Development of a clinically feasible translational medical approaches in the treatment of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* persistent biofilm infections.

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การพัฒนาวิธีการเพื่อนำมาใช้ในทางคลินิกในการรักษาการติดเชื้อแบบเรื้อรังจากการสร้างไบโอฟิล์มของ *Pseudomonas aeruginosa* และ *Acinetobacter baumannii*

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การพัฒนาแบบแผนในการยับยั้งไบโอฟิล์มที่เป็นไปได้ในทางคลินิกสำหรับการทดสอบความไวของยา

Development of the clinically feasible anti-biofilm platform for drug susceptibility testing

แม้จะมีการรักษาด้วยยาต้านจุลชีพที่มีฤทธิ์สูง การติดเชื้อ *Pseudomonas aeruginosa* และ *Acinetobacter baumannii* ที่สร้างไบโอฟิล์มนั้นมีความเกี่ยวข้องกับการพยากรณ์โรคที่ไม่ดีและตัวเลือกในการรักษาที่จำกัด การประเมินยาปฏิชีวนะสำหรับแบคทีเรียที่เป็นแพลงโทนิกเซลล์อาจเกิดความผิดพลาดได้จากการติดเชื้อในสภาวะไบโอฟิล์ม ปัจจุบันได้มีการให้ยาปฏิชีวนะในการรักษาโรคติดเชื้อในสภาวะไบโอฟิล์มโดยไม่ได้คำนึงถึงความไวของไบโอฟิล์มก่อนเริ่มรักษา การรักษาที่มีประสิทธิภาพเพื่อกำจัดการติดเชื้อในสภาวะไบโอฟิล์มจำเป็นต้องมีการประเมินประสิทธิภาพของยาปฏิชีวนะที่ใช้กันทั่วไปกับไบโอฟิล์ม คณะผู้วิจัยจึงขอนำเสนอวิธีที่มีประสิทธิภาพและง่าย เพื่อประเมินประสิทธิภาพของยาปฏิชีวนะต่อไบโอฟิล์ม ในการศึกษานี้ผู้วิจัยได้ทำการปรับระยะเวลาในการบ่มช่วงการตรวจจับ และรูปแบบการอ่านฟลูออเรสเซนซ์สำหรับการทดสอบโดยวิธีการย้อมไบโอฟิล์มด้วยรีซาซูรินในถาดหลุม 96-well-plate และกำหนดความเข้มข้นต่ำสุดที่สามารถกำจัดไบโอฟิมล์ (Minimal biofilm eradication concentrations :MBECs) สำหรับเชื้อ *P. aeruginosa* and *A. baumannii* ที่แยกมาจากผู้ป่วยติดเชื้อเรื้อรัง จากการใช้วิธีทดสอบที่กล่าวมานี้แสดงให้เห็นถึงการตอบสนองของยาปฏิชีวนะซึ่งมีรูปแบบที่แตกต่างและไม่ซ้ำกันภายในไบโอฟิล์มที่สร้างขึ้นจากตัวอย่างทางคลินิก ค่า MBEC-50 และ 75 มีอำนาจจำแนกเหนือกว่าค่า MIC (Minimal inhibitory concentration) สำหรับแพลงโทนิกเซลล์เพื่อแยกความแตกต่างของประสิทธิภาพโดยรวมของยาปฏิชีวนะในการกำจัดไบโอฟิล์มได้อย่างมีนัยสำคัญ การทดสอบในปัจจุบันเป็นแบบแผนที่เหมาะสมสำหรับการประเมินประสิทธิภาพของยาปฏิชีวนะต่อไบโอฟิลม์ในหลอดทดลองเพื่อปูทางสำหรับการรักษาที่มีประสิทธิภาพมากยิ่งขึ้น

การจำแนกลักษณะและการประเมินผลของเปปไทด์ชนิดใหม่ในการรักษาการติดเชื้อไบโอฟิลม์ของ *Pseudomonas aeruginosa, Haemophilus influenzae* และ *Acinetobacter baumannii*

พฤติกรรมชุมชนของการติดเชื้อแบคทีเรียที่สร้างไบโอฟิลม์อาจนำไปสู่การเกิดโรคที่เพิ่มขึ้น การเพิ่มสูงขึ้นของอัตราการเสียชีวิตและอัตราการเจ็บป่วยมีความสัมพันธ์กับโรคปอดอักเสบที่ได้มาจากชุมชนและโรงพยาบาล โรคปอดอักเสบจากเครื่องช่วยหายใจ โรคปอดอุดกลั้นเรื้อรัง โรคซิสติกไฟโบรซิสโรคหอบหืดและโรคหลอดลมอักเสบ รวมถึงวิกฤติทั่วโลกอันเกี่ยวเนื่องกับการดื้อยาปฏิชีวนะ ทำให้เกิดการค้นหากลยุทธ์การักษาแบบใหม่เพื่อต่อสู้กับการติดเชื้อไบโอฟิลม์ในปอด ฤทธิ์ของเปปไทด์ชนิดใหม่ทั้ง 17 ชนิดในการยับยั้งไบโอฟิลม์ถูกนำมาประเมินครั้งแรกกับเชื้อ *P. aeruginosa, A. baumannii* และ nontypeable *H. influenzae* ที่แยกได้จากสิ่งส่งตรวจผ่านการทดสอบด้วยการวิเคราะห์ plate-based assay คู่กับการใช้กล้องจุลทรรศน์แบบคอนโฟคอลโดยใช้การย้อมสีแบคทีเรียที่มีชีวิต/ ตาย ความสามารถของเปปไทด์ในการกำจัดไบโอฟิลม์ในเซลล์ปฐมภูมิเยื่อบุทางเดินหายใจที่ได้จากเด็กที่เป็นโรคซิสติกไฟโบรซิส (CF) ได้รับการประเมินโดยใช้แบบจำลองของเซลล์เพาะเลี้ยงแบบ air-liquid interface (ALI) ร่วมกับแบคทีเรียที่ได้รับการถ่ายโอนยีน GFP เปปไทด์ 6 ชนิด (HDP- 25, 26, 43, 101, 102, and 103) มีฤทธิ์ในการกำจัดเชื้อไบโอฟิลม์ของ *P. aeruginosa, A. baumannii* และ nontypeable *H. influenzae* ที่ความเข้มข้นต่ำ (16-128μg/ml) ยาปฏิชีวนะในปัจจุบันที่ระดับความเข้มข้นสูง (512-1024μg/ml) ไม่สามารถกำจัดไบโอฟิลม์เหล่านี้ได้ HDP 102 เป็นเปไทด์ที่มีศักยภาพสูงสุดซึ่งมีฤทธิ์มากกว่า 90% ในการลดปริมาณมวลชีวภาพในเซลล์เยื่อบุผิวทางเดินหายใจ และลดการติดยึดของแบคทีเรียกับเซลล์เหล่านี้ได้ถึง 74% การค้นพบนี้เน้นให้เห็นถึงศักยภาพของเปปไทด์ต้านจุลชีพซึ่งเป็นทางเลือกใหม่ในการรักษาโรคติดเชื้อแบบเรื้อรังจากการสร้างไบโอฟิลม์ในปอด ข้อมูลเชิงลึกที่ได้รับจากงานนี้อาจเสนอวิธีแก้ปัญหาที่ตรงเป้าหมายในการกำจัดไบโอฟิลม์ของแบคทีเรียและให้ผลการรักษาที่ดีขึ้นในผู้ป่วยติดเชื้อในปอดแบบเรื้อรัง

สาขาวิชา	วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต .....
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Dhammika Leshan Wannigama : Development of a clinically feasible translational medical approaches in the treatment of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* persistent biofilm infections.. Advisor: TANITTHA CHATSUWAN, Ph.D. Co-advisor: Cameron Paul Hurst, Ph.D., Peter Monk, Ph.D.

Development of the clinically feasible anti-biofilm platform for drug susceptibility testing

Despite strengthened antimicrobial therapy, biofilm infections of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are associated with poor prognosis and limited therapeutic options. Assessing antibiotics on planktonic bacteria can result in failure against biofilm infections. Currently, antibiotics to treat biofilm infections are administered empirically, usually without considering the susceptibility of the biofilm objectively before beginning treatment. For effective therapy to resolve biofilm infections it is essential to assess the efficacy of commonly used antibiotics against biofilms. Here, we offer a robust and simple assay to assess the efficacy of antibiotics against biofilms. In the present work, we carefully optimized the incubation time, detection range, and fluorescence reading mode for resazurin-based viability staining of biofilms in 96-well-plates and determined minimal biofilm eradication concentrations (MBECs) for *P. aeruginosa* and *A. baumannii* isolates from patients with chronic infection. By applying this assay, we demonstrated that antibiotic response patterns varied uniquely within the biofilm formation of various clinical samples. MBEC-50 and 75 have significant discriminatory power over minimal inhibitory concentrations for planktonic suspensions to differentiate the overall efficiency of an antibiotic to eradicate a biofilm. The present assay is an ideal platform on which to assess the efficacy of antibiotics against biofilms *in vitro* to pave the way for more effective therapy.

In-vitro characterization and evaluation of novel peptide mediated therapeutic approach in the treatment of biofilm infections by *Pseudomonas aeruginosa, Haemophilus influenzae* and *Acinetobacter baumannii*

The community behavior of bacterial biofilm infections may contribute towards enhanced disease pathogenesis. The consequently high mortality/morbidity rates associated to community and hospital-acquired pneumonia, ventilator-associated pneumonia, chronic obstructive pulmonary disease, cystic fibrosis, asthma, and bronchitis in conjunction with the global crisis of antibiotic resistance has promoted the search for novel therapeutic strategies to fight biofilm infections in the lung. The action of 17 novel anti-biofilm peptide candidates were firstly evaluated against clinical isolates of *P. aeruginosa, A. baumannii* and nontypeable *H. influenzae* via a high-throughput plate-based assay, coupled with confocal microscopy using live/dead bacteria staining. The ability of candidate peptides to eliminate biofilm on human primary airway epithelial cell cultures derived from children with CF were assessed using an air-liquid interface (ALI) cell culture biofilm model together with GFP tag bacteria. Six candidate peptides (HDP- 25, 26, 43, 101, 102, and 103) were active at eradicating *P. aeruginosa, A. baumannii* and nontypeable *H. influenzae* biofilms at relatively low concentrations (16-128μg/ml). High doses of current conventional antibiotics (512-1024μg/ml) were unable to eradicate these biofilms. HDP 102 was the most potent peptide, driving >90% bio-volume reduction in airway epithelial cells and a 74% reduction of bacterial attachment to these cells. These findings highlight the potential of host defence peptides as a novel option to treat chronic bacterial biofilm infections in lung. Insights gained through this work may offer solutions for targeted approaches to treat bacterial biofilms and improve the outcome of patients with chronic lung infections.

Field of Study:	Medical Sciences	Student's Signature .....
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LIST OF PUBLICATIONS

- Simple fluorometric-based assay of antibiotic effectiveness for *Acinetobacter baumannii* biofilms. Dhammika Leshan Wannigama, Cameron Hurst, Lachlan Pearson, Thammakorn Saethang, Uthaibhorn Singkham-in, Sirirat Luk-in, Robin James Storer & Tanittha Chatsuwan Nature - Scientific Reports volume 9, Article number: 6300 (2019).

- FIGHTING PSEUDOMONAS AERUGINOSA AND NONTYPEABLE HAEMOPHILUS INFLUENZAE BIOFILMS WITH HOST DEFENCE PEPTIDE AS A NOVEL STEP FORWARD IN THE TREATMENT OF CHRONIC LUNG INFECTIONS. D.L. Wannigama, A. Kicic, C. Hurst, P.N. Monk, R.J. Storer, T. Chatsuwan, S.M. Stick, A. Cf -CHEST , Volume 155 , Issue 4 , 73A (2019).

- A SIMPLE ANTIBIOTIC SUSCEPTIBILITY ASSAY FOR PSEUDOMONAS AERUGINOSA AND ACINETOBACTER BAUMANNII BIOFILM COULD LEAD TO EFFECTIVE TREATMENT SELECTION FOR CHRONIC LUNG INFECTIONS. D.L. Wannigama, C. Hurst, L. Pearson, T. Saethang, U. Singkham-in, S. Luk-in, R.J. Storer, T. Chatsuwan, - CHEST , Volume 155 , Issue 4 , 77A (2019).

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- OVERCOMING BIOFILM MEDIATED RESPIRATORY INFECTIONS THROUGH EXPLOITATION OF PATHOGEN AND HOST-DIRECTED NOVEL PEPTIDES. Dhammika Leshan Wannigama, Cameron Hurst, Peter Monk, Anthony Kicic, Stephen Stick, Tanittha Chatsuwan - Respirology 23(S2): 67-68, (2018).

- Overcoming biofilm mediated respiratory infections through exploitation of pathogen and host-directed novel peptides. Dissertation - Dhammika Leshan Wannigama: 22410824; Outbound Research and Academic Exchange University of Western Australia (2018).

LIST OF AWARDS

- Outstanding Abstract and Research Award in ICIC & ISAAR 2019

Won the outstanding research and abstract award in ICIC (4th International Interscience Conference on Infection and Chemotherapy) & ISAAR (12th International Symposium on Antimicrobial Agents and Resistance) in Gyeongju, Korea from September 26 to 28, 2019

- Outstanding Post Graduate Student Award in Medicine 2018-2019

Faculty of Medicine and King Chulalongkorn Memorial Hospital, Chulalongkorn University – August, 2019

- TSANZSRS 2019 Special Interest Group Best Research and Oral Presentation Award

Won the best research presentation award in Special Interest Group cell, Immunology & Molecular Biology of the Lung, The Annual Scientific Meeting for Leaders in Lung Health & Respiratory Science of The Thoracic Society of Australia and New Zealand (TSANZSRS 2019), Gold Coast Convention and Exhibition Centre, 29th March - 2 April 2019.

- Icomos2019 International Consortium of One Medicine One Science Travel Fellowship

Travel Fellowship to attend 4th International Conference on One Medicine One Science, International Consortium of One Medicine One Science, Chiang Mai, Thailand - February, 2019

- 2018 APSR Young investigator Travel Awards - Asian Pacific Society of Respirology

Travel award to attend 23rd Congress of the Asian Pacific Society of Respirology, Taipei, Taiwan - November 2018

- Australian Awards postgraduate research exchange fellowship

The University of Western Australia, Telethon Kids Institute, Perth Children Hospital and Government of Western Australia - March 2018

- 2018 Spring Outbound Doctoral Exchange Fellowship (Cross institutional Exchange)

The University of Western Australia - March 2018

- Overseas Research Experience Scholarship for Graduate Student

Graduate School Chulalongkorn University - February 2018

- Outstanding Excellent Achievement Award for Graduate Research Competition in Medicine

Gradate Research Competition, Graduate Affairs, Faculty of Medicine, Chulalongkorn University – August 2017

LIST OF PATENT

- Host defense peptide as an innovative novel therapy for *Pseudomonas aeruginosa* biofilm infections in the cystic fibrosis lung peptides – Pending approval

POSTERS

- A simple biofilm-guided antibiotic test for patients with chronic respiratory infections (Abstract selected to publish in Infection & Chemotherapy Journal, Volume 51, Sup 1, S93)
- Dhammika Leshan Wannigama, Anthony Kicic, Cameron Hurst, Peter N Monk, Robin James Storer,Tanittha Chatsuwan, Stephen M Stick, AREST CF
- ICIC (4th International Interscience Conference on Infection and Chemotherapy) & ISAAR (12th International Symposium on Antimicrobial Agents and Resistance) in Gyeongju, Korea (September 26 to 28, 2019)
- Towards effective treatment of complicated bacterial respiratory infections with host defence peptides (Abstract selected to publish in Infection & Chemotherapy Journal, Volume 51, Sup 1, S90)

- Dhammika Leshan Wannigama, Anthony Kicic, Cameron Hurst, Peter N Monk, Robin James Storer,Tanittha Chatsuwan, Stephen M Stick, AREST CF
- ICIC (4th International Interscience Conference on Infection and Chemotherapy) & ISAAR (12th International Symposium on Antimicrobial Agents and Resistance) in Gyeongju, Korea (September 26 to 28, 2019)

- A simple antibiotic susceptibility assay for *Pseudomonas aeruginosa* and *Acinetobacter baumannii biofilm* could lead to effective treatment selection for chronic lung infections peptides **(**Abstract selected to publish in CHEST Journal CHEST , Volume 155 , Issue 4 , 77A **)**

- *Dhammika Leshan Wannigama, Cameron Hurst, Lachlan Pearson, Thammakorn Saethang, Uthaibhorn Singkham-in, Sirirat Luk-in, Robin James Storer, Tanittha Chatsuwan*

- CHEST World Congress 2019, co-hosted by American College of Chest Physicians and the Thoracic Society of Thailand, Bangkok, Thailand **(**10-12 April, 2019**)**

- Fighting *Pseudomonas aeruginosa* and Nontypeable *Haemophilus influenzae* biofilms with host defence peptide as a novel step forward in the treatment of chronic lung infections **(**Abstract selected to publish in CHEST Journal CHEST , Volume 155 , Issue 4 , 73A**)**

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- A Simple and Reliable Way to Test Antibiotics on Biofilm could lead to Adequate Therapy Selection in Chronic *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Infections

- *Dhammika Leshan Wannigama, Cameron Hurst, Lachlan Pearson, Thammakorn Saethang, Uthaibhorn Singkham-in, Sirirat Luk-in, Robin James Storer, Tanittha Chatsuwan*
- 4th International Conference on One Medicine One Science, International Consortium of One Medicine One Science, Chiang Mai, Thailand (11-14 February, 2019)

ORAL PRESENTATIONS

- Host defense peptide as an innovative novel therapy for *Pseudomonas aeruginosa* biofilm infections in the cystic fibrosis lung (Abstract selected to publish in Journal of Respirology (2019), Abstract. Respirology, 24: 22-102)
- *Dhammika Leshan Wannigama, Anthony Kicic, Cameron Hurst, Peter N Monk, Tanittha Chatsuwan and Stephen M Stick & AREST CF*
- The Annual Scientific Meeting for Leaders in Lung Health & Respiratory Science of The Thoracic Society of Australia and New Zealand (TSANZSRS 2019), Gold Coast Convention and Exhibition Centre (29th March - 2 April 2019).
- Overcoming biofilm mediated respiratory infections through exploitation of pathogen and host-directed novel peptides (Abstract selected to publish in Journal of Respirology 23(S2): 67-68, 2018).
- *Dhammika Leshan Wannigama, Cameron Hurst, Peter Monk, Anthony Kicic, Stephen Stick, Tanittha Chatsuwan*
- The 23rd Congress of the Asian Pacific Society of Respirology, Taipei, Taiwan (November 29- December 2, 2018)

LIST OF ABBREVIATIONS

ATCC = American Type Culture Collection;

CLSI = Clinical and Laboratory Standards Institute;

cfu = colony-forming units;

EUCAST = European Committee on Antimicrobial Susceptibility Testing;

MHIIB = Müller-Hinton II broth;

MBEC = minimum biofilm eradication concentration;

MIC = minimum inhibitory concentration;

MBEC-75 = 75% non-viable cells;

MBEC-50 = 50% non-viable cells;

OD = optical density;

RFU = relative fluorescence units

S/B = signal-to-background;

S/N = signal-to-noise;

SD = standard deviations.

CHAPTER 1

Biofilm Infections

Like the majority of us who live in the urban community, bacteria also know that living together is the key to survival. Although they can be made up of a mixture of cells with complex structural molecules known as biofilms. Biofilms are predominantly composed of bacteria growing on both living and non-living foundations [1]. Bacteria whip their tail-like flagella to overcome the forces of the environment stream and anchoring on their chosen biotic or abiotic surface [2, 3]. This twitching motility event heralds the early establishment of the biofilm community to stacking bacteria cells on top of each other to create a series of tower block micro colonies [4]. And the matrix that holds these cells intact together is a gelatinous substance of exopolysaccharide (EPS) produced by the bacteria [4]. EPS is vital as it protects the biofilm from hostile environmental conditions [4]. In fact it has been found that the stronger the EPS, more impenetrable to antibiotics and immune cells [4, 5]. Like most crowded communities, biofilms also have a seedy part of colony to facilitate exchange of genetic material more efficiently between cells [4]. Such exchanges are contributed for antibiotic resistance, making the infection nearly impossible to eradicate [5]. Some bacterial cells within the biofilm morphed into temporarily dormant state or hibernation, sometimes for months at a time, during which time they would be insusceptible to antibiotics and immune cells

[6]. However, all these processes are a series of highly orchestrated events coordinated by a chemical language system termed quorum sensing [7]. This communication allows the millions of bacteria cells are in the biofilm to co-ordinate their behavior by regulating expression of certain genes and production of various virulence factors that allow the bacteria to invade an infected host [7, 8].

However, bacteria growing in this manner can have more serious implications for human health owing to their near invincibility to antibiotic treatments. Biofilm infections afflict millions of people around the world every year [9]. Those who suffer most tend to be high risk group patients and patients with immunocompromised conditions [10]. The more serious infections they can cause include soft tissue and wound infections in patients with burns and diabetics, bacterial endocarditis or infection of heart valves, colonization of the lungs of cystic fibrosis, COPD, asthma, ventilator and community associated pneumonia patients, cornel or eye infections, middle ear infections, bladder and kidney infections, catheter and surgical device infections [10-13]. Biofilm infection can stay with people for many years and wreak havoc with their lives [10]. Yet the ability to correctly diagnose and treat them remains limited [10]. When they grew on biofilms, along with host tissues, it displayed a range of alarming tactics to evade antibiotic treatment and host immune responses [10]. Patients with biofilm infections are typically prescribed a short course of antibiotics [14]. However, 80-90% of patients do not respond to these therapy and develop a chronic recalcitrant

infection that is difficult to eradicate [10]. Because bacteria cells that are floating free in the infection are killed off during short antibiotic therapy, but a reservoir might remain inside biofilm, contributing to relapse the infection [10]. Also, who suffer recurrent infections end up with antibiotic resistant strains. Several studies have shown that biofilm infection contributing to the vicious cycle of inflammation, infection, and disease progression [10].

Pseudomonas aeruginosa and *Acinetobacter bramuniiei* are among the most frequently isolated bacteria in biofilm infections [13, 15]. Both are an important opportunistic Gram-negative bacterium that infects critically ill, hospitalized patients, immunocompromised adults and other individuals [10, 13, 15, 16]. These patients suffer from highly recurrent chronic infections. And recurrent episodes are often caused by the same strains that caused the first infection, suggesting that presence of biofilm [10, 13, 15, 16]. Also secondary bacteremia and septic shock are associated with a poor prognosis [10, 13, 15, 16]. However, both are often present in patients with upper and lower respiratory tract infections [17] contributing to the maintenance of the vicious cycle of inflammation and progressive airways destruction, worst outcomes and morbidity [18]. But, *P. aeruginosa* and *A. bramuniiei* can also cause suppurative biofilm infections in other organs [19]. Both have been a major cause of infection is among wound, soft tissue, and invasive (blood and bone) infections in patients with traumatic injuries [10, 13, 15, 16]. Hospital-acquired or community-acquired *P. aeruginosa* and *A. bramuniiei* are most common clinical manifestations associated with

frequently multilobar and complicated biofilm [10, 13, 15, 16]. Also, multidrug-resistant *P. aeruginosa* and *A. bramuniiei* biofilm infections can be lethal for critically ill patients in intensive care units [10, 13, 15, 16]. Aspiration of air droplets of Pseudomonas and Acinetobacter are directly into the alveoli of mechanically ventilated patients through endotracheal tube, allowing for establishment of biofilm infection in respiratory tissue. Multiple studies have also shown that biofilm has a major impact on the organisms' virulence and strongly associated with increased mortality for community onset infections [10, 13, 15, 16]. Case fatality rates associated with chronic *P. aeruginosa* and *A. bramuniiei* infections are 20 to 60% [12].

Trends in the use of antibiotics and prevalence of treatment failure in biofilm infections

Antibiotics have long been used as all-purpose weapons against infectious. This tendency has caused many bacteria to develop intrinsic resistance to most of the currently used antibiotics. However, the tolerance of biofilms to antimicrobials is part of its unique structural and physiological mechanisms [9]. Such as, a restricted penetration of the antibiotics by matrix, restricted growth at low-oxygen stress, expression of biofilm-specific virulence genes and the presence of persisters or slow growing cells [10]. All the aforementioned mechanisms in biofilm are contributed to the failure of desired antibiotic activity in different manner. Such as the presence of polymorphonuclear leukocytes (PMN) at the site of biofilm infection consume oxygen creating low oxygen tension and restricting bacterial growth in chronic infection [9, 10, 20]. Also, biofilm itself have a deeper layer

of cells with slow growth. Therefore, reduced cell growth evidently enables them to escape the activity of antibiotics that target fundamental cellular processes (beta-lactam, quinolones, and aminoglycosides) [9, 10, 20]. Besides impairment of bacterial growth, low oxygen stress inhibits the reactive oxygen species (ROS) dependent effect of bactericidal antibiotics (fluoroquinolones, beta-lactams, and aminoglycosides) [9, 10, 20]. Moreover, restricted penetration of antimicrobials through the biofilm matrix components such as extracellular DNA, contribute to the antimicrobial tolerance (aminoglycosides) [9, 10, 20]. Also enzymes present in the matrix such as beta-lactamases inactivate the beta-lactam antibiotics [4, 9, 10, 20]. The different levels that antibiotics have to pass to reach the biofilm bacterial cells also contributed to large variation in the pharmacokinetics of antibiotics (clarithromycin, aminoglycosides) that may cause treatment failure [9, 10, 20]. Also, sub-inhibitory concentration of antibiotics exposer at the biofilm site contributed to antibiotic resistance development through acquisition of certain chromosomal mutations [4, 9, 10, 20]. The bactericidal effect of ciprofloxacin [21] on biofilm was reducing due to the selection of resistant mutants, despite adequate drug exposure. Differential expression of specific genes in biofilms sequester the bacteria cells form antibiotic, such as ndvB gene in *P. aeruginosa* biofilm which encodes an enzyme that is involved in the synthesis of periplasmic glucans that binds tobramycin [22].

Therefore, recalcitrance of biofilms to antibiotics is connected to the biofilm mode of growth and fundamentally different from antimicrobial intrinsic resistance bacteria in planktonic culture [10]. If bacteria originating from a biofilm are grown in planktonic culture, they may display susceptibility to antimicrobial activities. Currently, diagnosis of biofilm infections depends on culturing the organism from the site of infection followed by antimicrobial susceptibility testing in planktonic culture [23]. However, the gold standard test for antimicrobial testing (minimal inhibitory concentration (MIC)) in planktonic culture is not fit for purpose of guiding antimicrobial treatment in biofilm-associated infections. Therefore, susceptibility breakpoints and the PK/PD parameters that predict therapeutic success, based on planktonically growing bacteria offer no guidelines for clinicians to treat biofilm infections. According to numerous clinical research which suggests that it fails to eradicate most biofilm infections due to default position of prescribing antibiotics to most patients based on planktonic culture [10, 12, 23, 24]. Those studies considered the biofilm as a third niche, after the tissue (second) and blood (first) that the antibiotics have to move through in order to reach their bacterial cell target. Moreover, the local biofilm antibiotic concentration is dependent on the size and location of the biofilm as well as on individual drug metabolism [10, 12, 23, 24]. This result in significant antibiotic overuse, with resultant adverse effects, increased costs and risk of resistance.

CHAPTER 2

Quantitative scoring of differential drug sensitivity to biofilm populations of *Pseudomonas aeruginosa*: optimized susceptibility testing

Origin of the proposal: Aims and Objectives

Bacteria in biofilms differ from planktonic bacteria [1,2], and have extreme tolerance to immune responses and antimicrobial therapy [2,3]. Biofilm formation is an obstacle to the treatment of chronic infections with *Pseudomonas aeruginosa*, most of which are associated with biofilms [1,3]. Despite the negative impact of biofilms, to our knowledge, no treatment that directly targets bacteria in biofilms has yet been developed [4,5].

Biofilm recalcitrance to antibiotics is based on a mixture of resistance and tolerance [1,6]. Clinical dosages of antibiotics are usually determined from MICs for planktonic bacteria and as a result, patients may suffer from persistent infection over the course of weeks, or even months, often with recurrence of even more aggressive exacerbations [1,3]. Patients harbouring bacteria within biofilms require higher doses of antibiotics and prolonged courses of treatment [1,6].

Patients with chronic infections treated with antibiotic regimens based on biofilm susceptibility-testing have better clinical outcomes than those treated with regimens based on methods measuring planktonic bacterial susceptibility [5,7].

To our knowledge, there are no definitive methods for assessing the efficacy of anti-biofilm agents.

Flow systems or 96-well-mircotitre-plate incubations combined with various staining methods (e.g. Crystal Violet (CI 42555), Syto9, and propidium iodide), and spectrophotometry or confocal laser scanning microscopy have been used to assess cell viability [8-10]. Conventional plating is commonly used to quantify bacteria in biofilms, but requires manual detachment and dispersal [5]. Specific equipment (e.g. the Calgary biofilm device) is used characterize the minimum biofilm eradication concentration (MBEC) of antibiotics *in vitro* [11]. However, problems include poor reproducibility and low throughput, analysis requires considerable expertise, and non-specific staining of the biofilm matrix occurs [5]. Only limited clinical samples and statistical attributes have been used to substantiate the integrity of these methods.

In the present study, we developed a simplified antibiotic susceptibility assay based on a standardized model to quantify viable cells in biofilms of *P. aeruginosa*. Our model is based on the quantitative measurement of metabolically active cells using PrestoBlue, a resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide)-based viability indicator. We evaluated technical parameters, antibiotic resistance profiles, biofilm producing capacity, and association between MIC and MBEC for various sample types and antibiotics. This approach combining relevant statistical attributes with diverse clinical isolates might clarify the level of susceptibility of biofilms to various antibiotics, and ultimately contribute to resolving problems with biofilm-related infections.

Materials and Methods

Ethics

After approval by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (No. IRB 414/60), clinical isolates used in this study were selected from *P. aeruginosa* strain repository at King Chulalongkorn Memorial Hospital in Bangkok, Thailand. Clinical strains were isolated during 2015–2016 from chronically infected patients in the standard course of their care, and this isolation was unrelated to the present study.

Bacterial strains and growth conditions

P. aeruginosa PA01 biofilm-positive strain ATCC 15692 and clinical isolates were cultured on Müller–Hinton agar plates at 37°C. Without preference, we selected 137 unduplicated clinical isolates representing 137 patients and 14 collection sites (urine, bile, corneal scrapings, nasal swabs, tissue, blood, device related, broncho–alveolar aspirates, ear swabs, eye swabs, conjunctival swabs, wound pus, endotracheal aspirates, and sputum). Strains from patients with multiple sites of infection, were excluded and include samples from patients with infection at only single site. The strains had various morphology and resistance profiles. All isolates were stored at −80°C in tryptic soy broth containing 15% glycerol until subsequent experiments.

Antibiotics and agents

The biofilm eradication activity of seven antibiotics was tested against a subset of isolates (n = 137) with reference strain PA01. We tested four antibiotics (gentamicin, amikacin, ciprofloxacin, and ceftazidime) widely used for the treatment of *P. aeruginosa* infections. Other antibiotics tested either lacked conventional activity against planktonic cells of *P. aeruginosa*, but may had anti-biofilm effects (ceftriaxone), or were not commonly used as anti-pseudomonal antibiotics, either because of their recent introduction to clinical practice (fosfomycin) or toxicity (colistin). Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin, and ceftazidime were all from Sigma-Aldrich. Susceptibility testing for fosfomycin (Wako Chemicals) was determined by supplementation with 25 μg/mL glucose-6-phosphate. Antibiotic stock solutions were prepared <24 h before use. Antibiotics were dissolved in cation-adjusted Müller-Hinton II broth (MHIIB) (Becton Dickinson) and sterilized by filtration. Serial dilutions of the stocks were prepared in MHIIB immediately before use.

Optimization of biofilm formation

P. aeruginosa PA01 was used as a model organism to optimize parameters for biofilm formation in a 96-well-microtitre-plate format [8] with modifications to increase the procedure's compatibility with routine laboratory practice. A single colony of *P. aeruginosa* PA01 was inoculated into 2 mL of MHIIB medium in a tube and incubated in an orbital shaker (200 rpm) at 37°C overnight for about

16 h. Subsequently, a subculture was prepared by diluting the overnight culture with fresh medium to an OD of 0.02 at 600 nm (5×10^7 cfu/ml), and 100 μL aliquots added to flat-bottomed 96-well polystyrene microtitre plates (SPL Life Sciences), with uninoculated medium (100 μL) as a negative control, the plates were incubated at 37°C for 24 h. We used this procedure for subsequent biofilm experiments, which were performed in triplicate and repeated three times.

Optimizing fluorescence signal quality

PrestoBlue was added to the plates (10 μL/well), then incubated in darkness at 37°C for 15, 20, 30, 60, 120, or 240 min. Fluorescence was measured (excitation 535 nm and emission 590 nm) using two optional reading modes (top and bottom reading) with a microtitre plate reading fluorimeter (Varioskan Flash Multimode Reader; Thermo Fisher Scientific). MHIIB medium and a blank control were used to correct for the background signal of each well. The relationship between the fluorescent signal generated and bacterial concentrations in the wells was analysed for both bacterial suspensions and biofilms as described previously [12]. We calculated the mean fluorescence for test strains and negative controls. The relationship between the fluorescent signal generated by the reduced resazurin and bacterial concentrations in the wells for both (planktonic) suspensions and biofilms of bacteria was analysed. First, for (planktonic) bacterial suspensions, dilutions of an exponential phase bacterial culture (from 2.80×10^3 to 2.80×10^8 CFU/mL) were

prepared in 96-well microtitre plates. PrestoBlue (Invitrogen) was added directly to the wells (10 μL/well) and the plates were incubated in darkness at room temperature for 20 min and then the fluorescent signal measured as described above. Second, various biofilm concentrations were achieved by incubating suspensions (exponentially grown, 2.80×10^3 CFU/ml, 100 μL/well) in 96-well plates for various times ranging from 1 to 24 h. Biofilm formation was confirmed by Crystal Violet staining (40), followed by confocal laser scanning microscopy using live/dead bacteria staining as described previously (15). Before staining, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium, and PrestoBlue was added (10 μL/well) as described above. The mean fluorescence values for test strains and negative controls were determined in triplicate and assays were repeated three times. To measure actual bacterial concentrations for planktonic suspensions and biofilm, CFU counts were quantified using conventional plating techniques from replicate wells. The number of CFU per biofilm in each well represented the number of bacteria cells within the biofilm after biofilm formation. Before counting the CFU, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium and biofilms were scraped vigorously from the well surface, serially diluted in MHIIB medium, and plated on MHIIA.

Testing susceptibility to antibiotics

The MIC were established according to criteria in the EUCAST (criteria of *Enterobacteriaceae* for ceftriaxone and fosfomycin only) [13] and CLSI guidelines [14]. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. To establish planktonic MIC for the antibiotics used, the antibiotics were serially diluted two-fold in 96-well microtitre plates and bacteria added. The plates were incubated at 37°C for 18 h. MBEC were established by adding the serially diluted antibiotics to mature biofilms and incubating at 37°C for 24 h before staining with PrestoBlue to calculate the viable cells within the biofilm. Before adding the antibiotics, any non-adherent cells were removed from the mature biofilms by three gentle washes with medium.

Biofilm quantification and classification

Two methods were used to quantify [15] and classify [16] the biofilm formation capcity based on biofilm structure by Crystal Violet staining. Mean absorbances and their SDs were calculated for all tested strains and negative controls, determined in triplicate and repeated three times. The clinical isolates were classified as described previously [16].

Calculation of assay optimization parameters

The signal window coefficient Z'-factor, signal-to-noise (S/N), signal-to-background (S/B), were calculated as described previously. [17] Cell viability (%) was calculated using the formula: ((mean

signal of corresponding well – mean signal of negative control well)/(mean signal of positive control well – mean signal of negative control well)) × 100. Two cut-off values (50% and 75% non-viable cells) were used to determine the MBEC. Pearson correlation of PrestoBlue reduction to cfu/mL was analysed using the R statistical package [18].

Statistical analyses

Continuous variables are summarized using means and SDs, and categorical variables as counts and percentages. Levels of *P. aeruginosa* drug susceptibility are represented as a continuous measure of concentration; and an ordinal categorical form representing biofilm formation (negative, weak, moderate, or strong); both of these outcomes were measured repeatedly for each isolate. Linear mixed modelling was used to compare concentrations between test types (MIC vs MBEC) over time. We then examined which test types (MIC vs MBEC) were more successful in allowing concentration to be used to distinguish between biofilm formations (negative, weak, moderate, or strong) using ordinal logistic mixed effects regression. Finally, we examined whether concentration could be used to predict biofilm formation (negative, weak, moderate, or strong) using multi-nominal logistic regression. All analysis was conducted using the R statistical package [18], linear mixed modelling was performed using the R library, lme4 [19], and ordinal logistic mixed effect

modelling using the R library, ordinal [20] and multi-nominal logistic regression using the R library, nnet [21]. $P < 0.05$ was considered significant for all inferential analysis.

Results

Relative ability of planktonic and biofilm bacteria to reduce resazurin

We observed significant fluorescent signals when bacteria concentrations (planktonic and biofilm) were $<10^5$ cfu (Figure 1a). Fluorescence increased as the number of cells increased in the linear range from 10^6–10^8 to 10^4–10^8 cfu per biofilm. In a comparative analysis, planktonic cells showed stronger fluorescent signals than biofilm ($p < 0.005$) (p= 3.41E-6).

Effect of incubation time and fluorescence reading mode on the anti-biofilm assay performance

When staining biofilms of *P. aeruginosa* with PrestoBlue, 20 min was the shortest time providing acceptable results for bottom mode reading ($Z > 0.7$) (Figure 1b). For the top reading mode, 30 min was the shortest time providing acceptable results with a high signal window coefficient ($Z > 0.8$). Extending the incubation time dramatically decreases the quality of fluorescent signals in both modes of reading (lower Z and S/B) (Figure 1b). As shown in Figure 1b, 20–30 min incubation time provided fluorescent signals with high sensitivity and a low limit of detection.

Correlation between cfu and fluorescent signals of the anti-biofilm assays

In a susceptibility test, PrestoBlue and cfu counts showed an extremely close correlation ($P < 0.005$; linear modelling analysis; Figure 3). Non-viable cells rapidly lose the ability to reduce resazurin and do not generate a fluorescent signal. Despite the effect of a high antibiotic concentration, some

cells were persistently viable and generated detectable signals (Figure 4) as confirmed by CFU counts.

Association between antibiotic resistance and biofilm formation

Most strains were susceptible to tested antibiotics (Figure 2a), considerable variations in resistance ratios of ceftriaxone, ceftazidime, ciprofloxacin, and fosfomycin were noticed. Most strains showed high susceptibility to colistin, amikacin, and gentamicin. No isolate was resistant to all seven antibiotics.

In total, 127 (92%) isolates were positive for biofilm formation, and 56 (46%) isolates formed a stronger biofilm than PA01. Antibiotic resistant isolates tended to form stronger biofilms than sensitive isolates (Figure 2b). Strong and moderate biofilms showed similar levels of resistance in all three antibiotic resistance groups.

Effect of clinical sample type on susceptibility tests

For urine, corneal scrapings, nasal swabs, tissue, broncho–alveolar aspirates, ear swabs, eye swabs, conjunctival swabs, wound pus, endotracheal aspirates, and sputum, the variation in the MBEC (50 and 75) was much higher than in the MIC test (Figure 3). However, for ear swabs, device-related samples, and corneal scrapings, MBEC-75 is very pronounced (almost two times) compared with MBEC-50. While for isolates from blood, tissue, and bile, the trend for variation

(MBEC75>MBEC50>MIC) among the three tests was relatively lower than for other samples.

Correlation between biofilm formation and susceptibility test type

We found that an overall MBEC susceptibility test significantly modifies the relationship between biofilm formation and antibiotic concentration ($P < 0.001$; Figure 4). Strong and moderate biofilms exhibited similar trends for all of the antibiotics tested. The trend is very pronounced for amikacin and fosfomycin (MBEC75>MBEC50>MIC). Variation of the strong and moderate biofilm in MBEC-75 is much more pronounced for amikacin, ceftriaxone, and ceftazidime than other antibiotics, particularly colistin, where variation was comparatively low. MIC tests did not show any differences in association with weak, moderate, or strong biofilms.

Relationship between susceptibility test types and antibiotics

A linear mixed model revealed a significant relationship between the type of susceptibility test and antibiotics (Z^2_{LRT} = 312.26, 12 df, $P < 0.001$) showing that the magnitude of differences between tests was modified by antibiotics. Figure 5 shows all antibiotics except ceftriaxone and colistin tended to have the same general pattern (MBEC75>MBEC50>MIC). Whereas with ceftriaxone, the difference between MIC and MBEC 50 is much more pronounced, and for colistin the difference is much less pronounced.

Association of susceptibility test types, biofilm formation, and antibiotic concentrations

The association between odd ratios of MIC, MBEC (50 and 75) and concentration attribute of each antibiotic is shown in Table 1. For this analysis, we employed standardized concentrations (*Z*-scores) to avoid higher (raw) values of concentrations making associations appear more trivial. For each antibiotic, the odds ratios from MBEC-50 and 75 tests are a reflection of the higher level of associations than with MIC, except for fosfomycin. Notably, for gentamicin and amikacin the odds ratio of MBEC-50 was higher than for MBEC-75, but the significance levels were similar. However, for colistin, a similar level of significance was observed for the association between MIC and MBEC-50.

For all the tested strains, the accuracy of biofilm classification was higher for both MBEC-50 and 75 tests compared with a MIC test for each antibiotic (Table 1). The MBEC-50 correctly predicted the biofilm formation in gentamicin, ciprofloxacin, ceftriaxone, and fosfomycin, followed by ceftazidime. MBEC-75 is able to predict biofilm formation for colistin, and for amikacin both MBEC-50 and 75 displayed similar levels.

Figure 1 (a) Relationship between PrestoBlue reduction (in relative fluorescence units, RFU) and bacterial concentration (in cfu or cfu/mL) measured for planktonic bacteria and biofilms. (b) Robustness of the incubation time with PrestoBlue on the antimicrobial susceptibility assay performance, as measured by signal window coefficient, Z′-factor; signal-to-noise (S/N) and signal-to-background (S/B) ratios. (c) Correlation between number of cfu per biofilm as determined by plating (cfu plating) and biofilm cell viability as a percentage of P. aeruginosa PA01 exposed to increasing concentrations of antibiotic. (d) Distribution of the PrestoBlue-stained viable cells (colored bar chart) and cfu counts (colored symbols) of antibiotic-treated biofilms of P. aeruginosa PA01.

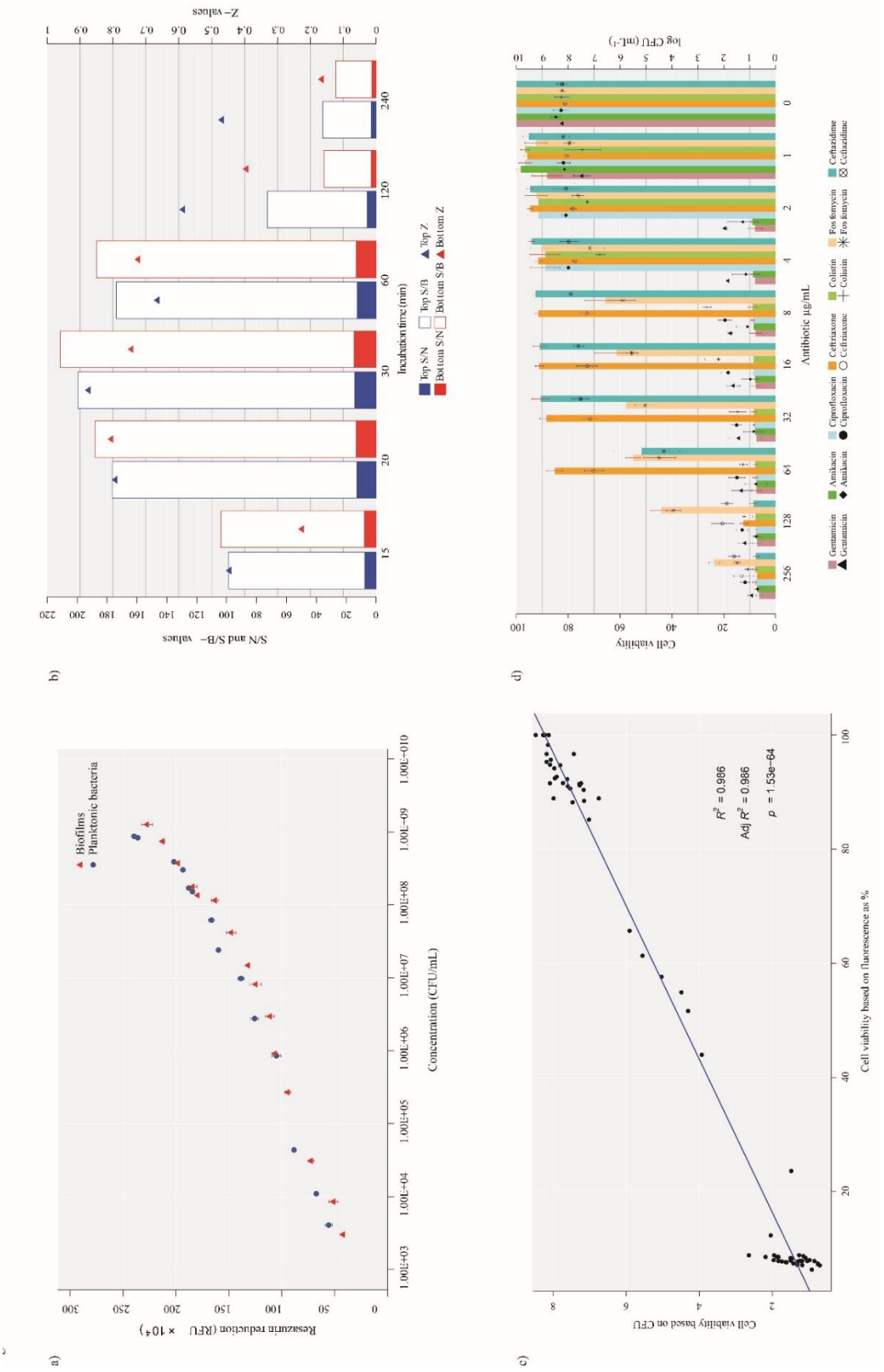

a)
Resazurin reduction (RFU × 10⁴)
Concentration (CFU/mL)
Biofilms
Planktonic bacteria
b)
S/N and S/B− values
Z− values
Incubation time (min)
Top S/N
Bottom S/N
Top S/B
Bottom S/B
Top Z
Bottom Z
c)
Cell viability based on CFU
Cell viability based on fluorescence as %
R² = 0.986
Adj R² = 0.986
p = 1.53e−64
d)
Cell viability
log CFU (mL⁻¹)
Antibiotic µg/mL
Gentamicin
Genta-micin
Amikacin
Ciprofloxacin
Ciprofloxacin
Ceftriaxone
Ceftriaxone
Colistin
Colistin
Fos fomycin
Fos fomycin
Ceftazidime
Ceftazidime

Figure 2 (a) Antibiotic susceptibility of clinical isolates of P. aeruginosa to 7 antibiotics. (b) Distribution of the susceptibility profile among various biofilm production capacities as a percentage.

