Final Report

The effects of modified-antimicrobial peptides and in combination with conventional antibiotic against *Burkholderia pseudomallei* biofilm

Asst.Prof.Sakawrat Kanthawong

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Abstract

Project Code: MRG5980045

Project Title: The effects of modified-antimicrobial peptides and in combination with conventional antibiotic against *Burkholderia pseudomallei* biofilm

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Abstract:

Melioidosis is a severe disease caused by *Burkholderia pseudomallei*. The growing *B. pseudomallei* in biofilm has been reported to induce resistance to several conventional antibiotics, a phenomenon that may be related to relapse cases in melioidosis patients. The aim of this study was to compare the antibiofilm activity of human cathelicidin antimicrobial peptides (LL-37, LL-31) and theirs D-enantiomeric form (D-LL-37, D-LL-31) with ceftazidime (CAZ) against 3 isolates of *B. pseudomallei* 1026b, H777 and biofilm mutant M10. Antibiofilm assay revealed that, a truncated variant of LL-37 lacking the six C-terminus residues in D-enatiomeric form, D-LL-31, revealed a strongest killing activity against all isolates of *B. pseudomallei* in dose-dependent manner. Moreover, the IC_{50} values of D-LL-31 was further investigated compared with CAZ against biofilm form of those isolates. The results showed that IC_{50} values of D-LL-31 were ranging from 1.07-5.55 μM, while, IC_{50} values of CAZ was higher than D-LL-31, about 45, 60, and 240 folds against biofilm form of *B. pseudomallei* M10, H777, and 1026b, respectively. In addition, D-LL-31/CAZ combination showed synergistic effects on biofilm of all isolates tested in static condition. Moreover, the D-LL-31/CAZ combination was applied to biofilm of *B. pseudomallei* under flow conditions and caused disruption of the biofilms. These results indicate that D-LL-31/CAZ combination not only disrupted preformed-biofilm but also exhibited potent killing activity against *B. pseudomallei* in biofilm form. Thus D-LL-31/CAZ combination

should be considered to enhance the efficacy of currently antibiotic against *B. pseudomallei* which is finally benefited to melioidosis patients.
Keywords: Anti-biofilm peptides, LL-37, LL-31, D-enantiomer peptide, *Burkholderia pseudomallei*

บทคัดย่อ

เมลิออยโดสิส เป็นโรคร้ายแรงที่เกิดจากการติดเชื้อ *Burkholderia pseudomallei* มีการรายงานว่าการสร้างฟิล์มชีวภาพของเชื้อ *B. pseudomallei* ส่งผลต่อการดื้อต่อยาปฏิชีวนะและยังเกี่ยวข้องกับการกลับเป็นซ้ำในผู้ป่วยโรคเมลิออยโดสิส งานวิจัยนี้มีวัตถุประสงค์เพื่อทดสอบประสิทธิภาพของเพปไทด์ต้านจุลชีพในกลุ่ม Cathelicidin (LL-37, LL-31) และเพปไทด์ดัดแปลง (D-LL-37, D-LL-31) ในการทำลายเชื้อ *B. pseudomallei* ในสภาวะที่สร้างฟิล์มชีวภาพ 3 สายพันธุ์ ได้แก่ *B. pseudomallei* 1026b, H777 และ biofilm mutant M10 โดยเปรียบเทียบกับยาปฏิชีวนะ ceftazidime ผลการทดลองพบว่าเปปไทด์ต้านจุลชีพ LL-37 ชนิดที่ถูกดัดแปลงโดยการใช้กรดอะมิโนในรูป D-enantiomer แทนและมีการตัดกรดอะมิโนที่ปลายด้าน C ออก 6 ตัว D-LL-31 มีประสิทธิภาพสูงที่สุดในการทำลายเชื้อในสภาวะที่สร้างฟิลม์ชีวภาพ แบบเปลี่ยนแปลงความเข้มข้น ยิ่งไปกว่านั้นการศึกษาหาค่าความเข้มข้นของเปปไทด์ต้านจุลชีพและยาปฏิชีวนะ ceftazidime ที่ทำลายเชื้อได้ 50% (Inhibition concentration 50) พบว่า ค่า IC_{50} ของ D-LL-31 ต่อเชื้อทั้ง 3 สายพันธุ์อยู่ในช่วง 1.07-5.55 ไมโครโมลาร์ ขณะที่ IC_{50} ของยาปฏิชีวนะ ceftazidime สูงกว่า D-LL-31 อยู่ที่ 40, 60 และ 240 เท่า ต่อเชื้อ *B. pseudomallei* 1026b, H777 และ biofilm mutant M10 ตามลำดับ นอกจากนี้พบว่าเปปไทด์ต้านจุลชีพ D-LL-31 เสริมฤทธิ์กับยาปฏิชีวนะ ceftazidime ในการลอปริมาณของพอลิแซ็กคาไรด์ (polysaccharides) ของฟิล์มชีวภาพ *B. pseudomallei* ภายใต้แรงเฉือนโดยเครื่อง BioFlux ได้ดีที่สุดเมื่อเปรียบเทียบยาปฏิชีวนะหรือเปปไทด์ต้านจุลชีพเพียงอย่างเดียว จากผลการทดลองทั้งหมดแสดงให้เห็นว่า D-LL-31 เป็นเปปไทด์ต้านจุลชีพที่มีประสิทธิภาพสูงสุดในการทำลายเชื้อ *B. pseudomallei* ในสภาวะที่สร้างฟิล์มชีวภาพ ทั้งแบบใช้อย่างเดียวและใช้ร่วมกับยาปฏิชีวนะ ceftazidime ดังนั้น D-LL-31 จึงควรนำไปศึกษาต่อเพื่อพัฒนาเป็นสารต้านจุลชีพชนิดใหม่ที่มีประสิทธิภาพในการต่อต้านเชื้อ *B. pseudomallei* ในสภาวะที่สร้างฟิล์มชีวภาพสำหรับผู้ป่วยโรคเมลิออยโดสิส

คำสำคัญ: เปปไทด์ต้านฟิล์มชีวภาพ, LL-37, LL-31, D-enantiomer peptide, *Burkholderia pseudomallei*

Exclusive summary

Introduction to Research

Melioidosis is a term describing a collection of serious and often-fetal diseases in humans and animals, arising from infection by *Burkholderia pseudomallei*. This disease is endemic in rainy season and mostly infects people who have direct contact with contaminated-wet soils and have an underlying predisposition to infection such as diabetes mellitus. *B. pseudomallei* infection has been reported in many countries including northern Australia, Papua New Guinea, Southeast Asia, most of the Indian subcontinent and southern China, Hong Kong and Taiwan. Northeast Thailand, northern Australia, Singapore and parts of Malaysia are currently recognized as 'highly endemic' locations where many cases are diagnosed each year.

B. pseudomallei is intrinsically resistant to many antibiotics, including penicillin, first- and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides, but is usually susceptible to amoxicillin/clavulanic acid (AMC), chloramphenicol (CL), doxycycline (DOX), trimethoprim/sulfa-methoxazole (SXT), ureidopenicillins, ceftazidime (CTZ) and carbapenems. However, CTZ or AMC-resistant *B. pseudomallei* have emerged, ultimate leading to treatment failure. Moreover, *B. pseudomallei* was reported to form biofilm and microcolonies. The growing *B. pseudomallei* in biofilm has been reported to induce resistance to several conventional antibiotics, a phenomenon that may be related to relapse cases in melioidosis patients. With the increasing development of antibiotic resistance, it is necessary to search for novel anti-infective and anti-biofilm agents against *B. pseudomallei*.

In view of the increasing resistance of bacteria and fungi to the commonly used antibiotics, there is growing interest in peptide antibiotics, driven by awareness of the potential therapeutic applications of these peptides or their synthetic analogues. Most peptides exert their antimicrobial effects by interacting with and destabilizing the microbial membrane, leading to cell death. A wide variety of human proteins and peptides have antimicrobial activity such as cathelicidin LL-37.

The inhibitory effects of LL-37 on biofilm formation as well as the initial attachment of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* have been reported (Hell E, et al, 2010, Overhage J, et al., 2008) Recently, we found that LL-37 and their derivative LL-31 have strong killing activity against *B. pseudomallei* biofilm (Kanthawong S et al, 2012). However, L-

amino acids of peptides (natural form) were very sensitive to enzymatic degradation by proteases in serum, and furthermore, these problems created difficulty in developing peptidic therapeutic agents. To overcome these problems, D-amino acids were substituted for the L-amino acids of antimicrobial peptides. It has been reported that this substitution can improve the antimicrobial activity and proteolytic stability (Hong et al., 1999). Thus, the substitution of D-amino acid could be a strategy for developing proteases-resistant antimicrobial peptides for clinical therapeutic use (Jung et al., 2007). Moreover, the antimicrobial peptide from venom of honey bee, melittin exhibited the antimicrobial activity against Gram-negative bacteria and showed it synergistic activity in combination with various antibiotics (Dosler *et al*., 2015).

In this study, the effect of human cathelicidin peptide (LL-37 and LL-31) and theirs modified peptides (D-LL-37 and D-LL31) were observed in the combination with conventional antibiotic for melioidosis treatment; ceftazidime (CAZ) against *B. pseudomallei* biofilm. The information obtained from this study may merit further investigation and exploration of modified antimicrobial peptides for the therapy of melioidosis which is finally benefited to melioidosis patients.

Literature review

1. Melioidosis

Melioidosis is a term describing a collection of serious and often-fetal diseases in humans and animals, arising from infection by *Burkholderia pseudomallei*. This disease is endemic in rainy season (Chaowagul et al., 1989) and mostly infects people who have direct contact with contaminated-wet soils and have an underlying predisposition to infection such as diabetes mellitus (Currie et al., 2000). *B. pseudomallei* infection has been reported in many countries including northern Australia, Papua New Guinea, Southeast Asia, most of the Indian subcontinent and southern China, Hong Kong and Taiwan. Northeast Thailand, northern Australia, Singapore and parts of Malaysia are currently recognized as ‘highly endemic’ locations where many cases are diagnosed each year (Wiersinga et al., 2012) (Fig 1). *B. pseudomallei* can be isolated from moist soil and water in endemic regions; inoculation of compromised skin, such as cuts, abrasion or wounds is thought to occur from rice paddies, palm fields, monsoon

drains, gardens and playground of endemic areas (Ellison et al., 1969; Leelarasamee et al., 1989).

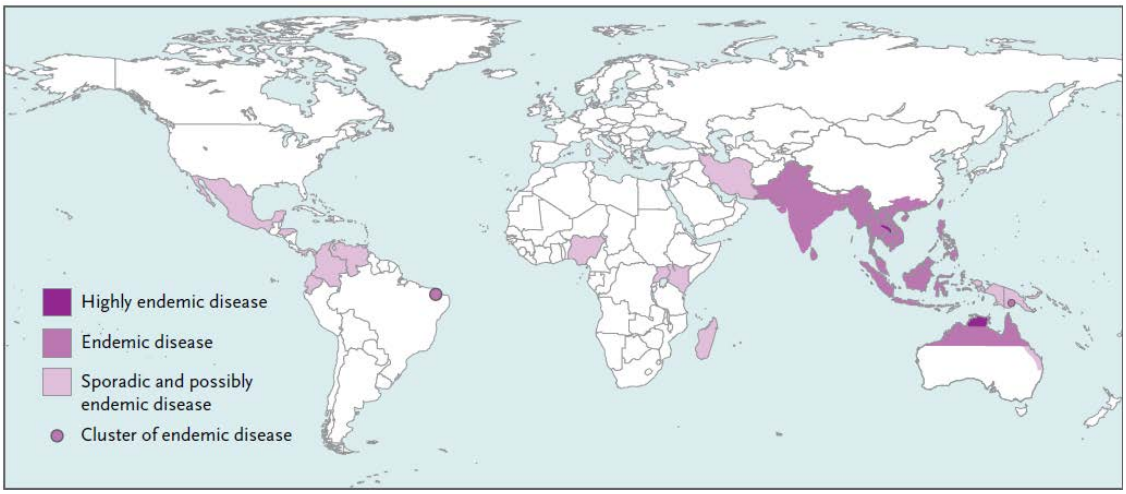


Figure 1 Global distribution of Melioidosis (Wiersinga et al., 2012)

2. *Burkholderia pseudomallei*

Burkholderia pseudomallei is a causative agent of melioidosis and has previously been named *Bacillus whitmori* (Whitmore, 1913). It is a motile, facultative anaerobic, non-spore forming, Gram -negative bacillus. *B. pseudomallei* is a free-living organism that can isolate from soil, clay and water in endemic regions (Leelarasamee et al., 1989). This bacteria is intrinsically resistant to many antibiotics, including penicillin, first- and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides, but is usually susceptible to amoxicillin/clavulanic acid (AMC), chloramphenicol (CL), doxycycline (DOX), trimethoprim/sulfa-methoxazole (SXT), ureidopenicillins, ceftazidime (CTZ) and carbapenems (Cheng et al., 2005; White, 2003). However, CTZ or AMC-resistant *B. pseudomallei* have emerged, ultimate leading to treatment failure (Thibault et al., 2004). Moreover, *B. pseudomallei* was reported to form biofilm and microcolonies. The growing *B. pseudomallei* in biofilm has been reported to induce resistance to several conventional antibiotics, a phenomenon that may be related to relapse cases in melioidosis patients. With the increasing development of antibiotic resistance, it is necessary to search for novel anti-infective and anti-biofilm agents against *B. pseudomallei*.

