log reduction	4X	0.5X	Log reduction
0	5.5	5.5	-	5.5	-	5.6	5.3	-
2	7.1	5.4	1.7	4.9	2.2	0.0	0.0	-
4	8.4	5.6	2.8	4.6	**3.8**	0.0	0.0	-
8	9.3	5.6	**3.7**	3.3	**6**	0.0	3.6	**5.7**
12	9.6	4.7	**4.9**	0.0	-	0.0	5.4	**4.2**
24	9.7	4.7	**5**	0.0	-	0.0	8.3	1.4

All values were tabulated in $\log_{10}$ CFU/ml. Bold numbers represent bactericidal activity as defined by reduction of ≥ 3 $\log_{10}$ CFU/ml. Different concentrations of MICs were represented by 1X, 2X, 4X and 0.5X

KB88 control

Time (hours)	ctrl (1)	ctrl (2)	ctrl CFU/ml (1)	ctrl CFU/ml (2)	MEAN CFU/ml
0	38	32	3.80×10^5	3.20×10^5	3.50×10^5
2	133	125	1.33×10^7	1.25×10^7	1.29×10^7
4	244	242	2.44×10^8	2.42×10^8	2.43×10^8
8	192	201	1.92×10^9	2.01×10^9	1.97×10^9
12	44	38	4.40×10^9	3.80×10^9	4.10×10^9
24	53	59	5.30×10^9	5.90×10^9	5.60×10^9

KB88 1X LMA Cd-N_3

Time (hours)	1X (1)	1X (2)	1X CFU/ml (1)	1X CFU/ml (2)	MEAN CFU/ml
0	43	26	4.30×10^5	2.60×10^5	3.45×10^5
2	28	22	2.75×10^5	2.20×10^5	2.48×10^5
4	44	34	4.40×10^5	3.40×10^5	3.90×10^5
8	46	36	4.60×10^5	3.55×10^5	4.08×10^5
12	43	59	4.30×10^4	5.90×10^4	5.10×10^4
24	47	53	4.70×10^4	5.25×10^4	4.98×10^4

KB88 2X LMA Cd-N_3

Time (hours)	2X (1)	2X (2)	2X CFU/ml (1)	2X CFU/ml (2)	MEAN CFU/ml
0	26	44	$2.55x10^5$	$4.35x10^5$	$3.45x10^5$
2	69	80	$6.85x10^4$	$8.00x10^4$	$7.43x10^4$
4	43	35	$4.25x10^4$	$3.50x10^4$	$3.88x10^4$
8	22	21	$2.20x10^3$	$2.10x10^3$	$2.15x10^3$
12	-	-	-	-	-
24	-	-	-	-	-

KB88 4X LMA Cd-N_3

Time (hours)	4X (1)	4X (2)	4X CFU/ml (1)	4X CFU/ml (2)	MEAN CFU/ml
0	43	31	$4.30x10^5$	$3.10x10^5$	$3.70x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

KB88 0.5X Polymyxin B

Time (hours)	0.5X (1)	0.5X (2)	0.5X CFU/ml (1)	0.5X CFU/ml (2)	MEAN CFU/ml
0	25.5	19	$2.55x10^5$	$1.90x10^5$	$2.23x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	63	1.5	$6.30x10^3$	$1.50x10^3$	$3.90x10^3$
12	31	17.5	$3.10x10^5$	$1.75x10^5$	$2.43x10^5$
24	33	44.5	$3.30x10^8$	$4.45x10^7$	$1.87x10^8$

Raw data of bactericidal activity of compounds towards MRSA (strain 080925) in time-kill assay

	MRSA080925			LMA Cd-N3				Vancomycin
Time (hours)	Control	1X	Log reduction	2X	Log reduction	4X	0.5X	Log reduction
0	5.6	5.6	-	5.7	-	5.1	5.8	-
2	6.4	4.8	1.6	0.0	-	0.0	4.9	1.5
4	7.6	0.0	-	0.0	-	0.0	4.0	**3.6**
8	8.9	0.0	-	0.0	-	0.0	2.7	**6.2**
12	9.3	0.0	-	0.0	-	0.0	0.0	-
24	9.6	0.0	-	0.0	-	0.0	0.0	-

All values were tabulated in $\log_{10}$ CFU/ml. Bold numbers represent bactericidal activity as defined by reduction of ≥ 3 $\log_{10}$ CFU/ml. Different concentrations of MICs were represented by 1X, 2X, 4X and 0.5X

MRSA080425 control

Time (hours)	ctrl (1)	ctrl (2)	ctrl CFU/ml (1)	ctrl CFU/ml (2)	MEAN CFU/ml
0	37	35	$3.70x10^5$	$3.50x10^5$	$3.60x10^5$
2	25	28	$2.50x10^6$	$2.80x10^6$	$2.65x10^6$
4	38	45	$3.80x10^7$	$4.50x10^7$	$4.15x10^7$
8	78	71	$7.80x10^8$	$7.10x10^8$	$7.45x10^8$
12	186	178	$1.86x10^9$	$1.78x10^9$	$1.82x10^9$
24	46	39	$4.60x10^9$	$3.90x10^9$	$4.25x10^9$