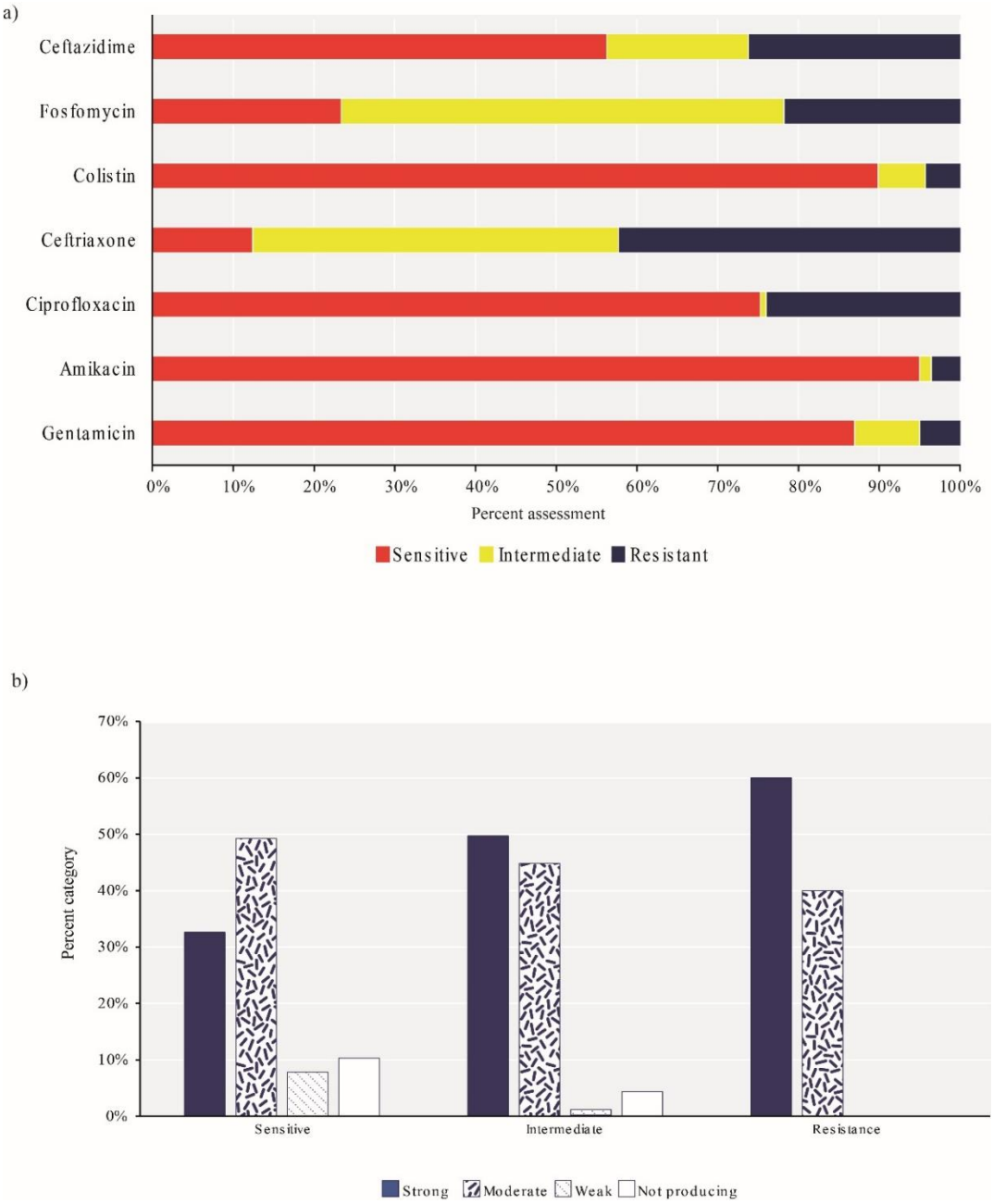


Figure 3 Relationship between susceptibility tests and type of clinical sample: (1) urine, (2) bile, (3) corneal scraping, (4) nasal swab, (5) tissue, (6) blood, (7) device related, (8) broncho–alveolar aspirate, (9) ear swab, (10) eye swab, (11) conjunctival swab, (12) wound pus, (13) endotracheal aspirate, and (14) sputum. MIC, minimum inhibitory concentration of planktonic cells based on conventional susceptibility test; and MBEC, minimum biofilm eradication concentration based on PrestoBlue cell viability indicator.

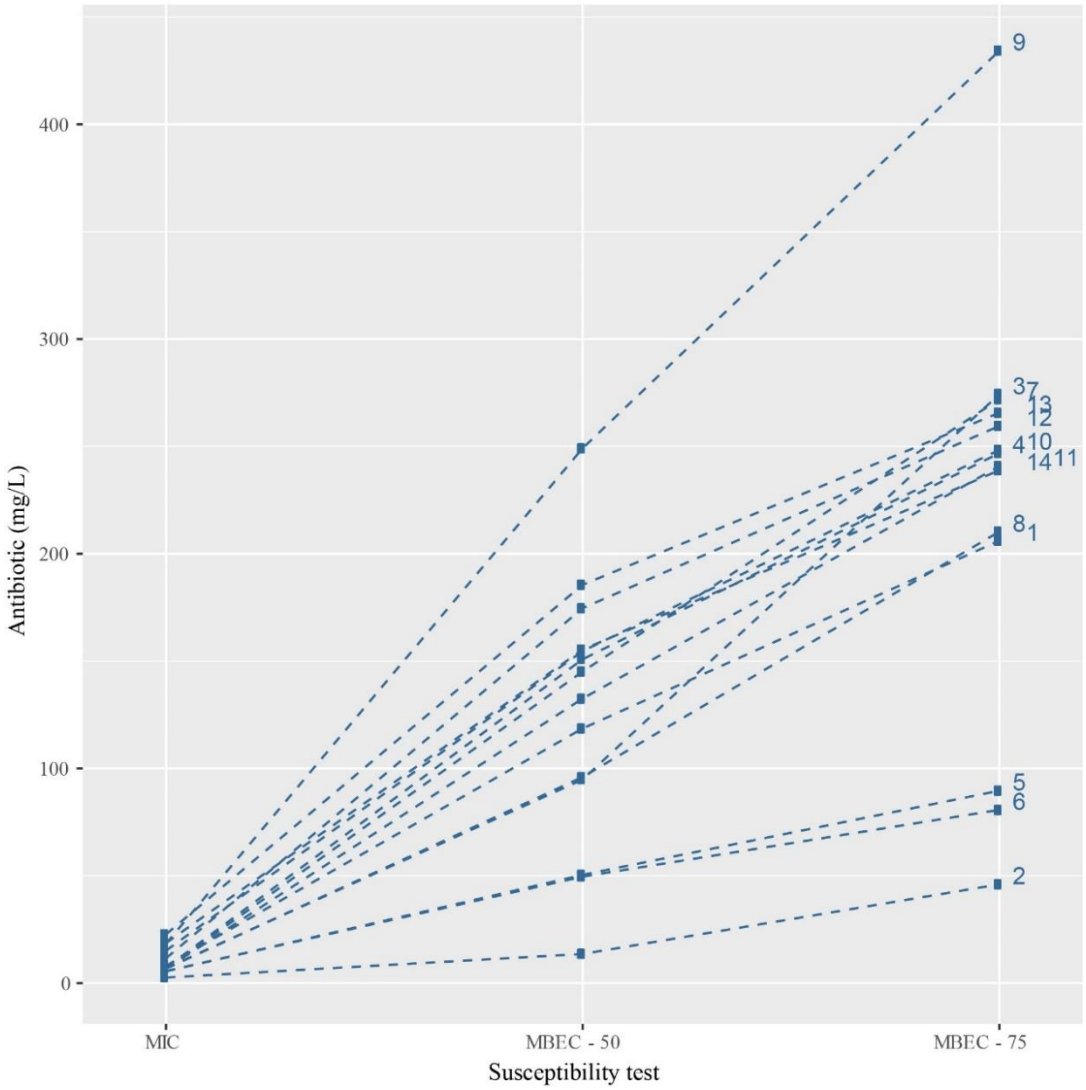


Figure 4 Association between the level of biofilm formation (negative, weak, moderate, or strong) and susceptibility test types to 7 antibiotics for P. aeruginosa clinical isolates. (a) gentamicin, (b) amikacin, (c) ciprofloxacin, (d) ceftriaxone, (e) colistin, (f) fosfomycin, and (g) ceftazidime. MIC, minimum inhibitory concentration of planktonic cells based on conventional susceptibility test; and MBEC, minimum biofilm eradication concentration based on PrestoBlue cell viability indicator.

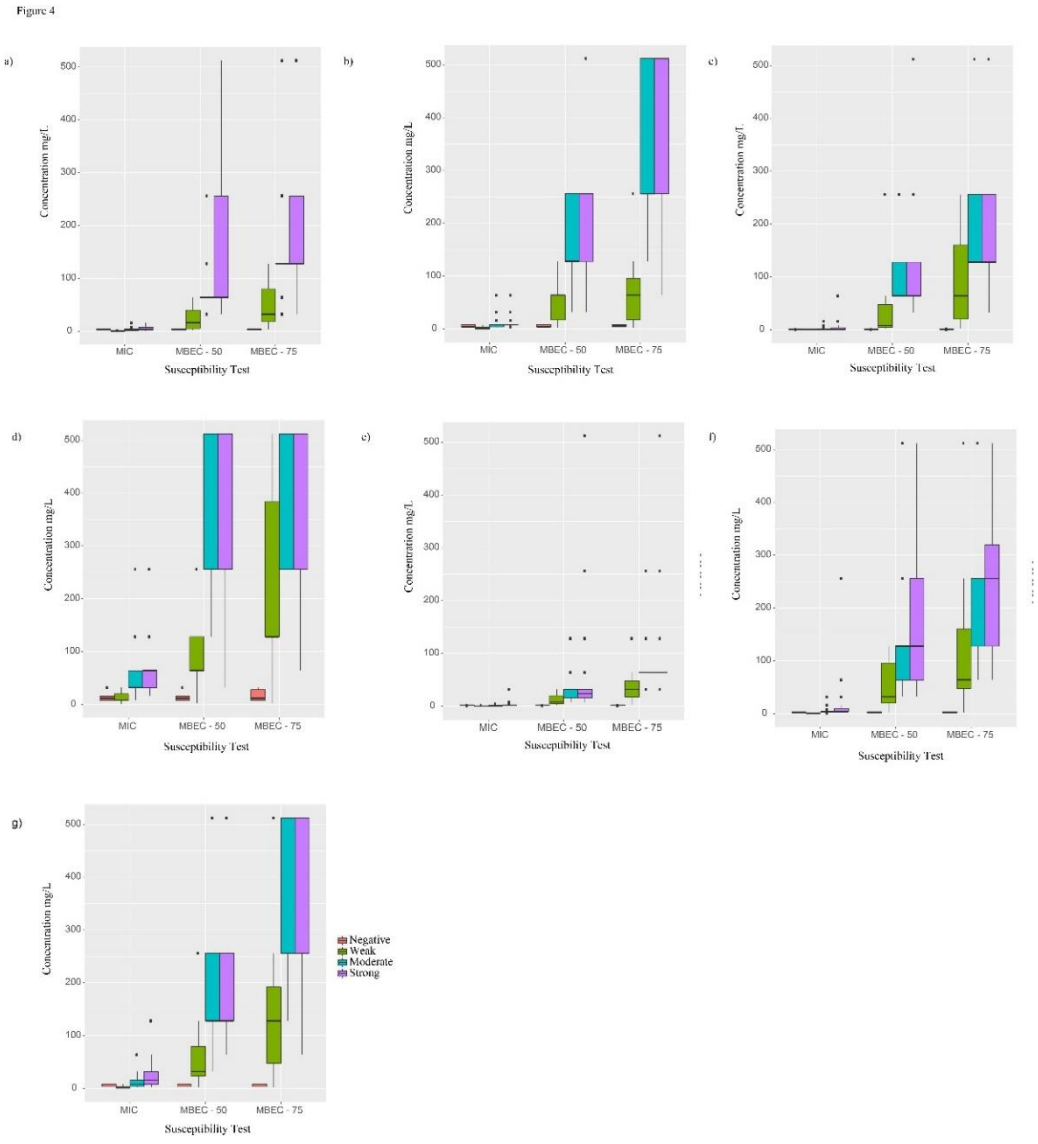


Figure 5 Relationship between susceptibility tests and 7 antibiotics for P. aeruginosa clinical isolates: (1) gentamicin, (2) amikacin, (3) ciprofloxacin, (4) ceftriaxone, (5) colistin, (6) fosfomycin, and (7) ceftazidime. MIC, minimum inhibitory concentration of planktonic cells based on conventional susceptibility test; and MBEC, minimum biofilm eradication concentration based on PrestoBlue cell viability indicator.

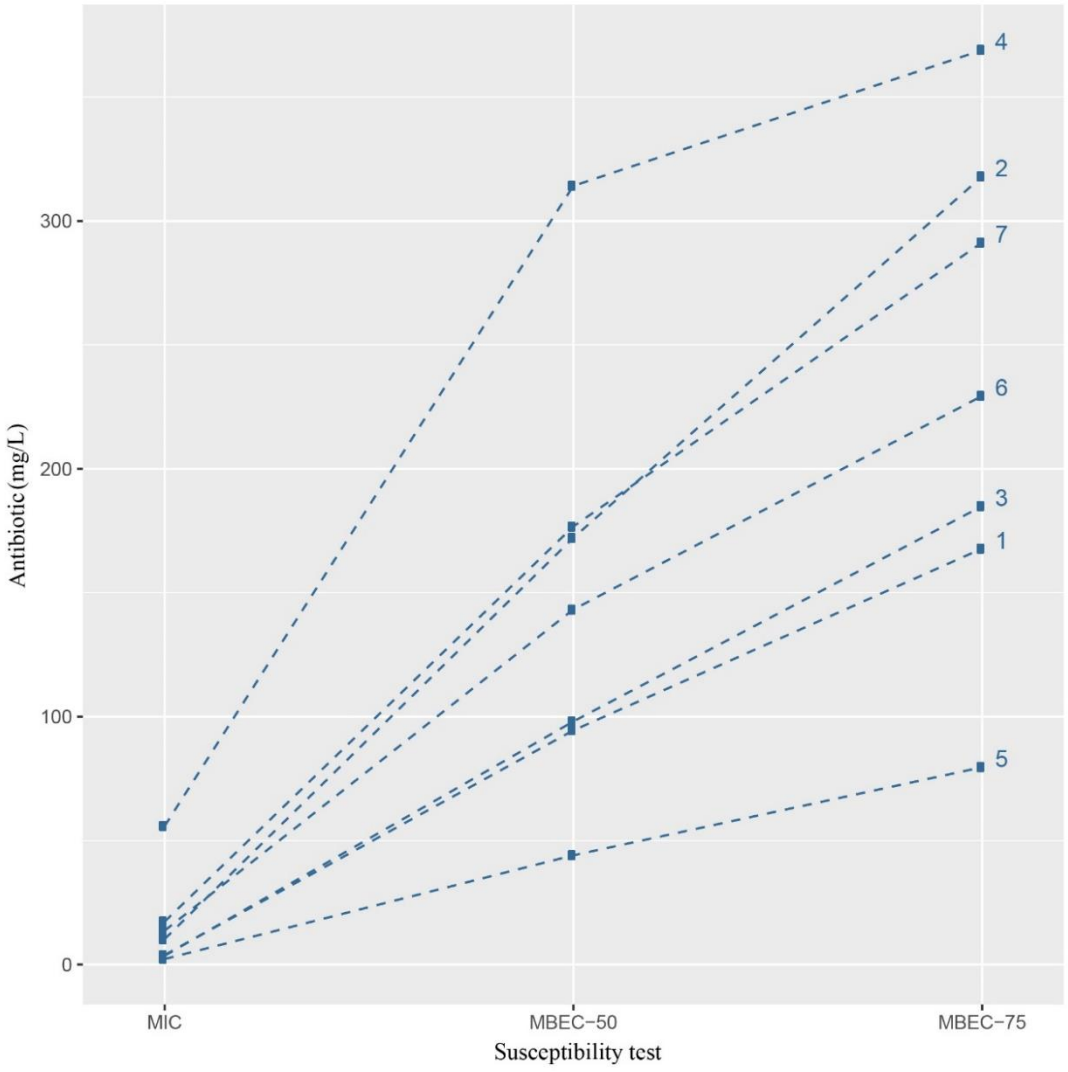


Table 1 ***Odds ratios with 95% CIs from ordinal mixed effect regression by susceptibility test types for each of the antibiotics based on standardized (Z-score) concentrations***

Antimicrobial agents	MIC†		MBEC-50‡		MBEC-75‡	
	OR (95% CIs)§	BFCA‖	OR (95% CIs)§	BFCA‖	OR (95% CIs)§	BFCA‖
Gentamicin	2.31** (0.00132, 0.0040(	61%	11.05*** (0.00390, 0.03132)	65%	4.18*** (0.00227, 0.00769)	62%
Amikacin	1.75* (0.00108, 0.00284)	50%	5.00*** (0.00251, 0.00996)	64%	4.06*** (0.00244, 0.00676)	64%
Ciprofloxacin	2.40 (0.00077, 0.00751)	51%	3.57*** (0.00196, 0.00650)	58%	5.40*** (0.00279, 0.01047)	55%
Ceftriaxone	2.49** (0.00135, 0.00459	50%	2.99*** (0.00196, 0.00456)	58%	2.62*** (0.00173, 0.00396)	54%
Colistin	8.66* (0.00153, 0.048.96	54%	7.49* (0.00154, 0.03636)	58%	18.66*** (0.00427, 0.08148)	58%
Fosfomycin	61.10* (0.00149, 2.50711	54%	2.91*** (0.00170, 0.00499)	58%	2.84*** (0.00182, 0.00444)	58%
Ceftazidime	1.54* (0.00103, 0.00231)	53%	2.20*** (0.00145, 0.00333)	56%	3.53*** (0.00216, 0.00576)	55%

†Minimal inhibitory concentrations (MIC, mg mL^{-1}) of planktonic cells.
‡Minimal biofilm eradication concentrations (MBEC, mg mL^{-1}) were categorized as responsive reaching about 50% and 75% of the total non-viable cells within a given antibiotic concentration range.
§Odds ratio with 95% confidence interval
‖Biofilm formation classification accuracy (negative, weak, moderate, and strong)

Discussion

Compared with conventional resazurin-based assays [22-26], we achieved a substantial decrease in incubation time while maintaining sensitivity and an accurate linear range for signal-detection, leading to increased efficiency and excellent reproducibility. A ratio of 10 μL PrestoBlue reagent to 100 μL cell culture volume resulted in a stronger fluorescent signals and weaker background fluorescence than other resazurin-based assays [22-26]. The stronger fluorescent signals found using the bottom mode with a shorter incubation time than for the top mode mean that detecting the fluorescence resulting from adherent biofilm viability is substantially more efficient using measurements made from the bottom of the well, because this approach shortens the distance between the biofilm and the sensor, minimizing interference from the growth medium. Further, this well-bottom reading approach uses at least two times less PrestoBlue than required in other approaches [7-13], substantially saving on reagent expenses. Resazurin-based reagents are designed for use in an end point assay and prolonged exposure to light will increase background fluorescence and decrease sensitivity [4,7-13], Therefore, the length of incubation is important for optimum results [4,7-13]. The present study confirmed that prolonged retention of PrestoBlue ultimately results in a weak fluorescent signal with poor assay quality [25]. However, other investigators have assumed that exposure of bacteria to resazurin while treating with a test drug may produce better fluorescent signals, especially for bacteria with low metabolism [22]. We found that this assumption

cannot be made. Early exposure to staining with a test drug only produces fluorescent signals during the first several hours, and decreases fluorescence at the end point [27].

When testing PA01 biofilm responses to antibiotics, the present assay was able to detect the presence of cells at $<10^4$ cfu. This sensitivity increases the lower limit of quantification for selecting a suitable MIC [28]. Not only is the assay sufficiently sensitive to detect low numbers of cells, it also produces a significantly higher fluorescence with a wider linear range. Calibration curves made with planktonic bacteria may not be able to estimate cfu in biofilms [7-13]. We found a notable difference in fluorescent signals generated by cells in suspension compared with biofilm. This is probably because of the low metabolic activity of persister cells and higher cell density within the biofilms [2]. However, we found a linear correlation between the fluorescent signal and the cfu counts in a PA01 biofilm susceptibility test.

Biofilms in human niches differ widely between sources of infections [1,2,4]. In the present study, most of the sample types displayed higher variation in MBEC than MIC. This is exemplified by infections that are directly associated with biofilms [1,3]. Device-related infection, otitis media, peritonitis, and corneal ulcers require higher concentrations of drugs to achieve 75% MBEC. This may be particularly relevant in the case of implanted devices, where there is a temporal loss of symptoms and late recurrence of infection with frequent treatment failure, requiring surgical removal of devices [1]. For planktonic bacteria, antibiotics must achieve a greater than 4 $\log_{10}$

reduction to fulfil performance standards [15]. However, for biofilms the reduction required to treat infection effectively is unknown [4,5]. We found that two MBECs (50 and 75) were important to monitor the impact of antimicrobials against clinically relevant attributes, and the MIC was unable to provide such information.

Recalcitrance of biofilms to antibiotics is reversible and transient [3]. In the present study, the planktonic state was not always resistant to tested antibiotics, while the moderately and strongly biofilm-forming strains dominated those with resistance. However, some strains were resistant to some antibiotics when not residing in biofilms, and this resistance was considered as being inherited. These findings highlight that in addition to inherited resistance mechanisms, biofilm-mediated tolerance also contributes to bacterial survival in high concentrations of antibiotics [17,19].

Strong and moderate biofilms required increasing the concentrations of amikacin, ceftriaxone, and ceftazidime to reach more than 75% of viable cells. These antibiotics are commonly used to treat chronic respiratory, urinary tract, sinus, and ear infections [29], and often fail to resolve them [1,3,6]. The effectiveness of ciprofloxacin is greater than ceftazidime, while both fluoroquinolones and β-lactams are less effective against biofilms than planktonic cells [30]. Such reduced effect may result from bacteria establishing biofilm infections within a protected niche and an altered physiological state to act as a barrier to delay the diffusion and action of antibiotics [1-3,6]. Gentamicin penetrates

moderate biofilms readily, but strong biofilms poorly. The odds ratio and biofilm formation effects in MBEC-50 highlighted impaired penetration of biofilm matrices by gentamicin and amikacin. Delayed penetration of negatively-charged matrices by positively-charged aminoglycosides may explain differences in drug effectiveness [1,30]. The reduced diffusion and slow growth in biofilms exposes bacteria to only low doses of antibiotics, and creates drug resistant reservoirs that ultimately succumb to infection [2,31]. The penetration of both moderate and strong biofilms by fosfomycin is significantly reduced, through contributes to the high levels of concentration to achieve MBEC 75. However, we noticed that fosfomycin has a higher odds ratio for MIC with lower levels of significance and accuracy. We consider that this may be the result of biofilm formation not being important in some strains with intrinsic fosfomycin resistance. The lack of information about intrinsic resistance levels of *P. aeruginosa* to fosfomycin in CLSI or EUCAST guidelines made it difficult to define the correct MIC for *P. aeruginosa* using breakpoints for *Enterobacteriaceae*. Further research into the most appropriate breakpoints of fosfomycin for *P. aeruginosa* is needed to provide the information. The need for a high dose of ceftriaxone for weak, moderate, and strong biofilms highlights that the most effective strategy for their recalcitrance is through the emergence of persister cells [2,32], illustrating how bacterial biofilms can actually thrive, rather than die, when antibiotics are given high doses [2,31-33]. Also these finding emphasized that it cannot be recommended as therapy in against *P. aeruginosa* biofilm, even it has some activity against

planktonic cells of some tested strains based on *Enterobacteriaceae* criteria. However, on the basis of our results and previous reports [13,14] it cannot be recommended as potential mono therapy for either biofilm or non-biofilm *P. aeruginosa* infections. Furthermore, colistin can diffuse through biofilms and is able to kill non-growing cells. The concentration of colistin to achieve 50% cell death in MBEC is similar to the MIC. However, to achieve 75% non-viable cells, colistin is needed at a high dosage. This observation suggests that relative effectiveness depends on particular antibiotics and biofilms [1,30,34]. Because the MIC is unable to differentiate *P. aeruginosa* sensitive to those antibiotics when tested under biofilm conditions, we can conclude that the present model is valid for predicting antibiotic sensitivity of biofilms. The key advantages of the present assay are first that it simplifies biofilm formation and viability assessment to provide accurate antibiotic selection in a clinically meaningful time frame. Second, the assay is a valid way to differentiate anti-biofilm effectiveness based on sample type. We observed marked differences between MBEC patterns of test samples to match various types of chronic infections. These findings suggest reconsideration of therapies currently used for various chronic infections with *P. aeruginosa* biofilms. Third, findings *in vitro* to assess the effectiveness of anti-biofilm therapies are transferable to situations *in vivo*. We showed the manner in which biofilm-induced tolerance and intrinsic resistance become integrated to promote biofilm-specific antibiotic resistance. The higher level of significant odds ratios with biofilm formation classification accuracy of MBEC-50 and 75 tests suggest

that they have better discriminatory power than a MIC test. The accuracy of biofilm formation classification shows that to exceed 50% cell death in a biofilm is crucial for the efficacy of particular antibiotics. This provides evidence for the lack of correlation between current conventional susceptibility testing and therapeutic success in chronic infections [4,5]. There are few prospective data demonstrating the clinical efficacy of biofilm specific susceptibility testing.

Summary of the strengths and limitations

In the presently described assay, the effect of antibiotics on biofilm were determined, although appropriate standard reference values required to clear infections in vivo remain unclear. The present results are specific to *P. aeruginosa* clinical isolates and the assay might need some modifications before it can be applied to other species or multispecies biofilms. The use of nutrient-rich media to generate biofilms may not precisely replicate the bacterial milieu in clinical situations. Nevertheless, the media are considered appropriate because standard MIC tests are performed in vitro using nutrient-rich medium as recommended by CLSI or EUCATS. Depending on the model of the incubator, heterogeneous evaporation of the samples may adversely affect the comparability of the generated biofilms with those in clinical circumstances. The presently described experiments included only 7 antibiotics. Nevertheless, those chosen represent a substantial proportion of currently used classes of antibiotics. Other drug classes may need to be evaluated in a similar fashion to determine their effectiveness. Combining the present quantitative screening of bacterial

biofilm-specific antibiotic resistance with clinical trials of antibiotics would clarify the clinical applicability of the assay.

Conclusions

In conclusion, the presently described quantitative screening assay of bacterial biofilm-specific antibiotic resistance assay is a versatile, easy to manage, and robust method that should help to improve treatment of infections that are threats in the clinic.

CHAPTER 3

A simple and reliable way to test antibiotics on *Acinetobacter baumannii* biofilm could lead to adequate therapy selection

Origin of the proposal: Aims and Objectives

Every medical procedure that depends on antibiotics to fight infections could become compromised by resistance to antibacterial drugs. Bacteria have acquired increasing resistance to antibiotics since their introduction and this causes extensive illness and deaths globally. Among the bacteria that are alarmingly prevalent are multi-drug resistant *Acinetobacter baumannii*, which causes some 60% of hospital-acquired or nosocomial infections [25]. These bacteria have become prevalent in communities, causing ventilator associated pneumonia, blood stream, and a variety of skin and tissue infections in both healthy and immune-compromised individuals [10, 25]. Indeed, the majority of infections are chronic biofilm-associated infections that are highly resistant to therapy, with 40%–60% mortality rates [10, 25].

The biofilm structure makes it difficult for antibiotics to kill the bacteria that form biofilms, and subsequent infection can persist up to weeks or months, develop even greater resilience against antibiotics and spreading to other organs [9, 10, 26]. The biofilms can be impenetrable to antibiotics and immune cells, and bacteria in the deeper portions of the biofilms are in a state of slow growth, which acts as structural and physiological protection against antibacterial agents [13, 26]. The

biofilm phenomenon is also responsible for producing various virulence factors to invade host immune systems to mount episodes of acute overexuberant inflammatory response [27].

Predisposing factors for antibiotic treatment failure are numerous, ranging from biofilm recalcitrance towards the treatments and lack of appropriate antibiotic selection tests [10]. The better selection of antibiotics for biofilm infection has long been enigmatic. Routine clinical selection of antibiotics are usually based on minimum inhibitory concentrations (MICs) for planktonic bacteria, rather than those for bacteria in biofilm growth states [10, 24, 28]. Therefore, rapid and accurate treatment is often difficult in routine clinical practice because physiological manifestation of biofilm conditions is not accurately represented in MIC testing procedures.

Nevertheless, antibiotic regimens based on biofilm susceptibility testing studies highlight the remarkable improvement in clinical outcomes compared with those based on standard MIC test results, and allow physicians to identify more rapidly the appropriate antibiotic for patients with chronic biofilm infections [29-32]. It is well recognized that, a simple, rapid antimicrobial susceptibility test for biofilms is crucial for better clinical decision making to control chronic biofilm infections with appropriate antibiotic therapy [23, 24, 32].

Currently, most common method to quantify biofilms is conventional plating and requires manual detachment and dispersal [33]. However, to our knowledge, there are currently no definitive, standardized, rapid methods to discriminate the efficacy of antibiotics between biofilm and non-

biofilm bacteria. Various research groups have developed various methods to characterize bacterial biofilm and antibiotic susceptibility *in vitro* [32]. Some methods involve staining (e.g. with Crystal Violet (C.I. 42555), Syto9, and propidium iodide) to evaluate cell viability in biofilms via spectrophotometric measures or using confocal laser scanning microscopy [34-36]. Other methods require specific equipment (e.g. the Calgary biofilm device and biofilm ring test) [32, 34, 36-38] to characterize the minimal biofilm eradication concentration (MBEC) for various antibiotics. These methods are not so simple for routine clinical use, and may require considerable expertise and expensive instruments to analyse the results, complex laboratory procedures, and may result in non-specific staining of the biofilm matrix rather than viable cells. Only a very limited number of equivalent biofilms can be produced at the same time with poor reproducibility [33]. Studies have often used only limited clinical samples and statistical attributes to claim the integrity of the methods.

Here we developed a simple fluorometric-based assay that rapidly quantified metabolically active bacterial cells in *A. baumannii* biofilm using PrestoBlue, a resazurin (7-hydroxy-3*H*-phenoxazin-3-one-10-oxide)-based viability indicator. We rendered this simple approach into a standard reliable test by carefully combining relevant statistical attributes with diverse clinical isolates to provide an accurate and precise quantitative analysis of MIC and MBEC for various clinical sample types and

antibiotics. The present assay represents a potentially definitive way of predicting how bacteria within biofilm will respond to antibiotic treatment.

Materials and Methods

Bacterial strains and growth conditions

After approval of the study protocol by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 414/60), *Acinetobacter baumannii* clinical isolates (n=138) with various morphology and resistance profiles were obtained without preference from a strain repository at the Department of Microbiology, King Chulalongkorn Memorial Hospital. Clinical strains had been isolated during 2016–2017 from 137 chronically infected patients and represented 7 collection sites (including urine, nasal swabs, tissue, broncho–alveolar aspirates, wound pus, endotracheal aspirates, and sputum) as part of standard care of the patients and was unrelated to the present study. Strains from patients with multiple sites of infection, were excluded and include samples from patients with infection at only single site. *A. baumannii (*ATCC 19606*)* biofilm-positive strain and clinical isolates were cultured on Müller–Hinton agar (Sigma-Aldrich) plates at 37°C. The cultures were stored at −80°C in tryptic soy broth (Sigma-Aldrich) with 15% glycerol until they were used in subsequent experiments in which they were suitably anonymised.

Antibiotics and agents

The biofilm eradication activity of ten antibiotics was tested against a subset of isolates (n = 137) with reference strain ATCC 19606. Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin,

ceftazidime, imipenem, meropenem, and sulbactam were purchased from Sigma-Aldrich. Susceptibility testing for fosfomycin (Wako Chemicals) was determined by supplementation with 25 μg/mL glucose-6-phosphate (Sigma-Aldrich). Antibiotic stock solutions were prepared less than 24 h before use. Antibiotics were dissolved in cation-adjusted Müller-Hinton II broth (MHIIB) (Becton Dickinson) medium and the supplemented medium sterilized by filtration through a membrane filter with 0.22-μm pores. Serial dilutions of the antibiotic stocks were prepared in MHIIB medium immediately before use.

Optimization of biofilm formation

*A. baumannii (*ATCC 19606*)* was used as a model organism to optimize parameters for biofilm formation in a 96-well-microtitre-plate format as described previously with modifications to make the procedure more compatible with routine laboratory practice [34]. Initially, a pure culture of a single colony of *A. baumannii* was inoculated into 2 mL of MHIIB medium in a tube and incubated in an orbital shaker (200 rpm) at 37°C overnight for about 16 h. Subsequently, a subculture was prepared from the overnight culture by diluting it with fresh MHIIB medium to an optical density (OD) of 0.02 at 600 nm (5×10^7 CFU/ml) and 100 μL aliquots were added in triplicate to flat-bottomed 96-well polystyrene microtitre plates (SPL Life Sciences), with uninoculated MHIIB medium (100 μL) in triplicate as a negative control, the plates were incubated at 37°C for 24 h.

After standardizing the conditions, we used the procedure to test the 117 biofilm-positive, and 20 biofilm-negative clinical isolates for their antimicrobial susceptibility profile under biofilm growth conditions. All experiments were performed in triplicate and repeated three times.

Optimizing fluorescence signal quality

The 96-well-microtitre plates were incubated in darkness at 37°C for six different times (15, 20, 30, 60, 120, and 240 min). The fluorescence of each well contents was measured (excitation 535 nm and emission 590 nm) using two optional reading modes (top from above the plate and bottom from below the plate) using a microtitre plate reading fluorimeter (Varioskan Flash Multimode Reader; Thermo Fisher Scientific). MHIIB medium and a blank control were used to correct for the background signal of each well. The parameters of signal window coefficient Z'-factor, signal-to-noise (S/N), and signal-to-background (S/B) were calculated using the corresponding formulae: S/N = (mean signal – mean background)/SD of background, S/B = mean signal/mean background, Z = 1 – ((3SD of sample + 3SD of control)/ (mean of sample – mean of control). The relationship between the fluorescent signal generated by the reduced resazurin and bacterial concentrations in the wells for both (planktonic) suspensions and biofilms of bacteria was analysed. First, for (planktonic) bacterial suspensions, dilutions of an exponential phase bacterial culture (from 2.80×10^3 to 2.80×10^8 CFU/mL) were prepared in 96-well microtitre plates. PrestoBlue (Invitrogen) was

added directly to the wells (10 μL/well) and the plates were incubated in darkness at room temperature for 20 min and then the fluorescent signal measured as described above. Second, various biofilm concentrations were achieved by incubating suspensions (exponentially grown, 2.80 × 10^3 CFU/ml, 100 μL/well) in 96-well plates for various times ranging from 1 to 24 h. Biofilm formation was confirmed by Crystal Violet staining (40), followed by confocal laser scanning microscopy using live/dead bacteria staining as described previously (15). Before staining, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium, and PrestoBlue was added (10 μL/well) as described above. The mean fluorescence values for test strains and negative controls were determined in triplicate and assays were repeated three times. To measure actual bacterial concentrations for planktonic suspensions and biofilm, CFU counts were quantified using conventional plating techniques from replicate wells. The number of CFU per biofilm in each well represented the number of bacteria cells within the biofilm after biofilm formation. Before counting the CFU, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium and biofilms were scraped vigorously from the well surface, serially diluted in MHIIB medium, and plated on MHIIA.

Testing susceptibility to antibiotics

The MIC were established using standard techniques according to criteria in the EUCAST (criteria for *Enterobacteriaceae* for fosfomycin only) [39] and CLSI guidelines [40]. *E. coli* ATCC 25922*, and P. aeruginosa* ATCC 27853 were used as quality control strains, with modifications as follows. To establish planktonic MIC for the antibiotics used, the antibiotics were serially diluted two-fold in 96-well microtitre plates (from 0.015 to 4098 μg/mL) and bacteria added. The plates were incubated at 37°C for 18 h. Minimal biofilm eradication concentrations (MBEC) were established by adding the serially diluted antibiotics to mature biofilms and incubating at 37°C for 24 h and then staining with PrestoBlue. Before adding the antibiotics, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium. Cell viability was calculated using the corresponding formula: cell viability (%) = ((mean signal of corresponding well – mean signal of negative control well)/ (mean signal of positive control well – mean signal of negative control well)) × 100. Two cut-off values (50% and 75% non-viable cells) were used to determine the MBEC. Pearson correlations of PrestoBlue reduction to CFU/mL was analysed using the R statistical package [41]. All experiments were performed in triplicate and repeated three times.

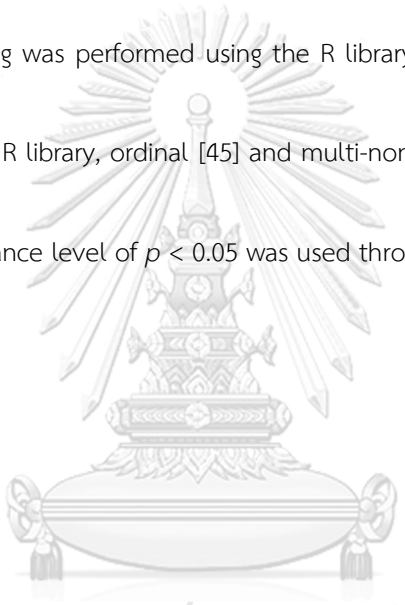
Biofilm quantification and classification

Two methods were used to quantify [42] and classify [43] the biofilm by Crystal Violet staining with modifications. The Crystal Violet (0.1%) stained biofilms were solubilized with 30% acetic acid followed by incubation at room temperature for 10–15 min. The absorbance (OD) at 550 nm was determined using a microtitre plate reading spectrophotometer with 30% acetic acid as a blank. Mean absorbances and their standard deviations (SD) were calculated for all tested strains and negative controls, performed in triplicate and repeated three times. The cut-off value (ODβ) was defined as 3SD above the mean OD of the negative controls: ODβ = average OD of the negative controls + 3SD of negative controls, and was calculated separately for each microtitre plate. The OD of a tested strain was expressed as the mean OD of the strain minus the ODβ (OD = mean OD of a strain – ODβ). The clinical isolates were classified as described previously [43].

Statistical analyses

Variables were described using standard deviations means and for continuous variables, and counts and percentages for categorical variables. The levels of drug susceptibility were represented in two ways: a continuous measure of concentration; and an ordinal categorical form representing biofilm formation (negative, weak, moderate, or strong), and both of these outcomes were measured repeatedly over time for each isolate, we employed mixed modelling to analyse these longitudinal

outcomes. Linear mixed modelling was used to compare concentrations (quantitative) between test types over time. We then examined which test better discriminated between biofilm formations (negative, weak, moderate, or strong) using ordinal logistic mixed effects regression. Finally, we examined whether concentration could be used to predict biofilm formation using multi-nominal logistic regression. All analysis was conducted using the R statistical package [41]. The linear mixed modelling was performed using the R library, lme4 [44], ordinal logistic mixed effect modelling using the R library, ordinal [45] and multi-nominal logistic regression using the R library, nnet [46]. A significance level of $p < 0.05$ was used throughout all inferential analysis.



Results

Fluorescent signals from planktonic cells are sturdier than those from cells in biofilm

The quantity of resorufin produced was linearly proportional to the viable bacterial cell concentrations in both planktonic and biofilm growth conditions (Fig. 6A). The linear range observed between 10^4–10^8 CFU per biofilm and while significant fluorescent measurements were detected when bacteria concentrations (planktonic and biofilm) were $<10^4$ CFU (Fig. 6A). The planktonic cells showed a stronger fluorescent signal than those within the biofilm ($p < 0.005$) (p= 3.98E-7).

Incubation time and fluorescence reading mode are important to optimise fluorescent signals

The minimum incubation period required to generate an adequate fluorescent signal above background was within the range 20–30 min (Fig. 6B). In the fluorescence reading mode from above the 96-well micro-titre plate (top mode), 30 min was the shortest time providing good results with a high signal window coefficient ($Z > 0.8$). A 20-min incubation period when using the top reading mode was sufficiently short to generate adequate sensitivity ($Z > 0.7$, higher S/B and S/N). For the bottom reading mode from the underside of the plate, 30 min was the shortest time providing good results with a high signal window coefficient ($Z > 0.8$). Changes in quality of fluorescent signals (lower Z and S/B) between modes of reading can be observed after one hour incubation suggest

interference at incubation times longer than 30 min (Fig. 6B).

The standard colony count correlated to fluorescent signals of PrestoBlue

Linear correlation ($p < 0.005$) (linear modelling analysis) between average fluorescence intensity of PrestoBlue and the CFU counts in biofilms were observed in a susceptibility test (Supplementary Fig. 12). We also found that some cells were able viable at high concentrations of antibiotics and emit the detectable fluorescence signals (confirmed by CFU counts) (Supplementary Fig. 13) without interference from background signals.

Differential responses to antibiotics by planktonic bacteria and those in biofilms are typically not a result of inherited resistance

The prevalence of the antibiotic resistance among the 138 resistant isolates included in the study are shown in Figure 2A. More than 50% of the isolates show high resistance to amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, and meropenem. Meanwhile, some isolates display considerable intermediate susceptibility to colistin, fosfomycin, and sulbactam (Fig. 7A).

We classified 119 isolates as biofilm positive, and 62 isolates as strong biofilm producers. However, within biofilm-positive isolates there was much heterogeneity in antibiotic susceptibility (Fig. 7B). We observed significant association between a strong and moderate level of biofilm production, and antibiotic resistance ($p < 0.001$). Strong biofilms were dominant in intermediate and resistant isolates ($p < 0.001$). By contrast, there was a significant association between antibiotic sensitive and

isolates forming moderate biofilms ($p < 0.001$).

Rationale for the anti-biofilm method for detecting differences in antibiotic susceptibility levels

A significant association (Z^2_{LRT} = 347.21, 18 df, $p < 0.001$) between the antibiotics and type of susceptibility test were confirmed by linear mixed modelling. Figure 8 shows immensity levels of discriminate power between each test type was modified by antibiotics. The pattern of MBEC75>MBEC50>MIC to tested antibiotics is more prevalent in all isolates (Fig. 8). In other instances, e.g., for colistin, the levels of discrimination between MIC and MBEC are much less prominent than for other antibiotics. Whereas for ceftriaxone, fosfomycin, imipenem, and meropenem the difference between MIC and MBEC50 is more pronounced. The MIC test has a relative paucity to differentiate antibiotic susceptibility in biofilms (Fig. 8). Because the overlapping set of antibiotic effective concentrations may constrained the possible basis for selection of appropriate antibiotics (Fig. 8).

The anti-biofilm test finds a correlation between level of biofilm formation and antibiotic responses

We conducted a comparative analysis of biofilm forming capacities of each isolate and three types of susceptibility tests for each antibiotic tested (Fig. 9). Of note, all isolates showed an MBEC susceptibility values were significantly modified by biofilm formation with similar direction for strong

and moderate biofilms ($p < 0.001$) (Fig. 9). In cases where an MIC test was unable to discern any relevant differences in association with weak, moderate, or strong biofilms. Eight antibiotics (gentamicin, amikacin, ciprofloxacin, ceftriaxone, fosfomycin, imipenem, meropenem, and sulbactam) showed an obvious tendency (MBEC75>MBEC50>MIC) for the required effective concentration to eliminate different biofilm forming capacity of the isolates (Fig. 9). Notably, isolates forming a strong and moderate biofilm had a pronounced difference in sensitivity to ciprofloxacin, ceftriaxone, and sulbactam in the MBEC75 test. This tendency was relatively different particularly for colistin, but also ceftazidime and fosfomycin, which displayed less dissimilarity in sensitivity between planktonic and biofilm states than the other antibiotics.

Anti-biofilm test reveals different susceptibility levels in clinical isolates exposed to the same antibiotic

The association between susceptibility test types and type of clinical isolates are illustrated for each antibiotic by 'spaghetti plots' (Fig. 10). Notably, most antibiotics had overlapping concentration lines for each type of clinical isolate in the MIC test, while MBEC50 and 75 discriminate between each type. The concentration of four antibiotics (colistin, fosfomycin, ceftazidime, ciprofloxacin) was skewed heavily towards MBEC75 (more than two-fold) for isolates from urine, nasal swabs, and broncho–alveolar aspirates. Meanwhile, isolates from nasal swabs display significant variation between MIC and MBEC 50 for sulbactam and fosfomycin ($p < 0.001$)

(Fig. 10). The variation between MBEC50 and 75 was less pronounced for isolates from wound pus: amikacin ceftriaxone and ceftazidime; tissue: ceftazidime and sulbactam; endotracheal aspirates: ceftazidime and fosfomycin; urine: sulbactam; and endotracheal aspirates: fosfomycin. Imipenem, and meropenem demonstrated a substantially similar pattern of variation (MBEC75>MBEC50>MIC) for all types of clinical isolates (Fig. 10).

Relationship between sample types and biofilm formation capacity is dissected by the anti-biofilm test method

In Figure 6 we plot a systematic comparison of biofilm formation capacities with each type of clinical isolate to underpin the efficacy of susceptibility tests. Our results show that when the level of biofilm formation is incorporated, nearly all types of clinical isolates exhibit consistent variation with either MBEC50 or 75. The moderate and strong biofilms show reasonable similarity for MBEC50 in nasal swabs, broncho–alveolar aspirates, endotracheal aspirates, and sputum. The isolates from urine, tissue and broncho–alveolar aspirates are predominant with strong and moderate biofilms, while only isolates from wound pus dominated with strong biofilm formation (Fig. 11).