3. Antimicrobial peptides

Innate immunity forms a first line of defense against infection by pathogenic microorganisms. An important part of innate immunity is a group of peptides with antimicrobial activity (De Smet et al., 2005). Then, a diversity of peptides with antimicrobial activity has been discovered throughout all living organisms, from bacteria to mammals and databases have been established (http://aps.unmc.edu/ AP/main.html) (Brahmachary et al., 2004). Most peptides exert their antimicrobial effects by interacting with and destabilizing the microbial membrane, leading to cell death. However, other modes of action are reported from several peptides, including inhibit synthesis of specific membrane proteins, arrest of DNA synthesis, breakage of single-strand DNA and interaction with DNA (De Smet et al., 2005). Example of human antimicrobial peptide is described in the following paragraph

Human cathelicidin peptide

Cathelicidins are a family of antimicrobial peptides derived from proteins. This peptide is expressed in leukocytes such as neutrophils, monocytes, NK cells, T cells and B cells, and in epithelial cells of the testis, skin, and the gastrointestinal and respiratory tract (De Smet *et al*., 2005). This antimicrobial domain varies in length (12-100 amino acid residues), sequence, and function. In humans, only one Cathelicidin has been characterized, LL-37 (Leucine-leucine 37), this peptide is derived by proteolytic activity of proteinase 3 from the C-terminal end of the human CAP18 protein (hCAP18) (Sorensen *et al*., 2001). This antimicrobial domain only acquires antimicrobial activity after cleavage from the N-terminal cathelin domain, the cathelin domain protects host tissue against cytotoxicity of the cathelicidin peptides (Zaiou *et al.*, 2003) (Fig 2).

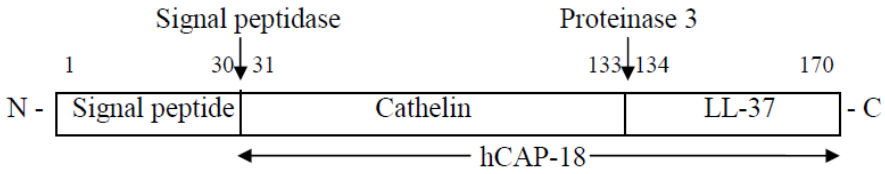


Figure 2 Schematic presentation of LL-37. After expression of cathelicidins, the signal peptide is cleaved by signal peptidase to yield hCAP-18 consists of an N-terminal cathelicidin domain and C-terminal domain, LL-37, which is released as the mature antimicrobial peptide by proteolytic activity of Proteinase 3 (den Hertog *et al*., 2006)

The inhibitory effects of LL-37 on biofilm formation as well as the initial attachment of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* have been reported (Hell E, et al, 2010, Overhage J, et al., 2008) Recently, we found that LL-37 and their derivative LL-31 have strong killing activity against *B. pseudomallei* biofilm (Kanthawong S et al, 2012). However, L- amino acids of peptides (natural form) were very sensitive to enzymatic degradation by proteases in serum, and furthermore, these problems created difficulty in developing peptidic therapeutic agents. To overcome these problems, D-amino acids were substituted for the L-amino acids of antimicrobial peptides. It has been reported that this substitution can improve the antimicrobial activity and proteolytic stability (Hong et al., 1999). Thus, the substitution of D-amino acid could be a strategy for developing proteases-resistant antimicrobial peptides for clinical therapeutic use (Jung et al., 2007).

Objective

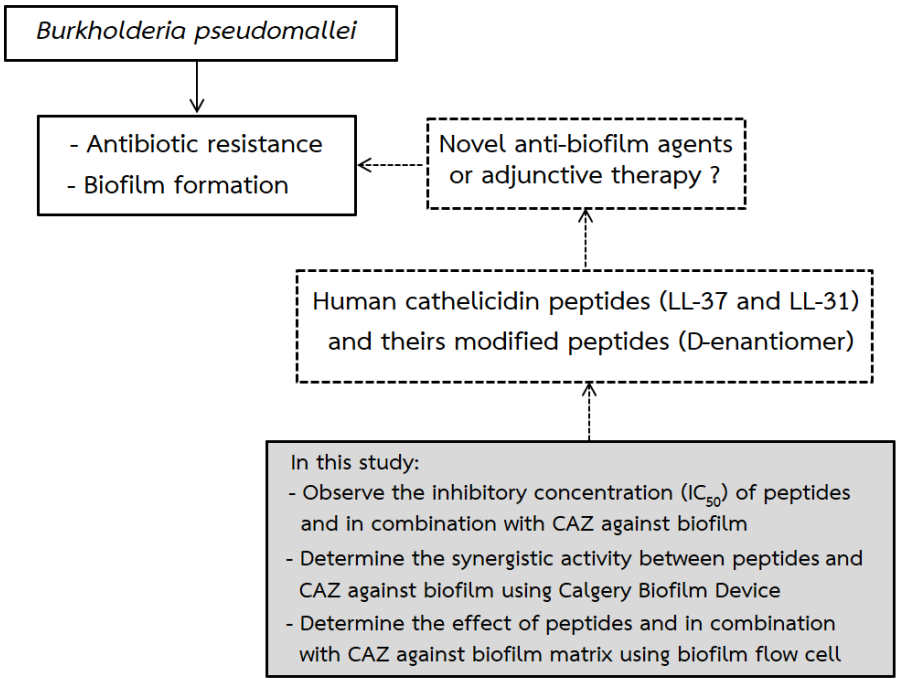
1. To observe the inhibitory concentration (IC_{50}) of modified antimicrobial peptides and conventional antibiotic (Ceftazidime) against *B. pseudomallei* biofilm.

2. To determine the synergistic activity between modified antimicrobial peptides and conventional antibiotic (Ceftazidime) against *B. pseudomallei* biofilm using Calgary Biofilm Device.

3. To determine the effect of modified antimicrobial peptides and in combination with conventional antibiotic (Ceftazidime) against *B. pseudomallei* biofilm matrix using biofilm flow cell.

Research methodology

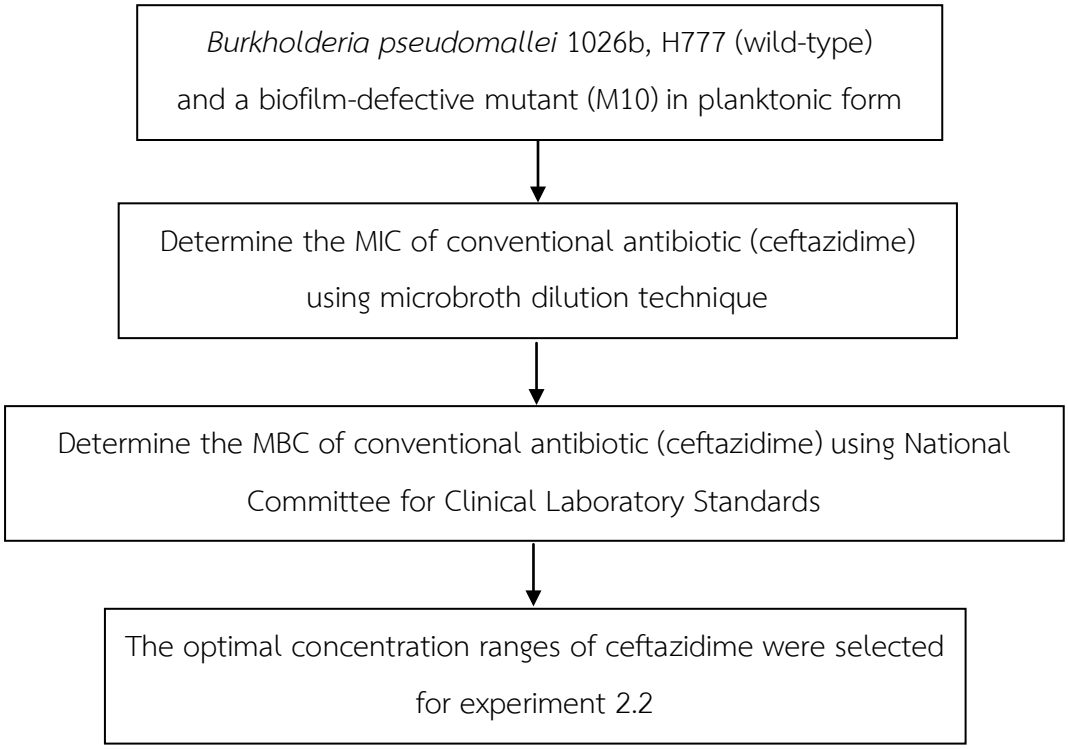
1. Conceptual framework



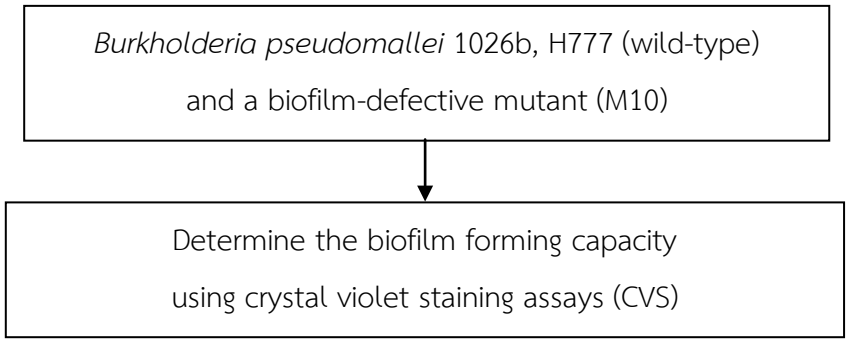
2. Experimental design

In this study, the effect of human antimicrobial peptides (LL-37 and LL-31) and theirs modified antimicrobial peptides (D-LL-37 and D-LL-31) and in combination with conventional antibiotic (ceftazidime) were determined against *B. pseudomallei* in static and flow biofilm condition. The experimental design for this project is shown below.

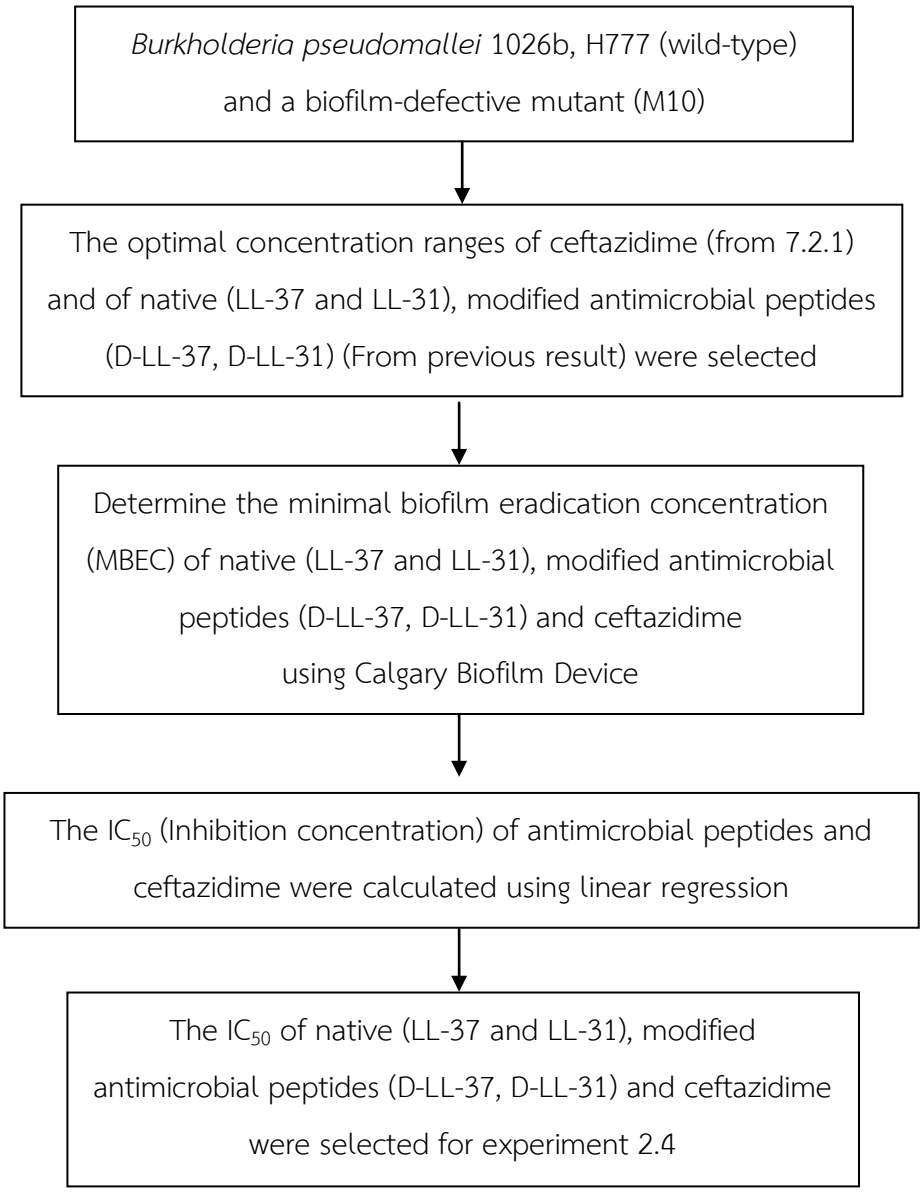
2.1. Determine the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of conventional antibiotic (ceftazidime)