MRSA080425 1X LMA Cd-N_3

Time (hours)	1X (1)	1X (2)	1X CFU/ml (1)	1X CFU/ml (2)	MEAN CFU/ml
0	39	40	$3.85x10^5$	$4.00x10^5$	$3.93x10^5$
2	64	63	$6.40x10^4$	$6.25x10^4$	$6.33x10^4$
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

MRSA080425 2X LMA Cd-N_3

Time (hours)	2X (1)	2X (2)	2X CFU/ml (1)	2X CFU/ml (2)	MEAN CFU/ml
0	39	62	$3.85x10^5$	$6.15x10^5$	$5.00x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

MRSA080425 4X LMA Cd-N_3

Time (hours)	4X (1)	4X (2)	4X CFU/ml (1)	4X CFU/ml (2)	MEAN CFU/ml
0	18.5	5	$1.85x10^5$	$5.00x10^4$	$1.18x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

MRSA080425 0.5X Vancomycin

Time (hours)	0.5X (1)	0.5X (2)	0.5X CFU/ml (1)	0.5X CFU/ml (2)	MEAN CFU/ml
0	53	64	$5.30x10^5$	$6.40x10^5$	$5.85x10^5$
2	59	9	$5.85x10^4$	$8.50x10^4$	$7.18x10^4$
4	107	117	$1.07x10^4$	$1.17x10^4$	$1.12x10^4$
8	6	5	$6.00x10^2$	$5.00x10^2$	$5.50x10^2$
12	-	-	-	-	-
24	-	-	-	-	-

Raw data of bactericidal activity of compounds towards *A. baumannii* (strain 08121) in time-kill assay

	AC08121	LMA Cd-N_3						Polymyxin B
Time (hours)	control	1X	Log reduction	2X	Log reduction	4X	0.5X	Log reduction
0	5.6	5.9	-	5.7	-	5.8	5.8	-
2	7.0	4.7	2.3	**3.8**	**3.2**	0.0	0.0	-
4	8.4	**3.8**	**4.6**	0.0	-	0.0	0.0	-
8	8.9	0.0	-	0.0	-	0.0	0.0	-
12	9.1	0.0	-	0.0	-	0.0	0.0	-
24	9.5	0.0	-	0.0	-	0.0	0.0	-

All values were tabulated in $\log_{10}$ CFU/ml. Bold numbers represent bactericidal activity as defined by reduction of ≥ 3 $\log_{10}$ CFU/ml. Different concentrations of MICs were represented by 1X, 2X, 4X and 0.5X

AC08121 control					
Time (hours)	ctrl (1)	ctrl (2)	ctrl CFU/ml (1)	ctrl CFU/ml (2)	MEAN CFU/ml
0	38	35	$3.80x10^5$	$3.50x10^5$	$3.65x10^5$
2	95	97	$9.50x10^6$	$9.70x10^6$	$9.60x10^6$
4	22	24	$2.20x10^8$	$2.40x10^8$	$2.30x10^8$
8	78	79	$7.80x10^8$	$7.90x10^8$	$7.85x10^8$
12	130	127	$1.30x10^9$	$1.27x10^9$	$1.29x10^9$
24	36	32	$3.60x10^9$	$3.20x10^9$	$3.40x10^9$

AC08121 1X LMA Cd-N_3					
Time (hours)	1X (1)	1X (2)	1X CFU/ml (1)	1X CFU/ml (2)	MEAN CFU/ml
0	72	74	$7.20x10^5$	$7.40x10^5$	$7.30x10^5$
2	77	32	$7.70x10^4$	$3.20x10^4$	$5.45x10^4$
4	87	44	$8.70x10^3$	$4.40x10^3$	$6.55x10^3$
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

AC08121 2X LMA Cd-N_3

Time (hours)	2X (1)	2X (2)	2X CFU/ml (1)	2X CFU/ml (2)	MEAN CFU/ml
0	44	66	$4.35x10^5$	$6.60x10^5$	$5.48x10^5$
2	21	95	$2.10x10^3$	$9.50x10^3$	$5.80x10^3$
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

AC08121 4X LMA Cd-N_3

Time (hours)	4X (1)	4X (2)	4X CFU/ml (1)	4X CFU/ml (2)	MEAN CFU/ml
0	57	68	$5.65x10^5$	$6.75x10^5$	$6.20x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-

AC08121 0.5X Polymyxin B

Time (hours)	0.5X (1)	0.5X (2)	0.5X CFU/ml (1)	0.5X CFU/ml (2)	MEAN CFU/ml
0	59	61	$5.90x10^5$	$6.05x10^5$	$5.98x10^5$
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
12	-	-	-	-	-
24	-	-	-	-	-