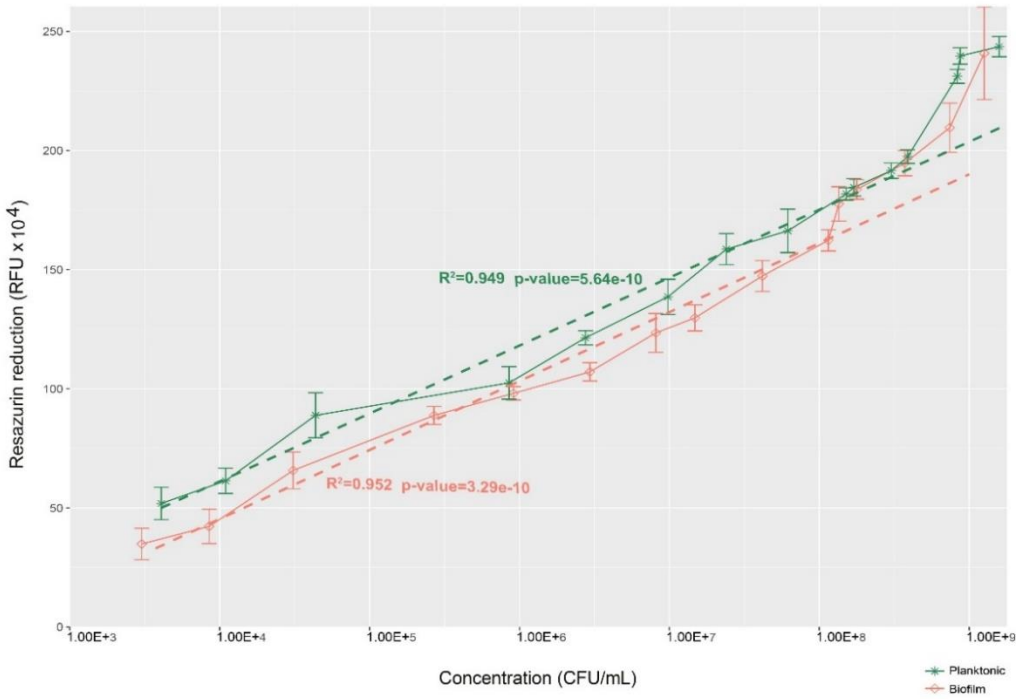
MBEC50 and 75 of antibiotics predicts the capacity of isolates to form biofilms

We used standardized values of concentrations (*Z*-scores) to avoid higher (raw) values of concentrations that make associations appear more trivial (Table 2). This analysis confirmed that both MBEC50 and 75 tests have significant capacity to classify biofilm formation accurately ($p <$

0.001) for the tested antibiotics compared with a MIC test. The MBEC50 and 75 data showed a high consistency to predict correctly the biofilm formation as “negative” or “weak” or “moderate” or “strong”. The MBEC75 tests clearly predicted biofilm formation more accurately than the MBEC50 test and higher divergence was seen for imipenem, meropenem, and sulbactam.

Figure 6 (A) Relationship between PrestoBlue reduction (in relative fluorescence units, RFU) and bacterial concentration (in colony forming units or CFU/mL) measured for planktonic bacteria and biofilms. (B) Robustness of the incubation time with PrestoBlue on the antimicrobial susceptibility assay performance, as measured by signal window coefficient, Z′-factor; signal-to-noise (S/N), and signal-to-background (S/B) ratios.

a)


Resazurin reduction (RFU x 10^4)
250
200
150
100
50
0
R²=0.949 p-value=5.64e-10
R²=0.952 p-value=3.29e-10
1.00E+3
1.00E+4
1.00E+5
1.00E+6
1.00E+7
1.00E+8
1.00E+9
Concentration (CFU/mL)
Planktonic
Biofilm

b)

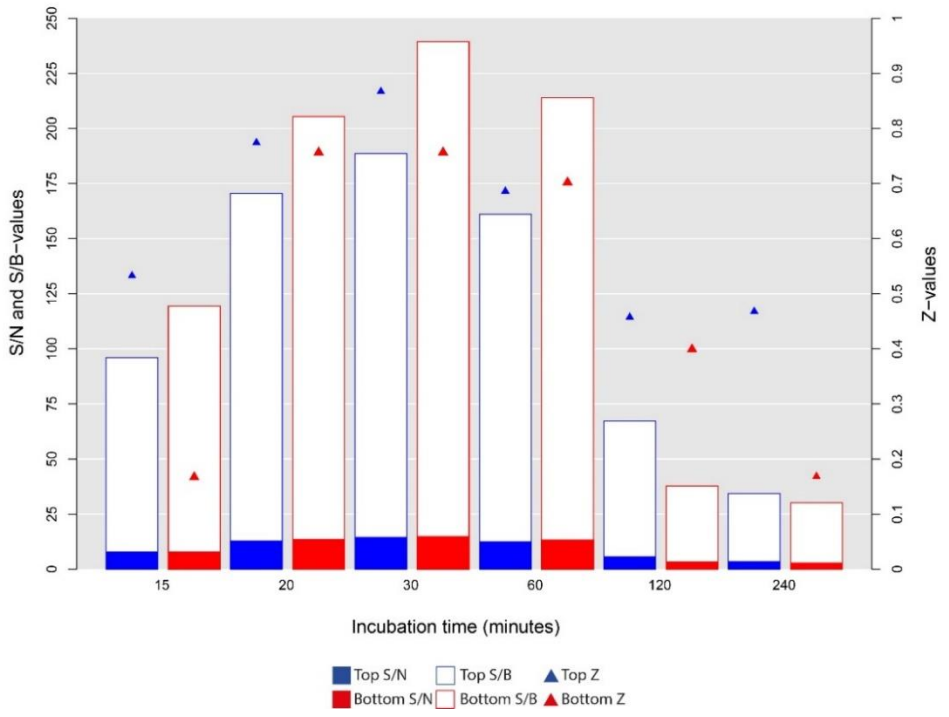
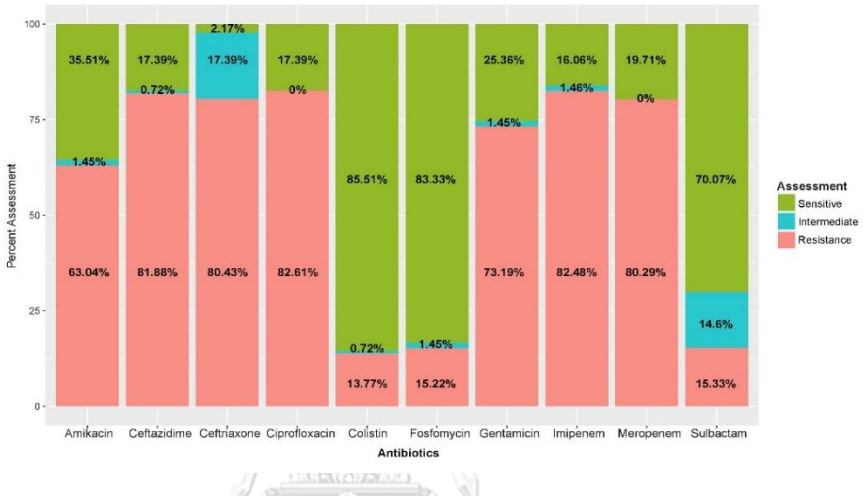

S/N and S/B−values
Z−values
0
25
50
75
100
125
150
175
200
225
250
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
15
20
30
60
120
240
Incubation time (minutes)
Top S/N
Top S/B
Top Z
Bottom S/N
Bottom S/B
Bottom Z

Figure 7 (A) Antibiotic susceptibility of clinical isolates of A. baumannii to seven antibiotics. (B) Distribution of the resistance among various biofilm production capacities as a percentage.

a)



b)

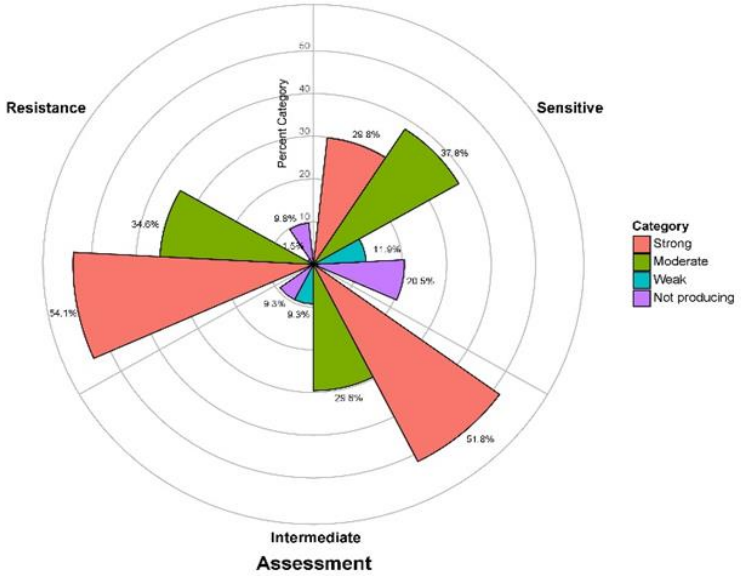


Figure 8 Relationship between susceptibility of A. baumannii clinical isolates and ten antibiotics ***(****1, gentamicin; 2, amikacin; 3, ciprofloxacin; 4, ceftriaxone; 5, colistin; 6, fosfomycin; 7, ceftazidime; 8, imipenem; 9, meropenem; 10, sulbactam****).***

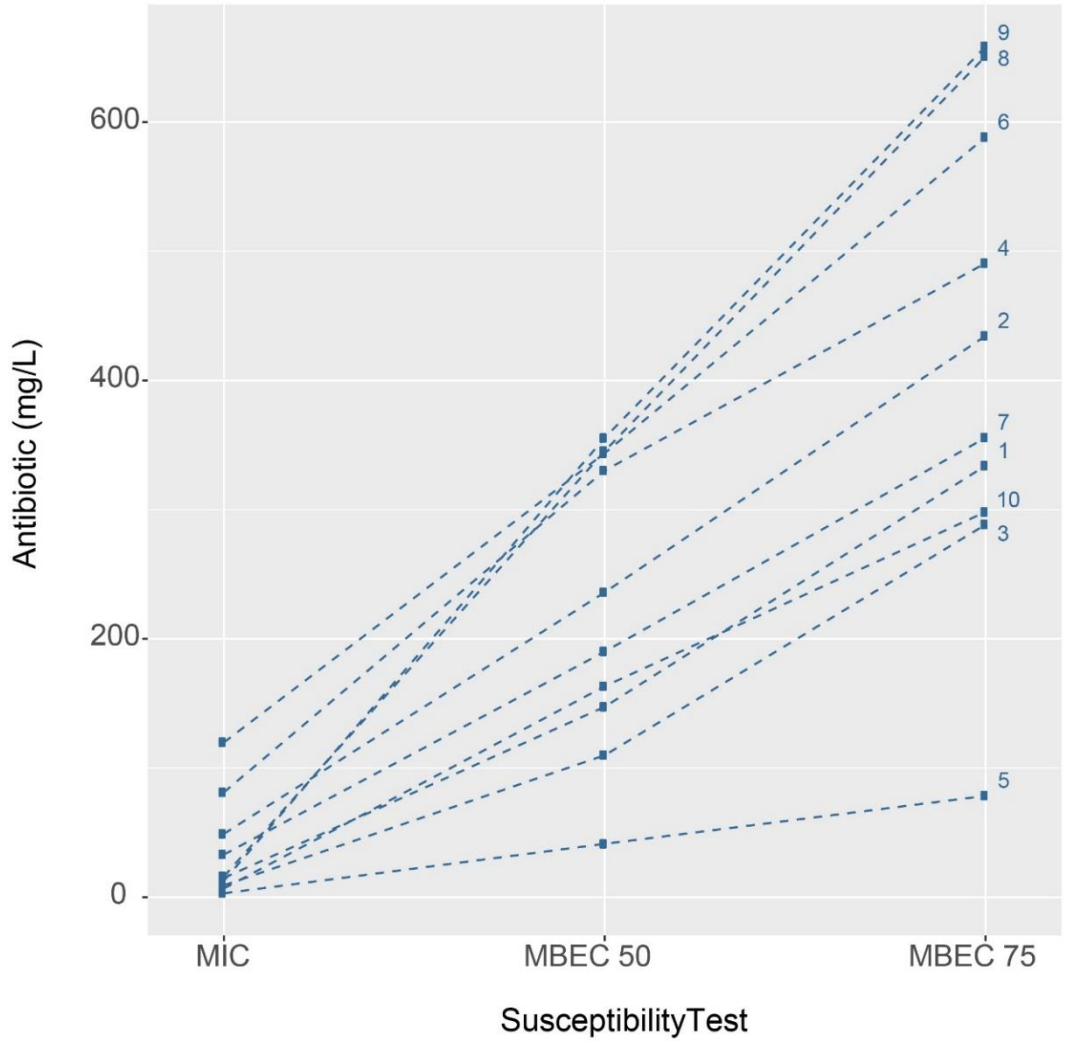


Figure 9 Association between the level of biofilm formation (negative, weak, moderate, or strong) and susceptibility of A. baumannii clinical isolates test results for ten antibiotics. (A) gentamicin, (B) amikacin, (C) ciprofloxacin, (D) ceftriaxone, (E) colistin, (F) fosfomycin, (G) ceftazidime, (H) imipenem, (I) meropenem, and (J) sulbactam.

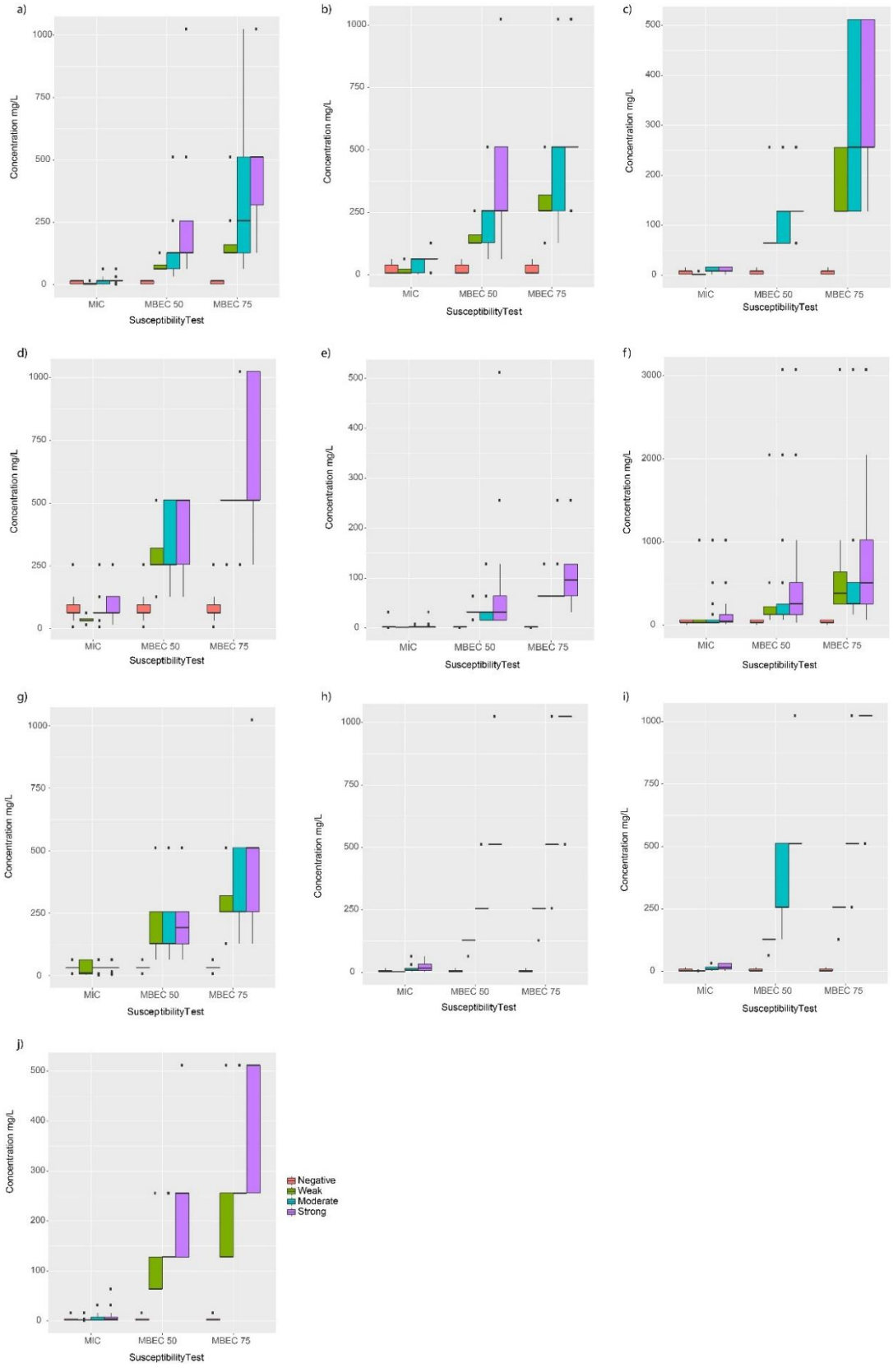
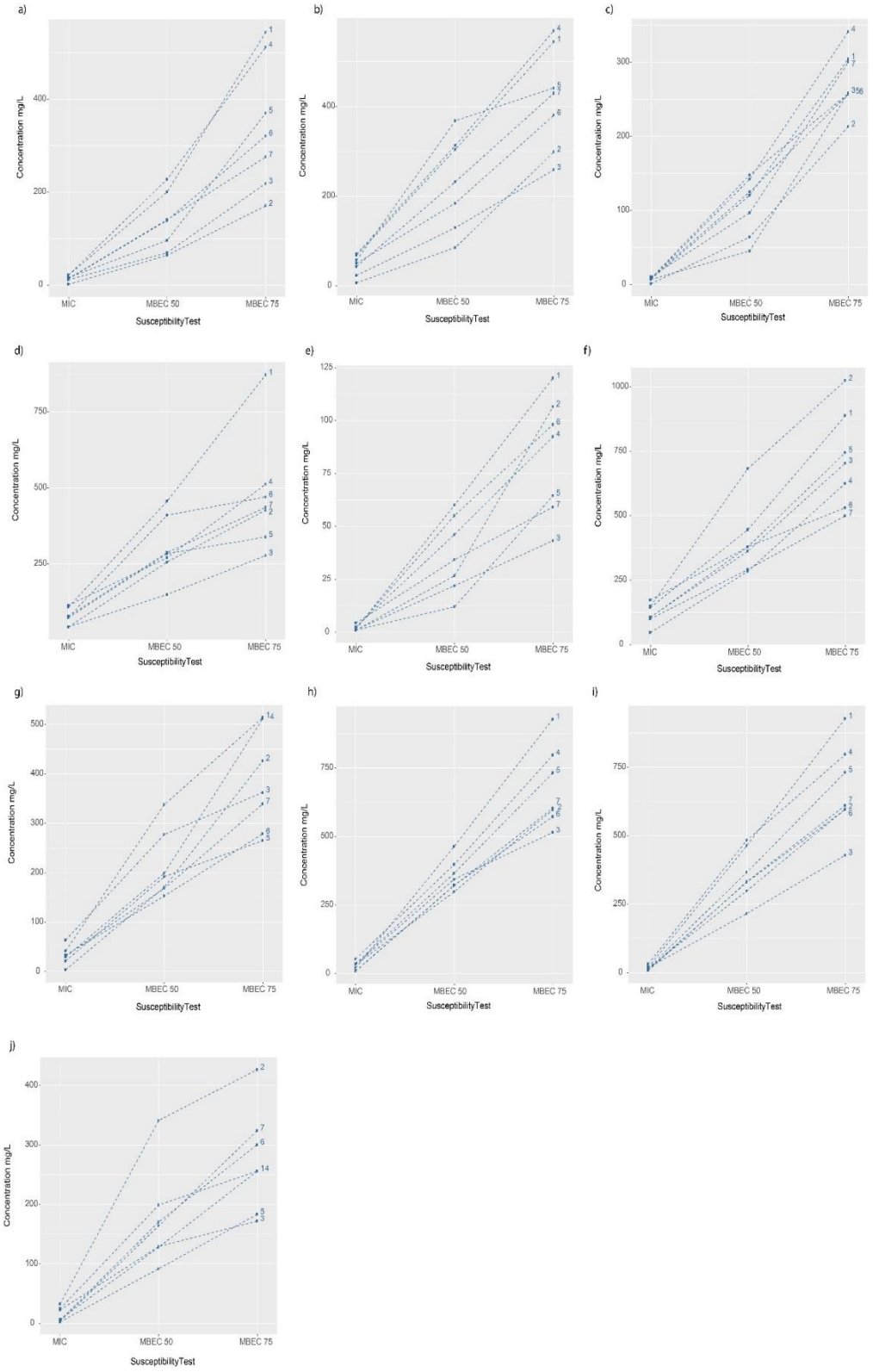

a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
Concentration mg/L
MIC
MBEC 50
MBEC 75
SusceptibilityTest
Negative
Weak
Moderate
Strong

Figure 10 Association between the type of A. baumannii clinical isolate sample (1, urine; 2, nasal swabs; 3, tissue; 4, broncho–alveolar aspirates; 5, wound pus; 6, endotracheal aspirates; and 7, sputum) and susceptibility to 10 antibiotics. (A) gentamicin, (B) amikacin, (C) ciprofloxacin, (D) ceftriaxone, (E) colistin, (F) fosfomycin, (G) ceftazidime, (H) imipenem, (I) meropenem, and (J) sulbactam.


a)
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j)
Concentration mg/L
MIC
MBEC 50
MBEC 75
SusceptibilityTest

Figure 11 Relationship between susceptibility test results, biofilm formation (negative, weak, moderate, or strong) and type of clinical sample (1, urine; 2, nasal swab; 3, tissue; 4-broncho–alveolar aspirate; 5, wound pus; 6, endotracheal aspirates; and 7, sputum).

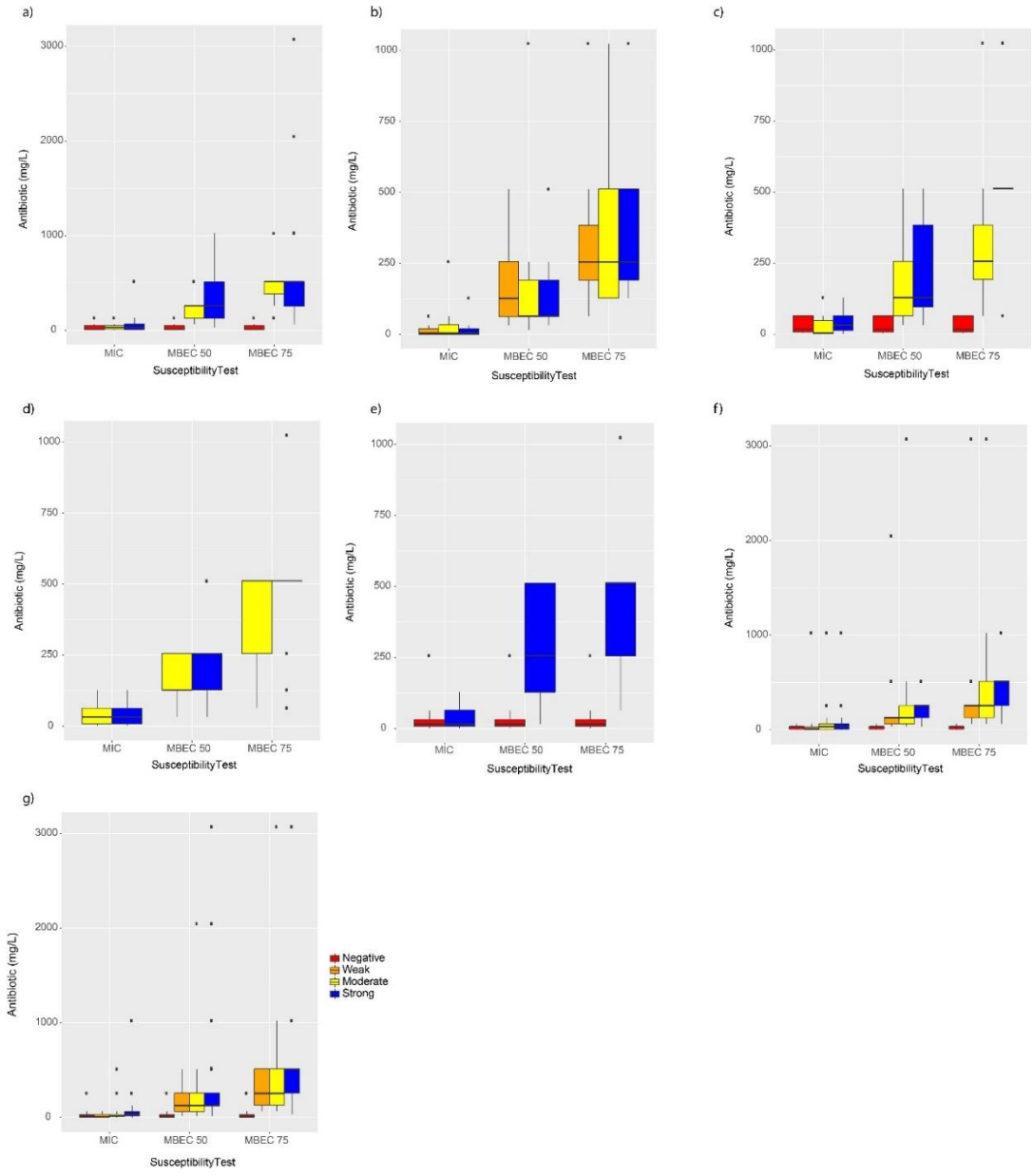


Table 2 Accuracy of biofilm classification ("negative" or "weak" or "moderate" or "strong") from ordinal mixed effect regression by susceptibility results for each of the antibiotics based on standardized (Z-score) concentrations.

Antibiotic	MIC†	MBEC50‡	MBEC75‡
Gentamicin	48.55%	65.22%	68.84%
Amikacin	54.35%	69.57%	68.84%
Ciprofloxacin	48.55%	64.49%	65.22%
Ceftriaxone	46.38%	52.17%	54.35%
Colistin	44.93%	52.17%	64.49%
Fosfomycin	44.93%	57.25%	64.49%
Ceftazidime	48.55%	60.87%	65.22%
Imipenem	57.24 %	94.92%	95.65%
Meropenem	63.04 %	86.95%	97.10%
Sulbactam	44.92%	74.63%	73.91%

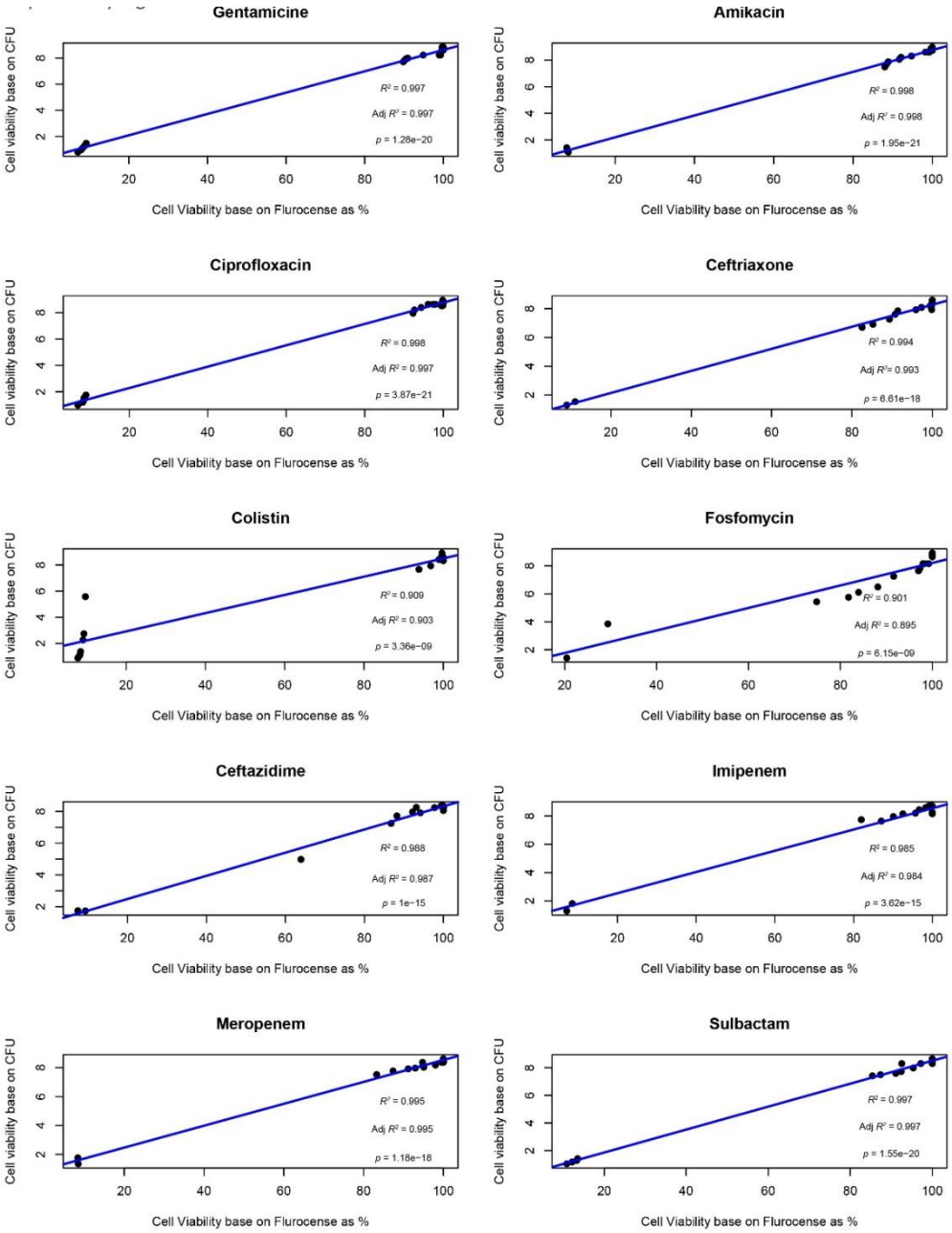
†Minimal inhibitory concentrations (MIC, μg mL ^{1}) for planktonic cells.
‡Minimal biofilm eradication concentrations (MBEC, μg mL^{-1}) were categorized as responsive reaching about 50% and 75% of the total non-viable cells within a given antibiotic concentration range.
The percentage (%) represent prediction of biofilm classification ("negative" or "weak" or "moderate" or "strong") accuracy for all tested antibiotics based on standardized (Z-score) concentrations.

Supplementary *Table 3 Betas with 95% confidence interval (95% CIs) from linear mixed modelling by compare concentrations (quantitative) between susceptibility test types for each of the antibiotics over time.*

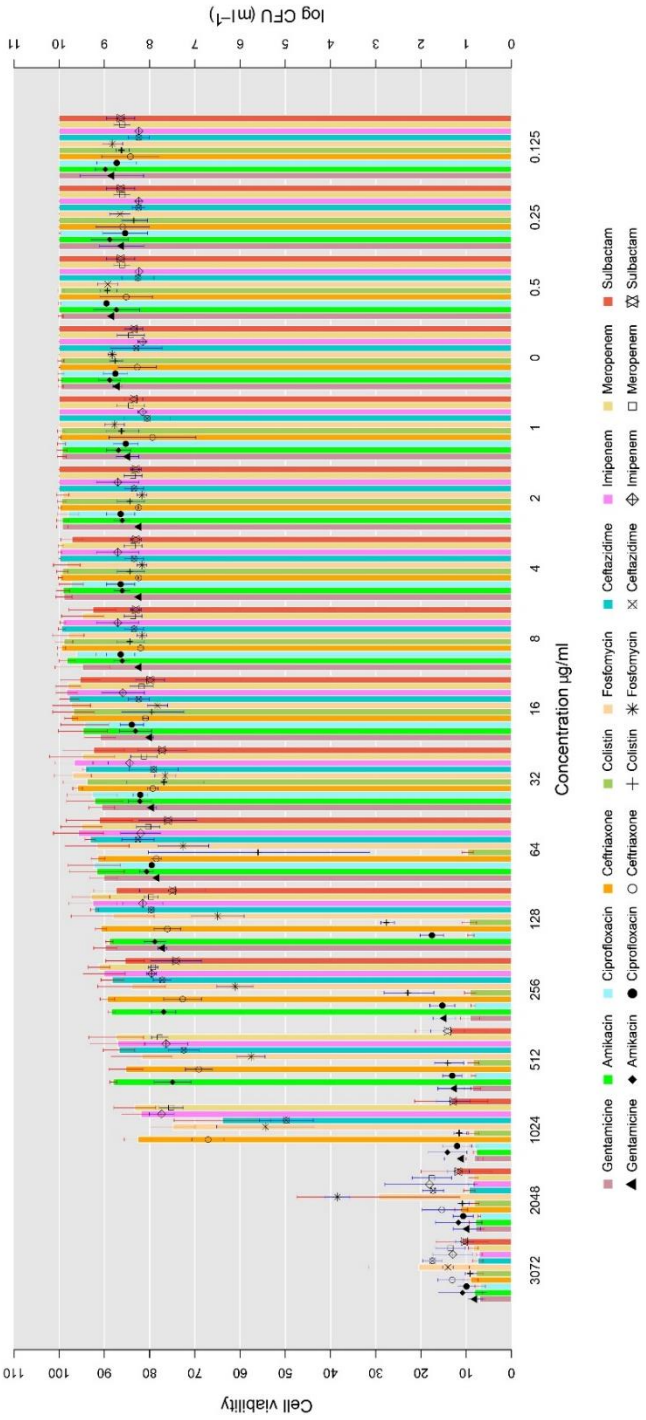
Antimicrobial agents	MIC†			MBEC-50‡			MBEC-75‡		
	95% CIs§		β‖	95% CIs§		β‖	95% CIs§		β‖
	L95	U95		L95	U95		L95	U95	
Gentamicin	-31.13618	72.81897	20.8414	96.11921	168.75035	132.4348	282.78588	355.41702	319.1014
Amikacin	-3.877788	104.7358	50.4290	145.527550	228.2985	186.9130	344.049289	426.8203	385.4348
Ciprofloxacin	-9.457032	27.81935	9.181159	74.293791	127.01056	100.652174	252.844516	305.56128	279.2029
Ceftriaxone	-6.432496	149.2263	71.39689	208.807024	289.3959	249.10145	369.270792	449.8596	409.56522
Colistin	-7.873465	16.84595	4.486242	27.226724	49.36748	38.297101	64.328174	86.46893	75.39855
Fosfomycin	42.45418	245.7491	144.1016	103.27316	344.6254	223.9493	347.84562	589.1979	468.5217
Ceftazidime	1.800552	96.66574	49.23314	122.490231	191.68368	157.08696	288.055448	357.24890	322.65217
Imipenem	-27.52334	101.6767	37.07669	273.24233	385.2794	329.26087	578.86551	690.9026	634.88406
Meropenem	-40.85145	95.59948	27.37401	285.77460	400.02250	342.89855	588.61518	702.86308	645.73913
Sulbactam	-32.55944	37.54344	2.491998	129.75117	183.27781	156.514493	264.70769	318.23434	291.471014

†Minimal inhibitory concentrations (MIC, mg mL^{-1}) of planktonic cells.
‡Minimal biofilm eradication concentrations (MBEC, mg mL^{-1}) were categorized as responsive reaching about 50% and 75% of the total non-viable cells within a given antibiotic concentration range.
§ 95% confidence interval
‖ Beta

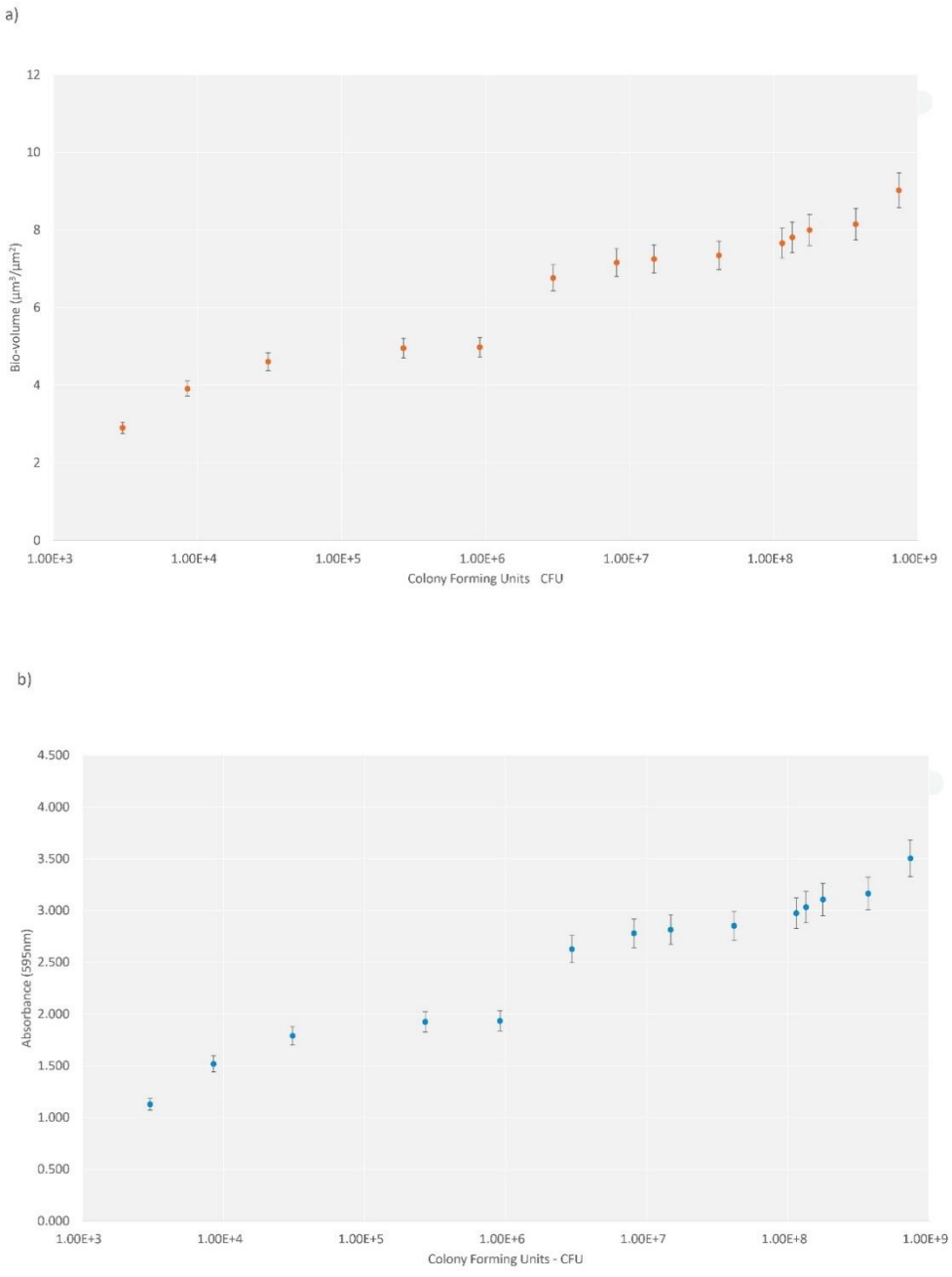
Supplementary *Figure 12 Correlation between the number of CFU per biofilm as determined by plating (CFU count) and biofilm cell viability as a percentage of A. baumannii ATCC 19606 exposed to increasing concentrations of antibiotics.*



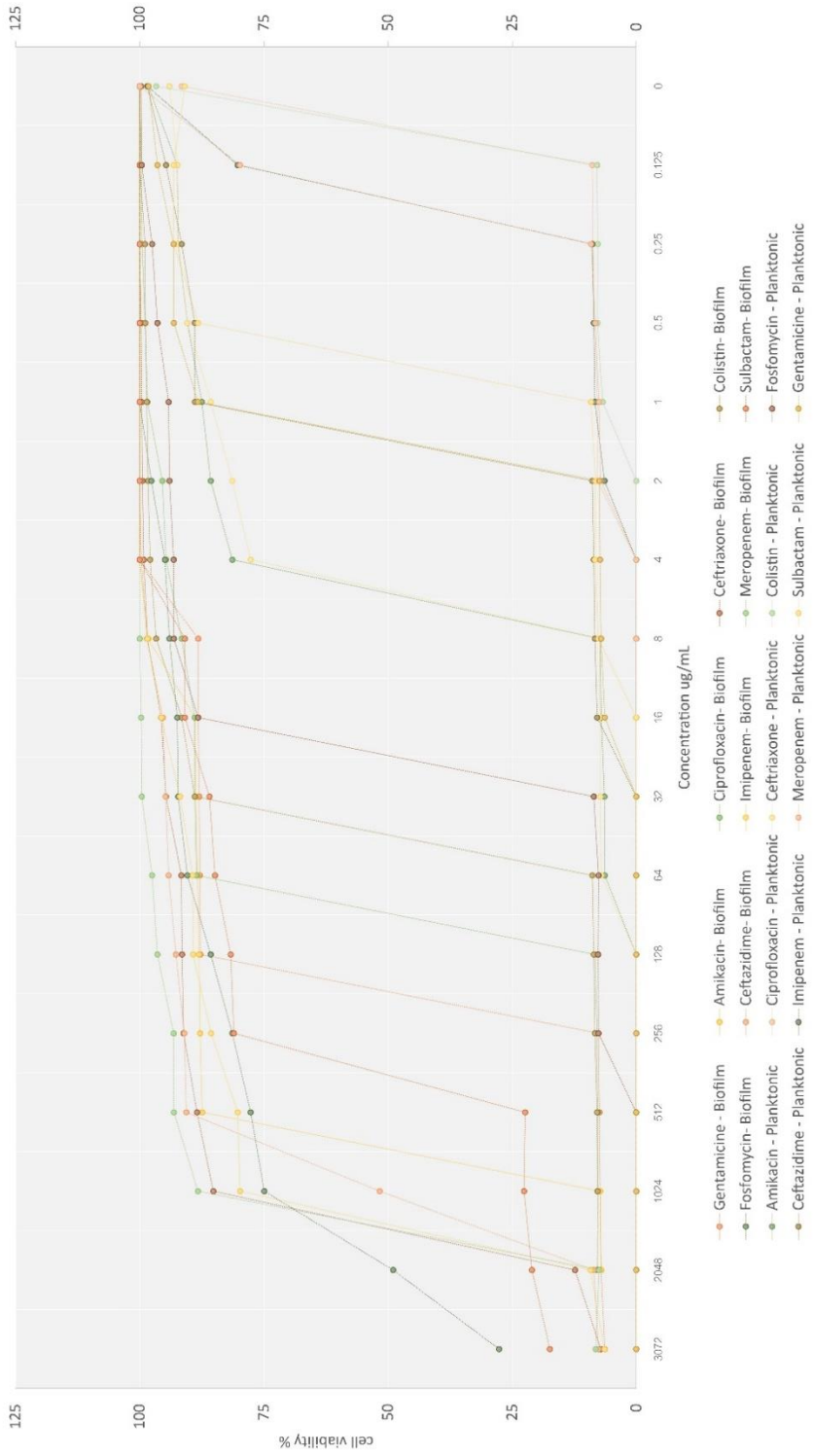
Supplementary *Figure 13 Distribution of the PrestoBlue-stained viable cells (coloured bar chart) and CFU counts (coloured symbols) of antibiotic-treated biofilms of P. aeruginosa PA01.*



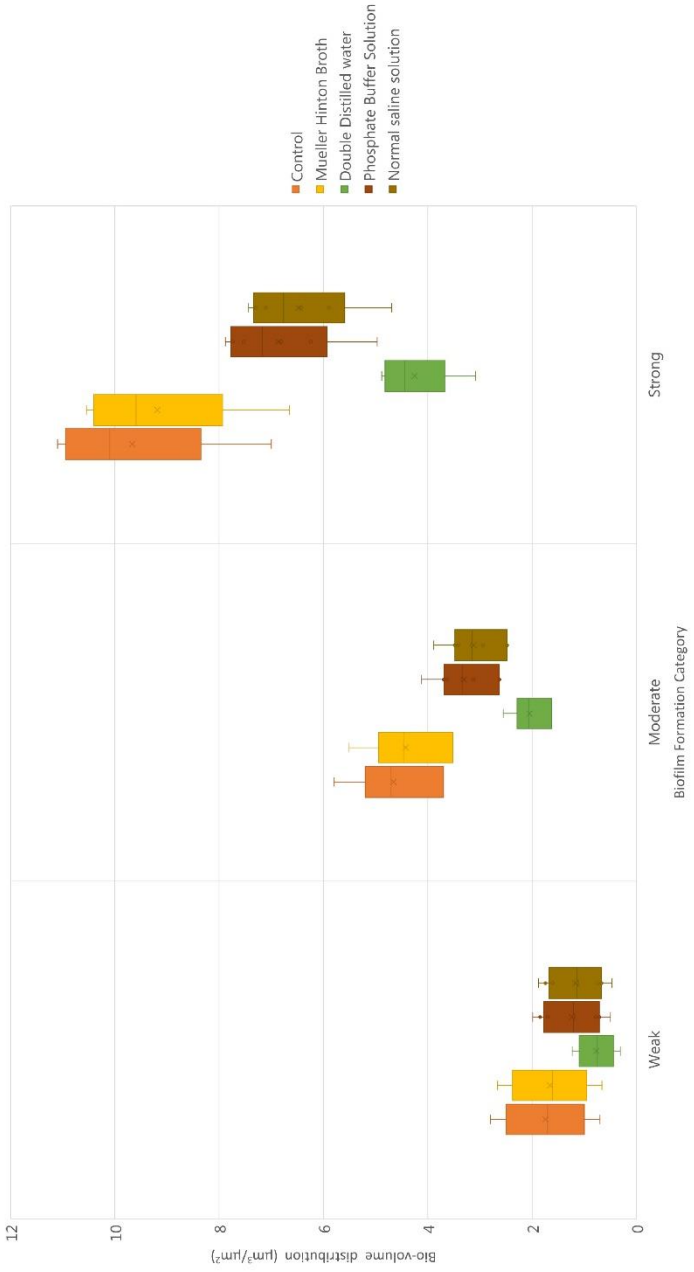
Supplementary *Figure 14 Distribution of biovolume and biomass within the biofilm in a 96-well plate in relation to initial bacterial concentration (CFU/mL) measured for planktonic bacteria and biofilms. (A) Biovolume data based on mean values of three independent replicates of z-stack measurements by confocal laser scanning microscopy, (B) Biomasses data based on staining with crystal violet and mean values of three independent replicates of absorbance (OD) measurement at 550 nm.*



Supplementary *Figure 15 Dose–response curves of antibiotics towards biofilm and planktonic cells were generated measuring PrestoBlue cell viability as a percentage in an assay validation step in A. baumannii ATCC 19606.*



Supplementary *Figure 16 Quantification of biovolume distribution in clinical isolates of A. baumannii biofilm after washing step using different washing solutions. Biovolume data are mean values of three independent replicates z-stack measurements by confocal laser scanning microscopy.*



Discussion

Identifying an accurate response of biofilms to antibiotics using a simple reliable assay has remained a major limitation in selecting adequate antibiotic therapy. At present, the level of biofilm formation is not generally considered when choosing antibiotics [33]. Accordingly, mimicking such conditions in a testing process is crucial to discriminate the efficacy of antibiotics between biofilm and planktonic bacteria. Therefore, the present study originated in the investigation of an inexpensive, standard, and clinically relevant anti-biofilm assay with which to determine antibiotic efficacy for biofilm infections accurately.