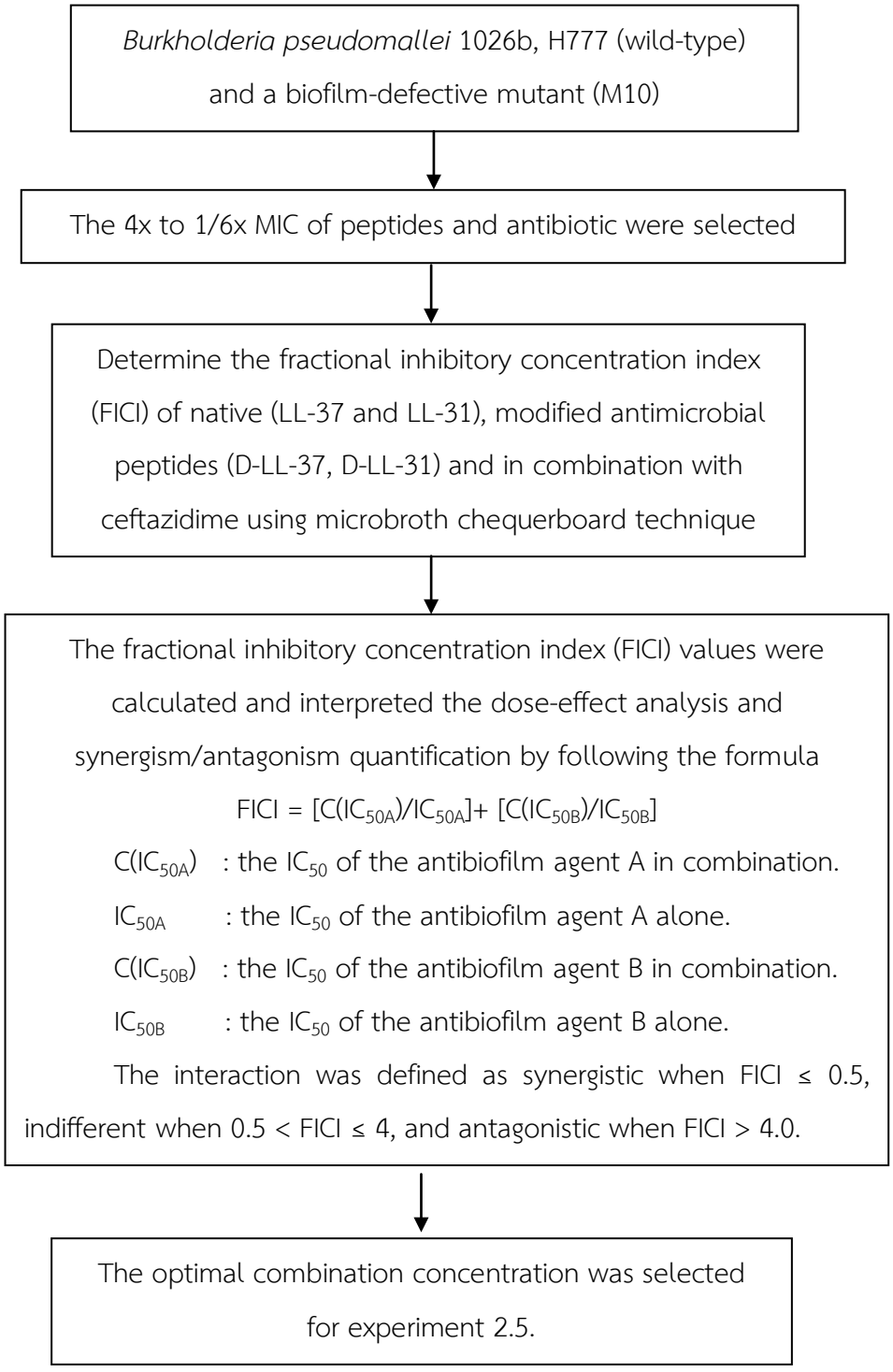
2.2. Determine the biofilm forming capacity of *B. pseudomallei* 1026b, H777 (wild-type) and a biofilm-defective mutant (M10)



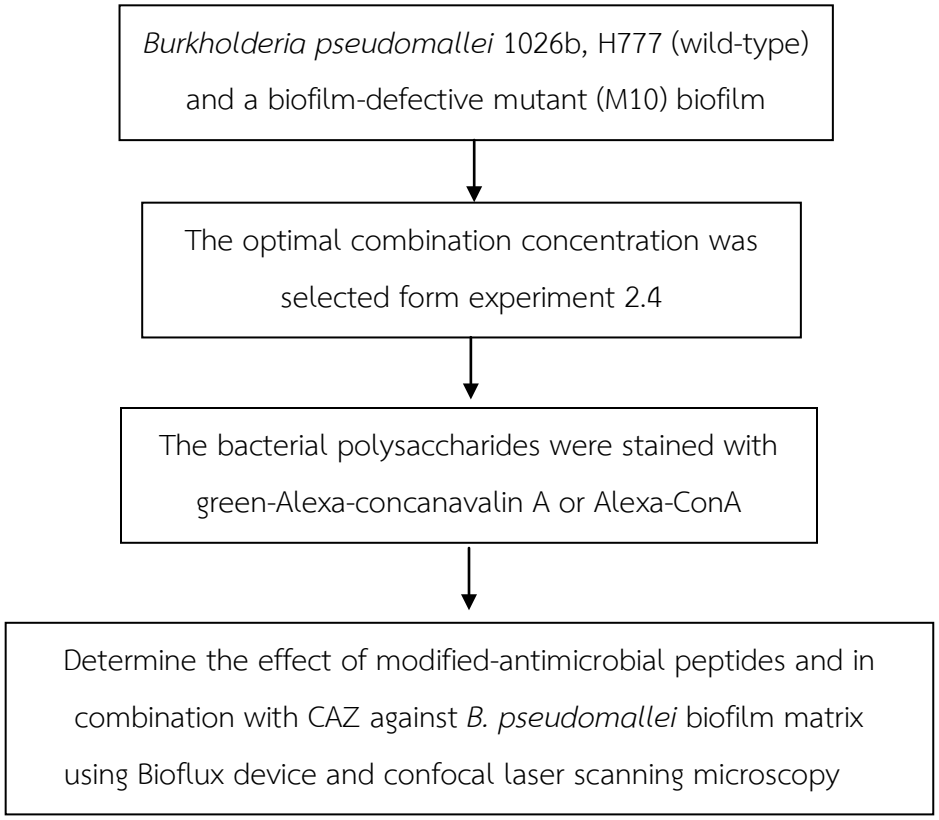
2.3. Determine the IC_{50} (Inhibition concentration) of modified antimicrobial peptides and ceftazidime against *B. pseudomallei* biofilm using Calgary Biofilm Device



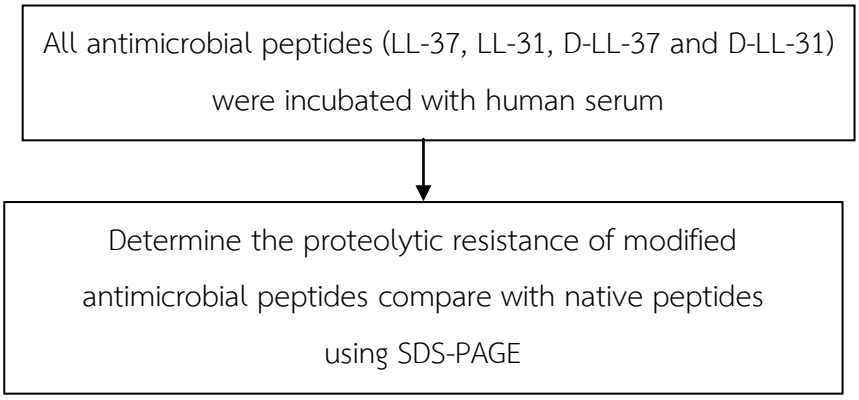
2.4. Determine the fractional inhibitory concentration index (FICI) of modified antimicrobial peptides and in combination with conventional antibiotic against *B. pseudomallei* biofilm



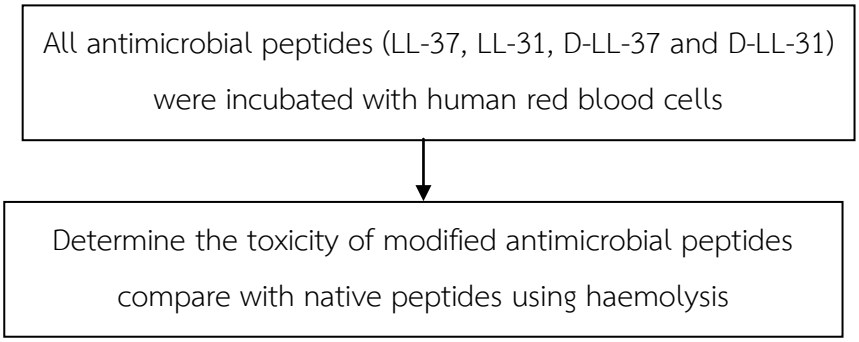
2.5. Determine the effect of modified antimicrobial peptides and in combination with conventional antibiotic against *B. pseudomallei* biofilm matrix



2.6. Determine the proteolytic resistance of modified antimicrobial peptides



2.7. Determine the toxicity of antimicrobial peptides to hRBCs



3. Materials

3.1. Peptide synthesis, purification

The cathelicidin antimicrobial peptide (LL-37 and LL-31) and theirs modified form (D-LL-37 and D-LL-31) were synthesized by fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry using a MilliGen 9050 Peptide Synthesizer (MilliGen/BioSearch, Bedford, MA) as described previously (van der Kraan *et al*., 2004). All peptides were of ≥95% purity as determined by reverse-phase high-performance liquid chromatography (RP-HPLC) (Jasco Corp., Tokyo, Japan). The sequences of all peptides investigated are shown in Table 1.

Table 1 The amino acid sequences of antimicrobial peptides

Peptides	sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
D-LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
LL-31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL
D-LL-31	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNL

3.2. Bacterial strains and growth conditions

Burkholderia pseudomallei 1026b, H777 (wild-type) and a biofilm-defective mutant (M10) werer selected in this study (Table 2). All bacterial strains were initially grown on Luria–Bertani (LB) agar or LB agar containing 15 μg/ml tetracycline (for biofilm mutants) then a single colony was inoculated into 10 mL of modified Vogel and Bonner's medium (MVBM), which is a chemically defined medium used to facilitate the formation of biofilm (Lam *et al.*,

1980) and incubated at 37 °C, 200 rpm for 16 h. Cells were re-suspended to the densities as indicated below.

Table 2 Bacterial strains were used in this study

Bacterial strains	Source	References
1026b	Blood	DeShazer, D. *et al*, 1999
H777	Blood	Taweechaisupapong S. *et al*, 2005
M10	Biofilm mutant	Taweechaisupapong S. *et al*, 2005

4. Methods

4.1 MIC and MBC determinations

Minimum inhibitory concentrations (MIC) of conventional antibiotic (ceftazidime; CAZ) against three *B. pseudomallei* strains were determined using microbroth dilution technique as previously described with some modifications (Steinberg et al., 1997). Briefly, serial two-fold dilutions of antibiotic (0-512 µg/ml) were prepared in 96 well polypropylene plates. Each well was inoculated with 100 µl of bacterial suspension to give final concentration of $5x10^5$ cfu/ml. The plates were incubated at 37 °C for 18-20 h and the MIC were observed as the lowest concentration of peptides or antibiotic producing complete inhibition of visible growth.

Minimal bactericidal concentrations (MBC) of antibiotic were determined at the end of the incubation period by collecting 10 µl samples from each well that showing no visible growth and plate on tryptic soy agar (TSA). The bacterial colonies were counted after 16-18 h incubation at 37 °C. The MBC were observed as the lowest concentration of antibiotic producing at least 99.9% killing of the initial inoculum (National Committee for Clinical Laboratory Standards, 1999). Each assay was performed on three separate occasions, with triplicate determinations each time.