In the first part of our work, we altered the several technical parameters to obtain optimum assay performance. For example, a ratio of 1:10 PrestoBlue reagent to cell culture volume resulted in an stronger fluorescent signal with a shorter incubation time, and more accurate signal-detection linear range than other resazurin-based assays [35, 47-51]. Furthermore, as twofold less staining reagent was used compared with other approaches [35, 47-51], this substantially reduced possible sources of false positives because of reduced fluorescence interference as well as false negatives and minimized reagent expense.

Assays requiring high fluorescence specificity are particularly vulnerable to inappropriate incubation times and reading modes [52, 53]. For *A. baumannii*, we have shown that similar fluorescent intensity can be obtained between a bottom and a top reading mode with a 30 min incubation

time. Therefore, this approach produces a more accurate and reproducible assay, where both reading modes could be used either sequentially or in an interleaved fashion. In the present experiments, the fluorescence intensity changes over a long incubation time for both reading modes. Reasons for this change include that resorufin photobleaches in which case the fluorescence would decrease over time because of its exposure to excitatory light or its reduction to the colourless non-fluorescent hydroresorufin over time [35]. Importantly, one study has assumed that rather than using an endpoint mode, addition of resazurin staining with the test drug can produce more stable fluorescent signals [47]. Our observations raised questions for such an assumption and the reproducibility of procedure. The capacity of resazurin to generate a fluorescent signal sufficiently adequate above background usually remains for up to 1–4 hours [52]. Therefore, early exposure to staining with a test drug only produces fluorescent signals during the first several hours and prolonged exposure to excitatory light a can decrease the signal-to-background ratio and sensitivity [52].

We noted a difference in fluorescent signals from bacteria in suspension compared with that from bacteria in biofilms, either owing to differences in the metabolic activity of the bacteria within the biofilms, such as the low metabolic activity of persister cells [26] or perhaps because of limited accessibility of fluorophores to bacteria within the biofilms, preventing or delaying some

fluorescence signal as a result of the physical structure of the biofilm. Our results indicated that the calibration curves with planktonic bacteria may not be relevant to estimate CFUs in biofilms. Our staining approach showed wider linear correlation between the fluorescent signal and the biofilm CFU than reported in the earlier studies. This correlation allowed detection of $<10^4$ CFU for *A. baumannii* biofilm across all the drugs tested. The technical validation provides data to support that the assay is sufficiently sensitive to detect low numbers of cells (low CFU) for selecting a suitable MBEC [53]. Therefore, such specificity for biofilms leads to higher sensitivity and precision than conventional resazurin-based assays [35, 47-51].

When the performance of an anti-biofilm assay is applied to the treatment of Acinetobacter infections, there is a need to assess the assay performance and reliability with clinical isolates. Our results showed the proposed anti-biofilm assay (MBEC50 and 75) was able to achieved and provide a clear delineation of antibiotic efficacy between the various capacity of biofilm formation (weak, moderate, or strong). These results re-emphasize the sensitivity of the assay and ability to resembling clinical situations. As a result, understanding how these different biofilm formation response to antibiotics afford the credibility for clinicians to drive accurate therapeutic strategies toward biofilm-associated infections. In particular our results, the relationship between biofilm formation capacity and susceptibility test results reflect that the bacterial cells within biofilms have

a large impact on antibiotic efficacy. This emphasized that in addition to their propensity to evolve and acquire inherited resistance to survive in high concentrations of antibiotics (32), clinical isolates that are inherently susceptible to tested antibiotics can also be phenotypically refractory to their action via biofilm-mediated tolerance (5). Also, our results clearly emphasized the assumption that previous studies made that every clinical isolate produces a similar amount of biofilm is not true, and they have not clearly proven whether their assay was able to mimic diversity of biofilm formation. This also raises questions about the credibility of the biofilm formation assay reported in previous studies (17, 19, 20, 22-24).

The proposed anti-biofilm assays (MBEC50 and 75) are able to provide a clear delineation of antibiotic efficacy between the various capacity of each different sample type to form a biofilm to afford the flexibility for clinicians to drive accurate therapeutic strategies toward biofilm-associated infections. Our MBEC results showed that at low concentrations (>MBEC75), the more commonly employed antimicrobials against Acinetobacter (ceftazidime, sulbactam, imipenem, and meropenem) [54] do not entirely kill the bacterial biofilm of most of the tested isolates. This property can result in a significant poor effect of antibiotics for clinical isolates from urine, nasal swabs, tissue, broncho–alveolar aspirates, wound pus, endotracheal aspirates, and sputum. Our results distinctly show that isolates that are susceptible to β-lactams or carbapenems are less effective against eradicating biofilm. However, carbapenems have 57%–83% clinical cure rates for

A. baumannii ventilator-associated pneumonia (VAP) for an adequate period of time with frequent recurrence (despite possible clinical improvement) [55-57]. Planktonic cells (which cause an acute exacerbation) respond briskly to carbapenems, but are resistant via biofilm formation to establish persistence infection [55]. A rapid biofilm test (MBEC50 and 75) should facilitate confirmation of biofilm susceptibility to specific carbapenems before their use.

At present, evidence from MIC does not allow for any firm conclusions about the effectiveness of colistin on Acinetobacter biofilms. However, polymyxins, such as colistin, are the agents most commonly used for Acinetobacter isolates resistant to first-line agents [58]. By contrast, our MBEC test results clearly emphasized the effective concentration of colistin varies by biofilm formation capacity and sample type. Colistin can diffuse through biofilms and is able to achieve 75% non-viable cells with relatively lower dosage than other antibiotics tested. This observation suggests that relative effectiveness depends on penetration of antimicrobials through the biofilm matrix and differential physiological activity of the bacteria in biofilms. Antibiotic effectiveness on biofilms may be reduced by restricted penetration of antibiotics through biofilms as may occur where the antibiotics bind to bacterial membranes or components of the biofilm matrix such as extracellular DNA, or inactivation by enzymes present in the matrix [9, 10, 59].

Their restricted penetration through biofilms may result in exposure of bacteria to low concentrations of antibiotics for long periods [60]. This exposure may fuel the emergence and

selection of antibiotic resistant mutants with a potential risk of systemic spread to other organs or nosocomial spread to patients [26, 60]. Our MBEC test results showed that diffusive capacity through biofilm varied with the type of antibiotic and sample. For example, gentamicin, amikacin, ciprofloxacin, and fosfomycin were shown to penetrate moderate biofilms readily compared with their penetration of strong biofilms, which is highlighted by the accurate predictability of MBEC50 and 75 results for biofilm formation. These antibiotics are commonly used to treat chronic respiratory, urinary tract, sinus, and ear infections [61] and often fail to resolve them [10, 13, 62]. In all isolates, substantially higher concentrations of antibiotics were needed to achieve 75% cell death (MBEC75) than for MIC. Moreover, MBEC50 results of antimicrobial activity on biofilm formation capacity with the various sample types and antibiotics reveal that ciprofloxacin, ceftazidime, gentamicin, amikacin, and fosfomycin are effective only against the (metabolically active) outer layers. Whereas colistin can kill biofilm cells in the inner layers preferentially (low MBEC75), which indicates unparalleled penetration and provides opportunities to establish combination therapy (such as with ciprofloxacin or the β-lactams).

Biofilm infections are associated with various human milieu [10, 24, 26] that allow various structural characteristics and complex resistance spectra [10]. Therefore, an anti-biofilm assay should be effective where the biofilm formative capacity of related infections differs between the sites of infections, so the antibiotic selection for each site will be more reliable. For example, our MBEC

results showed the samples from people with chronic lung infections or the endotracheal tube infection may produce a different profile of responses to antibiotics and displayed significant variation compared with MIC results. This suggests current MIC antibiotic testing may not lead to appropriate antibiotic choices for infections that are associated with biofilm infections [10, 13] and result in recurrence of symptoms after treatment. This may be relevant particularly for soft-tissue or wound infections and device-associated nosocomial infections, which often persist for many weeks to months with serious treatment implications and failure, followed by multiple rounds of antibiotic and surgical treatment [10].

Examining anti-biofilm efficacy of antibiotics using our assay will increase the selection of effective therapy for chronic biofilm infections. First, the assay provides for rapid, simple, and precise identification of anti-biofilm sensitivity patterns allowing the most potent and effective drug to be selected. The optimal selection may also contribute to minimize bacterial resistance and spread of further infection or recurrence. Selection of a specific antibiotic therapy can contribute to preserve the healthy gut flora, supporting immunity and health. Second, having a detailed understanding of the anti-biofilm effectiveness based on sample type guides implementation of the therapy best suited for a particular infection site or type (e.g. local or systemic). Third, the accurate classification of biofilm formation from MBEC data demonstrates the utility of an anti-biofilm test. We acknowledge that empirical antibiotic therapy for chronic *Acinetobacter infections*,

should be selected based on patterns of biofilm-specific susceptibility and addition of a biofilm-specific susceptibility assay will facilitate appropriate treatment selections. We observed strong agreement across the clinical isolates that MBEC50 provides an important base line for predicting the efficacy of antibiotics for biofilm eradication. A 50% reduction of bacterial viability within a biofilm may be beneficial for patients with chronic and recurrent pneumonia because of high *Acinetobacter* load because such a scenario would facilitate more immune cells to access the bacteria within the biofilm and contribute to bacterial clearance [10]. However, patients with an immunodeficiency disorder are unable to effectively resolve infections or other complications related to their immune system, such as peritoneal dialysis, and so the MBEC75 may be necessary to control their infections, such as chronic and recurrent pneumonia. Therefore, integration of the anti-biofilm assay in clinical settings will aid the application of accurate and effective antimicrobial therapy.

Summary of the strengths and limitations

The anti-biofilm approach presented here offers the potential of broad applicability to determine the efficacy of antibiotics through their effects on biofilms. Yet, appropriate standard reference values required to clear infections *in vivo* remain unclear. To obtain a clinical effect on planktonic bacteria, antibiotics must achieve a >4 $\log_{10}$ reduction to fulfil performance standards (12, 19). However, there is no such kind of standard requisite log reduction value that best indicates

therapeutic efficacy for biofilm infections (12). Standardization of such values would improve therapeutic outcomes, and we welcome efforts in this direction. The microenvironments of infection sites where biofilms grow may not replicate precisely the nutrient-rich media used *in vitro* under assay conditions. Nevertheless, both the EUCATS (42) and CLSI (43) criteria use nutrient-rich medium methods for standard MIC drug testing *in vitro*. The experiments presented here are limited in that they have focused only on clinical isolates of *A. baumannii* and the assay may need various modifications before it can be applied to other species of bacteria. Moreover, only 10 antibiotics in current clinical practice were examined. More extensive testing in a similar fashion with other antibiotics would strengthen the credibility of the present assay.

Conclusions

In conclusion, our assay may be advantageous for the treatment of chronic infections with *A. baumannii*, but clinical trials are required to confirm this assertion. The assay is a valid, simple, reliable, and yet robust testing platform on which to dissect the antibiotic sensitivity of biofilms of *A. baumannii*.

CHAPTER 4

A simple and reliable way to test antibiotics on biofilm could lead to adequate therapy selection in patients with chronic respiratory infections: a retrospective preliminary analysis

Origin of the proposal: Aims and Objectives

Approximately 80-90% of patients with biofilm infections in respiratory tract have a relapse after initial antibiotic therapy, and majority of patients have exposed to high dosage of several different antibiotic class during the treatment [63]. Lengthy antibiotic treatment places burdens on public health systems with high cost and increases the risks of toxicity, community or hospital acquired co-infections, declining lung function and development of drug resistance [11, 63, 64]. Thus, the standard antibiotic therapy that is recommended for the biofilm treatment heavily depends on the minimum inhibitory concentration (MIC) of planktonic bacteria [11, 64]. Therefore, no measures are currently available for reliably assigning patients with biofilm infections to more accurate antibiotic treatment at first place and reduce the treatment durations [64].

We postulated that antibiotic susceptible of biofilm might have a capacity to distinguish the efficacy of antibiotics on biofilm infections and that could be used to determine the initial antibiotic therapy. Therefore, we developed a simple and reliable assay which able to differentiate antibiotic efficacy on biofilm of *A. baumannii* and *P. aeruginosa* clinical isolates [65]. If the minimum biofilm

eradication concentration (MBEC) level of an antibiotic is below the standard resistance breakpoint, and able to achieved 50% or 75% bacterial cell death within the biofilm the antibiotic is considered to be effective on biofilm infections. If the MBECs above the breakpoint, the antibiotic is considered to be biofilm inactive.

In previous study, we assessed MBEC values (*in vitro*) of most commonly used antibiotic on *A. baumannii* and *P. aeruginosa* clinical isolates obtained from patients who had a biofilm infections in respiratory tract with relapse (development cohort) [65]. Our results were clearly demonstrate the significant discriminatory power of the assay (MBEC) to differentiate antibiotic efficacy on biofilm compare to current MIC base assay [65, 66]. However, we combined this analysis with clinical, and other laboratory data to generate predictive models for antibiotic failure or success in biofilm infections based on MIC selection and then validated the MBEC selection using data from patients who had a relapse or were cured with antibiotic treatment (validation cohort). We postulated that by analyzing pretreatment isolates of *A. baumannii* and *P. aeruginosa* obtained from patients who subsequently had a relapse or were cured, we could determine how accurately MBEC assay able to determine the successful antibiotic at first place compare to the MIC base assay on biofilm and the relapse risk after treatment.

Materials and Methods

Sample Selection for validation cohort

We randomly obtained *A. baumannii* and *P. aeruginosa* clinical isolates with cultures from all 170 patients with chronic respiratory infection and a confirmed relapse, for whom a frozen isolate of the pre-treatment sample could be located and regrown by the previous study. We excluded 20 of the 170 after retesting because of missing clinical data (n=11) or culture contaminated (n=4) or failed cultures (=5), which left 140 isolates (*A. baumannii* (n = 70) and *P. aeruginosa* (n = 70) for further analysis. All these strains were stored at a Department of Microbiology, King Chulalongkorn Memorial Hospital strain repository collection after standard characterization and identification, including 16S rRNA sequencing. Clinical strains cohort used in this study had been isolated during 2016–2017 from patients with chronic infections as part of the standard care of the patients and was unrelated to the present study.

Bacterial strains and growth conditions

The biofilm-positive reference strains *P. aeruginosa* PA01 (ATCC 15692), *A. baumannii (*ATCC 19606*)* and randomly selected (n = 70) clinical isolates of both strains were cultured on Müller–Hinton agar (Sigma-Aldrich) plates at 37°C. The strains were stored at −80°C in tryptic soy broth (Sigma-

Aldrich) with 15% glycerol until they were used in subsequent experiments in which they were suitably anonymised.

Antibiotics and chemotherapy reagents

The biofilm eradication activity of ten antibiotics was tested against a subset of isolates (n = 70) with reference strain ATCC 15692 and ATCC 19606. Gentamicin, amikacin, ciprofloxacin, sulperazon, piperacillin/tazobactam, ceftriaxone, ceftazidime, imipenem, meropenem, trimethoprim-sulfamethoxazole, levofloxacin, doripenem, colistin, and fosfomycin were all from Sigma-Aldrich. Susceptibility testing for fosfomycin (Wako Chemicals) was determined by supplementation with 25 μg/mL glucose-6-phosphate (Sigma-Aldrich). The concentrations of all antimicrobials were adjusted to the susceptibility breakpoint concentrations recommended by the Clinical and Laboratory Standards Institute (CLSI). Antibiotic stock solutions were prepared less than 24 h before use. Antibiotics were dissolved in cation-adjusted Müller-Hinton II broth (MHIIB) (Becton Dickinson) medium and the supplemented medium sterilized by filtration through a membrane filter nominally with 0.22-μm pores. Serial dilutions of the antibiotic stocks were prepared in MHIIB medium immediately before use.

Biofilm formation

Biofilm formation in a 96-well-microtitre-plate format was performed as described previously (20). Initially, a pure culture of a single colony of *A. baumannii* and *P. aeruginosa* were inoculated into 2 mL of MHIIB medium in a tube and incubated in an orbital shaker (200 rpm) at 37°C overnight for about 16 h. Subsequently, a subculture was prepared from the overnight culture by diluting it with fresh MHIIB medium to an optical density (OD) of 0.02 at 600 nm (5×10^7 CFU/mL) and 100 μL aliquots were added in triplicate to flat-bottomed 96-well polystyrene microtitre plates (SPL Life Sciences), with uninoculated MHIIB medium (100 μL) in triplicate as a negative control, the plates were incubated at 37°C for 24 h.

Minimal inhibitory concentrations for planktonic cells

The MIC were established using standard broth microdilution techniques, according to criteria in the EUCAST (criteria for *Enterobacteriaceae* for fosfomycin only) (42) and CLSI guidelines (43). *E. coli* ATCC 25922*, and P. aeruginosa* ATCC 27853 were used as quality control strains. Minimal biofilm eradication concentrations (MBEC) were established as described previously by adding the serially diluted antibiotics to mature biofilms and incubating at 37°C for 24 h and then staining with PrestoBlue (Thermo Fisher Scientific). Before adding the antibiotics, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium. Two cut-off values

(50% - MBEC 50and 75% - MBEC 75 non-viable cells) were used to determine the MBEC as described previously. All experiments were performed in triplicate and repeated three times.

Biofilm quantification and classification

Two methods were used to quantify (45) and classify (46) the biofilm by Crystal Violet staining with modifications. The Crystal Violet (0.1%) stained biofilms were solubilized with 30% acetic acid followed by incubation at room temperature for 10–15 min. The absorbance (OD) at 550 nm was determined using a microtitre-plate-reading spectrophotometer (Varioskan Flash Multimode Reader; Thermo Fisher Scientific) with 30% acetic acid as a blank. Mean absorbances and their standard deviations (SD) were calculated for all strains and negative controls tested, performed in triplicate and repeated three times. The cut-off value (ODβ) was defined as 3SD above the mean OD of the negative controls: ODβ = average OD of the negative controls + 3SD of negative controls, and was calculated separately for each microtitre plate. The OD of a tested strain was expressed as the mean OD of the strain minus the ODβ (OD = mean OD of a strain – ODβ). The clinical isolates were classified as described previously (46).

Alginate measurement assay

To measure the amount of alginate produced by *P. aeruginosa* clinical isolates used in this study were assayed as described previously. The isolates were grown in 5 ml of LB broth with orbital

shaking at 200 rpm at 37°C until the culture reached an OD600 of 2.0. The bacterial cells were then collected by centrifugation at 7000 × g for 20 min and suspended in 1 ml of PBS buffer. Simultaneously, another culture was used to correlate OD600 2.0 with the dry cell weight. To remove any contaminants such as RNA and DNA from the alginate, the samples were treated with RNAse A (Promega) and DNAse I (Sigma). The samples were then incubated at 37°C for 1 h. To remove the cells, the mixture was vortexed and centrifuged at 8000 × g for 20 min. The alginate remaining in the supernatant was precipitated with 25 ml of 95% ethanol. The alginate precipitates were collected by centrifugation at 10000 × g for 30 min and suspended in 2 ml of 0.85% NaCl. The uronic acid concentration was determined by a standard colorimetric assay. All experiments were performed in triplicate and repeated three times.

Quantification of pyocyanin generated in biofilms

The amount of pyocyanin produced by *P. aeruginosa* clinical isolates biofilm were quantitatively measured by method described previously with modifications. Briefly, the isolates were grown in 5ml of LB broth culture at 37°C with shaking (200 rpm) for 16 h and centrifuged (7000 × g for 20 min). The resultant supernatant was then used for pyocyanin quantification. In brief, 600 µl of chloroform was added to 1 ml of supernatant, and the tube was vortexed twice for 10 seconds. The tubes were centrifuged at 10,000 rpm for 10 min, and the bottom phase (600 µl) was

transferred to a new tube containing 300 µl of 0.2 N HCl. The tubes were vortexed twice for 10 s each time and centrifuged at 10,000 rpm for 2 min. The OD_{520} of the top phase was measured and multiplied by 17.072 to calculate the micrograms of pyocyanin per milliliter. All experiments were performed in triplicate and repeated three times.

Measurements of extracellular DNA (eDNA) in biofilms

The eDNA concentration in *A. baumannii* and *P. aeruginosa* biofilms were determined by PicoGreen fluorescent staining (Quant-iT Invitroge) in a 96-well-microtitre-plate biofilm formation method as described previously with some modifications. A freshly prepared solution PicoGreen dye diluted in TE buffer (1:200) was added to the each well in the ratio 1 : 1 and eDNA concentration was measured on a fluorospectrometer (Varioskan Flash Multimode Reader; Thermo Fisher Scientific), using 470 nm excitation and 525 nm detection. To verify reproducibility eDNA production in the biofilm cultures were also quantified by laser scanning fluorescence microscopy (Zeiss Axiovert 200M) after staining with propidium iodide (BacLight Live/dead staining kit) during all experiments. All experiments were performed in triplicate and repeated three times.

Statistical analyses

Analysis is this section was predominately descriptive, although some inferential analysis is included. Unstacked (side-by-side) bar graphs were used to display the incidence of successful and unsuccessful antibiotic treatment events. In addition the impact of biofilm structure (moderate and strong), and organism (*A. baumannii* and *P. aeruginosa*) on mortality, length of hospital stay and intensive care unit stay were examine using Kaplan-Meier stimates of survival curve, the equality of which were tested using log-rank tests. Finally, the association of successful treatment with various patient and clinical characteristics was examined using bivariate binary logistic regression analysis. All analysis was conducted using the R statistical package [41].

Results

Study Patients

Of the 140 patients who were enrolled in study average of age 47.72 for patents with *P. aeruginosa* biofilm infection and 52.62 for patents with *A. baumannii* biofilm infections. A significant number of ($p< 0.001$) *P. aeruginosa* biofilm infection patents have either ventilator associated pneumonia (VAP), or hospital associated pneumonia (HAP) (Figure 17). Interestingly multidrug resistance infections, mortality and adverse events were significantly higher ($p< 0.001$) in patents with *A. baumannii* biofilm infections (Figure 17). Patents were belong to different underlying diseases based on diagnosis on hospital admission (Table 4), male and female ratio, biofilm categories (moderate and strong) (Figure 18).

Clinical isolates display diverse sensitivity to current antibiotics

A significant portion of ($p< 0.001$) clinical isolates from patents with *P. aeruginosa* biofilm infection displayed higher dug susceptibility to current antibiotic treatment regime (Figure 19) compare to the clinical isolates from patents with *A. baumannii* biofilm infections (Figure 20). The β-lactam antibiotic imipenem resistance were high prevalence in patents with *P. aeruginosa* biofilm infection (Figure 19). Interestingly, ceftriaxone, colistin and fosfomycin are the most common susceptibility drugs in patents with *A. baumannii* biofilm infections (Figure 20).

Patents with *P. aeruginosa* and *A. baumannii* biofilm infections in respiratory tract tend to have a less co-infections

The *Klebsiella pneumoniae* co-infection significantly ($p< 0.001$) associated in patents with *P. aeruginosa* biofilm infection (Figure 21). Interestingly, the patents with *A. baumannii* biofilm infections significantly ($p< 0.001$) associated with *P. aeruginosa* co-infections (Figure 22). However, the significant ($p< 0.001$) portion of patents with *P. aeruginosa* and *A. baumannii* biofilm infections in respiratory tract does not displayed any kind of co-infections (Figure 21 and 22).

Biofilm infections associated with different adverse events

The significant ($p< 0.001$) portion of patents with *P. aeruginosa* and *A. baumannii* biofilm infections in respiratory tract suffer from diarrhea and abdominal pain (Figure 23). However, the patents with *A. baumannii* biofilm infections also significantly associated with diarrhea, abdominal pain, and nausea or vomiting (Figure 23). Interestingly, close amount in both patents with *P. aeruginosa* and *A. baumannii* biofilm infections not associated with any kind of adverse events.

Risk factors associated with relapse

Characteristics that were significantly ($p< 0.001$) associated with relapse were biofilm category (moderate and strong), ventilator associated pneumonia (VAP) or hospital associated pneumonia (HAP), extracellular DNA content in the biofilm matrix (eDNA), and age (Table 5). However, the

having co-infection in addition to biofilm infection, gender, and infected with multidrug resistance strain does not displayed any significant association on relapse.

Antibiotic treatment selection based on minimum inhibitory concentration (MIC) associated with lengthy hospital and ICU days with more relapse.

The probability of selecting biofilm related antibiotic choices in first episode for patents with *P. aeruginosa* and *A. baumannii* biofilm infections in respiratory tract were significantly ($p< 0.001$) lower in current MIC base selection (Figure 24). Moreover, such selection associated with significant ($p< 0.001$) days of stay in hospital or intensive care unit (Figure 25) and prolong relapse compare to the cure (Figure 26). Especially, the significant ($p< 0.001$) portion of patents with *P. aeruginosa* biofilm infections associated with long hospital and intensive care unit stay compare to the patents with *A. baumannii* biofilm infections (Figure 25).

Minimum biofilm eradication assay (MBEC) able to narrow down the antibiotic selection towards the treatment success in biofilm infection

In 93 out of 140 cases (i.e. 66.4%) MBEC50 identified at least one antibiotics in the successful list at the first incidence of biofilm infection in contrast to MIC which chose 0 out of 140 correctly. However, that is, in 84/140 cases *(i.e. 60%)* MBEC50 identified antibiotics which were all in the

successful list at the first incidence of infection in contrast to MIC which chose 0/140 correctly for both bacteria biofilm infections.

When breakdown to the organism level, that is, in 50/70 cases (i.e. 71.4%) MBEC50 identified at least one antibiotics in the successful list at the first incidence of *P. aeruginosa* biofilm infection in contrast to MIC which chose 0/140 correctly. That is, in 46/70 cases (i.e. 65.7%) MBEC50 identified antibiotics which were all in the successful list at the first incidence of *P. aeruginosa* biofilm infection in contrast to MIC which chose 0/140 correctly. That is, in 43/70 cases (i.e. 61.4%) MBEC50 identified at least one antibiotics in the successful list at the first incidence of *A. baumannii* biofilm infection in contrast to MIC which chose 0/70 correctly and that is, in 38/70 cases (i.e. 54.28%) MBEC50 identified antibiotics which were all in the successful list at the first incidence of biofilm infection in contrast to MIC which chose 0/70 correctly. There is a significant ($p< 0.001$) difference in success of MBEC50 to select antibiotic between *A. baumannii* and *P. aeruginosa* biofilm infections.

That is, in 83/140 cases *(i.e. 59.3%)* MBEC75 identified at least one antibiotics in the successful list at the first incidence of infection in contrast to MIC which chose 0/140 correctly. That is, in 58/140 cases *(i.e. 41.3%)* MBEC75 identified antibiotics which were all in the successful list at the first incidence of infection in contrast to MIC which chose 0/140 correctly.

That is, in 45/70 cases *(i.e. 64.3%)* MBEC75 identified at least one antibiotics in the successful list at the first incidence of *P. aeruginosa* biofilm infection in contrast to MIC which chose 0/70 correctly. That is, in 27/70 cases *(i.e. 38.6%)* MBEC75 identified antibiotics which were all in the successful list at the first incidence of *P. aeruginosa* biofilm infection in contrast to MIC which chose 0/70 correctly. That is, in 38/70 cases *(i.e. 54.2%)* MBEC75 identified at least one antibiotics in the successful list at the first incidence of *A. baumannii* biofilm infection in contrast to MIC which chose 0/70 correctly. That is, in 31/70 cases *(i.e. 44.3%)* MBEC75 identified antibiotics which were all in the successful list at the first incidence of *A. baumannii* biofilm infection in contrast to MIC which chose 0/70 correctly. There is a significant ($p< 0.001$) difference in success of MBEC75 to select antibiotic between *A. baumannii* and *P. aeruginosa* biofilm infections.

Minimum biofilm eradication assay (MBEC) able to enhance the odds of treatment success in biofilm infection

In other words, if MBEC50 identifies any antibiotics that works at biofilm infection at the first incidence, then a patient has 152 times the odds of (eventually) being cured (OR=152.04, 95%CI: 40.23, 574.68, $p<0.001$). Moreover, if MBEC50 identifies antibiotics which were all in the successful list at the first incidence, then a patient has 55 times the odds of (eventually) being cured (OR=54.667, 95%CI: 16.871, 177.134, $p<0.001$).

For MBEC75 identifies any antibiotics that works at biofilm infection at the first incidence, then a patient has 115 times the odds of (eventually) being cured (OR=114.667, 95%CI: 24.713, 532.037, p<0.001) and if if MBEC50 identifies antibiotics which were all in the successful list at the first incidence, then a patient has 66 times the odds of (eventually) being cured (OR=66.000, 95%CI: 8.567, 508.469, p<0.001).

Biofilm matrix components dominated with strong biofilm producing clinical isolates from respiratory tract

The pyocyanin and alginate content were significantly (p< 0.001) higher in strong biofilm producing clinical isolates from patents with *P. aeruginosa* biofilm infection (Figure 27 A and B). Similar results were observed in extracellular DNA content (eDNA) in the biofilm matrix of clinical isolates from patents with *P. aeruginosa* and *A. baumannii* biofilm infections (Figure 27 C). Interesting, the *P. aeruginosa* clinical isolates displayed higher eDNA in moderate biofilms compare to the *A. baumannii* clinical isolates (Figure 27).

Success or unsuccessfulness of current antibiotic towards the biofilm reflected necessity of MBEC assay

Both successful and unsuccessful antibiotics for patents with *P. aeruginosa* and *A. baumannii* biofilm infections were overlap with smiler antibiotic groups (Figure 28 and 28). However, colistin

significantly ($p< 0.001$) dominated in successful antibiotic for patents with *A. baumannii* biofilm infections compare to *P. aeruginosa* (Figure 28). For *P. aeruginosa* biofilm infections the meropenem and piperacillin/tazobactam were most frequently successful antibiotic for treatment (Figure 28). Interestingly, meropenem, ceftazidime, and piperacillin/tazobactam significantly ($p< 0.001$) dominated in unsuccessful antibiotic for patents with *A. baumannii* and *P. aeruginosa* biofilm infections (Figure 29).

Figure 17 Comorbidities associated with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

Figure 18 Characteristic of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

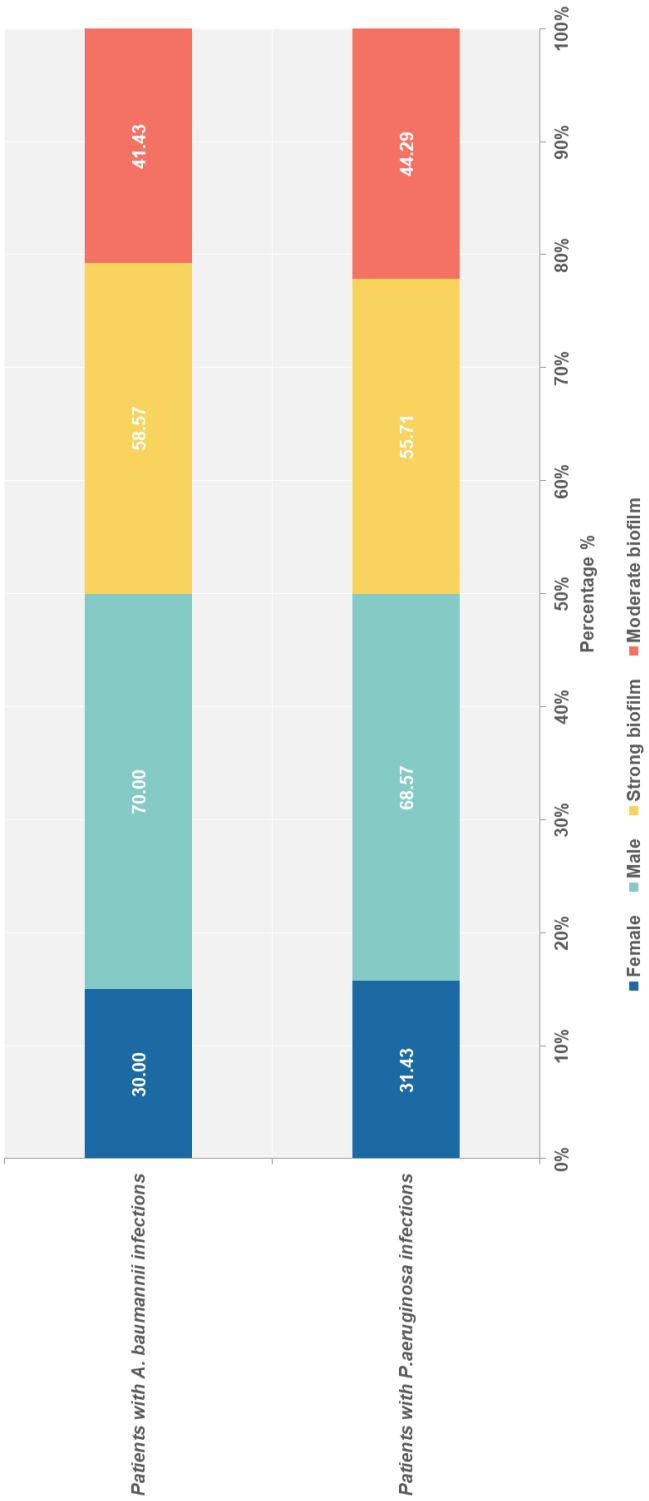


Figure 19 Antibiotic senility patterns of clinical isolates form patients with P. aeruginosa biofilm infections in respiratory tract.

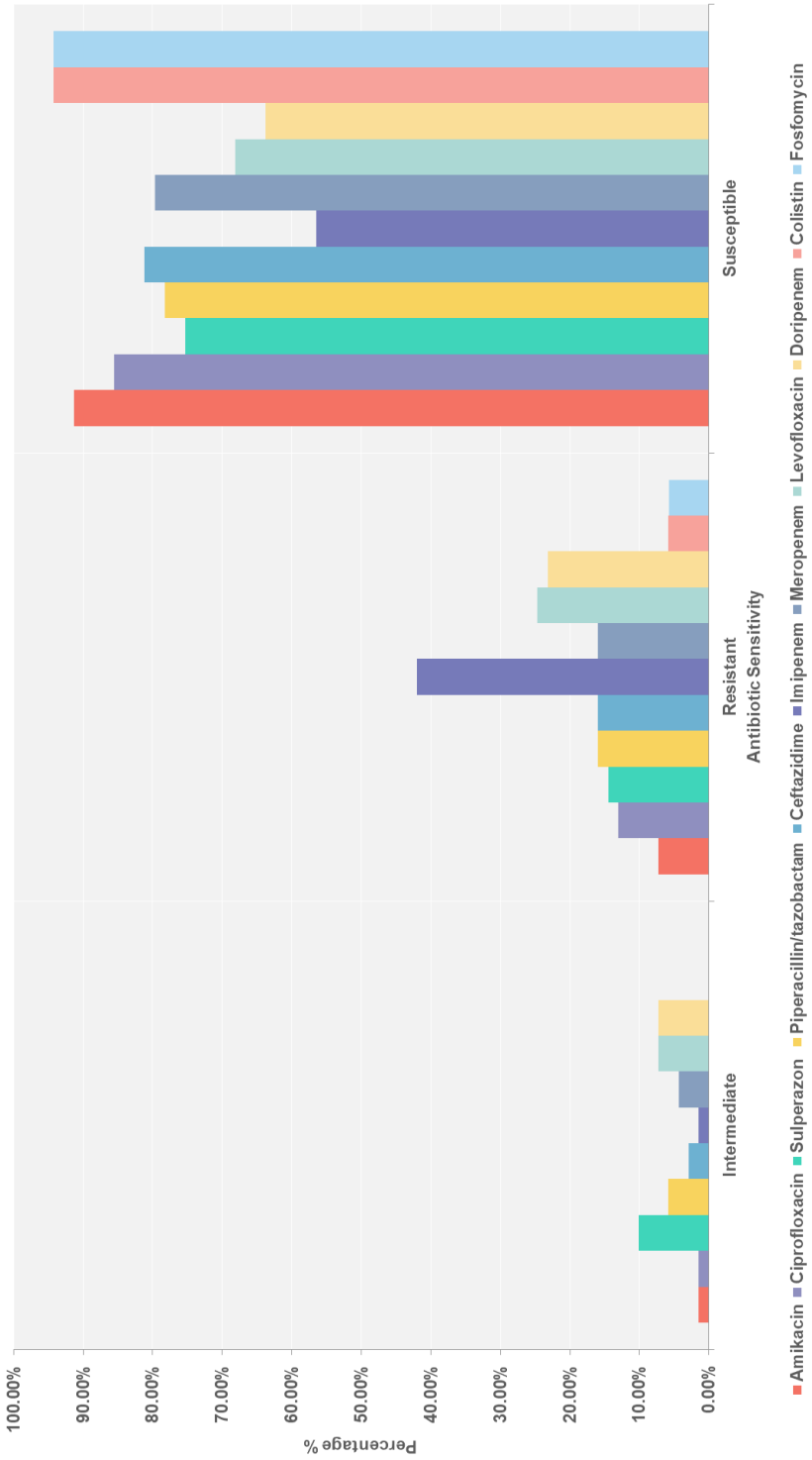


Figure 20 Antibiotic senility patterns of clinical isolates form patients with chronic A. baumannii biofilm infections in respiratory tract.

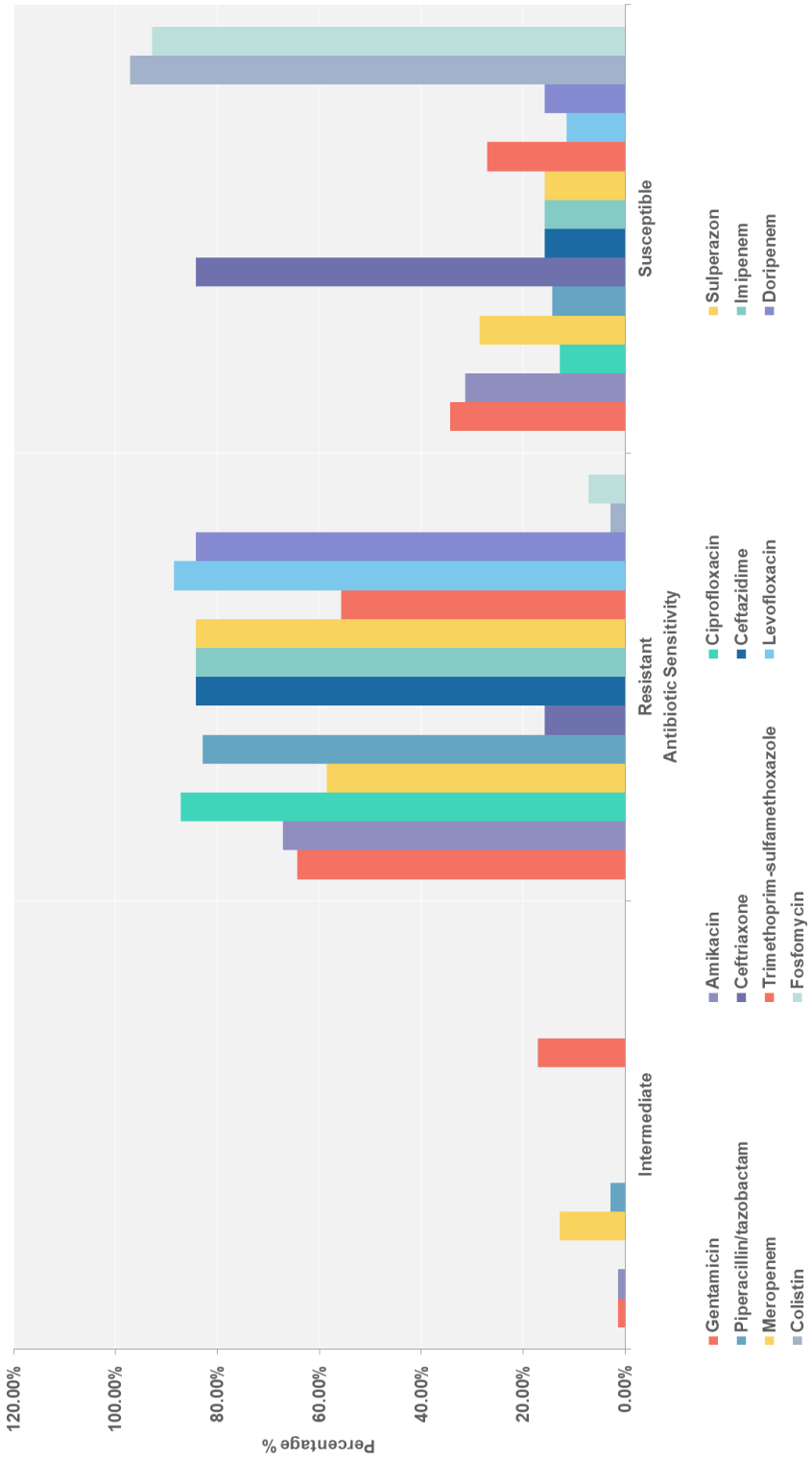


Figure 21 Co-infection characteristic form patients with chronic P. aeruginosa biofilm infections in respiratory tract.

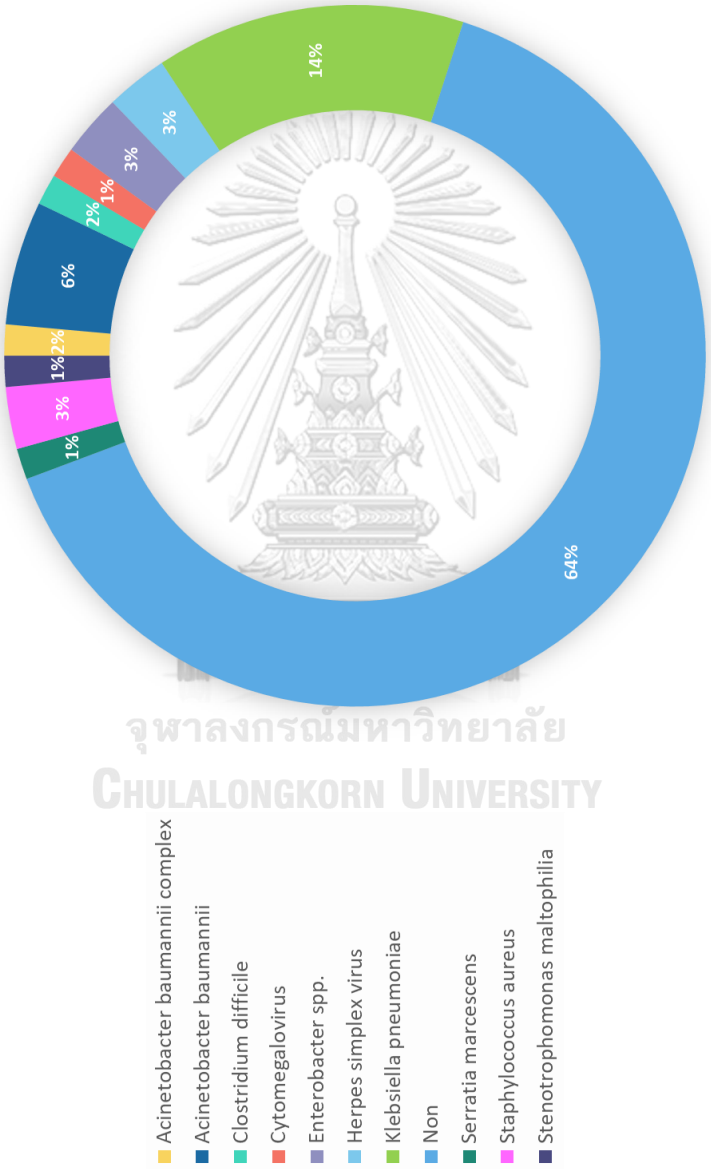


Figure 22 Co-infection characteristic form patients with chronic A. baumannii biofilm infections in respiratory tract.

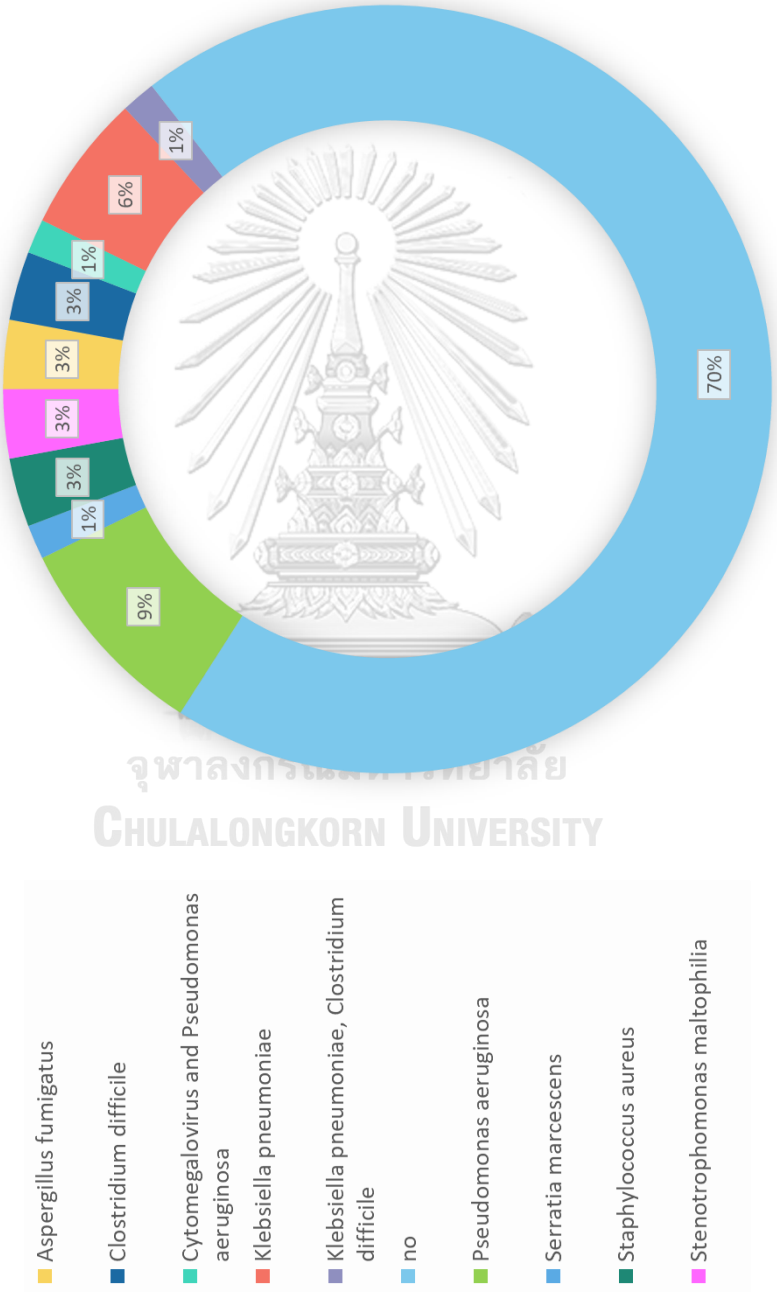


Figure 23 Adverse effect in patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

Table 4 Diagnosis on hospital admission in patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

Diagnosis on hospital admission	%
Acute bronchitis	0.71%
Acute myocardial infarction	0.71%
Advanced Ca nasopharynx	0.71%
Advanced stage lung cancer	3.57%
Advanced stage thyroid cancer	0.71%
AIDS with disseminated cryptococcosis	0.71%
AIDS with nocardiosis	0.71%
AIDS with TB lung	0.71%
Anaplastic large cell lymphoma	1.43%
Anterior cerebral artery aneurysm ruptureartery	0.71%
Arterial injury	0.71%
Biliary atresia	0.71%
Brain stem stroke	0.71%
Brain tumor	0.71%
Brian stem stroke	0.71%
Bronchitis	2.14%
Ca thyroid with lung metastasis	0.71%
CBD stone with cholangitis	0.71%
Cerebellar stroke	1.43%
Cerebral palsy with acute tracheitis	0.71%
Cholangiocarcinoma	0.71%
Chronic asthma	0.71%
CIDP with hypercapnic respiratory failure	0.71%
Closed Fx Lt proximal tibia	0.71%
Coarctation of aorta	0.71%
Cognitive cytomegalovirus with epilepsy	0.71%
Congenital and Perinatal Cytomegalovirus Infection	0.71%
Coronary artery disease and diabetes mellitus	0.71%
Coronary artery disease with congestive heart failure	0.71%

Corrosive agent ingestion	0.71%
Cryptogenic organizing pneumonia (COP) with acute respiratory distress syndrome (ARDS)	1.43%
Diffuse Large B-Cell Lymphoma	1.43%
Diffuse Large B-Cell Lymphoma DLBCL	0.71%
Drug induced hepatitis	0.71%
Encephalitis, unspecified	1.43%
Epilepsy	0.71%
Epileptic seizure	0.71%
Floor of mouth cancer	1.43%
Fulminant myocarditis	2.14%
Gastrointestinal stromal tumor with acute bleeding	0.71%
Heart failure	0.71%
Heart failure with Cardiogenic shock	1.43%
Hepatic ischemia reperfusion injury	0.71%
Hypoplastic left heart syndrome	0.71%
Hypovolemic hyponatremia	0.71%
Iliac artery aneurysm	0.71%
Iliac artery aneurysm infection	0.71%
Infected bedsore	1.43%
Infected hematoma left hand	0.71%
Infected Lt dry gangrene ulcer	0.71%
Interstitial lung disease ILD	0.71%
Invasive pulmonary aspergillosis	1.43%
Ischemic bowel	0.71%
Large cerebral infarction	0.71%
Lateral tongue cancer	0.71%
Lobar pneumonia	0.71%
Locally advanced esophageal cancer with neutropenia	0.71%
low transverse cesarean section with acute kidney injury	0.71%

Lung cancer	0.71%
Lung cancer with brain metastasis	0.71%
Major degree burn (62%)	0.71%
Mandible cancer	0.71%
Mantle cell lymphoma	0.71%
Mitral stenosis with regurgitation	0.71%
Myocardial infarction	0.71%
Necrotizinf fasciitis Rt forearm	1.43%
Necrotizinf fasciitis Rt leg	0.71%
Nocardiosis	0.71%
Ohtahara syndrome with aspiration pneumonia	2.14%
Pancreatic cancer	0.71%
Parkinson's disease with pneumonitis	0.71%
Patent ductus arteriosus PDA	2.86%
Perforated DU	0.71%
Pituitary macroadenoma	0.71%
Pneumonia	3.57%
Post TB bronchitis	0.71%
Preterm labor	0.71%
Pulmonary tuberculosis	0.71%
Pulmonary venous anomaly	0.71%
Ruptured A com aneurysm	1.43%
Ruptured abdominal aortic aneurysm	2.14%
Ruptured basilar artery aneurysm	1.43%
Ruptured posterior communicating artery	0.71%
Secretion obstruction	0.71%
Seizure with acute renal failure	2.14%
Severe mitral regurgitation	0.71%
Severe mitral regurgitation (MR) with mitral stenosis (MS) and valvular atrial fibrillation (AF)	0.71%

Status epilepticus	2.14%
Status epilepticus (SE)	0.71%
Subdural hematoma (SDH)	0.71%
Supraglottic cancer with bronchitis	0.71%
Thyroid cancer	2.14%
Tongue cancer	0.71%
Total pulmonary venous anomaly	3.57%
Tracheobronchitis	0.71%
Traumatic subarachnoid hemorrhage SAH	0.71%
Triple vessel disease with congestive heart failure	0.71%
UTI with septic shock	0.71%
Viral pneumonia	0.71%

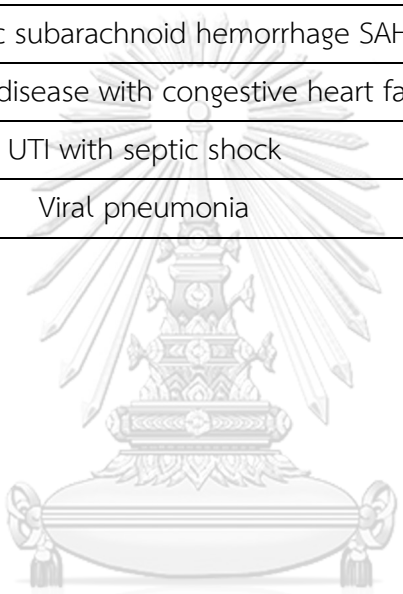


Table 5 Co-relation of comorbidities associated with infection outcome as a relapse in chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

Comorbidities	**OR**	**L95**	**U95**	**P.val**
Coinfection	1.158	0.556	2.410	0.69313
Organisms	0.942	0.475	1.867	0.86262
Biofilm Category	0.294	0.144	0.601	0.00072
MDR	1.544	0.759	3.141	0.22653
VAP	6.352	2.599	15.524	0.00004
HAP	4.235	1.251	14.341	0.01929
eDNA Content	3.387	1.710	6.711	0.00042
Sex	0.906	0.435	1.888	0.79007
Age	0.986	0.974	0.997	0.01541

Figure 24 Comparison of the antibiotic selection based on MIC compare to the MBEC choices in treatment of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

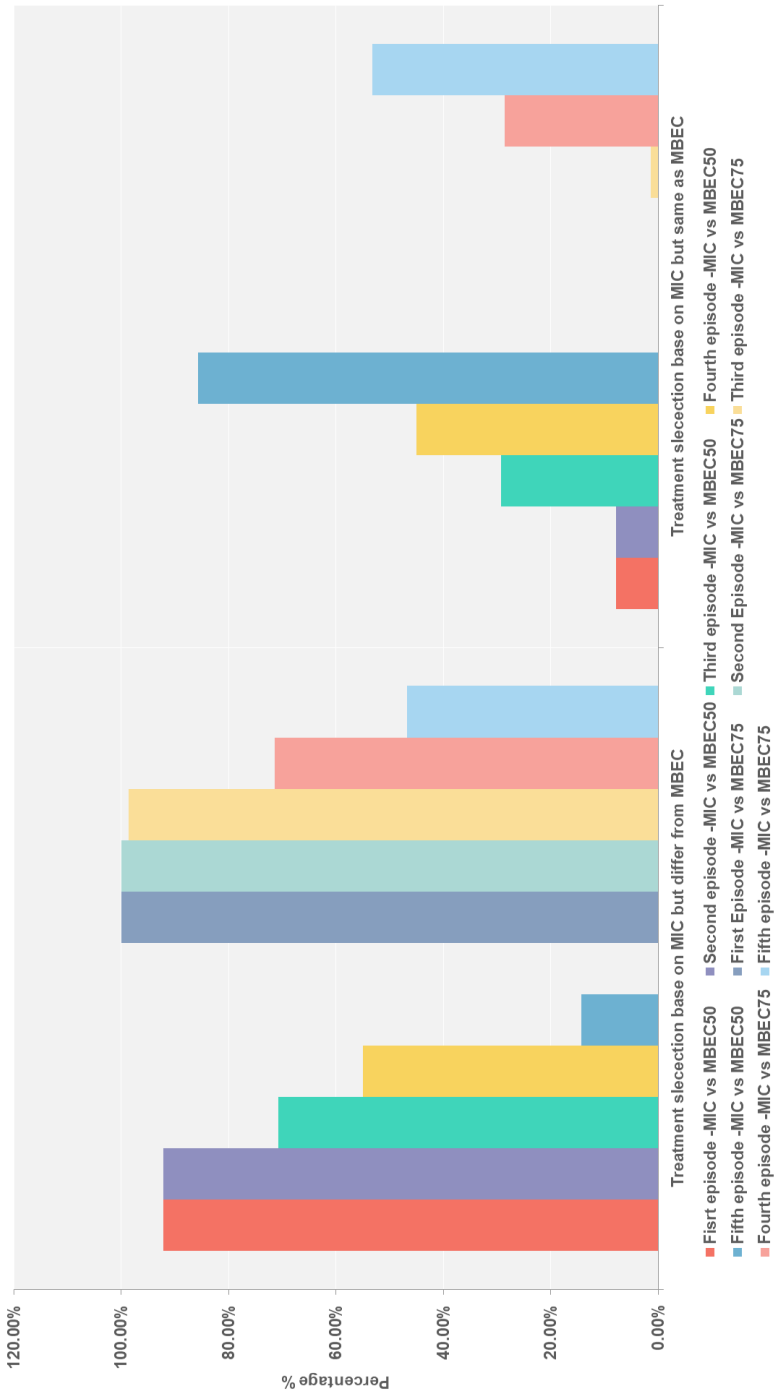


Figure 25 Average length of hospital and intensive care unit stay of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

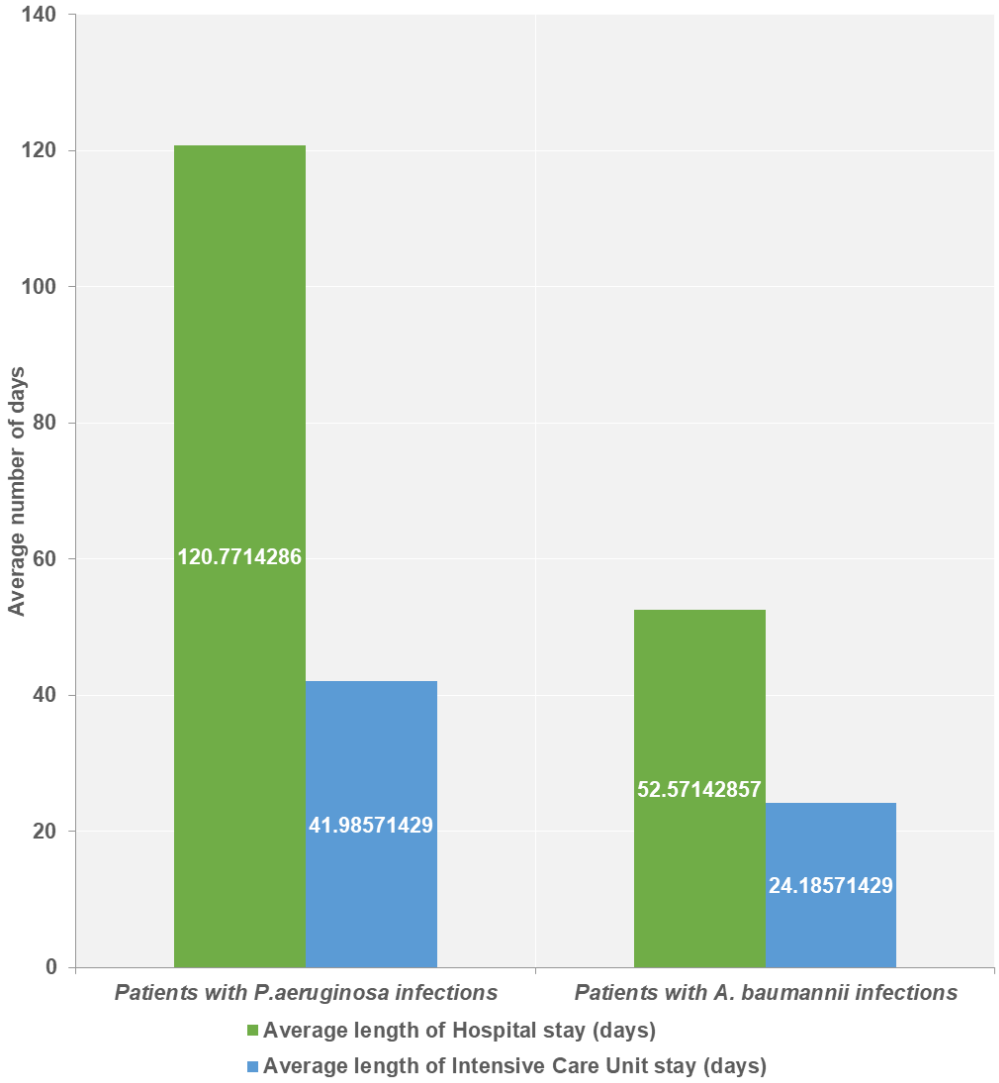


Figure 26 Relationship of treatment outcome after every episode in patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

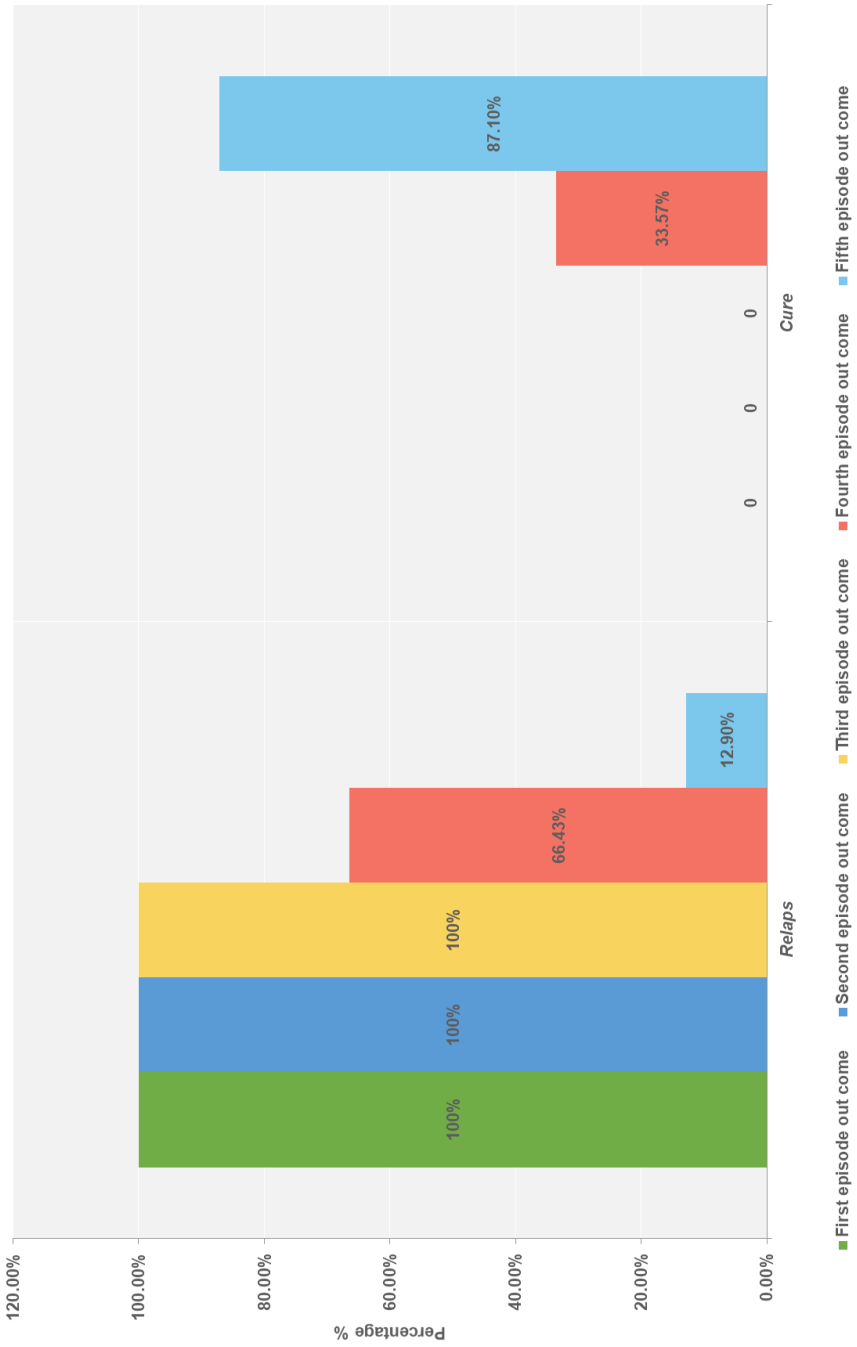


Figure 27 Characterization of biofilm matrix components in clinical isolates of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

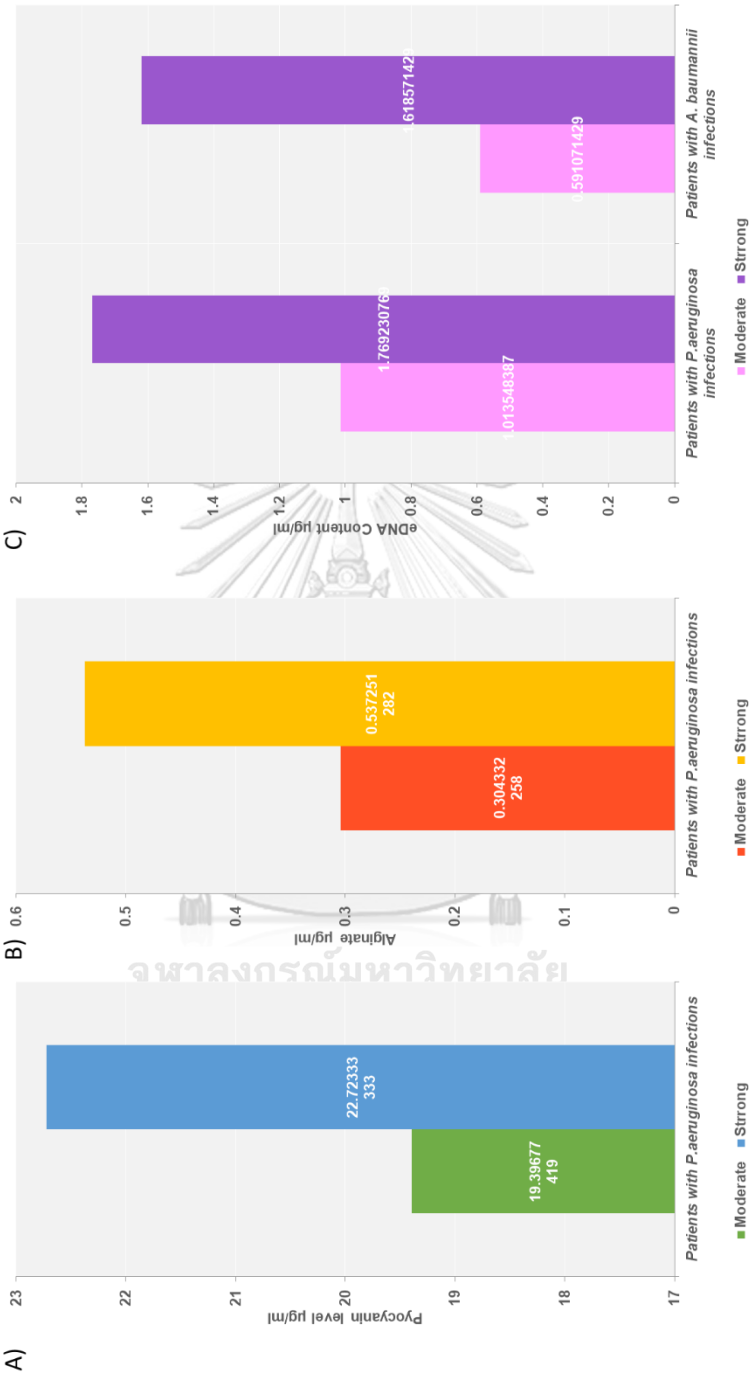


Figure 28 List of successful antibiotic in treatment of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.

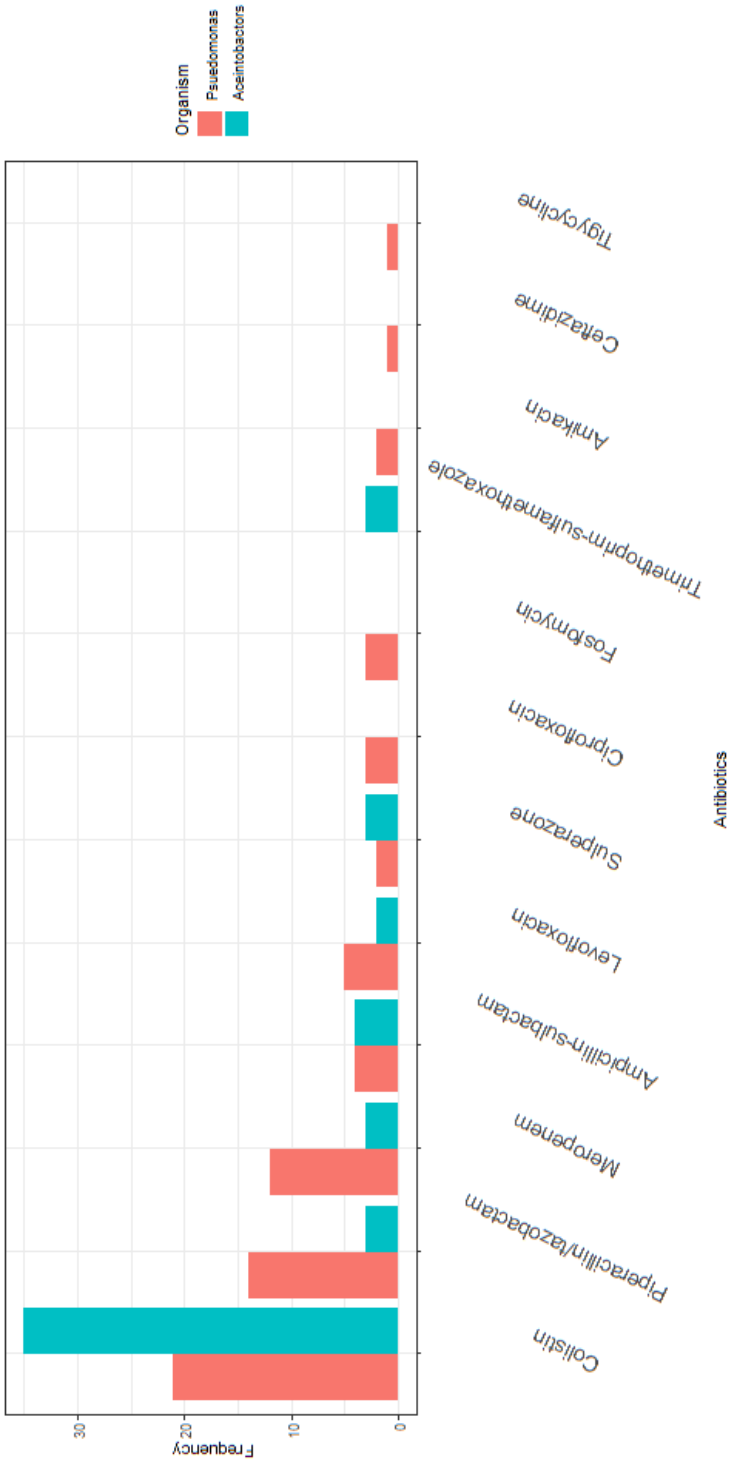
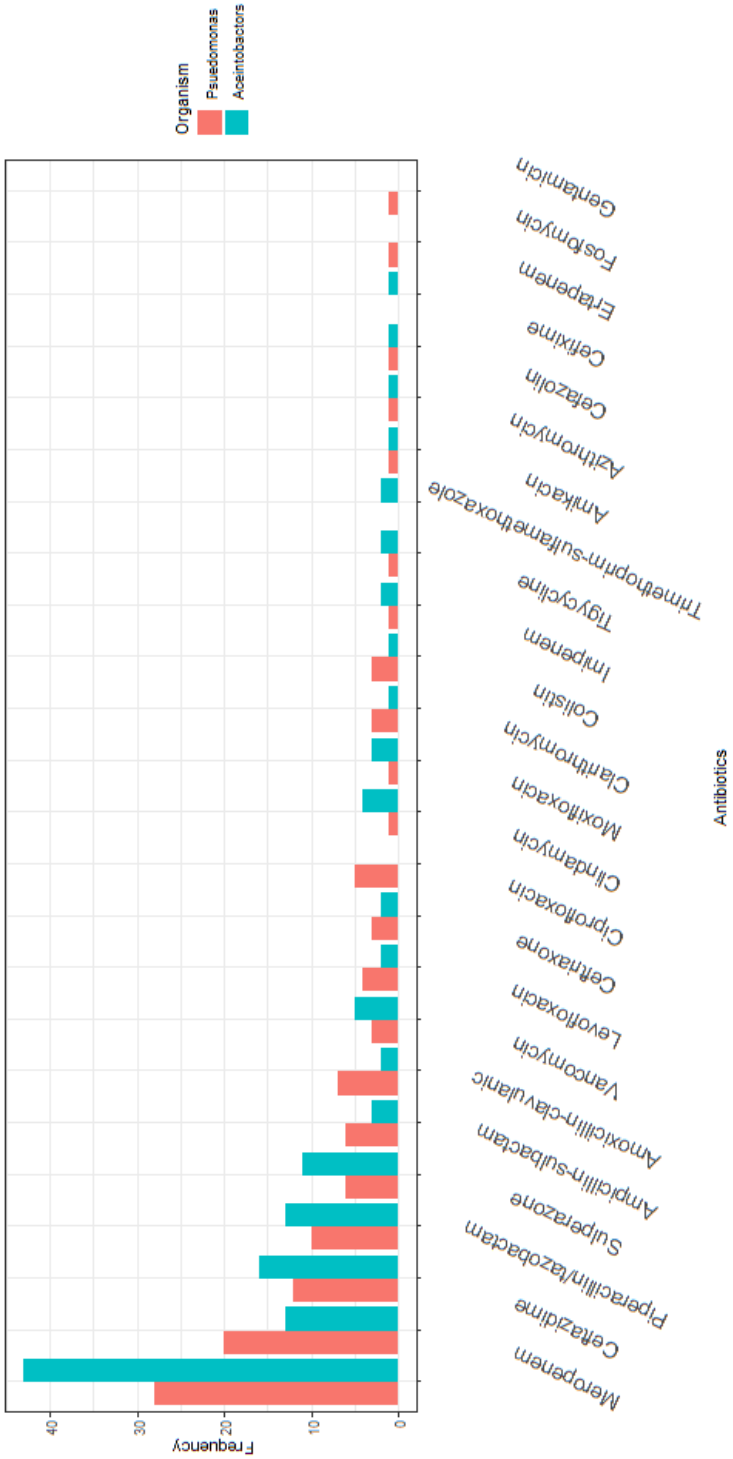


Figure 29 List of unsuccessful antibiotic in treatment of patients with chronic A. baumannii and P. aeruginosa biofilm infections in respiratory tract.



Discussion

Antibiotics are mainstay treatment for patients with chronic biofilm related bacterial infection in respiratory tract with an acute exacerbation that includes increased sputum purulence and worsening shortness of breath [63, 67, 68]. Although such treatment is associated with short term clinical benefit, treatment failure and relapse rates may be high, particularly in cases of inadequate antibiotic therapy through incomplete resolution of the initial exacerbation and persistent bacterial biofilm infection [11, 63, 67, 68]. These aspects have led to recommendations for a stratified approach to antibiotic therapy based on patient characteristics associated with increased risk factors like biofilm for failure.

Our results clearly suggest that biofilm infection related patients at greatest risk for poor outcome (i.e., those with strong biofilm isolates, VAP or HAP, age) are likely to derive greatest benefit from early treatment with accurate antibiotics. The validation of our MBEC assay in isolates obtained from patients with chronic *A. baumannii and P. aeruginosa* biofilm infections in respiratory tract suggests that MBEC measurements may be useful in selecting antibiotics which can be safely used in treatment with shorter-duration.

In our study the MIC based antibiotic treatment has been shown to enhance episodes of relapse or exacerbations and long hospitalizations. In line with our finding previous studies strongly warned that the increase could be linked to an incapability of MIC testing to filter the antibiotic selection

based on biofilm [63, 67-70]. And therefore decrease of probability on the prescribing of accurate antibiotics by clinicians base on guidelines and own personal experiences, resulting in greater risk of relapse, adverse events, co-infections, mortality and some patients may developing antimicrobial resistance (AMR) infections that future deuterated the quality of life. However, currently there are no clear guidelines for how to treat biofilm infections and there is disagreement among clinicians [11, 63, 67, 69]. Numerous studies have described the difficulty in getting biofilm infection diagnosed and navigating between traditional health care systems to get appropriate antibiotic treatment clinicians [11, 63, 67, 69].

We selected MBEC 50 cut-offs for antibiotic selection in the assay with the goal of detecting a sensitivity of assay to differentiate the diffusion ability of antibiotics in biofilm structure, which may have a high specificity in moderate biofilm producing clinical isolates. However, higher cut-offs MBEC 75 could be selected to future enhance sensitivity in strong biofilm producing clinical isolates with some risk of selecting antibiotics with toxicity (i.e., colistin). Differences in MBEC 50 and 75 values may also reflect more fundamental differences in the biologic characteristics in biofilm structure and metabolic or physiological factors of the *A. baumannii* and *P. aeruginosa* biofilm infections in respiratory tract that were not account on MIC testing because of their physiological variability. With a more in-depth understanding of these biofilm related factors, treatment can be improved by new anti-biofilm agents and our study result reflect that current antibiotics are

inadequate to treat biofilm infections.

Our results of successful and unsuccessful antibiotics, combined with the findings of other studies clinicians [11, 63, 67, 69] showing an association between inadequate drug levels and a worse outcome in patients being treated for biofilm infections in respiratory tract, suggest that MBEC assay that include higher-potency drugs at right time could be beneficial. Compared with successful or unsuccessful antibiotics with relapse and cure, during MIC based selection treatment a much larger percentage of patients have exposed to majority of antibiotic class in addition to significantly higher concentrations in long duration. However, treatment with appropriate antibiotics for biofilm at first place significantly decreases such high antibiotic exposer, subsequently lowering the bacterial burden (and frequently eradicates the organisms that are sensitive) and reduces clinical failure and the risk of progression to more severe infections, such as pneumonia. These effects can be achieved by implementing the MBEC assay to reducing bacterial load in the airways in biofilm infections and improving immune related clearance. Among the major goals of chronic bacteria treatment in the current guidelines is the prevention of acute exacerbations clinicians [11, 63, 67, 69]. Clinical studies have shown that long-term continuous or intermittent use of antibiotics has some beneficial effect of reducing exacerbation frequency and extending the time to the next exacerbation in biofilm related infections clinicians [11, 63, 67, 69]. The mechanism underlying this improvement is unclear and most of these patients later develop resistance to those antibiotics clinicians [11, 63, 67, 69].

It is possible that the benefit of long-term antibiotic treatment may be due to eradication of colonizing bacteria with in the biofilm, although evidence supporting this hypothesis is limited. We believed that such effect due to clearness of planktonic or single living bacteria, not due to eradication of biofilm. In our study the increased exacerbation or relapse during treatment is reduced after selection of correct antibiotic treatment towards the biofilm with in short period, this resolution has been shown to be dependent on bacterial eradication with in the biofilm. The incomplete resolution of the initial exacerbation and persistent bacterial infection appear to be important determinants of the risk of relapse and MBEC assay would help to overcome such scenario.

Although systemic antibiotics are likely to remain the core treatment for patients with biofilm infection in respiratory tract clinicians [11, 63, 67, 69], we believed that inhaled antibiotics may represent a more optimal and realistic approach for the treatment and prevention of relapse in the future. However, in this study cohort none of the patents have received any antibiotics through inhalation. Regardless of the route of administration, MBEC selection of antibiotics are required to eliminate the biofilm infection and reduce the long-term hospital or ICU days, adverse events of antibiotics and the development of bacterial resistance. Further clinical trials are needed to determine the ideal antibiotic selection cut-offs of MBEC and treatment duration for patients who have been classified as being at high risk for biofilm infections. However, an antibiotic selection for

treatment based on more than MBEC 50 cut-offs may be warranted in this study cohort.

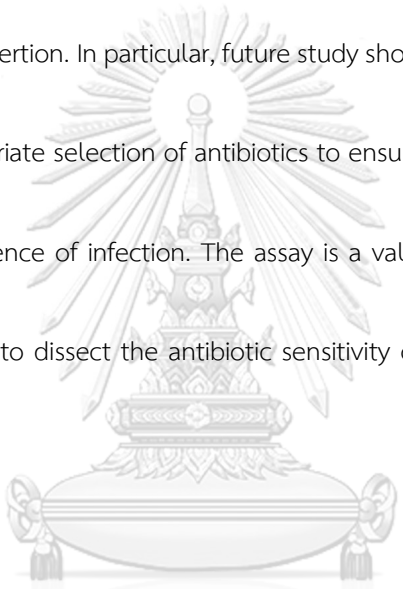
Summary of the strengths and limitations

Our clinical study is limited by its relatively small size and retrospective design. However, we were able to confirm the predictive ability of our MBEC assay in a validation cohort, based on clinical data. Another limitation of our study is that we could not test the MIC values of drugs in the primary *P. aeruginosa* and ten for *A. baumannii* culture made directly from the patient's sputum isolate. Instead, isolates were sub cultured at least three times before MIC and MBEC testing. This period of in vitro culture might have allowed isolates to acquire new mutations that altered the MIC or MBEC values. However, the isolates were not exposed to tested antibiotics during any of these subcultures; thus, it is unlikely that repeated culturing of isolates affected the MIC or MBEC results. Another potential limitation is that data for patients who were lost to follow-up (5% in Study cohort) excluded from our analyses. These results could have been competing risks for the primary outcome. Combining the present quantitative screening of bacterial biofilm-specific antibiotic resistance with clinical trials of antibiotics would clarify the clinical applicability of the assay.

Conclusions

In conclusion, we have addressed several aspects related to the effect and selection of appropriate antimicrobial therapy for biofilm infections using the present anti-biofilm assay. We found that

current MIC based antibiotic selection on biofilm infections were inadequate and had an influence on treatment outcomes, with the risk of relapse increasing together with antibiotic resistance. In addition, we confirmed these findings in isolates obtained from patients with *P. aeruginosa* and *A. baumannii* biofilm infection in a validation cohort. Our assay may be advantageous for the treatment of chronic infections with to *P. aeruginosa* and *A. baumannii*, but clinical trials are required to confirm this assertion. In particular, future study should clarify the possible applicability of the assay to the appropriate selection of antibiotics to ensure adequate antimicrobial coverage and to prevent the recurrence of infection. The assay is a valid, simple, reliable, and yet robust testing platform on which to dissect the antibiotic sensitivity of biofilms of *P. aeruginosa* and *A. baumannii*.



CHAPTER 5

The burden of chronic biofilm associated bacterial respiratory Infections

There is an alarming rise in persistence infections caused by bacteria that are resistant to common antibiotics. A particular problem is respiratory Infections. Approximately 40-60 million people a year in world contract respiratory Infections, and between 3 and 4 million die from the disease [18] . The lower and upper respiratory infections are the largest cause of disease burden among public health, result in the loss of lots of disability-adjusted life years [18]. The prevalence of chronic respiratory infections is particularly high in patients with high risk group or weakened immune systems [18]. Such as condition like chronic pneumonia associated with biofilm related bacterial infections are frequently effects elderly people, mostly in the develop nations [18]. But in the developing countries pneumonia is the biggest killer of children [69]. In 2016, it cost the lives of an estimated 880,000 children, most of them were less than two years old and more than for malaria and diarrhoea combined [69]. Not only are patients with high risk particularly susceptible to such infections, patients with respiratory disorders who need ventilator support are usually unable to clear foreign material from their lungs, therefore causing mucus to aggregate and become a favourable for bacterial infections [69]. Bacteria can grow biofilms on the surface of the lungs as well as on the surface of respirator tubes, which can lead to chronic lung infections [18, 69, 71]. In

addition, recent research in air pollution and climate changes has made the link between exposure to particles from vehicle exhausts or air pollution and increased risk of susceptibility to the chronic bacteria chest infection, which can be fatal [18, 69, 71].

Respiratory infections by bacteria are usually categorized as acute or chronic depending upon the rate at which they cure after antibiotic therapy [18, 69, 71]. Acute infections are responding rapidly to antibiotic treatments and usually able clear the most of the bacteria at infection site [18, 69, 71]. In contrast, chronic respiratory infections are characterized by periods of stability punctuated by periods of exacerbation. Such as, the most common symptom of a chronic bacterial lung infection is associated with persistent, severe cough and will often bring up phlegm or mucus when coughing. Patients also experience fever and sometimes sweats, a tight feeling across the chest, or sometimes sharp stabbing pain (pleurisy), shortness of breath which may involve wheezing and fatigue [18, 69, 71]. Symptoms will vary in severity from person to person, but they can be result in significant morbidity and loss of lung function if not treated. A large number of patients with disruptions in lung immunity, or mucosal clearance, suffer from chronic infections that typically don't resolve even with antibiotic treatment and exacerbations are common [18, 69, 71]. As most bacteria involved in these infections have survived treatment as biofilm formation, and may continue to persist lifelong in some patients. It is for this reason chronic respiratory infections may be better classified as biofilm related infections [18, 69, 71].

Table 6 Biofilm-associated infections (BAI)

Infection type	Reference example
Cystic fibrosis lung infections	[11, 18, 69, 71-74]
Chronic otitis media	[11, 18, 69, 71-74]
Chronic Rhinosinusitis	[11, 18, 69, 71-74]
Chronic tonsillitis	[11, 18, 69, 71-74]
Catheter and shunts	[11, 18, 69, 71-74]
Ventilator-associated pneumonia	[11, 18, 69, 71-74]

The bacteria living with in the biofilm contributions to an exacerbation have traditionally been determined by planktonic culture results and antibiotic selection is based on these cultures [11, 18, 69, 71-74]. However there are a number of clinical situations that challenge the utility of this traditional approach [11, 18, 69, 71-74]. Most clinicians who treat for individuals with biofilm related airway infections will encounter many instances where a patient's course is not predicted by planktonic sputum cultures [11, 18, 69, 71-74]. Many patients will not show a clinical response to antibiotics directed at the planktonic bacteria cultured in their sputum [11, 18, 69, 71-74]. On the contrary, some patients may respond to aerosolized antibiotics that could penetrate biofilms at the air-liquid interface in the mucosal lining [11, 18, 69, 71-74]. In the cases of antibiotic

ineffectiveness, it was presumed antibiotics could not reach every niche of the biofilm, leaving some bacteria untreated. Despite these approaches bacterial biofilm, ineffectiveness of antibiotics, and unpredictable clinical responses have continued to frustrate clinicians [11, 18, 69, 71-74].

The chronic lung infections are typically a consequence of underlying diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis, acute or chronic asthma, tuberculosis, scarring after acute infection, primary ciliary dyskinesia, immune deficiencies and lung cancer [11, 18, 69, 71-74]. From all causes results in the progressive production of thick, purulent secretions of mucus, dyspnea, wheezing, cough, progressive inflammation and an advancing destruction of the airways and lung parenchyma [11, 18, 69, 71-74]. Patients who suffer such conditions experience poorer airway clearance and provide passive environment for biofilm infections [11, 18, 69, 71-74]. Studies relying on sputum culture predict 50% of COPD exacerbations are due to bacterial lung infection and 70–80% are chronic biofilm infections [11, 18, 69, 71-74].

COPD is an inflammatory, non-reversible obstructive lung disorder caused primarily by exposure to tobacco smoke or other toxic fumes due to air pollution [11, 18, 69, 71-74]. Acquisition of a strain of *Haemophilus influenzae*, *Pseudomonas aeruginosa, Moraxella catarrhalis*, *Acinetobacter baumannii* or *Streptococcus pneumoniae* in the lung is associated with an increased risk of exacerbation in COPD patients [11, 18, 69, 71-74]. These pathogenic bacteria often form the biofilm infections and typically relapse between exacerbations. *P. aeruginosa* and *H. influenzae* infection

is also a serious problem in cystic fibrosis (CF) patients [11, 18, 69, 71-74]. CF is a genetic disease that results in bronchiectasis from the inability to clear dehydrate mucous from the airways [11, 18, 69, 71-74]. The mortality rate in such patients is very high as the bacterial biofilm causes chronic lung infections [11, 18, 69, 71-74]. In other conditions, the relationship with biofilm infection is more complex. *P. aeruginosa* infections are often associated with blue-green pus lead to life-threatening sepsis in long-term hospitalized patients who rely on ventilator support [11, 18, 69, 71-74]. And *H. influenzae*, is a typical cause of chronic pneumonia in children's and adults asthma [11, 18, 69, 71-74]. Asthma is a syndrome of intermittent airway inflammation that causes wheezing and shortness of breath [11, 18, 69, 71-74]. Studies demonstrated that patients with asthma have a high risk of biofilm infections resulting progressive inflammatory response leading to further respiratory function decline. *Acinetobacter baumannii* biofilm infections are most frequently dominate the sputum and airway microbiota of both patients with aspiration pneumonia and bronchiectasis [11, 18, 69, 71-74]. Common pathogens isolated by culture from patients with HAP and VAP are include *Haemophilus influenzae*, *Pseudomonas aeruginosa, Moraxella catarrhalis*, *Acinetobacter baumannii* and *Streptococcus pneumoniae* [11, 18, 69, 71-74]. Mixed or polymicrobial biofilm infections occur in patients who are immunocompromised, lung cancer or suffering from influenza, rhinoviruses or other viral and *Aspergillus* infections, posing further therapeutic challenges [11, 18,

69, 71-74]. Therefore, specific measures need to be put in place in order to tackle the problem of biofilm infections efficiently.

Current therapeutic strategies for chronic biofilm related bacterial respiratory infections

The chronic respiratory tract infections are the most frequent causes of antimicrobial prescription in primary care, the hospital setting, and health care facilities. The respiratory tract has become one of the biggest reservoirs of biofilm infections with multi drug resistance (MDR) variants of bacteria such as *H. influenzae, P. aeruginosa, M. catarrhalis, A. baumannii and S. pneumonia* [11, 18, 69, 71-74]. Currently antibiotics are the mainstay of treatment for patients with biofilm related chronic respiratory infections. They are usually given orally with occasional inhalation and, ther are no clear evidence that systemic or inhale antibiotics are effective in those patients [11, 63, 64, 75-79]. The initial antibiotic treatment needs to be empiric based on guidelines, and as the causative organism or organisms are known antibiotic choice depends upon the likely organism [11, 63, 64, 75-79].

Antibiotic prophylaxis (every day) is often used in the management of such infections by *H. influenzae*, *P. aeruginosa, M. catarrhalis*, *A. baumannii* and *S. pneumonia*. In addition, intermittently (three times per week) or pulsed (e.g. for five days every eight weeks) antibiotic

treatments are used for treatments [11, 63, 64, 75-79]. But antibiotic prophylaxis against staphylococci has no clear benefit and may increase carriage of *P. aeruginosa* or *H. influenzae* and risk of relapse [11, 63, 64, 75-79]. Also early targeted eradication of *P. aeruginosa* and *A. baumannii*, similarly fashion, has no definite beneficial effect on mortality or morbidity [11, 63, 64, 75-79]. The impact of pulsed antibiotics remains uncertain and in contrast intermittently treatment is only able to show certain significant clinical outcomes with reduce exacerbations [11, 63, 64, 75-79].

Beta-lactams were the most commonly prescribed drugs of first choice for the chronic respiratory infections [11, 63, 64, 75-79]. The monotherapy was more common than combination therapy, but recent studies showed that sharp increaser of combination therapy [11, 63, 64, 75-79]. Unfortunately, these differences in antibiotic recommendations are not based on bacteria biofilms; it's mostly ascribed to prevalence of antibiotic-resistant pathogens, patient condition and guideline recommendations [11, 63, 64, 75-79]. Such as in CF patients, the most commonly used are the extended-spectrum penicillins, aminoglycosides, cephalosporins, fluoroquinolones, polymixins and the monobactams [11, 63, 64, 75-79]. An aminoglycoside with a beta-lactam penicillin is usually considered to be the first choice and not any particular combination therapy shown any significant clinical advantage [11, 63, 64, 75-79]. The pipericillin, piperacillin/tazobactam and meropenem have strong activity against *P. aeruginosa* biofilms and is thought to prevent recurrent exacerbations, reduce relapse and maintain lung function, particularly in patients with CF [11, 63, 64, 75-79].

Antibiotics are used frequently in COPD patients in order to treat chronic respiratory infection associated with an acute exacerbation [11, 63, 64, 75-79]. The most common bacterial organisms isolated in COPD remain *H. influenzae, P. aeruginosa* and *S. pneumoniae* [11, 63, 64, 75-79]. However, *A. baumannii and Enterobacter* species are increasingly being seen particularly in developing countries [11, 63, 64, 75-79]. The first antibiotic choice would be amoxicillin–clavulanate where beta-lactamase production by *H. influenzae, A. baumannii* and *P. aeruginosa* is prevalent or a fluoroquinolone or sometimes extended-spectrum macrolides [11, 63, 64, 75-79]. According to the recent studies, the use of continuous prophylactic antibiotics, or intermittently or pulsed were results in a clinically significant benefit and reducing exacerbations in COPD patients [11, 63, 64, 75-79]. Based on 14 randomized controlled trials (RCTs) involving 3932 participants between 2001 and 2015, for every eight participants treated, one person would be prevented from suffering an exacerbation by use of antibiotics [11, 63, 64, 75-79]. Unfortunately, not all the antibiotic regimens had the same impact on exacerbations and hence the noted benefit mostly applies only to the use of macrolide antibiotics prescribed at least three times per week [11, 63, 64, 75-79]. The results suggested that antibiotics given at least three times per week may be more effective than antibiotics given daily for a few days followed by a break of several weeks [11, 63, 64, 75-79]. On the other hand, use of antibiotics did not significantly affect the number of deaths due to any cause, the frequency of hospitalization due to relapse, or the loss of lung function in COPD [11,

63, 64, 75-79]. The COPD patients colonized with moxifloxacin-sensitive *P. aeruginosa* at initiation of therapy rapidly became resistant with the quinolone treatment [11, 63, 64, 75-79].