4.2 Determine the biofilm forming capacity of *B. pseudomallei*

The biofilm forming capacity of *B. pseudomallei* 1026b, H777 (wild-type) and a biofilm-defective mutant (M10) were determined. Three strains of B. *pseudomallei* were cultured and then diluted to give $1x10^5$ CFU/200 µl in MVBM. The bacterial suspension was added to each well of 96-well plate. After incubation, the biofilm mass was observed using

microtiter dish biofilm formation assay (O'toole, 2001) with some modifications. Briefly, the media was removed and washed 3 times with 250 µl phosphate buffer saline (PBS) solution to remove unattached bacteria. 200 µl of 99% methanol was added per well for fixation 15 min and then aspirate. Wells were stained with 200 µl of 0.1% crystal violet for 5 min. Excess stains were gently rinsed off with tap water and re-solubilized in 125 µl of 30% acetic acid. The plate was incubated at room temperature for 10-15 min. The optical density at 550 nm was measured using 30% acetic acid in water as the blank. Each assay was performed on three separate occasions, with triplicate determinations each time.

4.3. Determine the IC_{50} (Inhibition concentration) of modified antimicrobial peptides and conventional antibiotic against *B. pseudomallei* biofilm using Calgary Biofilm Device

B. pseudomallei 1026b, H777 and M10 were grown as biofilm using a modification of the Calgary Biofilm Device. This method of biofilm culture has been previously validated and shown to be reproducible (Ceri *et al.*, 1999). Each isolate (ca. 10^7 CFU/mL) was placed into a 96-well microtitre plate, then a transferable solid-phase (TSP) pin lid (NUNC, Roskilde, Denmark) was placed into the microtitre plate, which was incubated in an orbital incubator at 37 °C, 150 rpm, for 24 h. Following the period of incubation, biofilms were rinsed by inserting the TSP pin lids into microtitre plates containing 200 µL/well of saline for 1 min to remove loosely adherent cells. The TSP pin lid with grown biofilms was placed into a new 96-well microtitre plate containing antimicrobial peptides (LL-37, LL-31, D-LL-37 and D-LL-31) and ceftazidime (CAZ) and then incubated at 37 °C for 12 h. Wells without antimicrobial agent were also included to control for growth due to the addition of media. After that, the TSP pin lid was removed again and rinsed with saline in another microtitre plate. After rinsing, the TSP pin lid was placed onto the new microtitre plate containing Muller–Hinton broth (recovery plate) and biofilms were disrupted from the TSP pin surface using an ultrasonic cleaner (SONOREX; Bandelin, Berlin, Germany) for 5 min. Viability of the biofilm bacteria was determined by plate counts. Colonies were counted after 24 h incubation at 37 °C. The percentage killing effects of each concentration of antimicrobial peptide and in combination with antibiotic was calculated using the formula [1 – (CFU sample/CFU control)] × 100%. Each assay was performed on three

separate occasions, with triplicate determinations each time. The IC_{50} (inhibitory concentration) value is the concentration that exhibit 50% killing activity of each agent against bacterial biofilm, was calculated by linear regression of plots (Saleem *et al*, 2014).

4.4. Determine the fractional inhibitory concentration index (FICI) of modified antimicrobial peptides and in combination with conventional antibiotic against B. pseudomallei biofilm.

The IC_{50} value of each antimicrobial agent was selected from previous experiment, the synergistic effects between CAZ and the most effective antimicrobial peptide (D-LL-31) were performed by broth microdilution checkerboard technique, which is the most widely used technique to test antimicrobial combinations with some modification (Moody, 1992). Briefly, the bacterial cell culture was diluted to provide a final inoculum density approximately 10^7 CFU/ml in MVBM, then diluted bacteria suspension was added to 96-well microtiter plate (Nunclon™), medium alone was used as the negative control. The plates were incubated for 24 hours at 37°C then the biofilm on the lids were washed by sterilized distilled water 3 times. The peg lids were transferred to a standard 96-well plate that contained serial dilution of CAZ and D-LL-31 diluted in 1 mM PPB at concentrations range from 4 times to 1/16 times of IC_{50} of each agent and antimicrobial agent-free well was also used as control for bacterial growth by adding only the PPB, then plates were incubated at 37°C for 24 hours. Subsequently, the lids were washed again and placed in a new 96-well plate that contain MHB (recovery media) (Himedai®), then the biofilm on the CBD pegs were removed by sonication (SONOREX) for 5 min and changed the new cover plate. Bacterial viability in each well of the bacterial challenged with antibiofilm agents was observed by plate count technique. The fractional inhibitory concentration index (FICI) values were calculated and interpreted the dose-effect analysis and synergism/antagonism quantification (Odds, 2003) by following the formula

$$FICI = [C(IC_{50A})/IC_{50A}]+ [C(IC_{50B})/IC_{50B}]$$

$C(IC_{50A})$: the IC_{50} of the antibiofilm agent A in combination.

IC_{50A} : the IC_{50} of the antibiofilm agent A alone.

$C(IC_{50B})$: the IC_{50} of the antibiofilm agent B in combination.

IC_{50B} : the IC_{50} of the antibiofilm agent B alone.

The interaction was defined as synergistic when FICI ≤ 0.5, indifferent when 0.5 < FICI ≤ 4, and antagonistic when FICI > 4.0.

4.5. Determine the effect of modified antimicrobial peptides and in combination with conventional antibiotic against *B. pseudomallei* biofilm matrix

The effect of D-LL-31, the most effective antimicrobial peptide and in combination with CAZ against *B. pseudomallei* biofilm in flow condition was observed using BioFlux device (Fluxion Biosciences, South San Francisco, CA) to cultivate biofilm under controllable shear force. The microfluidic channels were primed with growth medium from the outlet ports (waste well) until the channels were completely filled. *B. pseudomallei* 1026b was diluted to 10^8 CFU/ml in MVBM and added to outlet well by pumping 2 dyne/cm^2. The bacterial cells were allowed to colonize for 3 hours at 37°C. The fresh media was pumped for inlet wells through the channel to outlet wells under the shear force condition at the flow rate of 0.5 dyne/cm^2 for 21 hours. Then, D-LL-31 alone and in combination with CAZ were applied for 15 hours at flow rate of 0.5 dyne/cm^2. The anti-biofilm activities were determined by visualization of biofilm formation process in real-time under flow to compare between control and agent-treated channels. After the biofilm were treated with agents, the bacterial polysaccharides were stained with green-Alexa-concanavalin A or Alexa-ConA with 0.5 dyne/cm^2 for 1 hour and observed the results by using confocal laser scanning microscopy (CLSM, FluoView 1000, OLYMPUS, Tokyo, Japan). Each assay was performed on two separate occasions, with duplicate determinations each time.

4.6. Determine the proteolytic resistance of modified antimicrobial peptides

To determine the proteolytic resistance of modified antimicrobial peptide (D-LL-37 and D-LL-31) compare with native peptides (LL-37 and LL-31), the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Each peptide (50 μM) was incubated at a peptide-to-protease ratio of 300:1 for 4 h at 37°C in Tris buffer. The samples were supplemented with loading buffer, and proteolysis was heat terminated by 3 min of boiling. Gel electrophoresis was done using polyacrylamide gels and Tris-Tricine-sodium dodecyl sulfate running buffer at 150 V for 35 min. The gels were subsequently stained for 1 h with 0.25% Coomassie brilliant blue, destained, and scanned. The gel bands from the resulting images were quantified with ImageJ v1.37 software.

4.7. Determine the toxicity of antimicrobial peptides to hRBCs

Haemolytic activity of all peptides were determined against human red blood cells (hRBCs) as described previously (Kanthawong *et al*, 2010). Briefly, fresh hRBCs were washed three times with phosphate-buffered saline (PBS) and 20 μM each peptide was added to 400 μl of the hRBC suspension, then incubated at 37 °C for 1 h. After incubation, the mixtures were centrifuged at 800×g for 10 min and the absorbance of released haemoglobin was measured at 540 nm (A540). Controls for zero haemolysis and 100% haemolysis consisted of hRBCs suspended in PBS and 2% Triton X-100 (v/v), respectively. The haemolysis percentage was calculated using the equation:

$$\% \text{ Haemolysis} = \frac{100 \times [\text{A540 in D-LL-31 solution} - \text{A540 nm in PBS}]}{[\text{A540 in 2\% Triton X-100} - \text{A540 in PBS}]}$$

4.8. Statistical analysis

The killing effects of peptides and ceftazidime against 3 strains of *B. pseudomallei* were presented as mean ± standard error (SE). The comparison between the average percentage killing activities in each AMPs at the same concentration was analyzed by using One-Way ANOVA, the SPSS software, version 16.0 (Chicago, IL). A P value of < 0.05 were considered as statistically significant.

Results

The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of conventional antibiotic (ceftazidime)

To achieve initial information of *B. pseudomallei* 1026b, H777 and M10 (biofilm mutant) planktonic form to CAZ, the antimicrobial susceptibility assay was performed and interpreted the result follow by CLSI criteria (Edition AS-E; CLSI document, 2013). The MICs and MBCs recorded for CAZ against all *Burkholderia* strains are summarized in Table 3. The MIC values of *B. pseudomallei* 1026b and M10 were 2 µg/ml, while MIC values ranging from 1-2 µg/ml was observed in *B. pseudomallei* H777. Moreover, the MBC values of all *B. pseudomallei* were generally two-fold higher than the MIC. The result demonstrated that all *Burkholderia* strains in planktonic form were susceptible CAZ.

Table 3 The MIC and MBC of CAZ against *Burkholderia* strains.

Isolate	MIC range (µg/ml)	MBC range (µg/ml)
B. pseudomallei		
1026b	2	4
H777	1-2	4
M10	2	4

The quantification of bacterial biofilm formation in 3 strains of *B. pseudomallei*

The biofilm crystal violet staining (2 days-biofilm formation) was performed to determine the biofilm-formation capacity of each bacterial stain in 96-well plate. The amount of biofilm produced in all strains of *B. pseudomallei* were shown in Table 4. All strains of *B. pseudomallei* species exhibited biofilm ability, except M10 (biofilm mutant strain). The most significant biofilm form was observed in *B. pseudomallei* H777 (range of OD_{620} = 1.242-2.096). Whereas, *B. pseudomallei* 1026b exhibited low biofilm producing activity (OD_{620} = 0.694).

Table 4 The quantification of bacterial biofilm formation in *B. pseudomallei* 1026b, H777, M10 (biofilm-defective mutant).

Isolates	OD_{620} nm (mean ± SD)	Biofilm-producing groups
B. pseudomallei		
1026b	0.694±0.312	Low
H777	1.242±0.350	Moderate
M10	0.002±0.015	Very-low

The 3 groups of biofilm forming capacity; low biofilm-producing (OD_{620} nm<1.00), moderate biofilm-producing (OD_{620} nm = 1.00-3.00) and high biofilm-producing groups (OD_{620} nm>3.00) (Sawasdidoln *et al*, 2010). The data represent the mean ± standard deviation of 8 replicates from three independent experiments.

The antibiofilm activity of CAZ and antimicrobial peptides (LL-37, LL-31, D-LL-37 and D-LL-31) against *B. pseudomallei* in biofilm form

The Calgary Biofilm Device (CBD) was used to determine the biofilm susceptibility testing. CAZ displayed a clear dose- and strain- dependent antibiofilm activity against all *B. pseudomallei* tested strains (Figure 3). The highest concentration (1,024 µg/ml) exhibited 61.31%, 71.35% and 82.84% against *B. pseudomallei* 1026b, H777 and M10, respectively.