Chronic pneumonia is a pulmonary parenchymal process been present for weeks to months rather than days mostly due to the biofilm infections [11, 63, 64, 75-79]. The therapeutic strategy in patients with chronic pneumonia of unknown etiology is to escalate the complexity and in most circumstances, empirical therapy is initiated once adequate diagnostic studies have been obtained [11, 63, 64, 75-79]. The combination of pipercillin/tazobactam or ampicillin/sulfabactam plus vancomycin or nafcillin is often used [11, 63, 64, 75-79]. However, these treatments are unable to prevent relapse and selection for antimicrobial resistance (AMR) in most cases [11, 63, 64, 75-79]. The microbiological causes of Hospital-acquired pneumonia (HAP) and Ventilator-associated pneumonia (VAP) vary by location, and MDR to these organisms is almost common [11, 63, 64, 75-79]. Broad-spectrum antibiotic therapy for HAP and VAP is given at the onset of disease based on guidelines [11, 63, 64, 75-79]. HAP and VAP are a common and serious nosocomial infection in mechanically ventilated patients and results in high antibiotic consumptions [11, 63, 64, 75-79]. The multidrug resistant *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*. play a dominant role in VAP etiology, and these organisms were frequently isolated in orotracheal tube or carrying a tracheostomy or endotracheal tube (ET) as a biofilm [11, 63, 64, 75-79]. In patients with ventilator-

associated tracheobronchitis (VAT), antibiotic treatment has been reported to be associated with significantly lower intensive care unit (ICU) mortality and subsequent VAP rates, as well as more mechanical ventilator-free days. However, this can be relapse at end of antibiotic treatment due to resilient nature of biofilm [11, 63, 64, 75-79].

In contrast, there is little evidence that antibiotics are effective on biofilm related respiratory infections in patients with chronic rhinosinusitis. Some studies show a modest improvement in disease-specific quality of life in adults with chronic rhinosinusitis without polyps receiving three months of an oral macrolide antibiotic [11, 63, 64, 75-79]. The improvement only seen at the end of the three-month continues treatment and three months later infections relapse with exacerbations [11, 63, 64, 75-79]. Chronic bronchitis is another condition associated with biofilm infections by *S. pneumoniae, H. influenzae, H. parainfluenzae M. catarrhalis* and *P. aeruginosa* [11, 63, 64, 75-79]. Most commonly used antibiotics is azithromycin followed by amoxicillin/clavulanate or antipseudomonal agent *(e.g.*, ciprofloxacin, levofloxacin, piperacillin/tazobactam, cefepime*)* and clarithromycin [11, 63, 64, 75-79]. Antibiotic selection is optimally made based on planktonic cultures, especially for patients with frequent or severe exacerbations and there for relapse are common in those patients [11, 63, 64, 75-79].

Providing high antibiotic concentrations through inhalation to the site of chronic respiratory infection improved pulmonary symptoms effectively and reduce the biofilm formation in sputum

[11, 63, 64, 75-79]. The effect of inhaled treatment at high concentrations with aztreonam and tobramycin on *P. aeruginosa* biofilms is showing their effect on biofilm structures and their ability to reduce bacterial load in chronic lung infections [11, 63, 64, 75-79]. In the case of nebulized tobramycin in CF patients, serum concentration after inhalation is under 1 mg/L whereas it reaches 1200 mg/L in the sputum and able to suppressive infections with absence of exacerbations [11, 63, 64, 75-79]. Furthermore, inhaled antibiotics in patients with stable non-CF bronchiectasis seem to be more effective than symptomatic treatment in chronic infections and minimized the risk of acute exacerbations without risk of developing antimicrobial resistance [11, 63, 64, 75-79]. However, the efficacy of inhaled antibiotics for treatment of ventilator-associated pneumonia (VAP) or hospital-associated pneumonia (HAP) due to chronic infections remains unclear [11, 63, 64, 75-79]. Some studies found a significant reduction in signs of respiratory infection compared to placebo while other works found no difference when comparing intravenous antibiotics with intravenous antibiotics combined with aerosolized antibiotics [11, 63, 64, 75-79].

Nevertheless, one clinical study has assessed the effect of nebulized gentamicin on the prevention of VAP by the most frequent pathogens demonstrating its efficacy in the prevention of endotracheal tube (ETT) biofilm formation [11, 63, 64, 75-79]. Other attempts to prevent VAP are based on the use of ETTs coated with antimicrobial agents, especially with silver which could

hinder biofilm formation and exert antimicrobial effects within the proximal airways. In addition, silver-coated ETTs reduce mortality in mechanically ventilated patients [11, 63, 64, 75-79].

Table 7 Summary of current inhale antibiotic treatment regimens [11, 63, 64, 75-79]

Site of infection	Antibiotic regimen	Duration	Route of administration
Lung infection in CF	0.5–2 MU colistin, twice daily	Continuous	Inhalation
	300 mg tobramycin, twice daily	28 days on/off cycles	Inhalation
	112 mg tobramycin dry powder, twice daily	On/off cycles 28 days	Inhalation
	75 mg aztreonam, three times daily	on/off cycles	Inhalation
	32.5 mg or 65 mg ciprofloxacin, once daily	28 days	Inhalation
	240 mg levofloxacin, twice daily	28 days on/off cycles	Inhalation
Lung infection in non-CF Bronchiectasis	1 MU colistin, twice daily	Continuous	Inhalation
	300 mg tobramycin, twice daily	28 days	Inhalation
	32.5 mg ciprofloxacin, twice daily	28 days	Inhalation

Site of infection	Antibiotic regimen	Duration	Route of administration
Rhinosinusitis	80 mg gentamicin, twice daily	Continuous	Inhalation
	3 drops ofloxacin 0.3%, three times daily	28 days	Nasal drops
	125 mg mupirocin + saline, twice daily	–	Rinonasal rinses
Endotracheal tubes	120 mg vancomycin HCL + 2 mL saline, three times daily	14 days	Inhalation
	80 mg gentamicin + 2 mL saline, three times daily	14 days	Inhalation

Combined and sequential antimicrobial therapies are another treatment strategy for chronic respiratory infections with biofilms. Similarly, inhaled or oral antibiotic combination might represent an optimal anti-biofilm strategy in this setting [11, 63, 64, 75-79]. Such as ciprofloxacin, tobramycin or the β-lactams that attack metabolically active layers with colistin that preferentially kill biofilm cells with low metabolic activity provides a rational approach for establishing combination therapy [11, 63, 64, 75-79]. Especially in CF and COPD patients, is routinely used with the aim of preventing of relapse [11, 63, 64, 75-79]. An interesting combination of inhaled formulations of a 4:1 (w/w) of fosfomycin/tobramycin has been found to be effective in Phase II clinical studies against both

gram-negative and gram-positive bacteria [11, 63, 64, 75-79]. However, there is insufficient evidence to determine the advantage of choosing antibiotics based on combinations in the treatment of pulmonary exacerbations with chronic *P. aeruginosa, H. influenzae*, *A. baumannii* and *S. pneumonia* infection [11, 63, 64, 75-79].

Even though antibiotics are associated with some clinical benefit, less exacerbations and improved quality of life in chronic respiratory infection patients, there are considerable drawbacks of taking antibiotics [11, 63, 64, 75-79]. First, there were specific adverse side effects associated with the antibiotics. Second, patients have to take antibiotics regularly for long duration (months or years), which resulting increase in antibiotic resistance, risk of hypersensitivity, treatment failure and relapse rates [11, 63, 64, 75-79].

Potential use of host defense peptides (HDPs) in biofilm related respiratory infections

Over the last decades an increasing number of disease-causing bacteria are rapidly evolving immunity to every existing antibiotic [80]. The widespread emergence of resistant bacteria already claims millions of lives globally [80, 81]. This has resulted in a number of infectious diseases, in some cases life-threatening, for which limited treatment regimens exists or without effective antibiotics [82]. The strategy for combating infections is not significantly changed and the problems related to resistant bacteria will escalate even further by presence of biofilm [81]. As a result,

development of new effective antimicrobial compounds and treatment alternatives is an important for fight against the rising threats from antimicrobial resistance [81, 82].

Over the last decade, anti-microbial peptides (AMPs) have emerged as a promising alternative for treatment of various infections [83]. A large variety of AMPs have been identified and isolated from different sources. AMPs are in general rather small (10-20, amino acid residues) cationic and amphiphilic compounds with a diversity in secondary structure which can disrupting the bacteria membranes [84]. In addition, AMPs have a significant immune-regulatory role including anti-inflammatory properties, by stimulating chemotaxis and chemokine production, wound healing, angiogenesis and dendritic cell activation [83, 84]. Therefore, the rationale behind this approach is to enable resolution of the infection through killing the bacteria cells by antimicrobial peptides and then synergized with conventional antibiotic treatment, couple with the host immune system will be given a greater windows of opportunity in which to clear the gram negative bacterial infections. In that sense, cationic host defense peptides (HDPs) have a desirable properties, which make them excellent prospects as novel anti-microbial peptide [85-88]. Those HDPs can used for the development of novel treatment for biofilm related chronic respiratory infections alone or together with other antibiotics without a significant resistance problem.

HDPs are short, cationic amphipathic peptides with diverse sequences that are produced by various cells and tissues, such as mammalian intestine, skin, respiratory and reproductive tracts [85-87, 89,

90]. Conventionally, amphipathic nature of such peptides are acts exclusively via membrane disruption and formation of stable pores on the anionic surfaces of bacterial and cancer cells [85]. In addition, they also prevent the microorganism's adhesion or interaction with host tissues [88]. Moreover, these tissues are prolong exposed to external flora of bacteria, viruses and parasites and also natural reservoir for indigenous communities of commensal microorganisms [88]. Therefore, they are key component of the innate immunity with variety of mechanisms to fight against infections and inflammation, including selective modulation of innate immunity, active against bacterial biofilms and direct antimicrobial activity[85]. Another property of HDPs is that they can bind and neutralize bacterial lipopolysaccharides which are potent activators of Toll-like receptors in different innate-immune cells [85]. Their release to the blood is the primary cause of septic shock.

The two major families of mammalian host defence peptides are, defensins and cathelicidins [91]. Among them, defensins are small cationic host defense peptides that can be found in both vertebrate and invertebrate animals, and function as a first line of defense [85]. Such so-called defensins are quite small, in the range of 18-50 amino acids, typically cationic and cysteine-rich [92]. The two main defensin subfamilies are alpha- and beta-defensins. They are different in the length of peptide segments between the frameworks of six disulphide [92]-linked cysteines. A main function is lysis of bacterial cell membranes leading to loss of metabolites, and ions [92]. Immune

cells and epithelial cells extensively use these peptides as tools in killing phagocytosed bacteria [93, 94].

Although the defensin families are well-studied AMP, much less is known about NK-lysin and its human orthologue, granulysin. NK-Lysin is a cationic host defense peptide with potent microbiocidal and tumor cytolytic activities [95]. It was originally isolated from porcine intestinal tissue and also present in the granules of T-lymphocytes and natural killer (NK) cells. Its ability to form pores in the cell membrane is similar to saposin-like proteins and also due to the presence of α-helical structure [95]. Compared with the other antimicrobial peptides, NK-lysin is 78 amino-acid residues large globular structure, which are rich in positively charged amino acids and include conserved cysteine residues that form intrachain disulfide bonds [95-97].

Among other HDPs, melittin is a 26 amino-acid, amphipathic, alpha-helical, hemolytic peptide, which is the principal toxic component in the venom of the European honey bee *Apis mellifera [98, 99]*. It is active against a wide range of microorganisms including Gram-negative and Gram-positive bacteria [98, 99]. A perfect amphipathic nature that constitute basic and hydrophobic residues into a polar and a nonpolar face is recognized as an essential for α -helical AMP activity [99]. Recently, numerous studies have suggested that substitution of the nonpolar face with positively charged residues to disrupt the α-helical amphipathic structure appears to be related to

reduce cytotoxicity while retaining antimicrobial activity [99]. Therefore, those HDPs are useful candidates for development of novel anti-microbial peptide therapies.

Based on existing evidence that host defense peptides can kill bacteria cells and also can inhibit the bacteria adhesion, this both as a rational approach to target gram negative bacterial biofilm infections. Therefore, combining the machine learning methods (algorithms) to predict the structural characteristics, descriptors, involved in anti-microbial peptide activity, help to develop a several novel short cationic antimicrobial peptides targeting gram negative bacterial biofilm infections. This will include chemical modifications of native peptide structure resulting to the change of the desired parameters such as net charge, helicity, hydrophobicity, amphipathicity and stability against degradation in biological fluids. The action of such peptides is dependent on their amphipathic alpha-helical conformation permitting them to interact specifically with the anionic components of the bacterial membranes by the way which triggers the cascade of reactions leading to biological responses, such as killing of bacteria by disrupting the bacteria membranes, jamming the bacteria quorum sensing, degrading the stress related molecules (e.g., (p)ppGpp, guanosine pentaphosphate) increase of the chemotaxis of neutrophils, activation of leucocytes in the inflammation site etc. Therefore bacteria are not prone to develop high level resistance towards these compounds in the same extent as towards conventional antibiotics. Then, such cocktail of

peptides can be synergizing with antibiotics in current practice to clear the remaining bacterial infection.

While those peptides protect host cells, bacteria also have another range of mechanisms that allow them to adhere tightly to host cell surface. Such host establishment requires successful colonization in tissues, followed by persistent biofilm formation. The attachment of *P. aeruginosa* and *A. baumannii* to human respiratory epithelium [11, 18, 100, 101] represents an important step in the development of persistence chronic lung infection. These strains interacts with epithelial cells via cell-surface divers of receptors, such as GM1 (asialo ganglio-N-tetraosylceramide), CD14 (cluster of differentiation 14), cystic fibrosis trans-membrane conductance regulator (CFTR) and Toll-like receptors (TLRs), to establish a complex signal transduction cascades [18, 69, 72, 86, 102]. In addition, components of the biofilm matrix including secreted extracellular proteins, cell surface adhesins and protein subunits of cell appendages such as flagella and pili, play diverse roles in attaching to host cell surfaces [72, 74, 86, 103]. Such scenario facilitates the development of biofilm matrix via interactions with exopolysaccharide and extracellular DNA, to create three-dimensional biofilm architectures [103]. Interestingly, most of these adhesion target molecules are associated with family of mammalian transmembrane proteins called tetraspanins.

The tetraspanins are 33 groups of molecular facilitator proteins, which share similar structural motifs with four transmembrane domains (TM1 to -4), a small extracellular loop (EC1), and a large

extracellular loop (EC2) [104]. Couple with different transmembrane receptors and also among themselves, tetraspanins are able to form a distinct class of membrane domains, called the tetraspanin-enriched microdomains (TEMs) [104]. TEMs are known regulators of cell migration, tumor progression and metastasis, cell adherence and fusion, membrane trafficking, endocytosis, leukocyte adherence and motility [104, 105]. Moreover, bacterial adhesion requires an indirect interaction with tetraspanins, through receptors embedded in TEM [104-106]. The *Escherichia coli* adhesin, FimH, binding directly to tetraspanin TSPAN21 has been shown to exploit tetraspanins in order to adhere to bladder cells during urinary tract infections. In addition, antibodies of tetraspanins CD9, CD63, and CD151 were reduced adherence of *Staphylococcus aureus, Neisseria lactamica, E. coli,* and *Streptococcus pneumoniae* to epithelial cells [105, 107]. Moreover, peptides derived from CD9, drastically inhibited the *Staphylococcus aureus* adhesion to keratinocytes [106]. The CD9-enriched microdomains also negatively regulate LPS-induced receptor formation by preventing CD14 from accumulating into lipid rafts [108]. Both serve as a major interface for host-pathogen cross-talk which involves in *P. aeruginosa* and *A. baumannii* in lung inflammation and infection [25, 103, 109]. Therefore, tetraspanins are also novel, intriguing drug target to prevent biofilm formation on host surface.

CHAPTER 6

Overcoming biofilm mediated respiratory infections through exploitation of pathogen and host-directed novel peptides: preliminary screening and development

Origin of the proposal: Aims and Objectives

Biofilms are an alternative growth state of bacteria, represent two thirds of all infections, and are resistant to antimicrobial treatment being 10- to 1,000 fold more resistant to most antibiotics [19]. This resistance is adaptive in that it depends on the biofilm group behaviour and although many explanations have been provided to explain it [16]. It is likely that changes in gene and/or protein expression in the biofilm state explain why organisms become resistant [16]. Such behaviour can prevent an antibiotic entering the bacteria cell at all, reduction of diffusion, altering or neutralizing the antibiotic so that they don't bind to the target molecules anymore, or enhancing the efficiency of efflux mechanisms within the biofilm which allow it to simply pump a drug back out again [19]. Intriguingly despite this problem, not a single antibiotic has been developed for treating biofilms. Meantime antibiotic consumption had increased by 40 – 50 % in the past decade with growing numbers of bacteria already fully resistant to every clinical available antibiotic [110]. Therefore, we have started to address this using as templates of the cationic host defence (antimicrobial) peptides, which are produced by virtually all organisms as a major part of their innate defences

against infection [83]. They are a key component of innate immunity and have multiple mechanisms that enable them to deal with infections and inflammation, including selective modulation of innate immunity, activity against bacterial biofilms (the cause of 65% of all human infections) and direct antimicrobial activity [84]. Peptides act against biofilms formed by multiple species of bacteria in a manner that is independent of activity vs. planktonic bacteria. Therefore, cationic host defense peptides (HDPs) have desirable properties, which make them excellent prospects as novel anti-biofilm agents [85-88].

We made the breakthrough observation that peptide derived from human NK-lysin was able to inhibit *P. aeruginosa, Haemophilus influenzae* and *A. baumannii* biofilms at one tenth of its MBEC concentration (preliminary study). This led us to extensively pursue cationic host defense peptides (Defensins, NK-lysin, Melittin and) as a potential templates for designing the synthetic short anti-biofilm peptides. The rationale behind this approach is to enable resolution of the infection through trapping bacteria cells in the antibiotic-susceptible planktonic growth mode. Then these approaches are synergized with conventional antibiotic treatment, couple with the host immune system will be given a greater windows of opportunity in which to clear the biofilm infections. Therefore, combining the machine learning methods (algorithms) to predict the structural characteristics, descriptors, involved in anti-biofilm peptide activity, we have now developed novel 10-12 amino-acid anti-biofilm peptides that kill multiple species of bacteria in biofilms, including

P. aeruginosa, Haemophilus influenzae and *A. baumannii.* The action of such peptides is dependent on their ability to trigger the degradation of the nucleotide stress signal ppGpp, or block the quorum sensing (such as N-acyl homoserine lactone analogues and PQS analogues) or other biofilm related virulence factors to collapse the structure of biofilm and mean time disrupt the bacteria membrane integrity. Then, such peptides will synergize with antibiotics in current practice to clear the remaining planktonic bacteria.

Materials and Methods

Bacteria strains

After approval of the study protocol by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (No. IRB 414/60), *P. aeruginosa* isolates (n=70), *A. baumannii* (n=70) and *Haemophilus influenzae* (n=30) with various morphology and resistance profiles were obtained without preference from a strain repository at the King Chulalongkorn Memorial Hospital in Bangkok, Thailand. The clinical strains have been isolated during 2010–2015 from respiratory tract (sputum or endotracheal aspirate samples) of chronically infected patients as part of standard care and was unrelated to the present study.

Culture Conditions

The *P. aeruginosa* PA01 biofilm-positive strain ATCC 15692, *A. baumannii* biofilm-positive strain ATCC 19606 and both strains clinical isolates were cultured on Mueller Hinton agar (MHA) plates at 37°C. The *H. influenzae* biofilm-positive strain ATCC 49247 and clinical isolates were cultured on Chocolate agar plates in 5% CO2 for 18 h at 37°C. All isolates were stored at −80°C in tryptic soy broth with 15% glycerol until they were used in subsequent experiments in which they were suitably anonymised.

Antibiotics and agents

We tested fifteen antibiotics (Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin, ceftazidime, tobramycin, amoxicillin-calvulanate, clarithromycin, levofloxacin, aztreonam, erythromycin, tetracycline, sulfamethoxazole/trimethoprim and ampicilline) widely used for the treatment of *P. aeruginosa, A. baumannii* and *H. influenzae* infections. Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin, ceftazidime, tobramycin, amoxicillin-calvulanate, clarithromycin, levofloxacin, aztreonam, erythromycin, tetracycline, sulfamethoxazole/trimethoprim and ampicilline were all from Sigma-Aldrich. Antibiotic stock solutions were prepared less than 24 h before use. Antibiotics were dissolved in cation-adjusted Müller-Hinton II broth (MHIIB) (Becton Dickinson) or brain heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with β-nicotinamide adenine dinucleotide (β-NAD; 15 μg/ml) and hemin (30 μg/ml) medium and sterilized by filtration through a membrane (0.22 μm pores). Serial dilutions of the stocks were prepared in MHIIB medium immediately before use.

Anti-biofilm Peptide design and synthesis

The HDP design based on the machine learning algorithm and uses Moon and Fleming scale [111] and the following physico-chemical characteristics of peptides: Hydrophobic moment, PI , PH, hydrophobicity, hydropathicity, Solubility, Aggregation, Stability, Half-life, Heat capacity, Toxic

motifs, charge density and depth-dependent potential [112]. Peptide should consist of 20 canonical amino acids and peptide length does not exceed 10 -12 amino acids range. Whole amino acid composition (%) and selected residues composition (%) were used as input features for developing machine learning algorithm (SVM and WEKA) [111]. The amino acid composition is the fraction of each amino acid in a peptide and converts a peptide sequence into a vector of 8000 dimensions. All peptides were synthesized using solid phase Fmoc chemistry (Commercially synthesized) and purified to 95% using reverse-phase high-performance liquid chromatography. Correct peptide mass were confirmed by mass spectrometry. Peptide concentration was determined using amino acid analysis. Scrambled peptides were randomly generated from the respective sequences (negative control).

Biofilm formation in 96-well microtitre plates

A single colony of *P. aeruginosa, A. baumannii* and *H. influenzae* were inoculated into 2 mL of MHIIB or brain heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with β-nicotinamide adenine dinucleotide (β-NAD; 15 μg/ml) and hemin (30 μg/ml) medium supplemented with or without low molecular weight salmon sperm (Fluka) in a tube and incubated in an orbital shaker (200 rpm) at 37°C overnight for about 16 h. Subsequently, a subculture was prepared by diluting the overnight culture with fresh medium to an OD of 0.02

at 600 nm (5 x 107 cfu/ml), and 100 µL aliquots added to flat-bottomed 96-well polypropylene microtitre plates (SPL Life Sciences), with uninoculated medium (100 µL) as a negative control, the plates were incubated at 37°C for 24 h. We used this procedure for subsequent biofilm experiments, which were performed in triplicate and repeated three times.

Testing susceptibility to antibiotics

The MIC were established using standard techniques according to criteria in the EUCAST [39]and CLSI guidelines [40]. *E. coli* ATCC 25922, *H. influenzae* ATCC 49247 and *P. aeruginosa* ATCC 27853 were used as quality control strains, with modifications as follows. To establish planktonic MIC for the antibiotics used, the antibiotics were serially diluted two-fold in 96-well microtitre plates and bacteria added. The plates were incubated at 37°C for 18 h. MBEC were established by adding the serially diluted antibiotics to mature biofilms and incubating at 37°C for 24 h before staining with PrestoBlue to calculate the viable cells within the biofilm. Before adding the antibiotics, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium.

Biofilm formation quantification and classification

Two methods were used to quantify [42] and classify [43] the biofilm structure by Crystal Violet staining with modifications . The Crystal Violet (0.1%) stained biofilms were solubilized with 30% acetic acid followed by incubation at room temperature for 10–15 min. OD at 550 nm was

determined using a microtitre plate reading spectrophotometer with 30% acetic acid as a blank control. Mean absorbances and their SDs were calculated for all tested strains and negative controls, determined in triplicate and repeated three times. The cut-off value (ODβ) was defined as 3SD above the mean OD of the negative controls: ODβ = average OD of the negative controls + 3SD of negative controls, and was calculated separately for each microtitre plate. The OD of a tested strain was expressed as the mean OD of the strain minus the ODβ (OD = mean OD of a strain – ODβ). The clinical isolates were classified as described previously [43].

Treatment of DNaseI and reduced glutathione (GSH)

In experiments where GSH and DNaseI [113] treatment was required, 5mM reduced glutathione (GSH) (Sigma), and 40 Kunitz units DNaseI (Sigma) were dissolve in PBS as described previously . Biofilms were then treated with GSH, and DNaseI in combination with antibiotics for 18 h to determine the MBEC as described previously.

Alginate measuring assay

To measure the amount of alginate produced, *P. aeruginosa* clinical isolates used in this study were grown in 5 ml of LB broth with orbital shaking at 200 rpm at 37°C until the culture reached an OD600 of 2.0 as described previously [113]. The bacterial cells were then collected by centrifugation at 7000 × g for 20 min and suspended in 1 ml of PBS buffer. Simultaneously, another

culture was used to correlate OD600 2.0 with the dry cell weight. To remove any contaminants such as RNA and DNA from the alginate, the samples were treated with RNAse A (Promega) and DNAse I (Sigma). The samples were then incubated at 37°C for 1 h. To remove the cells, the mixture was vortexed and centrifuged at 8000 × g for 20 min. The alginate remaining in the supernatant was precipitated with 25 ml of 95% ethanol. The alginate precipitates were collected by centrifugation at 10000 × g for 30 min and suspended in 2 ml of 0.85% NaCl. The uronic acid concentration was determined by a standard colorimetric assay. All experiments were performed in triplicate and repeated three times.

Pyocyanin level Quantitative Analysis

The amount of pyocyanin produced, *P. aeruginosa* clinical isolates biofilm were quantitatively measured by method described previously [114]. Briefly, the isolates were grown in 2ml of LB broth culture at 37°C with shaking (200 rpm) for 16 h. Then, supernatant was collected by centrifugation at 7000 × g for 20 min for pyocyanin quantification. In brief, 600 µl of chloroform was added to 1 ml of supernatant, and the tube was vortexed twice for 10 s. The tubes were centrifuged at 10,000 rpm for 10 min, and the bottom phase (600 µl) was transferred to a new tube containing 300 µl of 0.2 N HCl. The tubes were vortexed twice for 10 s each time and centrifuged at 10,000 rpm for 2 min. The OD_{520} of the top phase was measured and multiplied by

17.072 to calculate the micrograms of pyocyanin per milliliter. All strains were tested four times. All experiments were performed in triplicate and repeated three times.

Measurements of extracellular DNA (eDNA) in biofilms

The eDNA concentration in *H. influenzae, A. baumannii* and *P. aeruginosa* biofilms were determined by PicoGreen fluorescent staining (Quant-iT Invitroge) in a 96-well-microtitre-plate biofilm formation method as described previously with some modifications [115]. A freshly prepared solution PicoGreen dye diluted in TE buffer (1:200) was added to the each well in the ratio 1 : 1 and eDNA concentration was measured on a fluorospectrometer (Varioskan Flash Multimode Reader; Thermo Fisher Scientific), using 470 nm excitation and 525 nm detection. To verify reproducibility eDNA production in the biofilm cultures were also quantified by laser scanning fluorescence microscopy (Zeiss Axiovert 200M) after staining with propidium iodide (BacLight Live/dead staining kit) during all experiments.

MIC, MBEC50 and MBEC75 Assays

The broth microdilution method with minor modifications for cationic peptides was used for measuring the MIC of all anti-biofilm peptides used as described previously [116]. MBECs leading to 50% and 75% decrease in biofilm (MBEC 50 and 75) was obtained using 96-well plate assays

and presto blue staining and crystal violet staining was used to determine whether the peptides could disperse preformed biofilms.

Inhibition of bacterial initial attachment

A bacteria attachment assay against biofilm-producing bacterial strains was performed using a previously described method with some modifications [117]. An overnight culture of bacteria was washed and re-suspend in Mueller Hinton broth (MHB) to an OD 600 = 0.05. Aliquots of 100 μl suspended bacteria were added to a 96-well plate containing 100 μ l MHB with peptides. The plate will be incubated at 37 ℃ for one hour without agitation to allow bacterial binding. Planktonic cells were then carefully removed by pipetting and the plate was washed thrice with 200 μ l PBS solution. The results were reported relative to untreated bacteria binding analysis by confocal laser scanning microscope.

Inhibition of biofilm formation

Biofilm formation an assay was performed as described for the inhibition of bacterial initial attachment with some alternations. Plates containing peptides at serial dilutions and bacteria were incubated for 24hours at 37 ℃ without agitation to allow biofilm formation. Wells without peptide were set up as positive controls. Presto blue viability reagent was added into each well of the microtiter plate follow by Incubate ≥30 minutes at 37° C and the fluorescence was read by using

in a plate reader (excitation 560nm and emission 590nm).The results was interpreted by the comparison of peptides on treated biofilms to untreated biofilms through viability and confocal laser scanning microscope and 3D reconstructions was generated using the Imaris software package. An experiment was performed in triplicate, and three independent experiments were performed for each of these assays.

Checkerboard assays

The checkerboard assay for the biofilms was performed as describe previously for levofloxacin, meropenem and piperacillin tazobactam [118]. The selection criteria for these three antibiotics were based on treatment data obtained from our previous study. The MBEC 50 and 75 values represent the concentration (or combinations of concentrations when using peptides in combination with antibiotic) at which 50-75 % biofilm eradication. The result was expressed as the fractional inhibitory concentration (FIC) index, calculated as follows: FIC = [A]/MBEC A + [B]/MBEC B , where MBEC A and MBEC B were the MBECs of peptides A and B alone and [A] and [B] were the MBECs of A and B when in combination with selected antibiotics (for levofloxacin, meropenem and piperacillin tazobactam). An FIC index of 0.5 was considered to indicate good synergy (representing the equivalent of a four-fold decrease in the MBEC of each compound when used in combination). An FIC index of 1.0 represents additive activity (a two-fold decrease

in the MBEC of each compound in combination), and an index of >4 would be indicative of antagonism.

Statistical analyses

Continuous variables are summarized using means and SDs, and categorical variables as counts and percentages. Levels of *P. aeruginosa, A. baumannii* and *H. influenzae* drug susceptibility are represented in two ways: a continuous measure of concentration; and an ordinal categorical form representing biofilm formation; both of these outcomes were measured repeatedly over time for each isolate. Linear mixed modelling was used to compare concentrations between drug types over time. We then examined which drug types were more successful in allowing concentration to be used to distinguish between biofilm formations using ordinal logistic mixed effects regression. All analysis was conducted using the R statistical package [41], linear mixed modelling was performed using the R library, lme4 [44], and ordinal logistic mixed effect modelling using the R library, ordinal[45]. $P < 0.05$ was considered significant for all inferential analysis.

Results

Respiratory isolates tend to form a diverse of biofilm

In this study, we employed 70- *P. aeruginosa,* 70 - *A. baumannii* and 30 - *H. influenzae* clinical strains isolated from 140 respiratory patients from a tertiary care facility. The age of the patients varied from newborn to 86 years and roughly 30% were female patients. Among the isolates, half of them were from sputum samples and the other half were isolated from the endotracheal aspirate. All the isolates displayed resistance to multiple antibiotics including broad-spectrum-β-lactams (data shown in previous study). We then investigated the biofilm formation capacity among the isolates by determining the presence of different biofilm category (Figure 30). All the isolates were dominated by moderate and strong biofilm producers with 0.8 – 4.5 μm^3 bio-volume.

Biofilm matrix component displayed complex nature of respiratory isolates biofilm

The observations have shown that level of biofilm matrix component of extracellular DNA level were significantly higher ($p < 0.001$) in strong biofilm of *P. aeruginosa, A. baumannii* and *H. influenzae* isolates compare to *P. aeruginosa* moderate ones (Figure 31). Moreover, *P. aeruginosa* biofilm specific matrix component of alginate and pyocyanin levels were also significantly ($p < 0.001$) higher in strong biofilm compare to the moderate ones (Figure 3). However, by removing

these molecules through addition of glutathione reductase (GSH) or DNase, the established *P. aeruginosa* biofilms were inhibited and biofilms were dispersed (Figure 31 and 32). Similar observation was found for *A. baumannii* and *H. influenzae* with DNase (Figure 31), confirming extracellular DNA is the major component of the nontypeable biofilm structure.

Host defence peptides exhibited broad-spectrum antibiofilm activity

A 16 short (12 amino acid range) HDP peptides were sleeted (top 16) among the 200 peptide candidates for *in vitro* testing based on computer based in silico predictions (selection algorithm data not shown due to intellectual property right restrictions). For those 16 HDPs, physiochemical parameters were characterized during the manufacture process as a part of standard quality control (Table 8). All tested peptides were displayed significant effect ($p < 0.001$) on cell viability (MBEC 90) of *P. aeruginosa, A. baumannii* and *H. influenzae* established biofilm with several peptides (PEP 25 and PEP 102) reducing the number of bacteria with in the biofilm more than 3 LOG (below the detection limit of 1000 CFU/ml) (Figure 33, 34, and 35) after overnight treatments. Therefore, PEP 25 and PEP 102 were selected for further experiments.

Host defence peptides alters the mature biofilm in a dose-dependent manner

Next, dose–response relation was assessed for all three strains in the presence of HDP PEP 25 and 102 (Figure 36, 37, 38). With increasing amounts of HDPs concentration, there was a significant (*p*

< 0.001) reduction in number of viable bacteria with in the biofilm at relatively low concentration range compare to clinically used antibiotic. Clearly, the dispersion or dismantle of established biofilm structure based on bio-volume were significant ($p < 0.001$) for PEP 25 and 102 based on concentration depended manner (Figure 39). However, in all the stariins PEP 102 able to displayed significant effect on biofilm eradication compare to the PEP 25 ($p < 0.001$).

Host defence peptide PEP 100 displayed significant effect on biofilm formation and eradication

The antimicrobial and ant biofilm activity of the peptide PEP 102 was summarized in Table 9. For *P. aeruginosa,* PEP 100 showed the wider concentration range 32-128 µg/ml antimicrobial effect on planktonic bacteria with multi drug resistant and 4-16 µg/ml on antibiotic susceptible isolates. Interestingly, similar range were observed to eradicate (32-128 µg/ml – Strong biofilms). For *A. baumannii* PEP 100 was able to eradicate established biofilm within 32-128 µg/ml for strong biofilms, which differ from *P. aeruginosa*, but both showed similar range 4-16 µg/m for moderate biofilms. However, the antibacterial activity for planktonic cells were range from 64-128 µg/ml for multi drug resistant and 8-16 µg/ml antibiotic susceptible isolates. *H. influenzae* displayed relatively narrow range of antibacterial concentration 16-32 µg/ml for planktonic cells with multi drug resistant and 4-8 µg/ml with antibiotic susceptible strains. But, to eradicate established biofilm within 24 hours relatively lower for *H. influenzae* (16-64 µg/ml – Strong biofilms and 4-16 µg/ml –

Moderate biofilms). *A. baumannii* and *P. aeruginosa* share the similar biofilm formation inhibition concentration range (2-16 µg/ml), but *H. influenzae* displayed lower concentration range 2-4 µg/ml.

Synergistic interactions between host defence peptides and conventional antibiotics to eradicate biofilms

This results revealed that PEP102 showed synergy with meropenem, levofloxacin and piperacillin/tazobactam for all three strains mature biofilms (Figure 40). Strong synergy is found for PEP102 and meropenem or levofloxacin or piperacillin/tazobactam against *H. influenzae* with FIC < 0.5. Whereas the MIC-value of meropenem itself is 512 µg/ml levofloxacin 256 µg/ml and piperacillin/tazobactam 256/64 µg/ml for strong biofilm and 128 µg/ml, 128 µg/ml and 128/32 µg/ml for moderate biofilm, in the presence of 8 µg/ml PEP 102, the MBEC 75 of meropenem and levofloxacin drops to 1-2 µg/ml followed by 8/4 µg/ml for piperacillin/tazobactam for both strong moderate biofilms. Similarly in the presence of 16 µg/ml PEP, the MBEC 75-value of meropenem and levofloxacin drops to 2 µg/ml followed by 4/1 µg/ml for piperacillin/tazobactam, range for both strong moderate biofilms of *P. aeruginosa* and *A. baumannii*. Interestingly, the required amount of the meropenem or levofloxacin or piperacillin/tazobactam alone to achieved MBEC 75-value was above the 128 µg/ml both strong moderate biofilms of *P. aeruginosa* and *A. baumannii*.

Figure 30 ***Characterization of the biofilm bio-volume of strong and moderate biofilm of A) P. aeruginosa B) A. baumannii C) H. influenzae; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy***

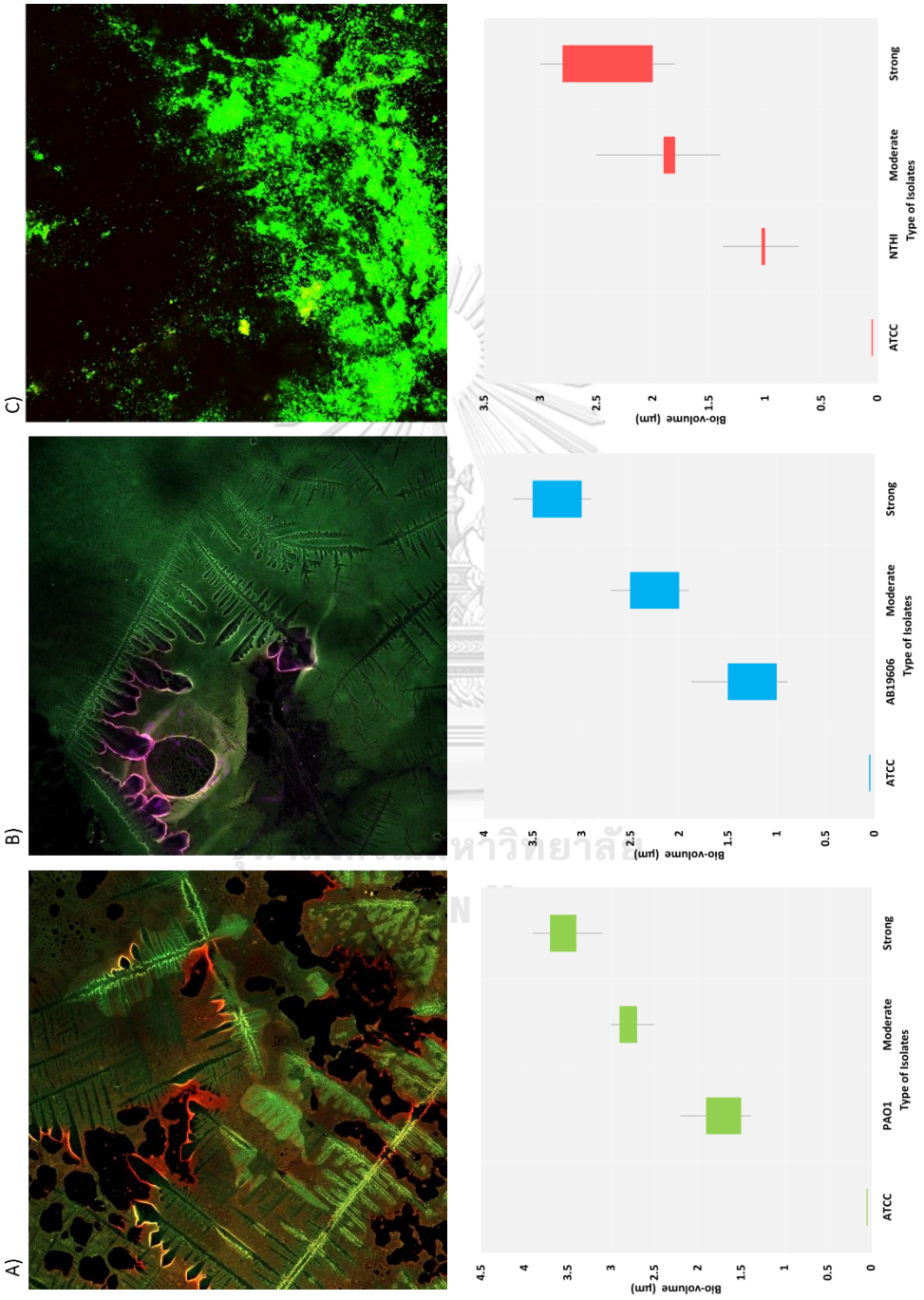


Figure 31 Characterization of biofilm matrix component based on extra cellular DNA (eDNA) for A) P. aeruginosa B) A. baumannii C) H. influenzae alone or in the presence of Dnase I: ATCC biofilm negative control and PAO1, Ab19606 and NtHi biofilm positive control.

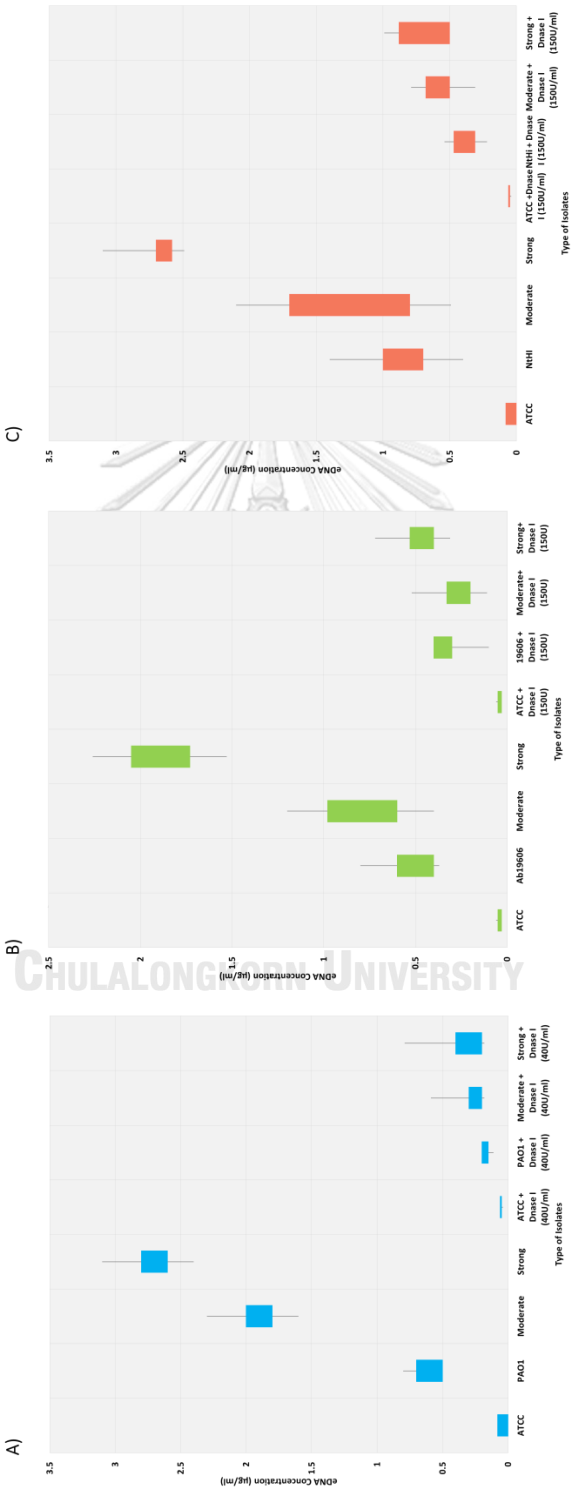


Figure 32 Characterization of P. aeruginosa biofilm matrix components based on A) Alginate production B) Pyocyanin (PCN⁻) production treated with Pyocyanin chelator glutathione reductase: ATCC biofilm negative control and PAO1 biofilm positive control.

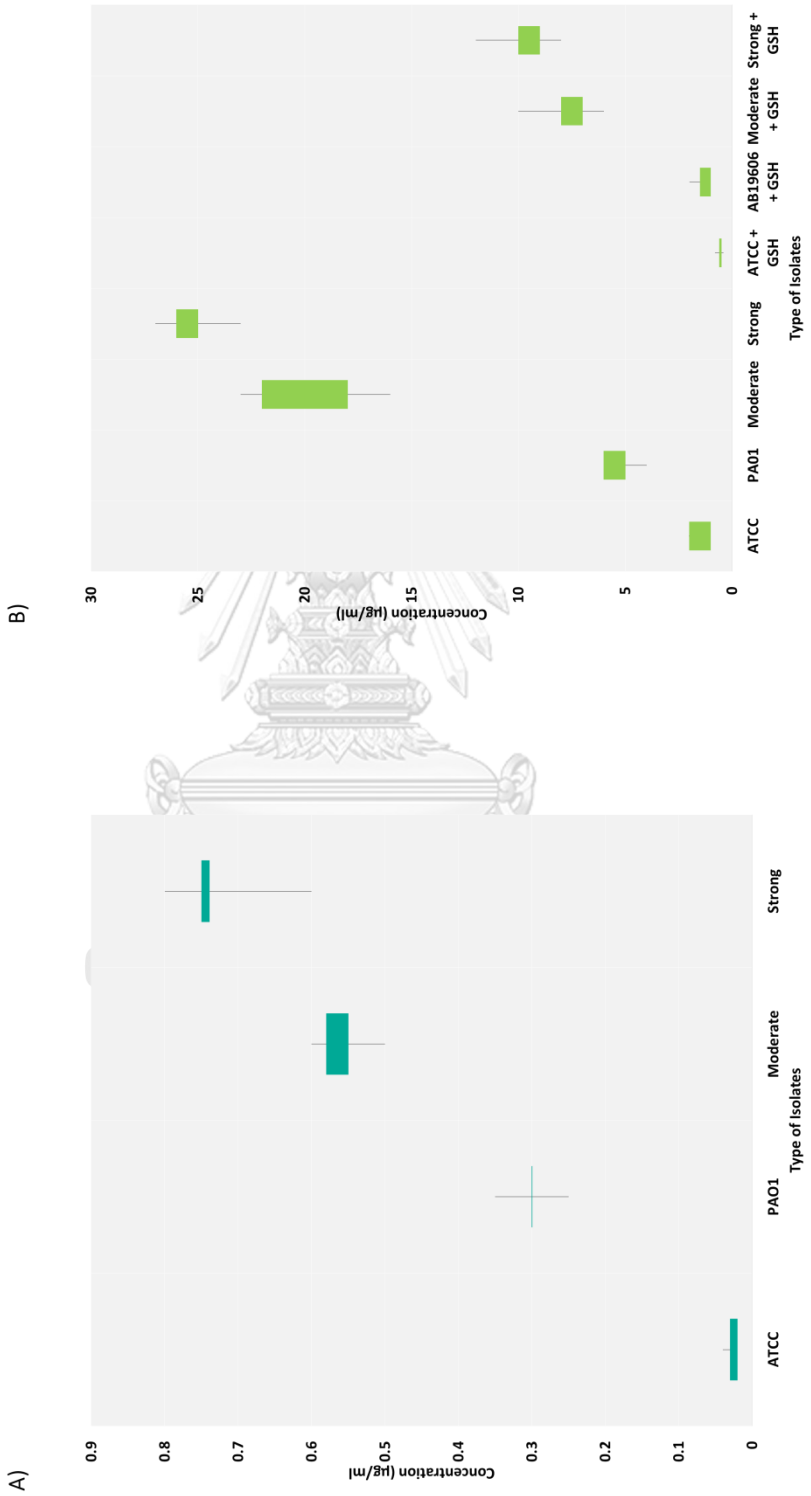


Figure 33 MBEC = minimum biofilm eradication concentration of P. aeruginosa biofilm were generated measuring bacteria cell viability as a percentage with 90% non-viable cells in the presence of anti-biofilm peptides

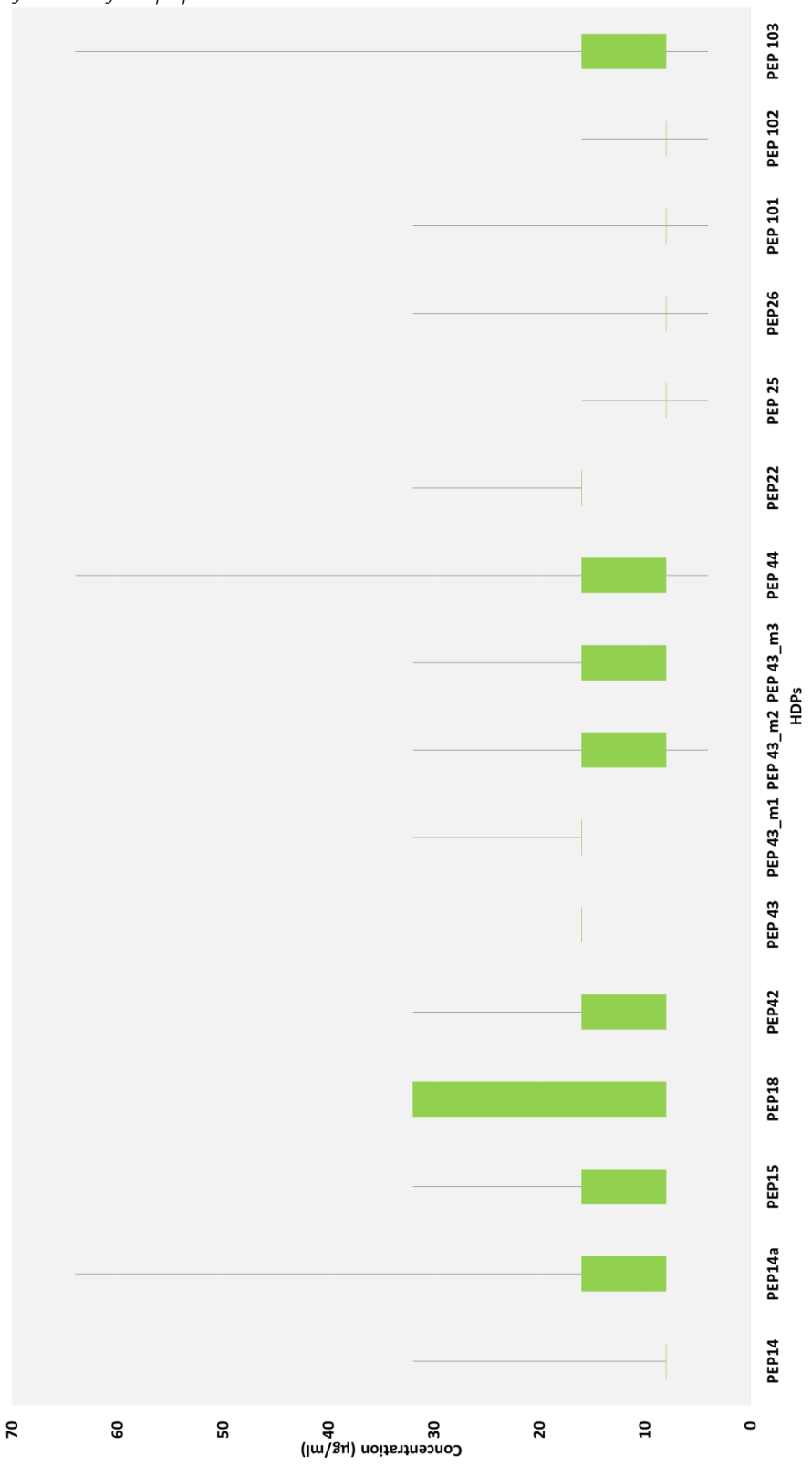


Figure 34 MBEC = minimum biofilm eradication concentration of A. baumannii biofilm were generated measuring bacteria cell viability as a percentage with 90% non-viable cells in the presence of anti-biofilm peptides

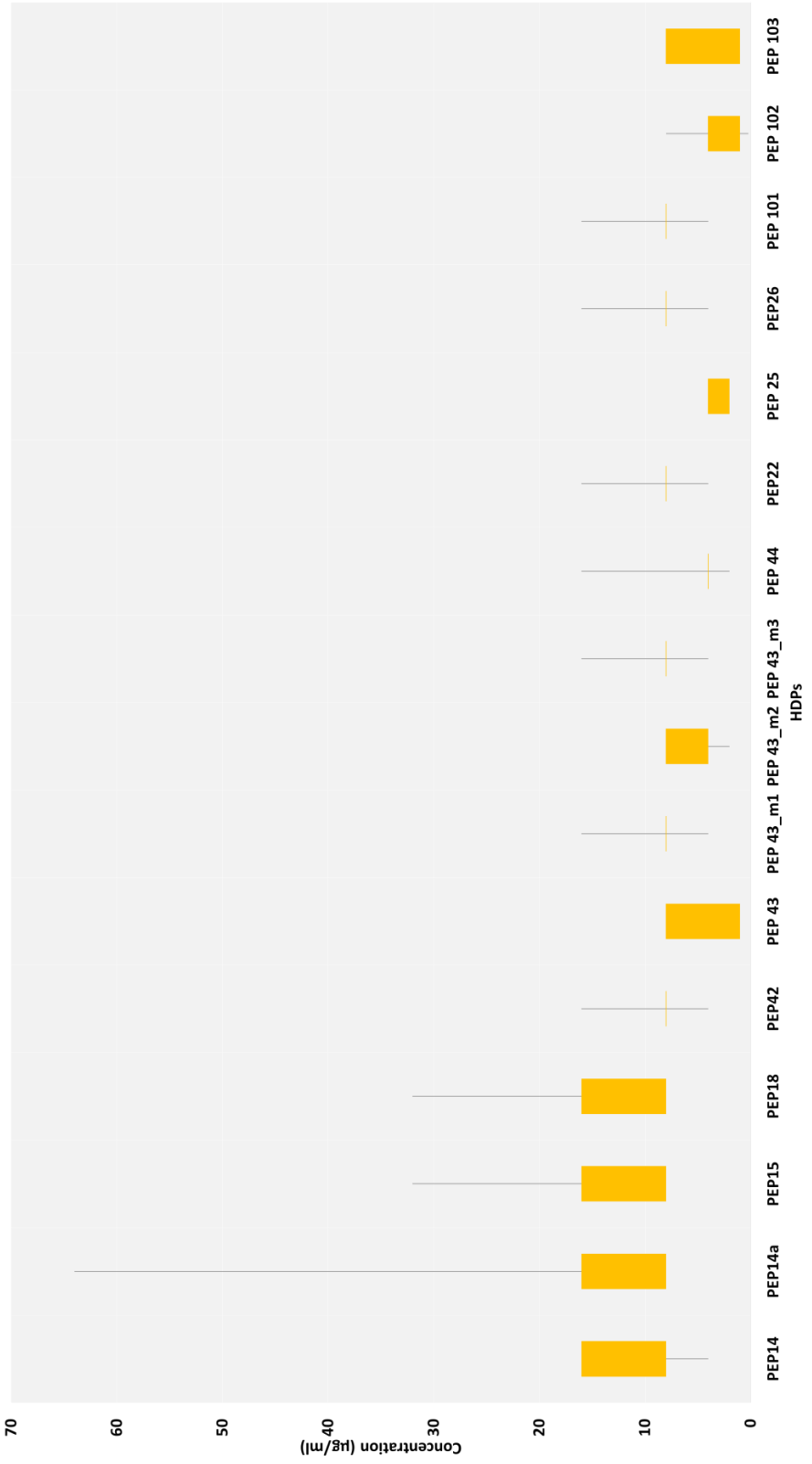


Figure 35 MBEC = minimum biofilm eradication concentration of H. influenzae biofilm were generated measuring bacteria cell viability as a percentage with 90% non-viable cells in the presence of anti-biofilm peptides

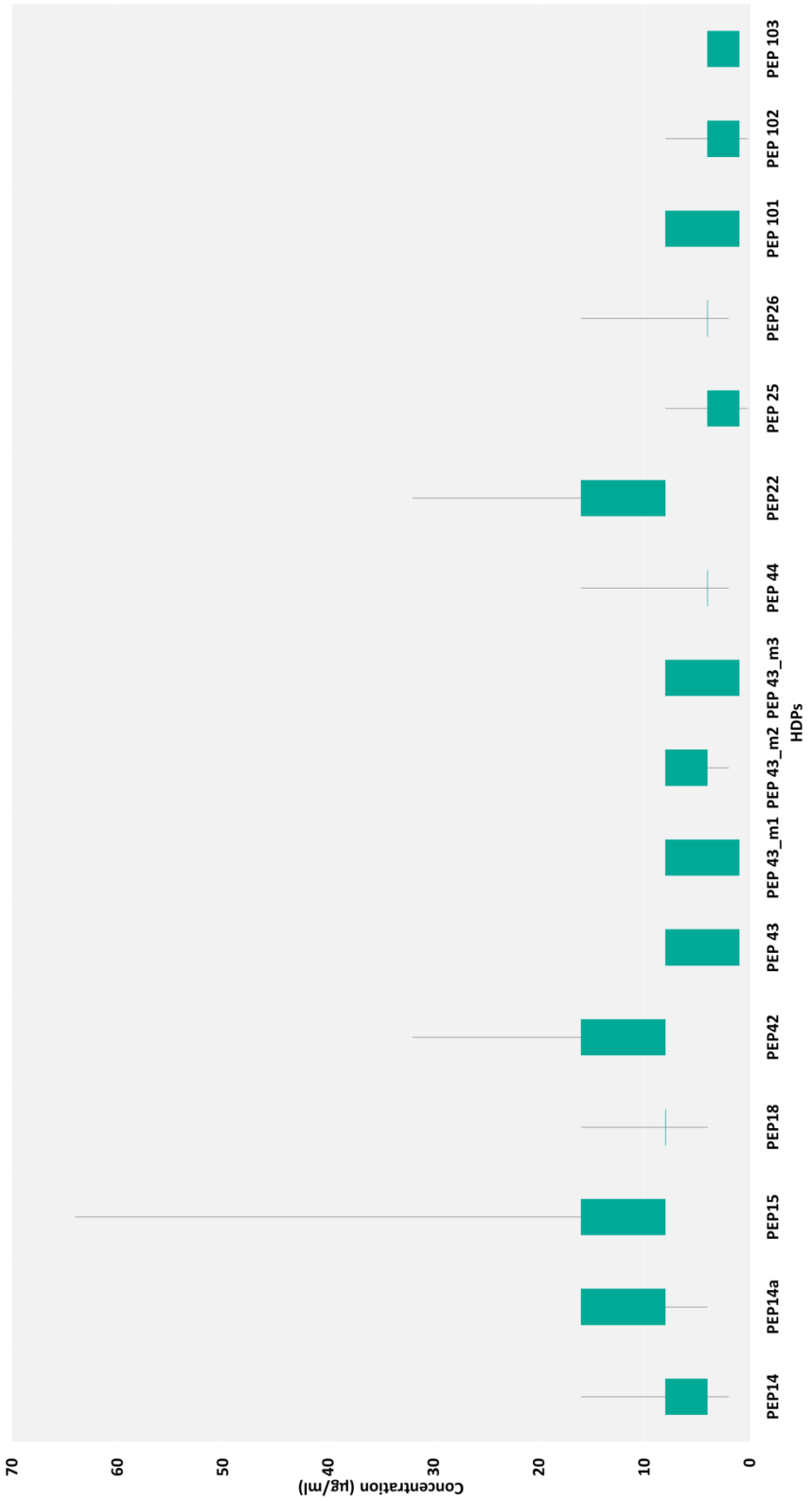


Figure 36 Dose–response curves of P. aeruginosa biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics and anti-biofilm peptide PEP 25 and PEP 102

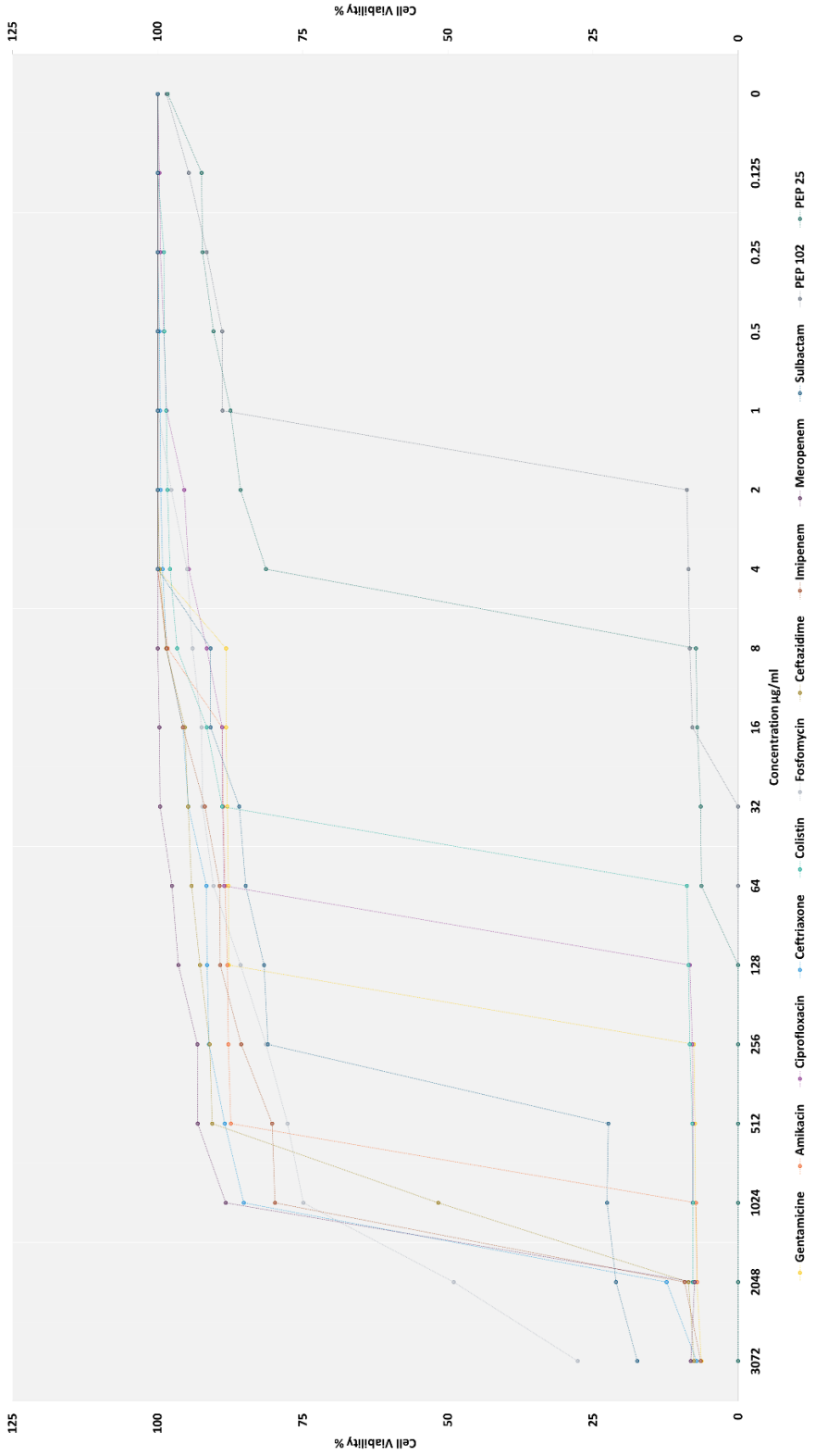


Figure 37 Dose–response curves of A. baumannii biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics and anti-biofilm peptide PEP 25 and PEP 102

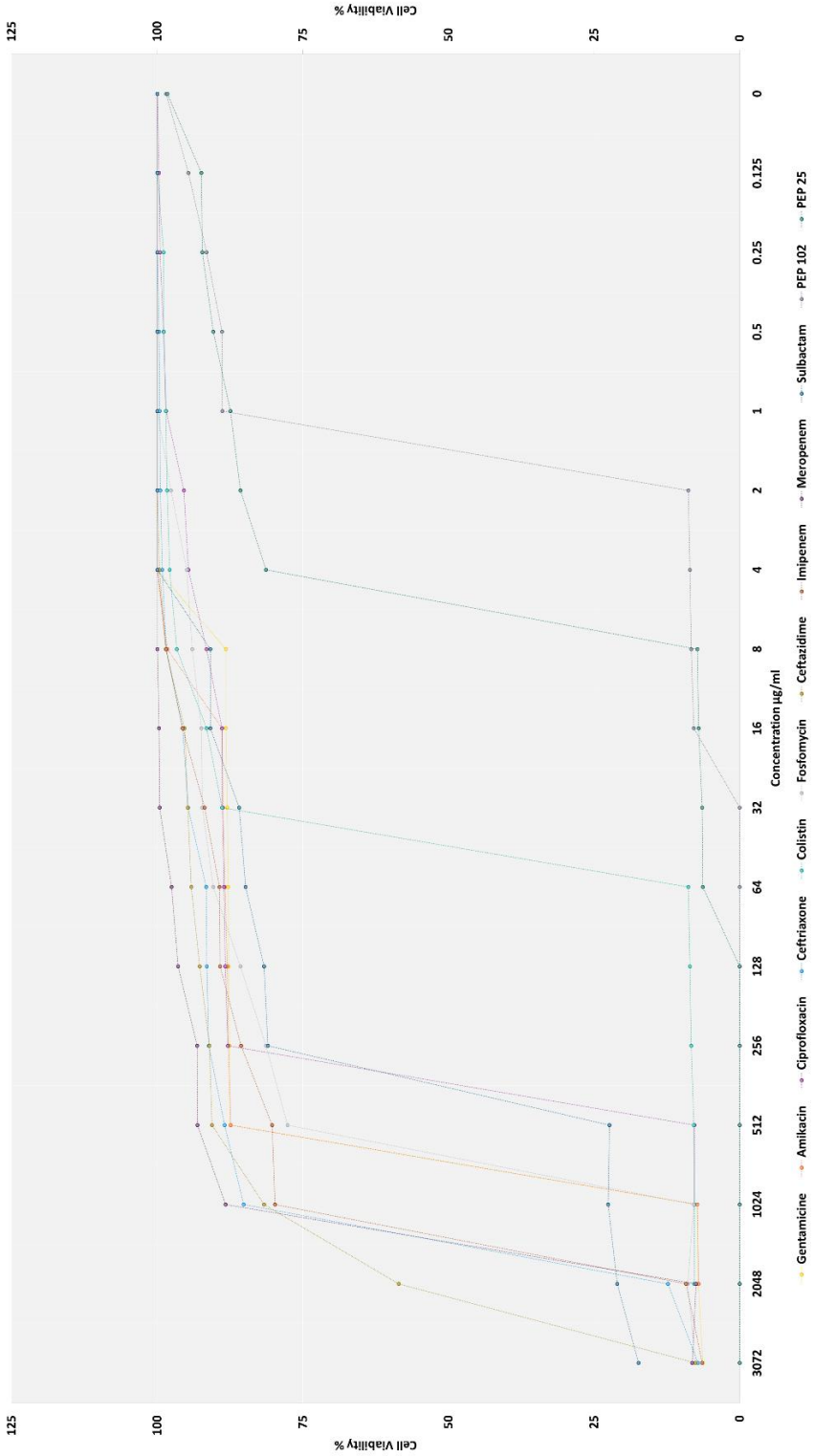


Figure 38 Dose–response curves of H. influenzae biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics and anti-biofilm peptide PEP 25 and PEP 102

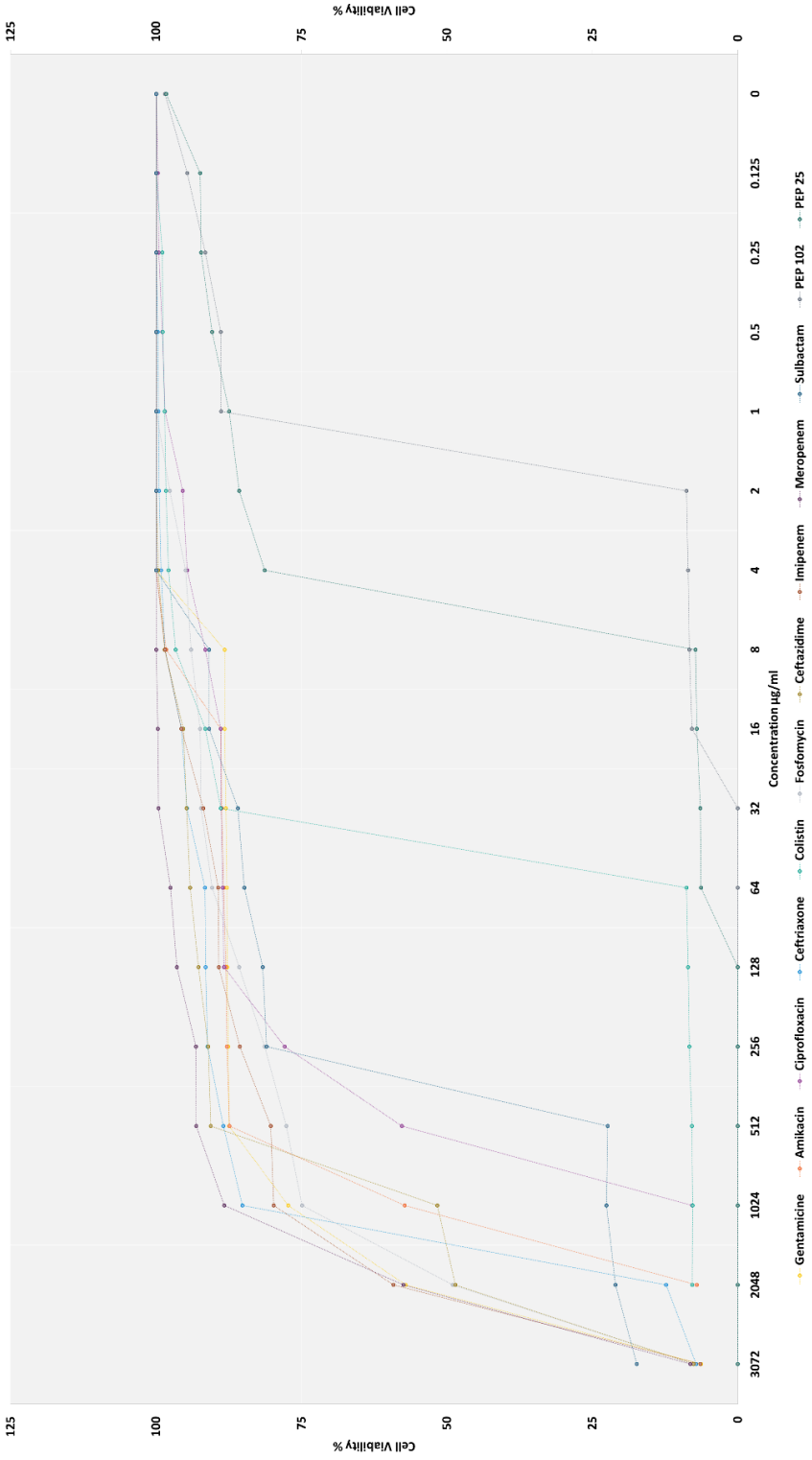


Figure 39 Correlation between the biofilm bio-volume and different concentration of anti-biofilm peptide PEP 102 and PEP 25 A) P. aeruginosa B) A. baumannii C) H. influenzae; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy

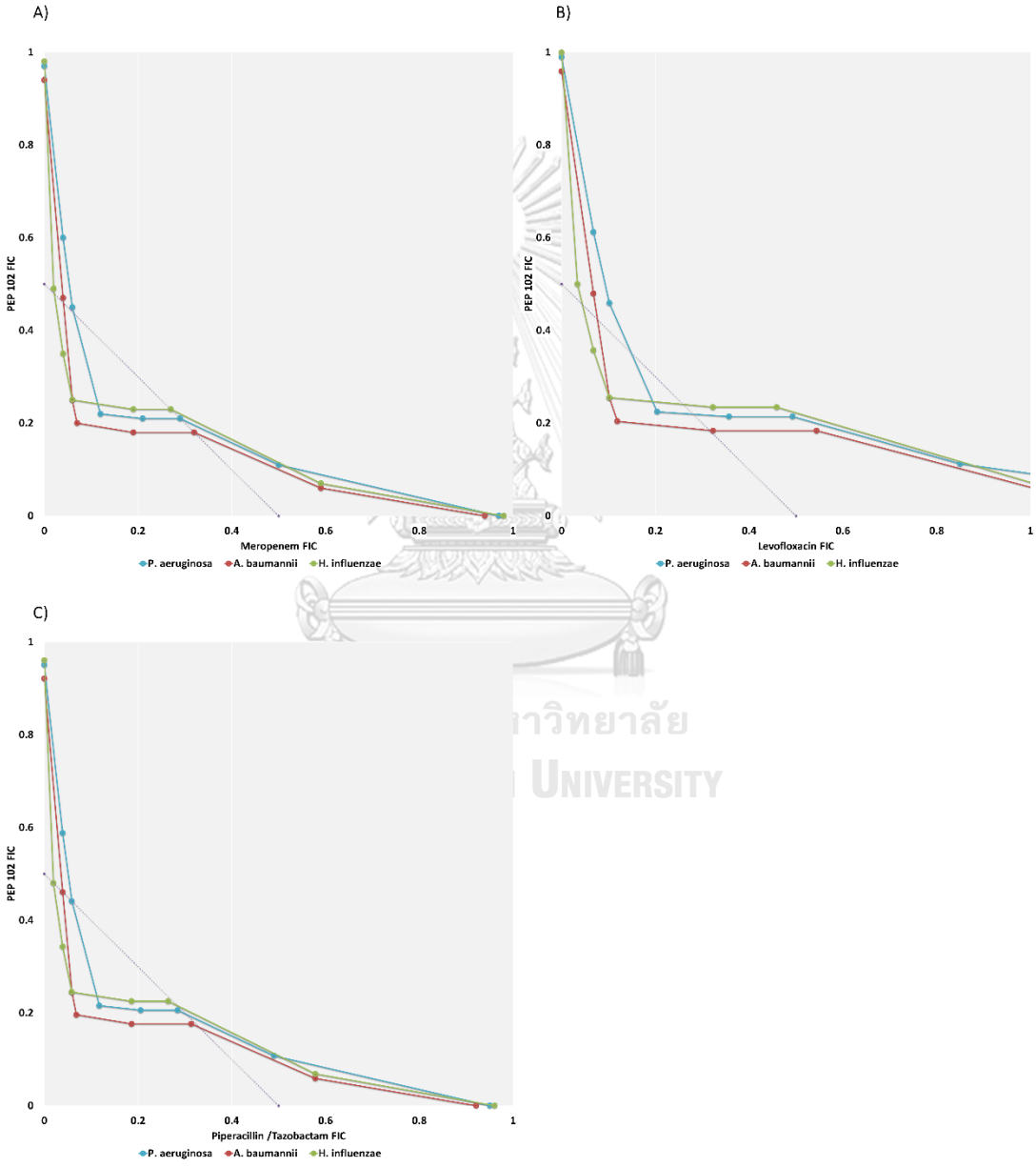
Table 8 Physiochemical Characters of the Anti-biofilm peptides based on manufactures quality control details.

Name	PEP14	PEP14a	PEP15	PEP18	PEP42	PEP 43	PEP 43_m1	PEP 43_m2	PEP 43_m3	PEP 44	PEP22	PEP 25	PEP26	PEP 101	PEP 102	PEP 103
Half-life [sec]	0.719	0.605	1.226	1.163	1.81	1.244	1.112	1.051	0.97	1.023	1.227	1.246	1.395	1.351	1.39	1.295
Stability	Normal	Normal	High	High	High	High	High	High	Normal	High	High	High	High	High	High	High
HPLC parameter	2.52	1.18	1.97	1.4	-0.72	0.5	1.21	-0.59	-0.95	-1.53	2.28	1.66	1.81	2.39	4.83	2.92
Hydrophobicity (KJ/mol)	25.02	18.7	24.94	18.96	10.12	16.57	23.57	17.75	12.35	12.98	14.93	17.36	15.89	16.31	43.66	36.08
pKa	21.18	21.37	21.22	21.23	22.45	22.26	21.72	22.46	22.05	22.6	23.11	21.91	21.92	21.94	21.73	22.02
pKb	91.8	92.36	91.78	93.7	94.17	93.61	91.15	91.89	92.38	92.56	92.63	92.46	92.37	92.44	90.76	91.38
Residue volume	1638.3	1625.6	1665.4	1564.7	1554.4	1567.1	1684.4	1736.5	1637.5	1702.3	1481.8	1591.2	1585.6	1532.5	1762.7	1721.2
Molecular weight	1334.7 8	1291.7 6	1348.8 1	1326.7 8	1171.7 1	1214.7 3	1377.8 5	1389.8 5	1305.7 9	1360.8 1	1143.5 9	1231.7	1203.6 9	1175.6 3	1467.9 2	1409.8 8
Isoelectric point	8.34	7.862	8.342	8.01	7.496	7.97	8.54	8.081	7.688	7.703	7.851	7.876	7.774	7.778	9.943	9.596
Surface Accessiblity	71.79	65.08	72.18	59.96	54.93	62.12	71.34	64.27	56.22	56.34	65.41	67.67	68.5	68.91	94.59	82.16
Flexibility	4.51	4.34	4.48	4.25	4.37	4.44	4.53	4.47	4.38	4.39	4.45	4.5	4.44	4.41	5.08	4.9
Charge	6	5	6	5	4	5	6	5	4	4	5	5	5	5	9	8
Polarity	309.84	258.29	310.16	262.24	203.48	255.33	310.9	259.72	209.68	210.35	250.05	254.46	251.96	251.83	464.03	411.26
Relative Mutability	632	607	598	591	518	487	667	622	716	640	536	586	577	603	651	650
Free Energy of Solution (in water,kcal/mole)	2.901	5.001	3.57	6.288	6.976	5.155	-1.858	0.907	0.364	1.308	1.252	1.707	2.737	1.968	-5.449	-5.378
Optical Rotation	3.033	0.683	1.37	-1.11	1.92	1.93	1.62	-0.76	0.713	-1.657	3.01	3.733	3.943	3.56	12.51	11.546
Entropy of Formation	2764.4 4	2655.6 3	2789.0 4	2665.8	2519.8 9	2627.6 9	2778.6 1	2705.1 7	2577.9 5	2612.3 1	2381.6 4	2550.8	2510.2 5	2456.9 8	3182.9 5	3062.1 8
Heat Capacity	428.28	449.94	435.96	411.04	461.17	442.54	417.95	424.18	419.83	407.43	467.09	459.4	490.13	479	373.54	353.12
Relative Stability	3.066	3.218	3.171	3.028	3.237	3.098	3.309	3.621	3.565	3.738	2.605	2.95	2.88	2.692	2.907	3.154
Water Solubility %	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Purity	97.34	96.14	99.01	98.65	97.8	99.01	99.57	97.45	98.9	98.05	99.03	97.36	98.59	99.13	98.45	99.1
Protease Stability	98.34	97.14	99.21	99.65	98.8	98.1	99.7	98.45	99.9	99.85	97.05	98.36	99.59	99.13	98.5	99.71

Table 9 Summary of the PEP 102 MIC - Minimum Inhibitory Concentration for planktonic bacteria. MBEC 100 – Minimum Biofilm Eradication Concentration based on 90% non-viable cells, MBIC - Minimum Biofilm Inhibitory Concentration.

Bacteria Strain	MIC	MBEC - 90	MBIC
P. aeruginosa	32 µg/ml - Multi drug resistant strains 4-16 µg/ml – Antibiotic susceptible strains	32-128 µg/ml – Strong biofilms 4-16 µg/ml – Moderate biofilms	2-16 µg/ml
A. baumannii	32 µg/ml - Multi drug resistant strains 8-16 µg/ml – Antibiotic susceptible strains	32-128 µg/ml – Strong biofilms 4-16 µg/ml – Moderate biofilms	2-16 µg/ml
H. influenzae	16-32 µg/ml - Multi drug resistant strains 4-8 µg/ml – Antibiotic susceptible strains	16-64 µg/ml – Strong biofilms 4-16 µg/ml – Moderate biofilms	2-4 µg/ml

Figure 40 Graphical summary of A) PEP102-meropenem B) PEP102-levofloxacin C) PEP102-piperacillin/tazobactam synergy by checkerboard methodology. Synergism is achieved with combinations of the two agents fully eradicating biofilm based on MBEC 75, where the sum of individual FIC values is <0.5 (diagonal dashed line).



Discussion

The increasing prevalence of antibiotic resistance bacterial infections in respiratory tract coupled with the fact that many chronic persistent infections are caused by bacterial biofilms emphasise the urgent need for the development of novel agents targeting biofilm. A number of short host defence peptides have recently been identified with broad-spectrum anti-biofilm activity, providing a foundation for a future therapeutic solution [119]. The present study utilized a simple computer based prediction algorithm approach to design a group of short host defence anti-biofilm peptides. The prediction algorithm was based on measured anti-biofilm activities of a diverse peptide library derived from a previously reported or anti-bacterial activity. Based on our findings this approach successfully predicted and correctly classified the investigated peptides as either biofilm active or inactive. These hypothetical peptide sequences were then used to screen *in silico* to future enhance the quality of selection and resulted in the development of a number of novel short defence anti-biofilm peptides. Overall, the results clearly exhibited enhanced activity against clinical isolates of *P. aeruginosa*, *A. baumannii* and *H. influenzae* biofilms *in vitro* and prove the usefulness of this approach to develop therapy for biofilm infections in respiratory tract.

It is important to note that the potential anti-biofilm peptide PEP 25 and PEP 102 identified in this study have all been optimized against diverse clinical isolates of *P. aeruginosa*, *A. baumannii* and *H. influenzae* biofilms than the currently used antibiotics. Even though the clinical relevance of

biofilms in respiratory tract is known, and these clinical isolates also displayed intrinsic antibiotic resistance with unable to resolved or eradicated the biofilm infections [11, 120, 121]. Therefore, developing short defence peptides with a broad-spectrum anti-biofilm potency would be desirable. This is one of the key significant hallmark of our study, because we were able to demonstrate the potency of peptide PEP 102 and 25 alone itself on biofilm with large number of clinically relevant isolates derived form patients with suspected chronic biofilm infections. Based on our knowledge, none of the previous studies used a large number of clinical isolate for screening of anti-biofilm peptides or other chemical molecules [62, 83, 89, 99, 122-125]. Importantly, peptide PEP 102 demonstrated a most potent broad spectrum anti-biofilm activity against a *P. aeruginosa*, *A. baumannii* and *H. influenzae* biofilms than other tested peptides [62, 83, 89, 99, 122-125]. Indeed, the PEP 102 exhibited a similar anti-biofilm effect as peptide 1018 against a *P. aeruginosa* and was able to disperse the biofilm structure [89, 126]. The peptide 1018 dispersing the biofilm via degradation of the nucleotide stress signal ppGpp, or block the quorum sensing (such as N-acyl homoserine lactone analogues and PQS analogues) [89, 126]. Therefore, we believed that PEP 102 may also trigger such a mechanisms to disperse the biofilm. However, in our results the peptide PEP 102 concentration differences between strong and moderate biofilm clearly showed that, the assumption many previous studies made that every clinical isolate produces a similar biofilm formation capacity is not true [62, 83, 89, 99, 122-125]. Therefore, testing the PEP 102 in clinical

isolates with strong and moderate biofilm showed its capability to overcome the different biofilm formations.

It is important to emphasize that our prediction strategy only classified peptides as “active” or “inactive” and it did not take into account anti-biofilm potency. Using our experimental approach, we were limited by the several physiochemical characters, such as solubility, half-life, and protease stability, and thus the highest peptide concentration that we evaluated in the microtiter assay was dictated by clinical isolates. Bio-volume results were highlighted the PEP 102 able to disperse the biofilm structure and dose response curves displayed theirs capability to penetrate deep in to the biofilm structure. We would expect that if we could assess the anti-biofilm activity with clinically relevant environment, such as presence of sputum and other host factors, PEP 102 will able to preserved similarly potency. We also anticipate that it should be possible to iteratively improve the accuracy of prediction for anti-biofilm peptides as more active sequences are reported in the literature based on clinical validation. Because for *A. baumannii* and *H. influenzae* there are limited number of active sequences are available and therefore its limit the prediction power. Also, the concentration differences between MIC values of multidrug resistance and susceptible strain may highlighted the intrinsic genetic resistance may limit the peptides damage to the bacteria cell membrane. However, such differences were reported in previous studies with low antibacterial activity than PEP 102 [62, 83, 89, 99, 122-125]. Moreover, the concentration differences between

P. aeruginosa, *A. baumannii* and *H. influenzae* biofilm may due to the presence of different biofilm structural components. Such differences only seen when tested with clinical isolates and therefore testing with clinical isolates validate the potency of peptides compare to the other studies [62, 83, 89, 99, 122-125].

Our result demonstrated that anti-biofilm peptides can synergize with conventional antibiotics. For example, when peptide was used in combination with various antimicrobial agents, it decreased the amount of drug required to treat biofilms formed by *P. aeruginosa*, *A. baumannii* and *H. influenzae*. The most common synergy was seen in the combination of 8 μg/ml PEP 102 with 1-2 μg/ml meropenem for *H. influenzae* and 16 μg/ml PEP 102 with 4.8 μg/ml meropenem or piperacillin/tazobactam for *P. aeruginosa*, and *A. baumannii*. Interestingly, meropenem and piperacillin/tazobactam are frequently used intravenously during treatments in patients with chronic respiratory infections [127]. In another study, peptide DJK-6 and 1018 demonstrated a potentiating effect on β-lactam antibiotics against multi-drug resistant carbapenemase-producing *Klebsiella pneumoniae, P. aeruginosa*, *Escherichia coli* and MRSA (among others) studies [62, 83, 89, 99, 122-125]. Importantly, main objective of our study is to develop antibiotic independent anti-biofilm peptide to treat biofilm infections and minimised the antibiotic resistance. It is likely that such potent effects would be conserved with our peptide PEP102, which should further decrease the amount of antibiotic required to treat biofilms formed by pathogenic bacteria.

Ultimately, the results of the current work clearly demonstrate the potential of using PEP 102 synthetic anti-biofilm peptides that bring a promise of combating life-threatening chronic bacterial infections in a variety of ways, including critical disruption of biofilms and direct killing of bacteria cells within the biofilm.

Summary of the strengths and limitations

The effectiveness of the biofilm targeted approach for HDPs was demonstrated here complete based on large number of clinical isolates selected form patients with chronic respiratory infections. Therefore, the concentration values of HDPs in this study may differ from other studies and reflect the true clinical scenario. However, the appropriate selection of *In vivo* biofilm infection model requires for further validation of PEP 102 efficacy.

Conclusions

HDPs may provide novel alternative therapeutics in the prevention of chronic *P. aeruginosa, A. baumannii* and *H. influenzae* biofilm infections in respiratory tract.

CHAPTER 7

Using Host Defense Peptides (HDP) as a novel therapy for *Pseudomonas aeruginosa* and nontypeable *Haemophilus influenzae* biofilm infections in the cystic fibrosis lung

Origin of the proposal: Aims and Objectives

Antimicrobial resistance is one of the most complex public health threat today **[**128**].** The illnesses like chronic bacteria respiratory infections are increasingly becoming challengeable to treat because of the emergence and spread of drug resistance bacteria **[**11**].** Such threat, are also becoming a key consideration for bacteria biofilm colonization on the respiratory tract in cystic fibrosis (CF) patients [11, 69]. The overall burden of biofilm infection is strongly linked to declining lung function, poorer outcomes –survival, exacerbations, prolonged ICU stay, slow recovery and prolonged rehabilitation in CF patients [11].The complexity of biofilm is a major challenge for prioritization of accurate treatment and they are up to 1,000 times more resistant to current antibiotics [69].

People with cystic fibrosis are susceptible to a range of bacterial infections and *Pseudomonas aeruginosa* and nontypeable *Haemophilus influenzae* are the most common pathogen isolated from cystic fibrosis patients [11, 69]. Caused by *P.aeruginosa* an obligate pathogenic bacterial species in the family Pseudomonadaceae and nontypeable *Haemophilus influenzae* family Pasteurellaceae, which both spread exclusively by airborne transmission[69]. An estimated

up to 60%–70% of adults with cystic fibrosis associated with chronic *P. aeruginosa* or nontypeable *H. influenzae* airway infections [69]. This type of infection is likely due to the inherent abnormalities of airway clearance due to CF and the bacteria ability to form biofilm communities largely resistant to antibiotics [11]. This situation has generated an urgent need for development of innovative, efficient and targeted treatments.

Over the last decade, host defence peptides (HDPs) or anti-microbial peptides (AMPs) have emerged as a promising alternative for treatment of various infections [84]. Those HDPs can used for the development of novel treatment for gram negative bacterial infections alone or together with other antibiotics without a significant resistance problem. Conventionally, amphipathic nature of such peptides are acts exclusively via membrane disruption and formation of stable pores on the anionic surfaces of bacterial cells [84, 122]. In addition, they also prevent the microorganism's adhesion or interaction with host tissues, selective modulation of innate immune responses, and active against bacterial biofilms [84, 122].

The tetraspanins are 33 groups of molecular facilitator proteins, which share similar structural motifs with four transmembrane domains (TM1 to -4), a small extracellular loop (EC1), and a large extracellular loop (EC2) [104]. Moreover, bacterial adhesion requires an indirect interaction with tetraspanins, through receptors embedded in TEM (Charrin, Jouannet et al. 2014, Halova and Draber 2016, Ventress, Partridge et al. 2016). The *Escherichia coli* adhesin, FimH, binding directly to

tetraspanin TSPAN21 has been shown to exploit tetraspanins in order to adhere to bladder cells during urinary tract infections. In addition, antibodies of tetraspanins CD9, CD63, and CD151 were reduced adherence of *Staphylococcus aureus, Neisseria lactamica, E. coli, and Streptococcus pneumoniae* to epithelial cells. Moreover, peptides derived from CD9, drastically inhibited the *Staphylococcus aureus* adhesion to keratinocytes (Ventress, Partridge et al. 2016).

Based on existing evidence that host defense peptides can kill bacteria cells and also targeting tetraspanin can inhibit the bacteria adhesion, we moved forward to use this both as a rational approach to target biofilm infection**.** Therefore, the present project in the Host-Pathogen Interactions (HPI) level that aims to identify, characterize, develop and evaluate novel prophylactic and therapeutic antimicrobial cationic peptides, based on the manipulation of biofilm virulence factors, and intervention on bacteria adaptation/anchoring. The present project focused on overcoming respiratory infection by two major pathogens in CF, *Pseudomonas aeruginosa,* and nontypeable *Haemophilus influenzae*.

The work produces synergies based on screening and evaluation of a number of different anti-biofilm peptide candidates (n=17) combined with peptides from tetraspanin to inhibit and disperse of bacterial biofilms in human airway epithelial cell cultures derived from the CF patients. An important aspect in this stage is to increase the stability and efficacy of the peptides as rational approach to target and breaking down the biofilms associated with airway epithelium. This will

offers the opportunity to study the effects of anti-biofilm peptides on biofilm infection and epithelial cell biology in the native pathological environment.

Specific objectives are:

1. *In vitro* Characterization of Biofilm production in *Pseudomonas aeruginosa* and nontypeable *Haemophilus influenzae* CF clinical isolates.
2. Correlation between the Biofilm formation and Antibiotic responses in the presence of exo DNA , Dnase I , EDTA and glutathione reductase of *Pseudomonas aeruginosa* and nontypeable *Haemophilus influenzae* CF clinical isolates.
3. *In vitro* characterization of number of different anti-biofilm new peptide candidates that inhibit and/or disperse *Pseudomonas aeruginosa,* and *Haemophilus influenzae* biofilms.
4. Preclinical validation of activity of novel anti-biofilm peptides on static co-culture biofilm model by using human airway epithelial cell cultures derived from the CF patients.

Materials and Methods

Bacteria strains

After approval of the study protocol by the Institutional Review Board of the Telethon Kids Institute, Division of Paediatric, School of Medicine, Faculty of Health and Medical Sciences, The University of Western Australia, *P. aeruginosa* isolates (n=30) and nontypeable *Haemophilus influenzae* (n=30) with various morphology and resistance profiles were obtained without preference from a strain repository at the Telethon Kids Institute, Division of Paediatric, School of Medicine, Faculty of Health and Medical Sciences, The University of Western Australia. *P. aeruginosa* clinical strains had been isolated during 2010–2015 from sputum samples of chronically infected cystic fibrosis paediatric patients as part of standard care. The nontypeable *H. influenzae* clinical strain had been isolated form paediatric patients as part of GROMIT study 2011 and was unrelated to the present study.

Culture Conditions

The *P. aeruginosa* PA01 biofilm-positive strain ATCC 15692 and clinical isolates were cultured on Mueller Hinton agar (MHA) plates at 37°C. The nontypeable *H. influenzae* biofilm-positive strain ATCC 49247 and clinical isolates were cultured on Chocolate agar plates in 5% CO2 for 18 h at 37°C. All isolates were stored at −80°C in tryptic soy broth with 15% glycerol until they were used in subsequent experiments in which they were suitably anonymised.