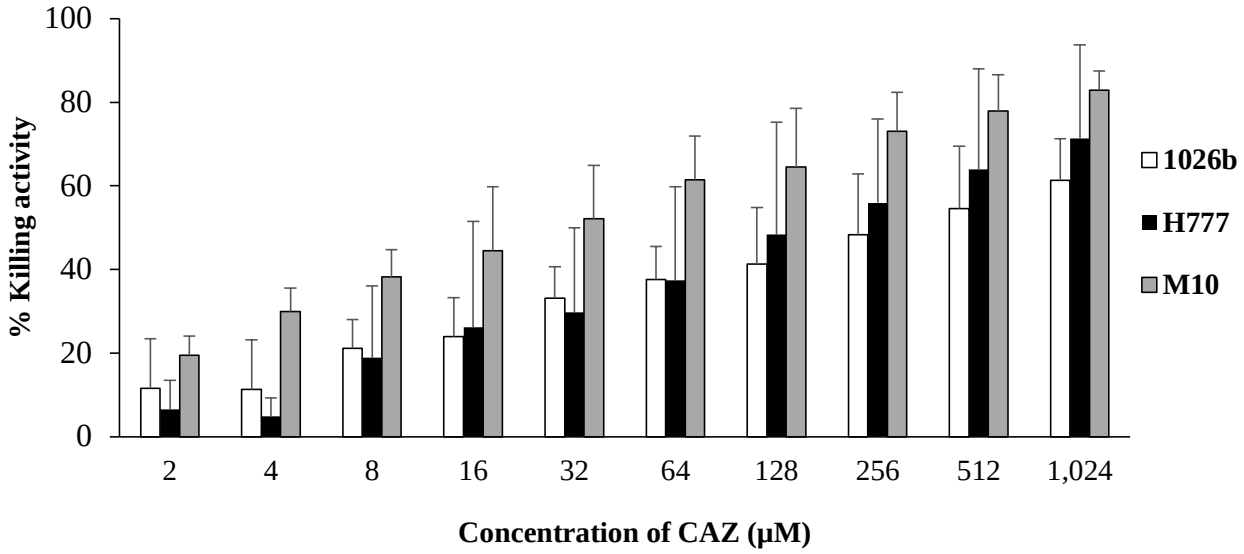
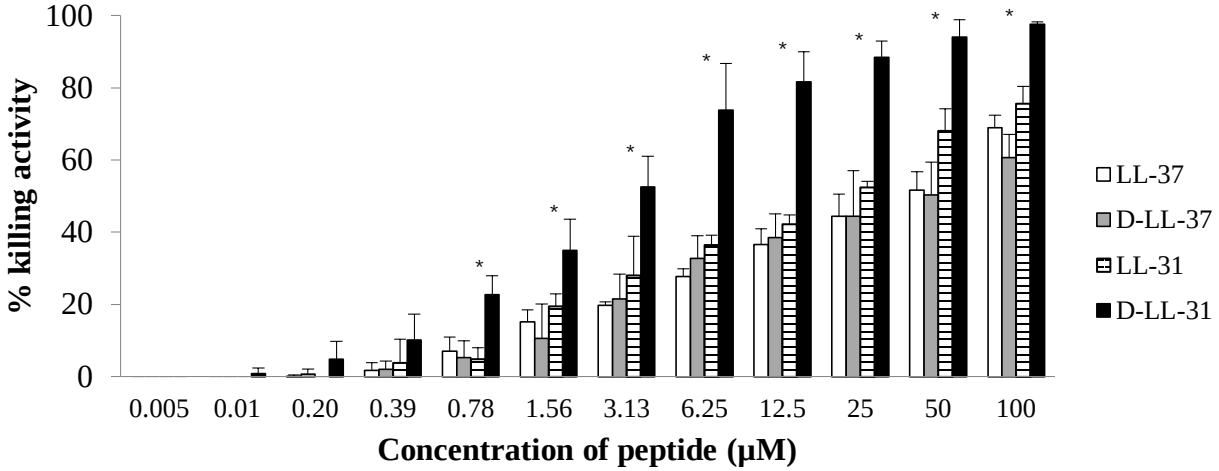


Figure 3 The antibiofilm activity of CAZ against *B. pseudomallei* 1026b, H777, and M10 (biofilm-defective mutant). The graph represents the mean ± standard deviation of duplicate from three independent experiments.

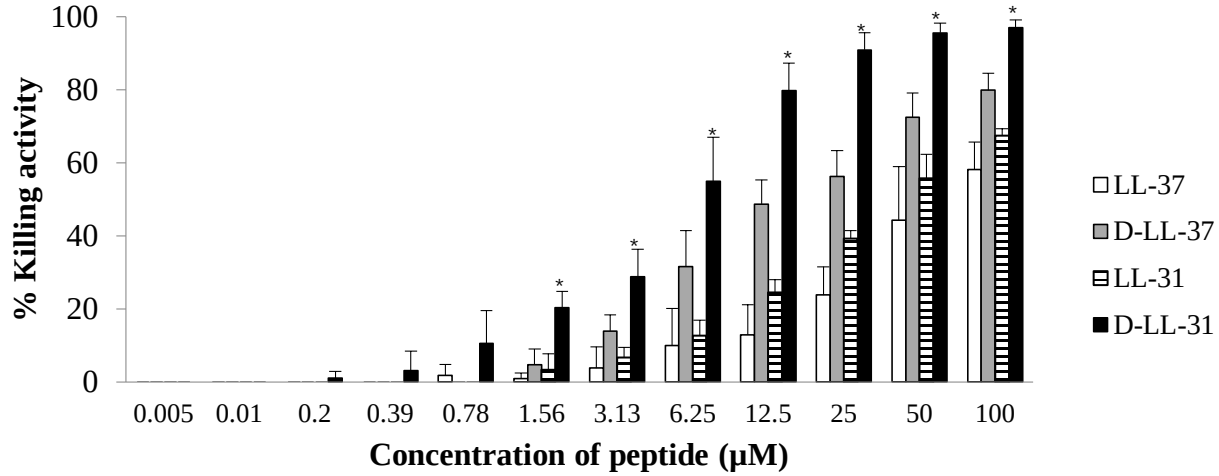
The antibiofilm activities of AMPs against 3 strains of *B. pseudomallei* biofilm are shown in Figure 4. After bacterial biofilm challenged with AMPs, the results showed that the bacterial viability was reduced in concentration-dependent manner in all AMPs. Of these four peptides, D-LL-31 appeared to be very strong against all tested strains. At 100 µM D-LL-31 exhibited the strongest antibioflm activity against all *B. pseudomallei* strains; H777 (97.02%), 1026b (97.62%) M10 (99.58%), while LL-37, LL-31 and D-LL-37 showed the percentage of inhibition lower than D-LL-31 at the same concentration.

Interestingly, higher antibiofilm activity than its L-peptide were recorded only for D-LL-31. While the antibiofilm activity pattern of L- and D- form of LL-37 are strain-dependent. No significant differences of susceptibility to both forms of LL-37 were evident for *B. pseudomallei* 1026b (Figure 4A). And the biofilm mutant strain *B. pseudomallei* M10 was sensitive to LL-37 but not to D-LL-37 (Figure 4C). In contrast, *B. pseudomallei* H777 wild-type expressed higher sensitive to D-LL-37 than its natural form (Figure 4B).

(A) 1026



(B) H777



(C) M10

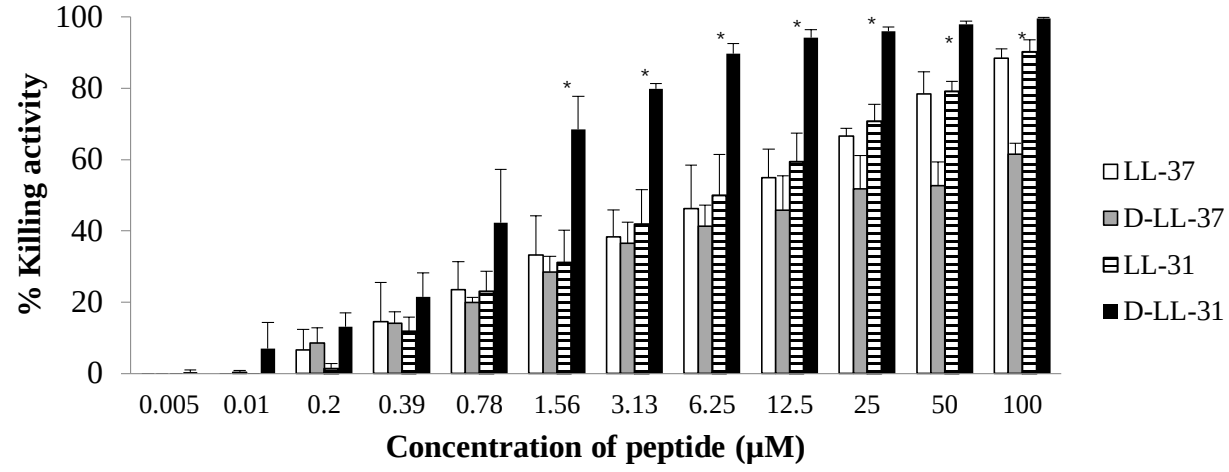


Figure 4 The antibiofilm activity of each AMPs against *B. pseudomallei* (A) 1026b, (B) H777, (C) M10 (biofilm-defective mutant). The graph represents the mean ± standard deviation of duplicate from three independent experiments. One-Way ANOVA was used for determine the statistical significance of the killing activities of each AMP in different concentrations compare with all of peptides (*$P < 0.05$).

Therefore, we further investigated the IC_{50} values of CAZ and D-LL-31 against 3 strains of *B. pseudomallei*. The graph represents the dose-response with variable slope of D-LL-31 and CAZ were shown in Figure 5 and 6, respectively. The IC_{50} value of each agent against biofilm of all *B. pseudomallei* strains were calculated by using the fitted line formula. The concentrations that provide the percentage of killing activity as the 50% were shown in (Table 5). The result showed that IC_{50} values of D-LL-31 displayed in biofilm-forming capacity dependent manner ranging from 1.07-5.55 μM, while IC_{50} values of CAZ was higher than D-LL-31 about 45, 60 and 240 folds against *B. pseudomallei* M10, H777 and 1026b, respectively. The biofilm-defective mutant *B. pseudomallei* M10 revealed to be the most susceptible to both CAZ and D-LL-31. Whereas, IC_{50} of CAZ combated *B. pseudomallei* H777 (moderate-biofilm producing) lower than *B. pseudomallei* 1026b (low-biofilm producing). This result suggested that D-LL-31 had a strong effect on *B. pseudomallei* biofilm. Further experiment was carried considering D-LL-31 antibiofilm properties combination with CAZ against *B. pseudomallei* biofilm.

Table 5 The IC_{50} values of D-LL-31 and CAZ against *B. pseudomallei* biofilm

Isolates	**IC_{50} value**	
	D-LL-31	**CAZ**
B. pseudomallei		
1026b	2.76 μM	365.10 μg/ml (667.95 μM)
H777	5.55 μM	184.44 μg/ml (337.44 μM)
M10	1.07 μM	26.38 μg/ml (48.26 μM)

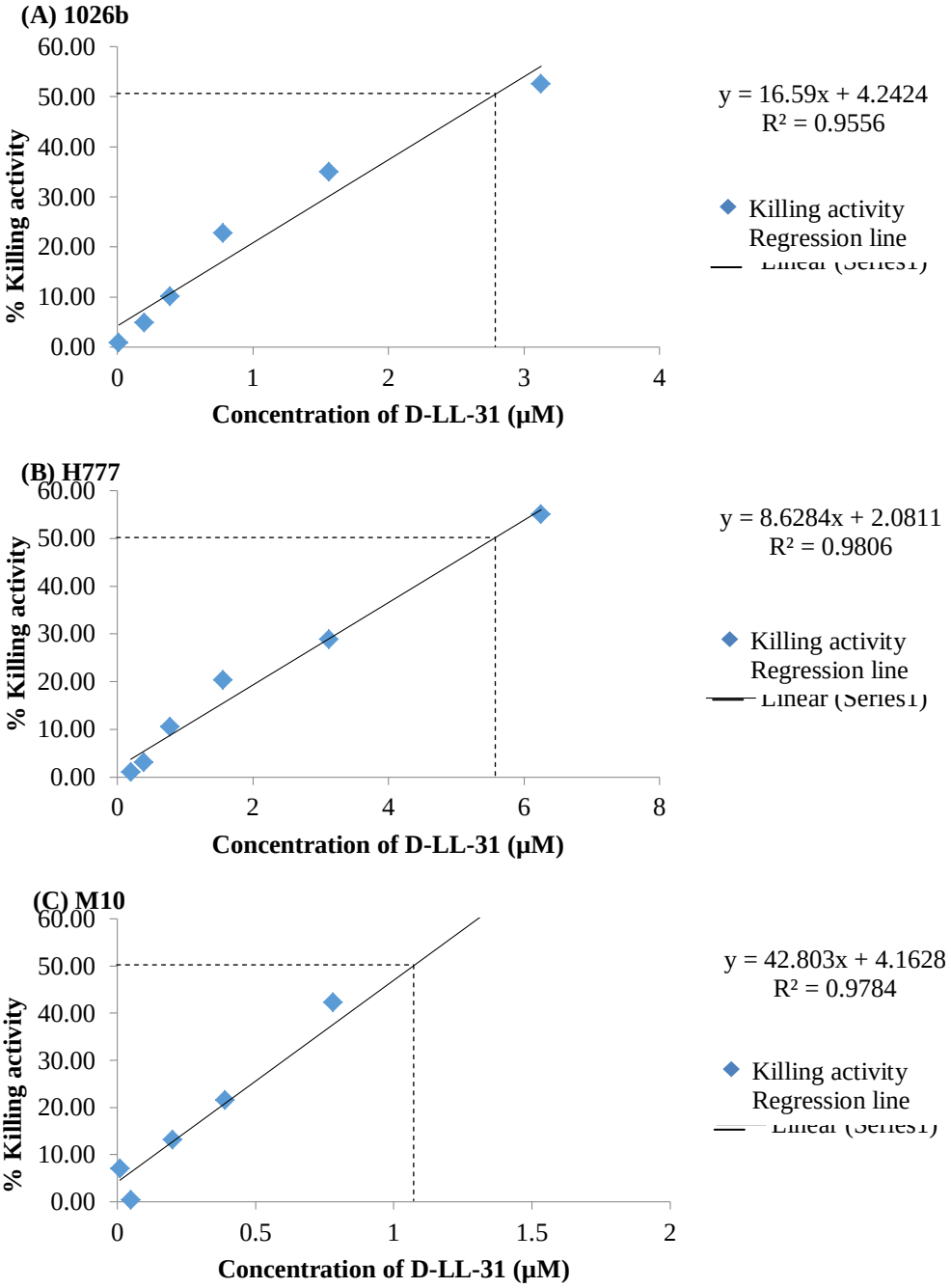


Figure 5 Linear regression graph of D-LL-31 against *B. pseudomallei* (A) 1026b, (B) H777, (C) M10 (biofilm-defective mutant) biofilm.

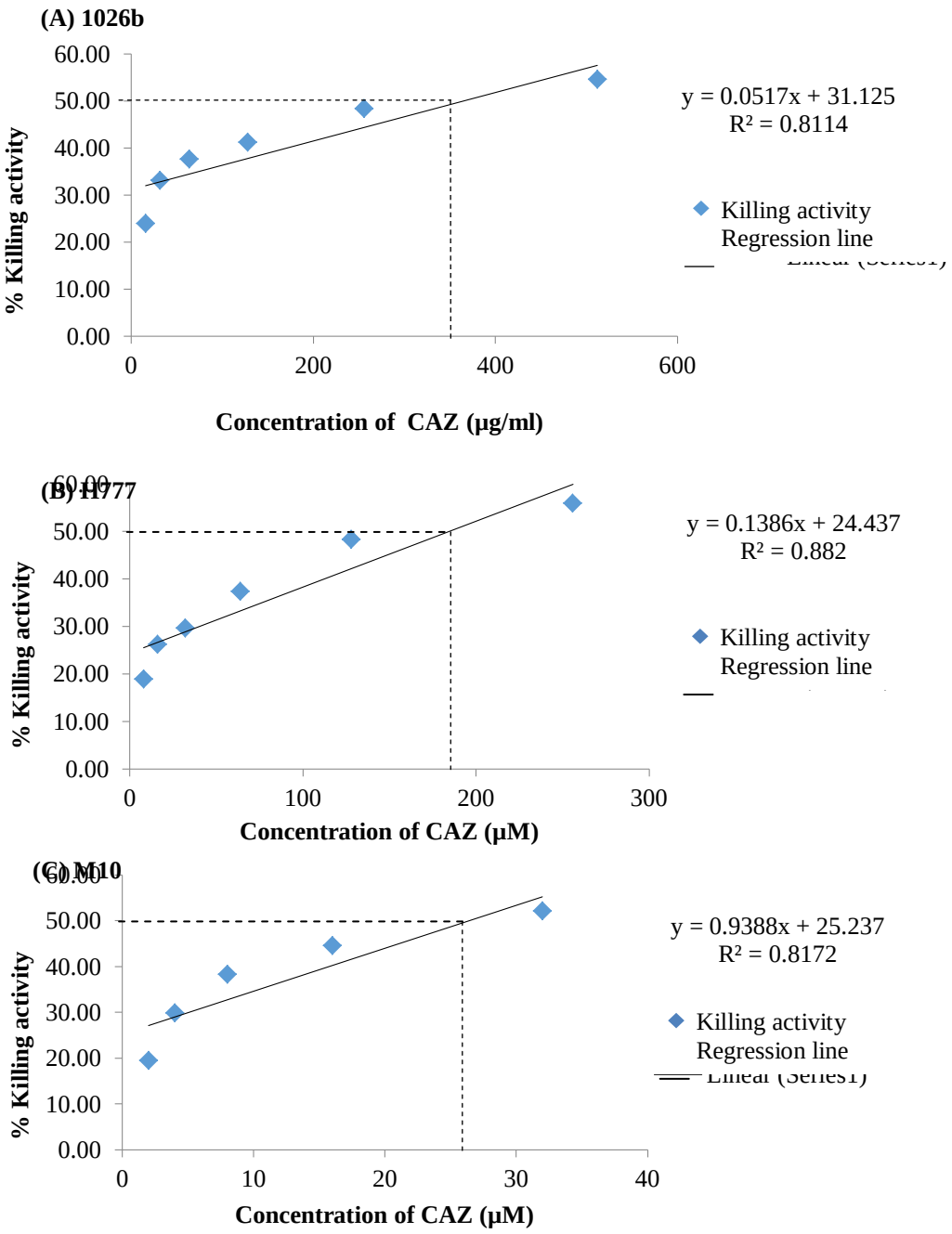


Figure 6 Linear regression graph of CAZ against *B. pseudomallei* (A) 1026b, (B) H777, (C) M10 (biofilm-defective mutant) biofilm.

The synergistic effect between the most effective antimicrobial peptide and CAZ against *B. pseudomallei* biofilm

The synergistic effect between D-LL-31 and CAZ at concentrations range from 4 times to 1/16 times of IC_{50} of each agent against 3 strains of *B. pseudomallei* biofilm was determined by using checkerboard technique and interpreted as fraction inhibitory concentration index (FICI) values. IC_{50} values in combination of both agents were shown in Table 6. The combined effect of D-LL-31 and CAZ against *B. pseudomallei* H777 and M10 biofilm reduced the IC_{50} values by 16-fold of both agents. Whereas, 8 and 16-fold drop in CAZ and D-LL-31 concentration in combination was found against *B. pseudomallei* 1026b. Moreover, the synergistic interaction between D-LL-31 and CAZ was observed against all 3 strains of *B. pseudomallei* according to FICI ≤ 0.5 (Table 6). This result suggested that D-LL-31 led to a substantial decrease in the concentration of CAZ required for antibiofilm activity. Thus, the synergistic effect of D-LL-31 with CAZ could be useful in combinational antibiotic therapy.

Table 6 The FICI values of D-LL-31 in combination with CAZ against *B. pseudomallei* biofilm.

Isolates	Synergistic concentrations		FICI values
	D-LL-31	Ceftazidime	
B. pseudomallei			
1026b	0.18 μM	45.64 μg/ml (83.50 μM)	0.188
H777	0.35 μM	11.53 μg/ml (21.09 μM)	0.125
M10	0.07 μM	1.65 μg/ml (3.02 μM)	0.125

The effect of D-LL-31 alone and in combination with CAZ against *B. pseudomallei* 1026b flow cell

The antibiofilm activities of D-LL-31 alone and in combination with CAZ against performed biofilm of *B. pseudomallei* 1026b were further observed under flow cell system using BioFlux device at the concentrations of both agents giving the synergistic result (D-LL-31: 0.18 μM and CAZ: 45.64 μg/ml or 83.50 μM) (Figures 7A-D). The image from microscopic indeed showed that the combination between D-LL-31 and CAZ obviously reduced biofilm mass (Figure

7D) when compared with untreated channel (Figure 7A), while the biofilm-treated with each agent alone at IC_{50} value (D-LL-31: 2.76 μM or CAZ: 365.10 μg/ml or 667.95 μM) was apparently not affected (Figures 7B and C).

Moreover, extracellular polysaccharides of *B. pseudomallei* 1026b were stained with Alexa-ConA (50 μM/ml), which is specific fluorescence for representative biofilm matrix molecules and observed the result by using confocal microscopic as shown in Figures 7E-H. The fluorescence micrographs revealed that the moderately decrease of exopolysaccharide matrixes of *B. pseudomallei* 1026b after treated with IC_{50} values of D-LL-31 and CAZ alone were observed (Figures 7F and G) when compared with agent-free channel (Figures 7E). However, a markedly disruption of *B. pseudomallei* 1026b pre-formed biofilm was still observed after combination treatment of D-LL-31 and CAZ (Figures 7H).

Furthermore, the results were confirmed by measuring the intensity mean values of green-fluorescent (Alexa-ConA) in flow cell chamber. The lowest fluorescence intensity mean value (3,602.18) was observed in the synergistic concentration between D-LL-31 and CAZ. Whereas, IC_{50} concentration of D-LL-31 revealed moderately reduced the intensity (5,913.45) compared with untreated channel (7,975.09). Even though imaging of biofilm production by fluorescence microscope after treated with IC_{50} value of CAZ-treated channel with IC_{50} value was slightly decrease (7,607.09).

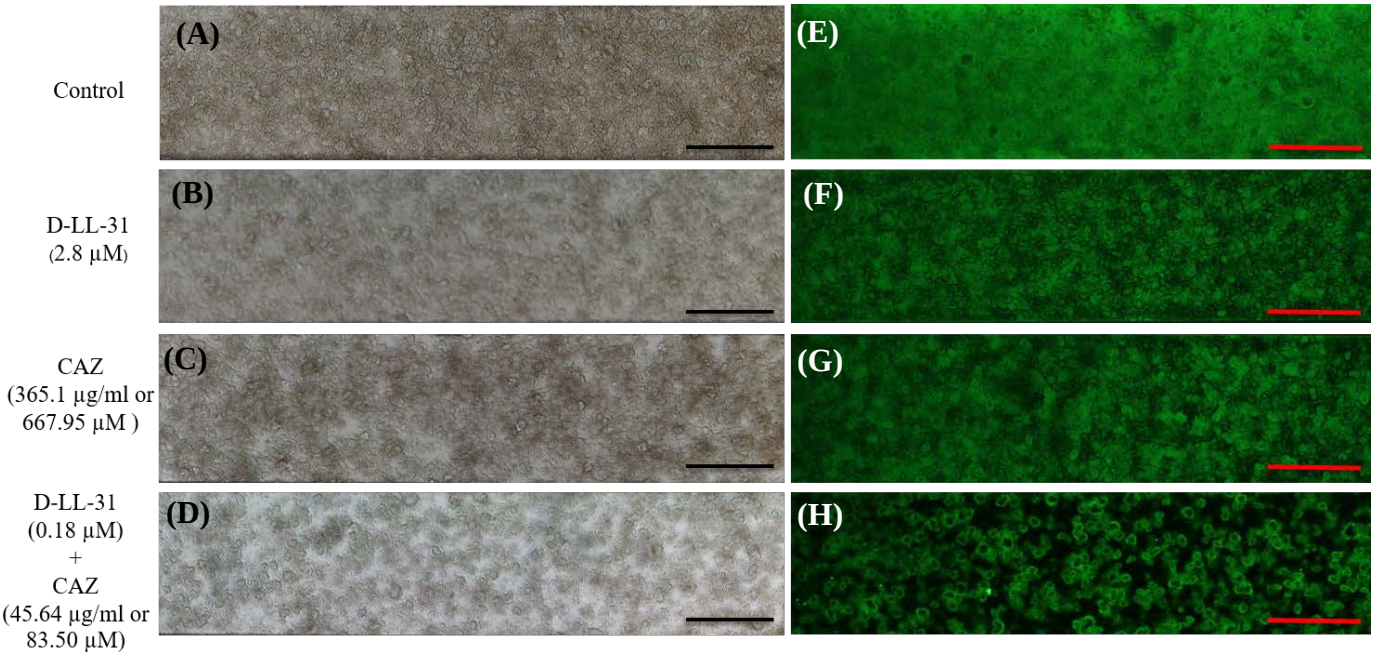


Figure 7 Effect of D-LL-31 alone and in combination with CAZ against *B. pseudomallei* 1026b flow cell. Each image contains; phase-contrast images (A-D) and Alexa-ConA (represent in green) stained biofilm under CLSM (E-H). The results were performed in two independent experiments. Scale bar, 200 µm.

In addition, the proteolysis resistance of D-LL-31 was determined (Figure 8). The SDS-PAGE protein profile showed that D-LL-31 exhibited the resistant to proteolytic activity after incubated with human serum for 4 h (Lane 5) compared with native peptide LL-31 (Lane 6). Moreover, the toxicity of all antimicrobial peptides were determined using haemolysis assay. D-LL-31 induced 1 1.1% haemolysis relative to the release induced by Triton X-100, which is lower than haemolysis activity induced by LL-37, D-LL-37 and LL-31 as 2.18%, 1.22% and 1.76% respectively.

This result suggested that D-LL-31 in combination with CAZ not only disrupt preformed-biofilm but is also found to be potent antimicrobial agent against *B. pseudomallei* in biofilm form which implies the therapeutic application of D-LL-31 as single or combination therapy.

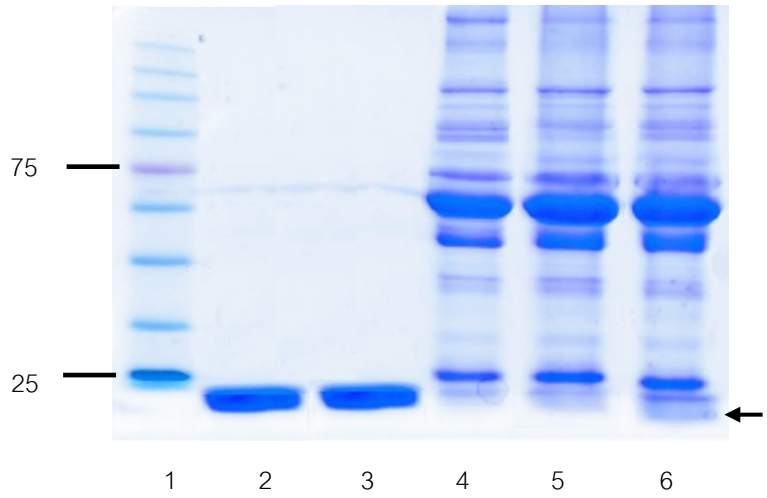


Figure 8 Proteolytic resistance of antimicrobial peptides LL-31 and D-LL-31 to human serum was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); 20 μM D-LL-31 (Lane 2) and 20 μM LL-31 (Lane 3), human serum alone (Lane 4), 20 μM D-LL-31 with human serum (lane 5) and LL-31 with human serum (lane 6). Lane 1 is a molecular mass standard.

Conclusion and Discussion

Melioidosis is a term describing a collection of serious and often fatal diseases in humans and animals, arising from an infection by *B. pseudomallei*. The *B. pseudomallei* growing in biofilm has been reported to induce resistance to several conventional antibiotics (Limmathurotsakul *et al*, 2014; Høiby *et al*, 2010). Previously we found that the truncation of LL-37 with six residues at the C-terminus (LL-31) possessed the strongest killing activity against *B. pseudomallei* in planktonic and biofilm form (Kanthawong *et al*, 2012). However, the sensitivity to enzymatic degradation of natural antimicrobial peptides has been recognized. Peptides consisting of L-amino acids are easily cleaved by proteases from mammalian hosts and numerous bacteria species (Walter *et al*, 2010; Sieprawska-Lupa *et al*, 2004). Previous report found that D-amino acids substitution of antimicrobial peptides can improve the proteolytic stability and thereby possibly the antimicrobial activity. The present study, we further

investigated the effect of LL-37, LL-31 and their D-enantiomers against *B. pseudomallei* in biofilm form. It was found that D-LL-31 showed stronger killing activity against *B. pseudomallei* in biofilm form than the L-form peptide.

The three *B. pseudomallei* strains used for this investigation were chosen according to their biofilm-forming capacity. From previous report, *B. pseudomallei* 1026b appeared low biofilm production, while moderate-biofilm producing strain, *B. pseudomallei* H777 (wild type) was included to test with its biofilm-defective mutant *B. pseudomallei* M10 (Kanthawong *et al*, 2012). We have demonstrated that all *B. pseudomallei* strains growing in biofilm-stimulating condition induced notably resistant to CAZ (up to 45 to 90 folds increased resistance compared to their planktonic form), even biofilm-defective mutant strain (up to 7 fold-increasing), as previously observed in other strains (Anutrakunchai *et al*, 2015). All three *B. pseudomallei* biofilms used in this study appeared to be very sensitive to the tested antimicrobial peptides, especially D-LL-31. This result suggested that though biofilm-forming of *B. pseudomallei* enhanced antibiotic resistance, antimicrobial peptides were still effective in killing biofilm bacteria.

The natural form of peptides (consisting of L-amino acids) are sensitive to enzymatic degradation by bacterial and human proteases and thereby hampering the development of peptidic therapeutic agents (Fjell *et al*, 2012). D-enantiomeric peptides have been used to resolve this problem. A stronger effect of D-peptides has been found against *Mycobacterium avium* (Silva *et al*, 2014), *P. aeruginosa* biofilm (De la Fuente-Núñez et al, 2015). In this study, we observed a similar effect with D-LL-31 for all *B. psuedomallei* strains tested, however only partially for D-LL-37. Other reports demonstrated no effect between L- and D-antimicrobial peptides against biofilm of *P. aeruginosa* (Dean *et al*, 2011), *Burkholderia thailandensis* (Blower *et al*, 2015) and *Staphylococcus aureus* (Dean *et al*, 2011).

The abuse or long term treatment of antibiotic have led to the development of widespread resistance in microorganism, with the result that single agents become less effective (O'Neil, 2014). Because of the highly potent effect of antimicrobial peptides and the lower probability to provoke resistance, they have been recognized as novel approaches to combat infections with resistant pathogens (Giacometti *et al*, 2004). However, production costs are often raised as an issue for antimicrobial peptides. Currently, the efficiency of antibiotic-based

combination therapy is recommended for severe of infections in order to enhance the rapid bactericidal activity, prevent and delay the dissemination of resistance which is crucial for the survival of critically ill patients (Dosler et al, 2016). Moreover, many studies have been found that antimicrobial peptides can be used in combination with currently antibiotics which leads to increase activity against several pathogens (Giacometti *et al*, 2004; Park *et al*, 2006). In this study, the ability of combinations of D-LL-31 and CAZ to eradicate pre-formed biofilm of *B. pseudomallei* was explored using the broth microdilution checkerboard method, which is the most widely used technique to test antimicrobial combinations (Breno *et al*, 2014). Our data showed that D-LL-31 in combination with CAZ had strong synergistic interaction against pre-formed biofilm of *B. pseudomallei* at 16-fold lower than those single treatments.

The possible mechanisms of bacterial biofilm contributing to antibiotic resistance have been reported such as i) biofilm matrix that might physically restrict the penetration of antibiotic ii) persister cells in biofilm which show slow growth rate and low metabolic activity caused by the limitation of nutrients and oxygen lead to prevent the action of antibiotic, especially beta-lactams that primarily target metabolically active cells (Lewis, 2001; Stewart, 2002). The general action mechanism of most antimicrobial peptides is membrane disruption leading to pore formation that efficiently kill various microorganism, even antibiotic resistance bacteria. Indeed, we have shown previously that LL-37 and truncated variants disrupted the membrane *B. pseudomallei* planktonic cells and caused leakage of intracellular molecules leading to cell death (Kanthawong *et al*, 2012). However, the mechanism of antimicrobial peptides or in combination with antibiotics against pre-formed of bacterial biofilm is still unclear. Possible mechanisms to explain this interaction may be disrupting biofilm matrix, altering the expression of biofilm-related genes and interfering intracellular signal system of biofilm formation (Reffuveille *et al*, 2014; Ribeiro *et al*, 2016). In this study, we focused on the effect of D-LL-31 and CAZ alone as well as in combination against the biofilm matrix of *B. pseudomallei* 1026b under flow condition using BioFlux device. This system provides a flow conditions to generate a flow biofilm, which mimic the hydrodynamic shear forces of the real biological conditions more than those formed in static culture of various microorganisms (Tao *et al*, 2011; Ding *et al*, 2014). Alexa-ConA was used to stain the polysaccharides in biofilm matrix. The highest ratio of green-fluorescent intensity (0.95) was observed after exposure of *B.*

pseudomallei biofilm to CAZ alone followed by D-LL-31 alone (0.74) and D-LL-31/CAZ combination (0.45) (Fig 7). These results indicated that the presence of D-LL-31 alone or in combination with CAZ exhibited more effective in reducing polysaccharide of *B. pseudomallei* biofilm compared with CAZ alone. Therefore, the higher killing activity of D-LL-31/CAZ combination than D-LL-31 or CAZ alone against *B. pseudomallei* in biofilm might be due to the capability of D-LL-31 in disruption of biofilm matrix and leads to an enhanced assessment of CAZ to bacterial cell.

In conclusion, we have demonstrated that D-LL-31 alone and in combination with CAZ not only exhibited strong killing effect against *B. pseudomallei* in biofilm-inducing condition but also disrupted the biofilm matrix of *B. pseudomallei*. In addition, D-LL-31 exhibited the proteolytic resistance to human serum and no cytotoxicity to hRBCs at concentration of D-LL-31 up to 20 μM. These finding suggested that, D-LL-31 should be further developed as novel antibiofilm agent in single therapy or used as adjunctive agent to enhance the efficacy of currently antibiotic for the therapy of melioidosis which is finally benefited to melioidosis patients.

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Output

1. International Journal Publication

- Manuscript จำนวน 1 ฉบับ (see in Appendix 1)

1) Wongkaewkhiaw S, Kanthawong S, Bolscher JGM, Nazmi K, Wongratanacheewin S, Suwimol Taweechaisupapong S. Antibiofilm activity of modified-antimicrobial peptide alone and in combination with ceftazidime against *Burkholderia pseudomallei.*

2. Research Utilization and Application

- เชิงวิชาการ: โครงการวิจัยนี้เป็นหัวข้อวิทยานิพนธ์สำหรับนักศึกษาระดับปริญญาโท จำนวน 1 คน ซึ่งสำเร็จการศึกษาแล้ว ได้แก่
    1) นายสหรัฐ วงศ์แก้วเขียว รหัส 575070018-6 ระดับปริญญาโท สาขาจุลชีววิทยาทางการแพทย์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

3. Others e.g. national journal publication, proceeding, international conference, book chapter, patent

- **Proceeding จำนวน 1 เรื่อง** (see in Appendix 2)

1) Wongkaewkhiaw S, Bolscher JGM, Nazmi K, Wongratanacheewin S, Suwimol Taweechaisupapong S and Kanthawong S. Effects of human cathelicidin antimicrobial peptides and their modified forms against *Burkholderia pseudomallei* biofilm (ในงานประชุม The 40th National Graduate Research Conference)

- **International Conference จำนวน 2 ครั้ง** (see in Appendix 3)

1) นำเสนอผลงานในรูปแบบ Poster presentation เรื่อง “Effects of human cathelicidin antimicrobial peptides and their modified forms against Burkholderia pseudomallei biofilm” ในงานประชุมวิชาการ MBSJ The 39th Annual Meeting of the Molecular Biology Society of Japan จัดโดย The University of Tokyo ณ เมืองโยโกฮ่า มา ประเทศญี่ปุ่น เมื่อวันที่ 1 ธันวาคม 2559
2) นำเสนอผลงานในรูปแบบ Poster presentation เรื่อง “Effect of D-enantiomer antimicrobial peptide against biofilm matrix of *Burkholderia pseudomallei*” ในงานประชุมวิชาการ 9th Asian community of glycoscience and glycotechnology conference ณ The University of Hong Kong ประเทศ Hong Kong เมื่อวันที่ วันที่ 17-20 ธันวาคม 2560

P05

Effect of D-enantiomer antimicrobial peptide against biofilm matrix of *Burkholderia pseudomallei*

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Abstract

Melioidosis is a severe disease caused by *Burkholderia pseudomallei*. The growing *B. pseudomallei* in biofilm has been reported to induce resistance to several conventional antibiotics, a phenomenon that may be related to relapse cases in melioidosis patients. The aim of this study was to compare the effect of human cathelicidin antimicrobial peptides (LL-37, LL-31) and theirs D-enantiomeric form (D-LL-37, D-LL-31) with ceftazidime (CAZ) against *B. pseudomallei* 1026b. A truncated variant of LL-37 lacking the six C-terminus residues in D-enatiomeric form, D-LL-31, revealed a strongest killing activity against all isolates of *B. pseudomallei* in dose-dependent manner. Moreover, IC_{50} values of D-LL-31 was 2.76 μM, while, IC_{50} values of CAZ was higher than D-LL-31, about 240 folds against biofilm form *B. pseudomallei* 1026b. Moreover, D-LL-31 was applied to biofilm of *B. pseudomallei* under BioFlux flowthrough conditions and caused disruption of the biofilms matrix. These results indicate that D-LL-31 not only disrupted biofilm matrix but also exhibited potent killing activity against *B. pseudomallei* in biofilm form. Thus D-LL-31 should be considered to develop as novel antibiofilm agent against *B. pseudomallei* which is finally benefited to melioidosis patients.

Effects of human cathelicidin antimicrobial peptides and their modified forms against *Burkholderia pseudomallei* biofilm

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Abstract

Melioidosis is a term of serious and often-fetal diseases in humans and animals, caused by the Tier 1 select agent *Burkholderia pseudomallei*. The growing *B. pseudomallei* in biofilm has been reported to induce resistance to several conventional antibiotics, a phenomenon that may be related to relapse cases in melioidosis patients. This study was aimed to determine the antibiofilm activity of human cathelicidin antimicrobial peptides (LL-37, LL-31) and their modified forms to improve the antimicrobial activity and proteolytic stability (D-LL-37, D-LL-31) compared with conventional antibiotic (ceftazidime) against *B. pseudomallei* biofilm. The results showed that all tested agents affected a clear dose- and strain-dependent killing activity against *B. pseudomallei* biofilms. 100 μM of LL-37, LL-31, D-LL-37 and D-LL-31 exhibited 68.87%, 75.72%, 60.66% and 97.62% killing activities against *B. pseudomallei* 1026b biofilm, respectively, while at the highest concentration of ceftazidime (1,024 μg/ml or 1,873 μM) showed lower activity than all antimicrobial peptides in a lower concentration used. These results indicated that D-LL-31 showed significantly higher killing activities against *B. pseudomallei* 1026b biofilm. Thus, D-LL-31 should be considered as the new therapeutics candidate in antimicrobial therapy for melioidosis patients.

Keywords: *Burkholderia pseudomallei* Antimicrobial peptide Antibiofilm activity

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Introduction

Melioidosis is a term describing a collection of serious and often-fetal diseases in humans and animals, arising from infection by *Burkholderia pseudomallei.* This disease is endemic in rainy season (Chaowagul, et al., 1989) and mostly infects people who have direct contact with contaminated-wet soils and have an underlying predisposition to infection such as diabetes mellitus (Currie, et al., 2000). *B. pseudomallei* infection has been reported in many countries , especially in Northeast Thailand, Northern Australia, Singapore and parts of Malaysia are currently recognized as 'highly endemic' locations where many cases are diagnosed each year (White, 2003).

This bacteria has been reposted resistant to many antibiotics such as doxycycline, ceftazidime, imipenem, and trimethoprim/sulfamethoxazole (Harris, et al., 2011). Moreover, *B. pseudomallei* was reported to form biofilm and microcolonies (Vorachit, et al., 1995). After these bacteria were induced to form biofilm, they were markedly resistant to various antimicrobial agents when compared with planktonic cells. Although, the biofilm formation was not correlated with *B. pseudomallei* virulence factor and the clinical outcome of infection, but involved with antibiotic resistance and relapse cases in melioidosis patients (Limmathurotsakul, et al., 2014).

Up to date, several researchers seek for the new therapeutic agents that can reduce mortality rate and duration times of antibiotic treatments. (Giuliani, et al., 2007). The human cathelicidin LL-37 antimicrobial peptide is one of therapeutic candidates in clinical trials. However, L- amino acids peptides (natural form) were highly sensitive to enzymatic degradation by proteases in serum (Walter, et al., 1975). To overcome this problem, D-amino acids were substituted for the L-amino acids of antimicrobial peptides for improve their antimicrobial activity and proteolytic stability. Dean and colleagues found that natural form LL-37 peptide was digested by trypsin, while the D-form showed no degradation after 1 hour incubated with trypsin (Dean, et al., 2011). In this study, the human cathelicidin antimicrobial peptide LL-37 and LL-31 and their modified peptides (D-LL-31 and D-LL-37) were observed to have antibiofilm activity in comparison with conventional antibiotic (ceftazidime) against *B. pseudomallei* 1026b biofilm. The information obtained from this study have a merit for further investigation and exploration of modified antimicrobial peptides for the therapy of melioidosis which is finally benefited to melioidosis patients.

Materials and Methods

Antimicrobial peptides

The human cathelicidin peptides LL-31, LL-37 and their D-sterioisomer (D-LL-31 and D-LL-37) were selected for this study. All peptides were synthesized from Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam (ACTA), Vrije Universiteit and Universiteit van Amsterdam, Amsterdam, The Netherlands, using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry

with a MilliGen 9050 peptide synthesizer (MilliGen-Biosearch, Bedford, MA) as described previously (Bolscher, et al., 2011).

Bacterial strain and growth condition

Burkholderia pseudomallei isolate 1026b was kindly provided by Melioidosis Research Center (MRC), Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. *B. pseudomallei* isolate 1026b was cultured in Ashdown's agar then incubated at 37°C for 48 hours. Afterward, a single colony was cultured aerobically in Modified Vogel Bonners Medium (MVBM) at 37°C, 200 rpm shaker incubator for 16-18 hours. In each experiment, bacterial suspension was diluted to the densities as indicate below.

Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)

MICs assay were performed in 96-well microtiter plates (Nunclon™, Roskilde, Denmark) to determine the antimicrobial susceptibility. The interpretation of the results followed the National Committee for Clinical Laboratory Standards NCCLS, 2013 (CLSI., 2013). The 2-fold serial dilution of ceftazidime (A6987, Sigma-Aldrich, St. Louis, MO) was done by dilution with Mueller Hinton broth (MHB) (Himedai®, Mumbia, India) to obtain the final concentrations range from 0.5-1,024 µg/ml. The bacterial suspension (~10^5 CFU/ml) was added to each well and incubated at 37°C for 24 hours. After that, plates were observed visually to obtain the MICs values and confirmed by microplate reader at OD_{620} (Sunrise™, TECAN, Australia). Then, Minimum Bactericidal Concentrations (MBCs) were further determined. The 10 µl of sample in each well at the end of incubation period without visible growth were plated onto Mueller Hinton agar (MHA) (Himedai®), then incubated at 37°C for 24 hours and the bacterial colonies were observed. Three independent experiments were performed in triplicate.

Antibiofilm assay.

The Calgary Biofilm Device (CBD) was selected to determine the susceptibility of biofilm as previously described with some modifications (Ceri, et al., 1999). The overnight bacterial culture were diluted to provide a final inoculum density of 10^7 CFU/ml in MVBM, 200 µl of bacterial suspension were added to flat-bottom 96-well microtiter plate (Nunclon™, Roskilde, Denmark) and the palate was covered by the peg (Nunc-TSP, Roskilde, Denmark), which is a modified polystyrene microtiter lid, then incubated at at 37 °C for 24 hours. After biofilms formed on peg lids, then lids were washed three times with sterilized distilled water and placed into the new microtiter plate that contained serial dilutions of antimicrobial agents in 1 mM potassium phosphate buffer (PPB), pH 7.0. Ceftazidime (A6987, Sigma-Aldrich, St. Louis, MO) was diluted range from 2-1,024 µg/ml, while LL-37, LL-31, D-LL-37 and D-LL-31 were diluted range from 0.05-100 µM. Then the plates were incubated at 37 °C for 24 hours. After that, the biofilm on lids were transferred to the new microtiter wells by sonication at room temperature for 5 min and determination of bacterial viability was performed by plate counts technique.

Statistical analysis

The killing effects of peptides and ceftazidime against *B. pseudomallei* 1026b are presented as mean ± standard error (SE). The comparison between the average percentage killing activities in each AMPs at the same concentration was analyzed by using Independent *t*-test, the SPSS software, version 16.0 (Chicago, IL, USA). A *P* value of <0.05 was considered as statistically significant.

Results

Antimicrobial susceptibility

To obtain initial information of *B. pseudomallei* 1026b, the antimicrobial susceptibility assay was performed and interpreted by CLSI criteria (CLSI., 2013). The MIC and MBC of ceftazidime were observed the antimicrobial activity at different concentrations. The result showed that MIC values of *B. pseudomallei* 1026b was 2 µg/ml while MBC was 4 µg/ml as shown in Table 1. These results demonstrated that *B. pseudomallei* 1026b in planktonic form was susceptible to ceftazidime.

Table1: The Antimicrobial susceptibility of ceftzidime against *B. pseudomallei* 1026b.

Strains/Isolate	MIC rang (µg/ml)	MBC rang (µg/ml)	Result
B. pseudomallei 1026b	2	4	Sensitivity

The Antibiofilm activities

The Calgary Biofilm Device (CBDs) was used to study the biofilm susceptibility assay. After bacterial biofilm challenged with AMPs and ceftazidime, the results showed that the bacterial viability were reduced in concentration dependent manner as shown in Figure 1A-B. However, antibiofilm activities between antimicrobial peptides and ceftazidime were distinctly different in *B. pseudomallei* 1026b.

All concentrations of ceftazidime showed lower antibiofilm activity than all tested AMPs. At 1,024 µg/ml (1,873 µM) of ceftazidime exhibited < 60% killing activity against *B. pseudomallei* 1026b biofilm (Figure 1A). While, 100 µM of D-LL-31, LL-31, LL-37 and D-LL-37 exhibited 97.62%, 75.72%, 68.87% and 60.66% killing activities, respectively (Figure 1B). Moreover, at the same concentration (100 µM), all AMPs showed the highest killing activities than ceftazidime (37.63%) as shown in Figure 1B. These results indicated that 100 µM of all AMPs showed the higher killing activity than ceftazidime at all concentrations used.

Interestingly, D-LL-31 (100 µM) exhibited the strongest antibioflm activity against *B. pseudomallei* 1026b (97.62%), while LL-37, LL-31 and D-LL-37 showed the percentage of inhibition lower than D-LL-31 at the same concentration. Therefore, D-LL-31 was the most effective peptide against *B. pseudomallei* 1026b biofilm.

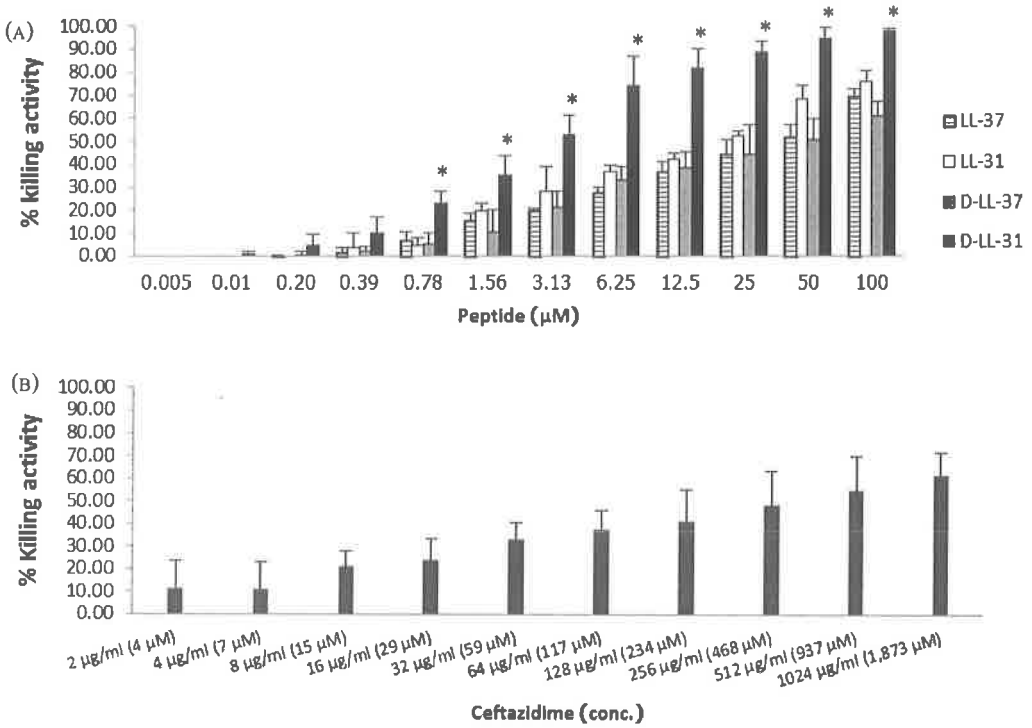

Figure1: The antibiofilm activity of each AMPs and ceftazidime against *B. pseudomallei* 1026b represent in Figure 1A and 1B. The graph represents the mean ± standard deviation of duplicate from three independent experiments (*P<0.05).

Discussion

Bacterial biofilms are population density of microorganisms, which are frequently attached to a solid surface and enclosed in an exopolysaccharide matrix (Mah, et al., 2001). The biofilm formation capacity of *B. pseudomallei* have been well documented in several publications, this component was also correlated with antibiotic resistance mechanism (Høiby, et al., 2010). Our results revealed that planktonic form of *B. pseudomallei* 1026b was shown to be susceptible to ceftazidime (MIC = 2 μg/ml and MBC = 4 μg/ml). While, this bacteria growing in a biofilm are highly resistant to ceftazidime at the highest concentration used (1,024 μg/ml), that was similar to previous reports of *B. pseudomallei* (Kanthawong, et al., 2012; Sawasdidoln, et al., 2010). Moreover, all AMPs (LL-37, LL-31, D-LL-37 and D-LL-31) exhibited strong killing activities against *B. pseudomallei* 1026b biofilm than that of ceftazidime at the same concentration.

Previous study of LL-31 in antibiofilm activities has revealed that at 100 μM of LL-31 exhibited >80% killing activities against *B. pseudomallei* 1026b (Kanthawong, et al., 2012). While, our results found that the enantiomer peptide (D-LL-31) was the most effective peptide against *B. pseudomallei* 1026b biofilm when compared with all tested AMPs (LL-37, LL-31 and D-LL-37). These results indicated that D-LL-31 had stronger antimicrobial activities against *B. pseudomallei*

than LL-31 in the previous study. In this study, it was found that D-and L-LL-37 displayed similar antibiofilm activity against *B. pseudomallei* 1026b biofilm, similar to previous observations against *Pseudomonas aeruginosa* (Dean, et al., 2011).

The possible mechanism of bacterial biofilm resistance to antibiotic is a slow growth rate and low metabolic activity that prevents the action of antibiotic, because most antimicrobial agents, especially beta-lactams, primarily target metabolically active cells (Lewis, 2001). In dissimilarity, AMPs have more potential to kill bacterial biofilm even slow-growing or low metabolic activities, because the main action mechanism of AMPs acts on bacterial cell membrane to cause pore formation and permeabilise within the cytoplasmic membrane (Pompilio, et al., 2012).

Conclusions and Recommendations

Therefore we concluded that D-LL-31 (D-amino acid peptide) was the most effect peptide against *B. pseudomallei* 1026b biofilm. Thus this peptide should be considered as the new therapeutics candidate in antimicrobial therapy for melioidosis patients.

Acknowledgement

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