Antibiotics and agents

We tested fifteen antibiotics (Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin, ceftazidime, tobramycin, amoxicillin-calvulanate, clarithromycin, levofloxacin, aztreonam, erythromycin, tetracycline, sulfamethoxazole/trimethoprim and ampicilline) widely used for the treatment of *P. aeruginosa* and nontypeable *H.influenza* infections. Gentamicin, amikacin, ciprofloxacin, ceftriaxone, colistin, ceftazidime, tobramycin, amoxicillin-calvulanate, clarithromycin, levofloxacin, Aztreonam, erythromycin, tetracycline, sulfamethoxazole/trimethoprim and ampicilline were all from Sigma-Aldrich. Antibiotic stock solutions were prepared less than 24 h before use. Antibiotics were dissolved in cation-adjusted Müller-Hinton II broth (MHIIB) (Becton Dickinson) or brain heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with β-nicotinamide adenine dinucleotide (β-NAD; 15 μg/ml) and hemin (30 μg/ml) medium and sterilized by filtration through a membrane (0.22 μm pores). Serial dilutions of the stocks were prepared in MHIIB medium immediately before use.

Anti-biofilm Peptide design and synthesis

The HDP design based on the machine learning algorithm and uses Moon and Fleming scale [111] and the following physico-chemical characteristics of peptides: Hydrophobic moment, PI , PH, hydrophobicity, hydropathicity, Solubility, Aggregation, Stability, Half-life, Heat capacity, Toxic

motifs, Charge density and depth-dependent potential [112]. Peptide should consist of 20 canonical amino acids and peptide length does not exceed 10 -12 amino acids range. Whole amino acid composition (%) and selected residues composition (%) were used as input features for developing machine learning algorithm (SVM and WEKA). The amino acid composition is the fraction of each amino acid in a peptide and converts a peptide sequence into a vector of 8000 dimensions. All peptides were synthesized using solid phase Fmoc chemistry (Commercially synthesized) and purified to 95% using reverse-phase high-performance liquid chromatography. Correct peptide mass were confirmed by mass spectrometry. Peptide concentration was determined using amino acid analysis. Scrambled peptides were randomly generated from the respective sequences (negative control).

Tetraspanin peptides

The tetraspanin peptide from EC2 region of CD9 was obtain form Professor Peter N. Monk research lab at Department of Infection, Immunity & Cardiovascular Disease University of Sheffield, United Kingdom, which was previously been tested against number of gram negative bacteria including *P. aeruginosa [106]*. Scrambled peptide was randomly generated from the cognate CD9 sequence. All peptides were synthesized using solid phase Fmoc chemistry (Genscript, New Jersey, USA).

Primary human airway epithelial (hAE)

The study was approved by the Perth Children Hospital and Telethon Kids Institute, Division of Paediatric, School of Medicine, Faculty of Health and Medical Sciences, The University of Western Australia Human Ethics Committees and written consent was obtained from each participant's legal guardian after being fully informed about the nature and purpose of the study. All experiments were performed in accordance with the relevant ethical committees' guidelines and regulations. Here, bronchial brushings were obtained from 12 children with cystic fibrosis (CF) during their routine clinical surveillance bronchoscopy as previously described. Cystic fibrosis transmembrane conductance regulator (CFTR) genotype was determined as part of standard care and unrelated to present study. On average, total cell yields were ~1.4×10^6 per child with CF. Here, 5,000 cells per cm^2 were used to establish a primary paediatric airway epithelial cell (AEC) cultures culture as described before, with the remainder used for cytospins, RNA, protein and/or establishing a traditional primary cell culture.

Biofilm formation in 96-well microtitre plates

A single colony of *P. aeruginosa* and nontypeable *H. influenzae* were inoculated into 2 mL of MHIIB or brain heart infusion (BHI) broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with β-nicotinamide adenine dinucleotide (β-NAD; 15 μg/ml) and hemin (30 μg/ml) medium

supplemented with or without low molecular weight salmon sperm (Fluka) in a tube and incubated in an orbital shaker (200 rpm) at 37°C overnight for about 16 h. Subsequently, a subculture was prepared by diluting the overnight culture with fresh medium of CF BAL fluid, mixed with mucin and minimum medium hydrogel (Mixture detail and technical details not shown due to IP restrictions) to an OD of 0.02 at 600 nm (5 x 107 cfu/ml), and 100 µL aliquots added to flat-bottomed 96-well polypropylene microtitre plates (SPL Life Sciences), with uninoculated medium (100 µL) as a negative control, the plates were incubated at 37°C for 24 h. We used this procedure for subsequent biofilm experiments, which were performed in triplicate and repeated three times.

Testing susceptibility to antibiotics

The MIC were established using standard techniques according to criteria in the EUCAST [39]and CLSI guidelines [40]. *E. coli* ATCC 25922, nontypeable *H. influenzae* ATCC 49247 and *P. aeruginosa* ATCC 27853 were used as quality control strains, with modifications as follows. To establish planktonic MIC for the antibiotics used, the antibiotics were serially diluted two-fold in 96-well microtitre plates and bacteria added. The plates were incubated at 37°C for 18 h. MBEC were established by adding the serially diluted antibiotics to mature biofilms and incubating at 37°C for 24 h before staining with PrestoBlue to calculate the viable cells within the biofilm. Before adding

the antibiotics, any non-adherent cells were removed from the mature biofilms by three gentle washes with MHIIB medium.

Biofilm formation quantification and classification

Two methods were used to quantify [42] and classify [43] the biofilm structure by Crystal Violet staining with modifications . The Crystal Violet (0.1%) stained biofilms were solubilized with 30% acetic acid followed by incubation at room temperature for 10–15 min. OD at 550 nm was determined using a microtitre plate reading spectrophotometer with 30% acetic acid as a blank control. Mean absorbances and their SDs were calculated for all tested strains and negative controls, determined in triplicate and repeated three times. The cut-off value (ODβ) was defined as 3SD above the mean OD of the negative controls: ODβ = average OD of the negative controls + 3SD of negative controls, and was calculated separately for each microtitre plate. The OD of a tested strain was expressed as the mean OD of the strain minus the ODβ (OD = mean OD of a strain – ODβ). The clinical isolates were classified as described previously [43].

Treatment of EDTA, DNaseI and reduced glutathione (GSH)

In experiments where EDTA, GSH and DNaseI treatment was required, 5mM reduced glutathione (GSH) (Sigma), 25 mmol/L EDTA (Sigma), 40 Kunitz units DNaseI (Sigma) were dissolve in PBS as

described previously. Biofilms were then treated with EDTA, GSH, and DNaseI in combination with antibiotics for 18 h to determine the MBEC as described previously.

Alginate measuring assay

To measure the amount of alginate produced, *P. aeruginosa* clinical isolates used in this study were grown in 5 ml of LB broth with orbital shaking at 200 rpm at 37°C until the culture reached an OD600 of 2.0 as described previously [129]. The bacterial cells were then collected by centrifugation at 7000 × g for 20 min and suspended in 1 ml of PBS buffer. Simultaneously, another culture was used to correlate OD600 2.0 with the dry cell weight. To remove any contaminants such as RNA and DNA from the alginate, the samples were treated with RNAse A (Promega) and DNAse I (Sigma). The samples were then incubated at 37°C for 1 h. To remove the cells, the mixture was vortexed and centrifuged at 8000 × g for 20 min. The alginate remaining in the supernatant was precipitated with 25 ml of 95% ethanol. The alginate precipitates were collected by centrifugation at 10000 × g for 30 min and suspended in 2 ml of 0.85% NaCl. The uronic acid concentration was determined by a standard colorimetric assay. All experiments were performed in triplicate and repeated three times.

Pyocyanin level Quantitative Analysis

The amount of pyocyanin produced, *P. aeruginosa* clinical isolates biofilm were quantitatively measured by method described previously [114]. Briefly, the isolates were grown in 2ml of LB broth culture at 37°C with shaking (200 rpm) for 16 h. Then, supernatant was collected by centrifugation at 7000 × g for 20 min for pyocyanin quantification. In brief, 600 µl of chloroform was added to 1 ml of supernatant, and the tube was vortexed twice for 10 s. The tubes were centrifuged at 10,000 rpm for 10 min, and the bottom phase (600 µl) was transferred to a new tube containing 300 µl of 0.2 N HCl. The tubes were vortexed twice for 10 s each time and centrifuged at 10,000 rpm for 2 min. The OD_{520} of the top phase was measured and multiplied by 17.072 to calculate the micrograms of pyocyanin per milliliter. All strains were tested four times. All experiments were performed in triplicate and repeated three times.

Measurements of extracellular DNA (eDNA) in biofilms

The eDNA concentration in nontypeable *H. influenzae* and *P. aeruginosa* biofilms were determined by PicoGreen fluorescent staining (Quant-iT Invitroge) in a 96-well-microtitre-plate biofilm formation method as described previously with some modifications [115]. A freshly prepared solution PicoGreen dye diluted in TE buffer (1:200) was added to the each well in the ratio 1 : 1 and eDNA concentration was measured on a fluorospectrometer (Varioskan Flash Multimode Reader; Thermo

Fisher Scientific), using 470 nm excitation and 525 nm detection. To verify reproducibility eDNA production in the biofilm cultures were also quantified by laser scanning fluorescence microscopy (Zeiss Axiovert 200M) after staining with propidium iodide (BacLight Live/dead staining kit) during all experiments.

MIC, MBEC50 and MBEC75 Assays

The broth microdilution method with minor modifications for cationic peptides was used for measuring the MIC of all anti-biofilm peptides used as described previously [116]. MBECs leading to 50% and 75% decrease in biofilm (MBEC 50 and 75) was obtained using 96-well plate assays and presto blue staining and crystal violet staining was used to determine whether the peptides could disperse preformed biofilms.

Inhibition of bacterial initial attachment

A bacteria attachment assay against biofilm-producing bacterial strains was performed using a previously described method with some modifications [117]. An overnight culture of bacteria was washed and re-suspend in Mueller Hinton broth (MHB) to an OD 600 = 0.05. Aliquots of 100 μl suspended bacteria were added to a 96-well plate containing 100 μ l MHB with peptides. The plate will be incubated at 37 °C for one hour without agitation to allow bacterial binding. Planktonic cells were then carefully removed by pipetting and the plate was washed thrice with 200 μ l PBS

solution. The result was reported relative to untreated bacteria binding analysis by confocal laser scanning microscope.

Inhibition of biofilm formation

Biofilm formation assays was performed as described for the inhibition of bacterial initial attachment with some alternations. Plates containing peptides at serial dilutions and bacteria was incubated for 24hours at 37 °C without agitation to allow biofilm formation. Wells without peptide were set up as positive controls. Presto blue viability reagent was added into each well of the microtiter plate follow by Incubate ≥30 minutes at 37° C and the fluorescence was read by using in a plate reader (excitation 560nm and emission 590nm).The results was interpreted by the comparison of peptides on treated biofilms to untreated biofilms through viability and confocal laser scanning microscope and 3D reconstructions was generated using the Imaris software package. An experiment was performed in triplicate, and three independent experiments were performed for each of these assays.

Static Co-culture Biofilm Model

CF primary human airway epithelial (hAE) cells were grown in vitro on porous supports at an air-liquid interface (ALI) with desired culture medium as described previously [130]. The gFP tag *Pseudomonas aeruginosa* and nontypeable *Haemophilus influenzae* was inoculated into 2 ml of

MHIIB or BHI broth supplemented with β-nicotinamide adenine dinucleotide (β-NAD; 15 μg/ml) and hemin (30 μg/ml) medium in a tube and incubated in an orbital shaker (200 r.p.m.) at 37 °C overnight (~18h) was typically reach a density of $5x10^9$ CFU/ mL. For bacterial inoculation, were inoculated with bacteria at a multiplicity of infection of approximately 1000:1 for *Pseudomonas aeruginosa* and MOI 25 for nontypeable *Haemophilus influenzae* relative to the number of primary cells originally seeded. Then, was incubated at 37°C with5% CO_2-95% air up to approximately 1 hours in orbital shaker (10 r.p.m.) and was analyzed the integrity of the cells using phase contrast microscopy. After 1 hours remove the all inoculation and carefully wash the epical surface with PBS one time to remove the free floating bacteria. After 24 Inoculation with bacterial strains that constitutively express GFP and CFP results in fluorescent biofilm micro colonies which can be visualized by confocal microscopy. After 24 hours', immerse and aerosol the double distil water containing different concentration ratios of anti-biofilm peptides for co-incubation at 37°C with 5% CO_2 for the 8hrs. After 8 hour of peptide or control treatment on CF airway epithelial cells was then fixed, and bacteria visualized by GFP and the epithelial cell nuclei by DAPI staining, and anti-biofilm effect was analyzed by confocal laser scanning microscope. Only inhibitory peptides were immerse and aerosol for 1hrs in to the airway epithelial cells prior to the bacterial infection. Infection cell model without peptide were set up as positive controls.

Bacteria adhesion inhibition in CF monolayer cells

Inhibition of bacteria adhesion by peptides were assayed by inoculation of cells with *P. aeruginosa* and nontypeable *H. influenzae* as described previously [106]. This assay differed from the biofilm disruption assay described above in that the peptides were added 1 hour prior to the inoculation and at the time of inoculation of the monolayer of airway cells by the bacteria. The assay was incubated for 30, 60, 180 and 300 minutes at 37 °C and 5% CO2 before adhesion were assessed as described previously [106].

Transepithelial electrical resistance measurements

Transepithelial electrical resistance was measured for pre infection and post treatment on air–liquid interface differentiated CF primary human airway epithelial (hAE) cells treated with different peptides using a Ag/AgCl electrode (EVOM meter).

Cell Viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to assess cell viability. CF primary human airway epithelial (hAE) cells were grown in a 96 well plate for 24 hours, then treated for a further 24 hours with different concentrations of peptides in media. Peptide was then removed and the tetrazolian MTT dye (Sigma-Aldrich, Poole, UK) added at a concentration of

0.5mg/ml in cell media for 1 hour. Cells were then lysed with 2-butoxyethanol (Sigma-Aldrich, Poole, UK) and the absorbance of supernatants measured at 562nm.

Expression of Tetraspanins receptors on CF primary human airway epithelial (hAE) cells

Gene expression was analysed by two-step RT-PCR reactions. cDNA was synthesised using hexanucleotide primers and Multiscribe™ Reverse Transcriptase (Applied Biosystems, CA, USA) in a final reaction volume of 20μL containing 1 X RT buffer, 5.5mM $MgCl_2$, 0.5mM of each of the dNTPs, 2.5μM random hexamers, 0.4U/μL RNase inhibitor, 0.5U/μL Multiscribe reverse transcriptase and 200ng RNA. The reactions are performed under the following conditions: initial primer incubation step at 25°C for 10 minutes followed by RT incubation at 48°C for 1h and ended by reverse transcriptase inactivation at 95°C for 5 minutes. The cDNA was then used in a final PCR reaction volume of 20μL containing 1 X TaqMan PCR master mix (Applied Biosystem) and specific TaqMan Gene Expression Assay (Table 2). The conditions for the PCR include initial incubation at 50°C for 2 minutes, AmpliTaq Gold DNA polymerase activation at 95°C for 10 minutes followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Gene expression was analysed using $\Delta\Delta^{CT}$ method and results presented as ratio to housekeeping gene cyclophilin A (PPIA).

Table 10 List of TaqMan Gene Expression Assay ID for a specific gene

Gene	TaqMan Gene Expression Assays
CD81	Hs201002167_m1
CD9	Hs01124022_m1
CD151	Hs00388381_m1
CD63	Hs01041238_g1
PPIA	Hs99999904_m1

Statistical analyses

Continuous variables are summarized using means and SDs, and categorical variables as counts and percentages. Levels of *P. aeruginosa* and nontypeable *H. influenzae* drug susceptibility are represented in two ways: a continuous measure of concentration; and an ordinal categorical form representing biofilm formation; both of these outcomes were measured repeatedly over time for each isolate. Linear mixed modelling was used to compare concentrations between drug types over time. We then examined which drug types were more successful in allowing concentration to be used to distinguish between biofilm formations using ordinal logistic mixed effects regression. All analysis was conducted using the R statistical package [41], linear mixed modelling was

performed using the R library, lme4 [44], and ordinal logistic mixed effect modelling using the R library, ordinal[45]. $P < 0.05$ was considered significant for all inferential analysis.

Results

P. aeruginosa and nontypeable *H. influenzae* CF isolates formed strong biofilms

To explore the biofilm development of CF clinical isolates of *P. aeruginosa* and nontypeable *H. influenzae*, under static conditions were examined using 96-well microtiter tray assay and confocal laser scanning microscopy. Biofilms were stained with LIVE/DEAD viability kit and visualized using confocal laser scanning microscopy (Figure 41). Results reveled that *P. aeruginosa* CF non-mucoid isolates tend to form dense macro colonies with higher biomass while mucoid ones form comparatively low biomass biofilm scattered micro colonies. For *Nontypeable H. influenzae* isolates form middle ear effusion displayed scattered smaller mature biofilm colonies while nasopharyngeal consist of thick mass of biofilm colonies (Figure 41 and 42).

CF isolates of *P. aeruginosa* and nontypeable *H. influenzae* formed diverse biofilm matrix components

The level of alginate production is significantly higher ($p < 0.001$) in mucoid isolates compare to non-mucoid ones (Figure 41). Also, confocal Images clearly showed that non-mucoid macro colony have more dead cells (red, orange or yellow) compare to mucoid ones (Figure 42).

The observations were shown that level of biofilm matrix component pyocyanin and extracellular DNA level were significantly higher ($p < 0.001$) in *P. aeruginosa* CF isolates compare to *P. aeruginosa*

PAO1 (Figure 43). However, by removing these molecules through addition of glutathione reductase (GSH) or DNase, the formation of *P. aeruginosa* biofilms were dispersed or dismantle (Figure 43).

For nontypeable *H. influenzae* isolates, with the addition of 150U DNase I, the biomass was decrease by threefold ($p < 0.001$) as compared to the control biofilms formed in the absence of added DNase I (Figure 43), confirming extracellular DNA is the major component of the nontypeable *H. influenzae* biofilm structure.

Biofilms formed on plastic could not be eradicated with high concentrations of antibiotics

In this study, eight antibiotics (Gentamicin, amikacin, ciprofloxacin, colistin, ceftazidime, tobramycin, levofloxacin, and aztreonam) for *P. aeruginosa* and eight (Ciprofloxacin, ceftriaxone, ceftazidime, levofloxacin, aztreonam, erythromycin, sulfamethoxazole/trimethoprim and ampicilline) for nontypeable *H. influenzae* CF clinical isolates were investigated for their effects on static biofilm by measuring biofilm biomass in 96-well microtiter tray assays (MBEC and MBIC). To determine the influence of antibiotics on the viability of planktonic cells (MIC and MBC), were also determined as described previously. For both *P. aeruginosa* (Figure 44) and nontypeable *H. influenzae* (Figure 45) CF clinical isolates the results were clearly indicate that therapeutically

impossible higher (significantly higher ($p < 0.001$)) concentration of antibiotics were required to eradicate the mature biofilms in comparison to current MIC levels.

The DNase I, GSH and EDTA together or alone enhances the efficacy of antibiotics in biofilm of *P. aeruginosa* and nontypeable *H. influenzae* CF isolates

These assays showed that addition of DNase I, GSH and EDTA together with antibiotics enhances the efficacy to disperse the mature biofilm structure of *P. aeruginosa* (Figure 46) and nontypeable *H. influenzae* (Figure 47). In contrast, the addition of exo DNA resulted in significantly ($p < 0.001$) enhanced of biofilm formation, whereas decrease the efficacy of antibiotic's on mature biofilm of *P. aeruginosa* (Figure 6) and nontypeable *H. influenzae* (Figure 47). This suggests that these molecules were likely to be dismantle the biofilm matrix components, such as extracellular DNA in both bacterial strains, pyocyanin in *P. aeruginosa* and divalent cations in *Nontypeable H. influenzae* biofilms.

The effects of anti-biofilm peptides are dose-dependent in biofilm of *P. aeruginosa* and nontypeable *H. influenzae* CF isolates

A dose response experiment was carried out to ascertain the anti-biofilm effects of peptides on *P. aeruginosa* (Figure 48) and nontypeable *H. influenzae* (Figure 51 and 54) *mature* biofilm on plastics. Results indicate that peptides alone were able to dismantle the mature biofilm with concentration

range from 16-128 μg/ml, meanwhile conventional antibiotics were unable to do that without supplemented with DNase I/GSH or EDTA for *P. aeruginosa* (Figure 46) and nontypeable *H. influenzae* (Figure 47). Future, the results indicate that the peptides PEP 25 and PEP 102 retain significant ($p < 0.001$) anti-biofilm function compare to the other peptides on *P. aeruginosa* at concentrations as low as 16 μg/ml, with range between 16 and 128 for both macro and micro mature biofilms (Figure 49). Similar significant ($p < 0.001$) results were observed with nontypeable *H. influenzae* mature biofilm, between 16-64 μg/ml (Figure 12 and 15) for PEP 25 and PEP 102. When comparison to the current MIC levels of tobramycin (*P. aeruginosa)* and ciprofloxacin (nontypeable *H. influenzae)*, PEP 25 and 102 able to achieved significantly effect on ($p < 0.001$) on biofilm eradication, with profound effect from PEP 102 (Figure 50, 53 and 56).

PEP 102 displayed drastic reduction of *P. aeruginosa* and nontypeable *H. influenzae* CF isolates biofilm

The biofilm bio-volume and confocal data of ***P. aeruginosa*** and nontypeable *H. influenzae* CF clinical isolates show that the PEP 102 peptide is fully effective at reducing the bio-volume significantly ($p < 0.001$) up to <5% at 16-128 μg/ml compare with non-treated ones, making promising candidate for future experiments (Figure 57 and 58). Also, results showed that PEP 102 can penetrate deeper in to the biofilm structure and future effecting small micro colonize of ***P. aeruginosa*** and nontypeable *H. influenzae* CF clinical isolates (Figure 57 and 58).

Treatment with anti-biofilm peptides decreases the mature biofilm volume and bacterial burden in a static co-culture CF biofilm model

After 24 hours *P. aeruginosa* and nontypeable *H. influenzae*, were localized to the surface of the epithelium, and formed the mature biofilm colonies (Figure 62 and 63), were subsequently treated with submerged treatment of PEP102 (124μg/ml) peptide up to 6hours. When submerged PEP 102 was significantly ($p < 0.001$) reduced or disrupted the 91.37% of biofilm bio-volume compare to the untreated control (Figure 59 and 60). However, when aerosol the PEP102 (124μg/ml) significant ($p < 0.001$) reduction was up to 88.74% for both bacteria strains (Figure 59 and 60). Interestingly, when PEP 102 combine with inhibitory peptide either submerged or aerosol (Figure 59 and 60) reduction was increased up to 95.67%. However, when inhibitory peptide alone (200μg/ml) (Submerged or aerosol), was unable to displayed any effect on mature biofilm structure of *P. aeruginosa* and nontypeable *H. influenzae* (Figure 59 and 60). Interestingly, when *P. aeruginosa* mature biofilm treated with tobramycin MIC value either submerged or aerosol there were drastic increase of the bio-volume compare to the untreated control (Figure 59) and unable to displayed any effect on mature biofilm. Similarly, for nontypeable *H. influenzae* (Figure 60) ciprofloxacin MIC concentration was unable to reduce the mature biofilm on CF epithelium.

Anti-biofilm peptide PEP 102 can help to retain CF epithelium integrity after dispersal of *P. aeruginosa* and nontypeable *H. influenzae* biofilm

Trans epithelial electrical resistance results of air way primary epithelium after post infection with nontypeable *H. influenzae* and *P. aeruginosa* CF isolates biofilms were significantly ($p < 0.001$) higher after treatment with PEP 102 (124µg/ml) alone or together with Inhibitory peptide –I compare to the non-treated air way primary epithelium (Figure 61). Also, PEP 102 treated air way primary epithelium able to restore the epithelial integrity 51.34% compare to the pre infections (Figure 61).

Tetraspanin peptides (inhibitory peptides) and anti-biofilm peptides can reduce the adherence of *P. aeruginosa* and nontypeable *H. influenzae* to CF airway epithelium and prevent the biofilm formation

In this study we tested the efficacy of anti-biofilm peptide PEP 102 and inhibitory peptide, to prevent, on *P. aeruginosa* and nontypeable *H. influenzae* biofilm formation via adherence to the CF airway epithelium. However, multiplicity of infection of approximately 1000:1 (0.5×10^7 CFU/ml) for *Pseudomonas aeruginosa* and MOI 25 (0.8×10^7 CFU/ml) for nontypeable *H. influenzae.* When pretreated with PEP 102 and inhibitory peptide together either submerged or aerosol, inhibition of biofilm formation or adherence to CF airway epithelium was significant ($p < 0.001$) than the non-treated ones with 91.54 % for *Pseudomonas aeruginosa* and 95.34% for nontypeable *H. influenzae* (Figure 64 and 65). However, PEP 102 and inhibitory peptide show significant ($p < 0.001$) inhibitory effect for nontypeable *H. influenzae* compare to the *Pseudomonas aeruginosa* (Figure 64 and 65).

Tetraspanin peptides (inhibitory peptides) PEP 102 can reduce the adherence of *P. aeruginosa* and nontypeable *H. influenzae* to CF airway epithelial cells in a competing manner

In this study we tested the efficacy of anti-biofilm peptide PEP 102 and inhibitory peptide, biofilm on *P. aeruginosa* and nontypeable *H. influenzae* to CF airway epithelial cell monolayer (Figure 66 and 67). When CF cells were infected with higher CFU (1.6×10^7) of non-mucoid or mucoid of *P. aeruginosa* and nasopharyngeal or middle ear effusion nontypeable *H. influenzae* CF clinical isolates, significant ($p < 0.001$) reductions in bacterial adherence were observed with combination of PEP102 and inhibitory tetraspanin-derived peptides in both pre-incubation inhibition adhesion assay and competing inhibition adhesion assay (Figure 66 and Figure 67). CF cells pre-treated with peptide PEP102 had a maximum reduction of adherence of *P. aeruginosa* mucoid strains of 55% compared to non-mucoid ones, and a maximum reduction of 70% was seen with PEP102 peptide and Inhibitory peptide combination either pre-incubation inhibition or competing inhibition adhesion (Figure 66). For nontypeable *H. influenzae* such differences wasn't observed between nasopharyngeal and middle ear effusion (Figure 67). Interestingly, the adherence of both bacterial strains to CF cells was significantly ($p < 0.001$) inhibited consistent manner throughout the time by either peptides alone or combination (Figure 26) for non-mucoid or mucoid of *P. aeruginosa* and

for nasopharyngeal or middle ear effusion nontypeable *H. influenzae* descended manner through time CF clinical isolates (Figure 67).

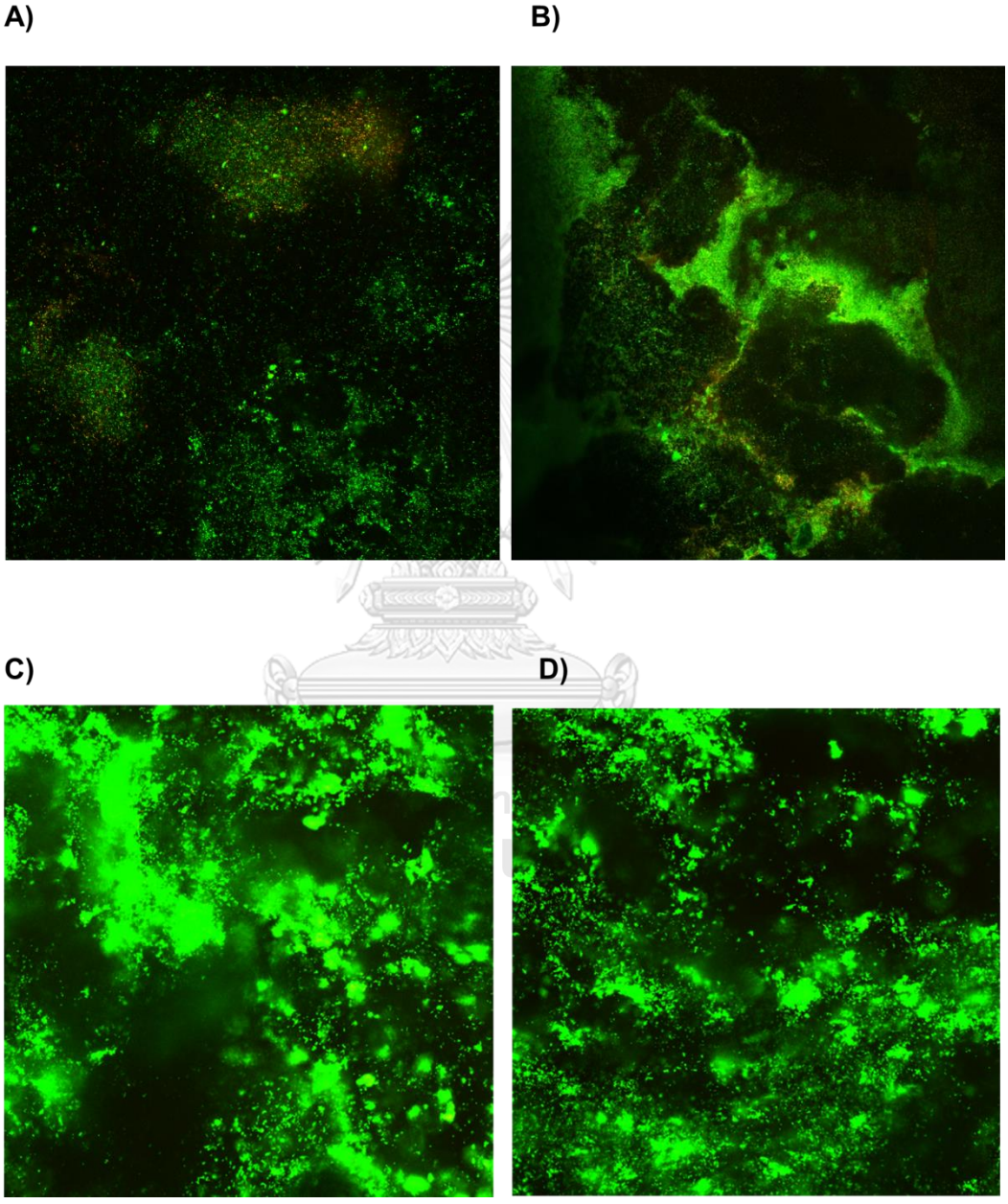
Anti-biofilm peptides do not affect CF airway epithelial cells viability

The peptides were also tested for any negative effects on CF airway epithelial cell viability and function. None of the peptides had any effect on CF airway epithelial cell metabolism when compared to their scrambled controls up to 256 μg/ml or when compared to a media alone using an MTT cell viability assay (Supplementary Figure 68). However, there was a small, non-significant decrease in the number of cells was observed when treated with 256 μg/ml PEP102 peptide, which need to be future characterized (Supplementary Figure 68).

Expression of common tetraspanins on CF airway epithelial cells

The expression of four common tetraspanins, CD9, CD63, CD81 and CD151, were assessed by RT-qPCR (Fig 23) to ensure genetic depletion of Cystic fibrosis trans regulator gene may not affect the common tetraspanins. The results revealed that CD9 and CD63 are highly expressed on CF tracheal and nasal cells than healthy ones (Supplementary Figure 69). Conversely, CD81 and CD151 are low expressed on both type of CF cells. The expression patterns confirmed the presence of tetraspanins CF airway epithelial cells and therefore those cells can be used to demonstrate inhibition of bacteria attachments via tetraspanin targeted inhibitory peptide.

Figure 41 Characterization of the biofilm bio-volume of P. aeruginosal *CF isolates; A) mucoid B) non-mucod with F) bio-volume and nontypable* H. influenzae *CF isolates; C) middle ear effusion D) nasopharyngeal with G) bio-volume ; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy. F) Characterization of* P. aeruginosa *biofilm matrix components based on alginate production; ATCC biofilm negative control and PAO1 biofilm positive control.*



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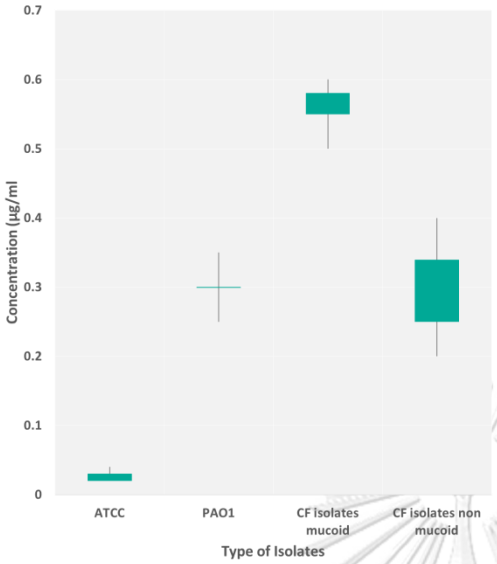
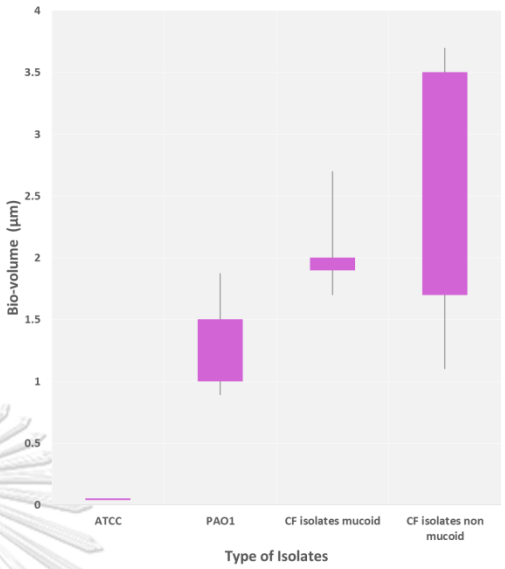


F)



G)

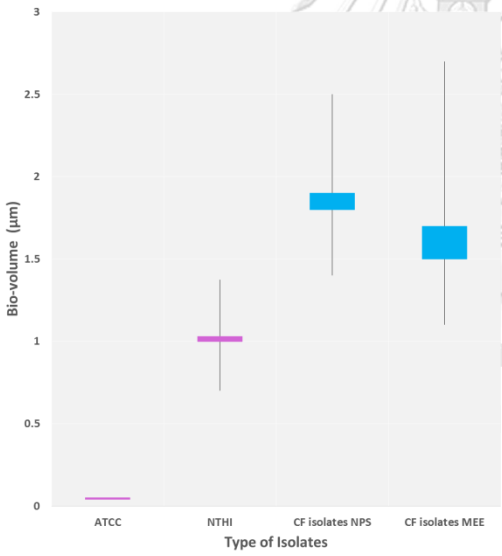


Figure 42 3D structure of the biofilm bio-volume of P. aeruginosal *CF isolates; A) mucoid B) non-mucoid and bio-volume of nontypeable* H. influenzae *CF isolates C) nasopharyngeal D) middle ear effusion ; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy.*

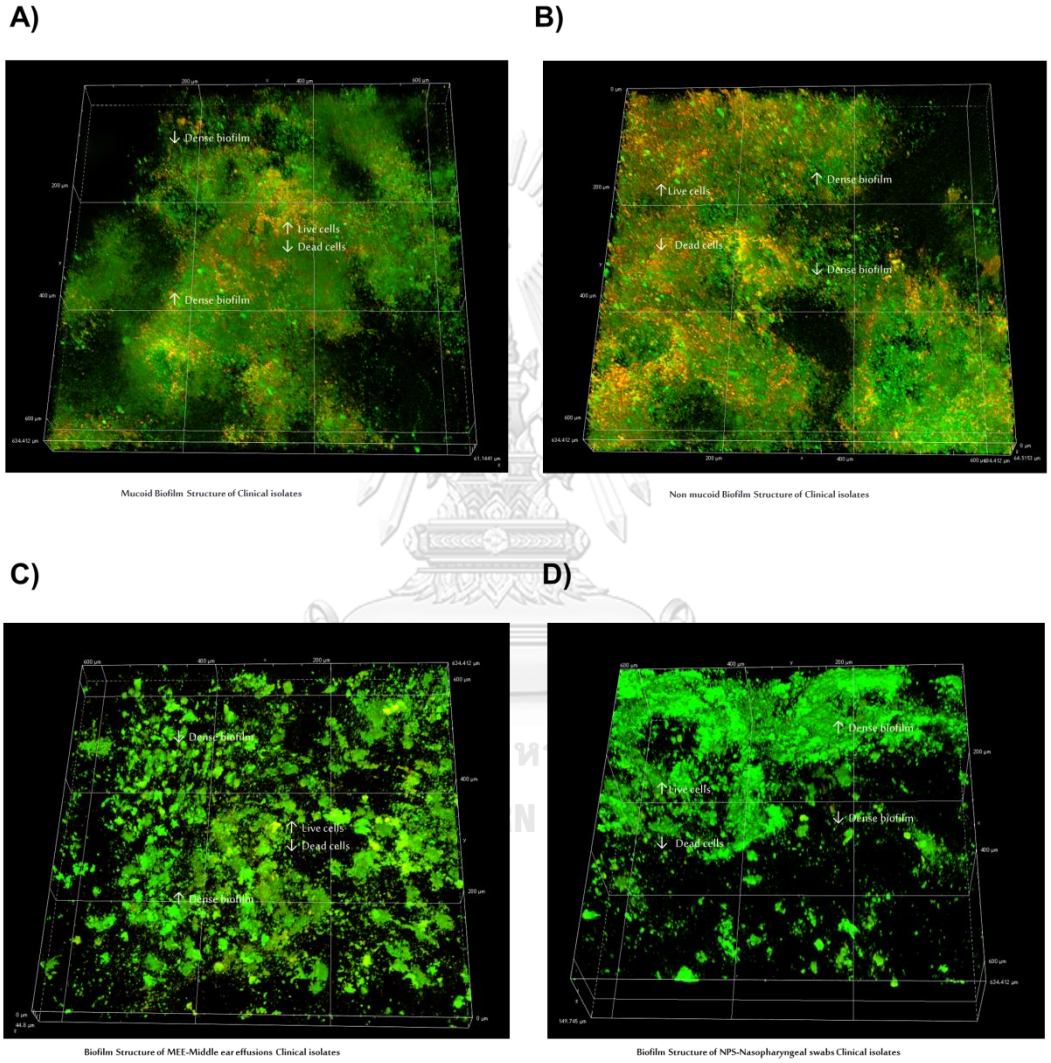


Figure 43 Characterization of biofilm matrix components based on A) Pyocyanin (PCN⁻) production of P. aeruginosa *treated with Pyocyanin chelator glutathione reductase B) extra cellular DNA (eDNA) of* P. aeruginosa *CF isolates and nontypeable* H. influenzae *CF isolates C) nasopharyngeal D) middle ear effusion; alone or in the presence of Dnase : ATCC biofilm negative control and PAO1 and NtHi as a biofilm positive control.*

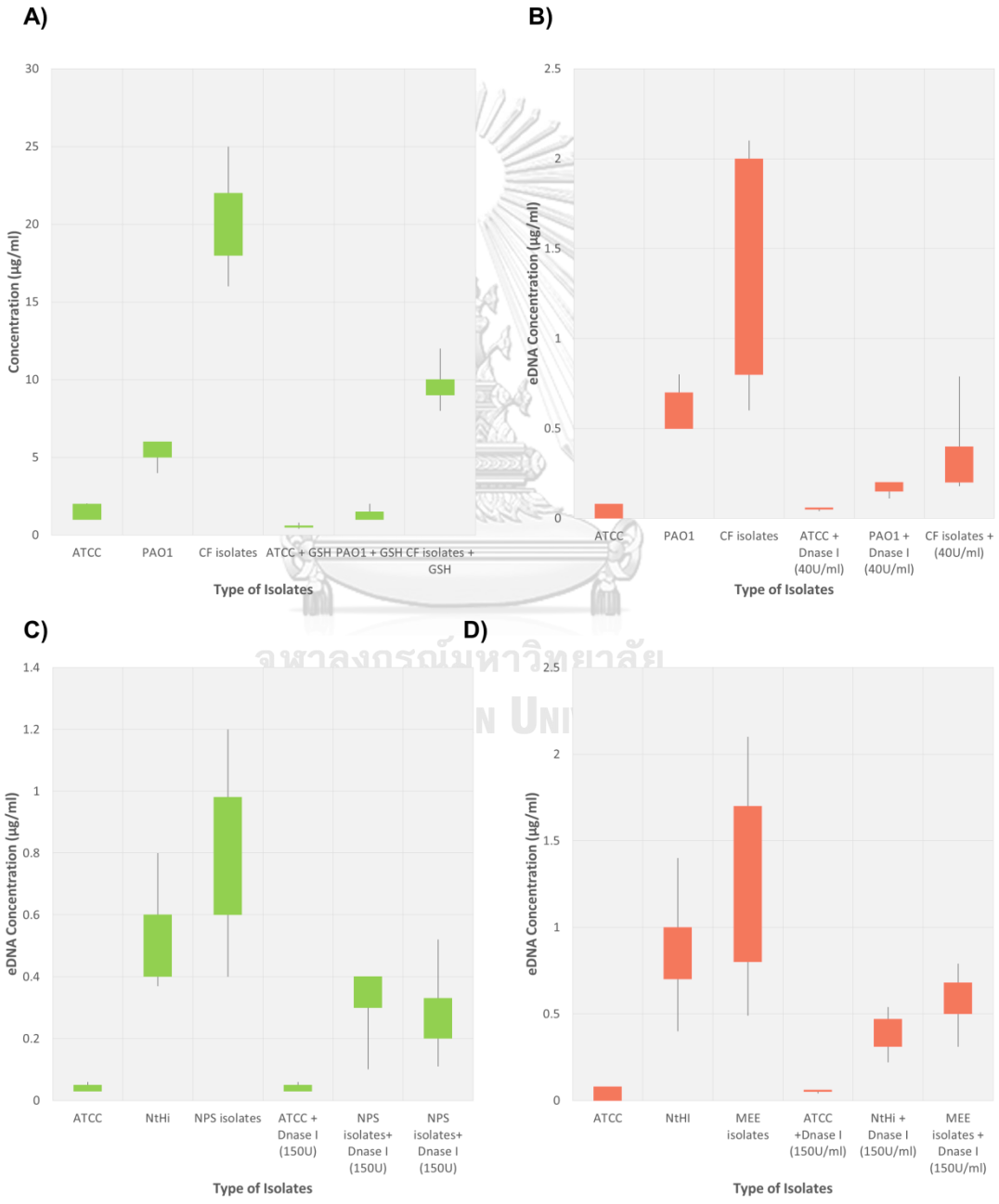


Figure 44 MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBEC = minimum biofilm eradication concentration; MBEC-75 = 75% non-viable cells; MBEC-50 = 50% non-viable cells; MBIC = minimum biofilm inhibitory concentration; of P. aeruginosa *CF isolates planktonic and biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics: A)Tobramycin B) Ciprofloxacin C) Gentamicin D) Amikacin E) Levofloxacin F) Colistin G) Ceftazidime H) Aztreonam*

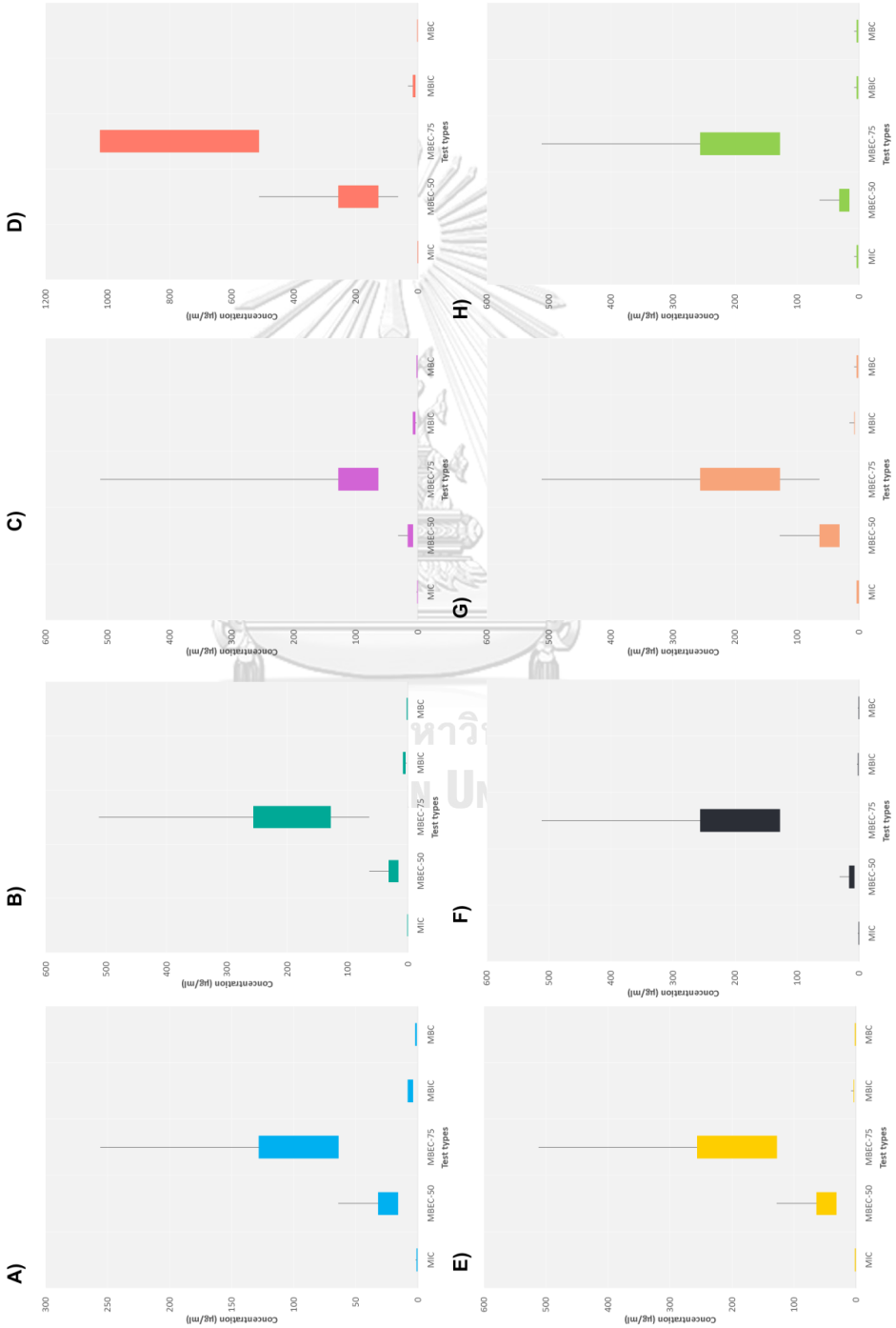
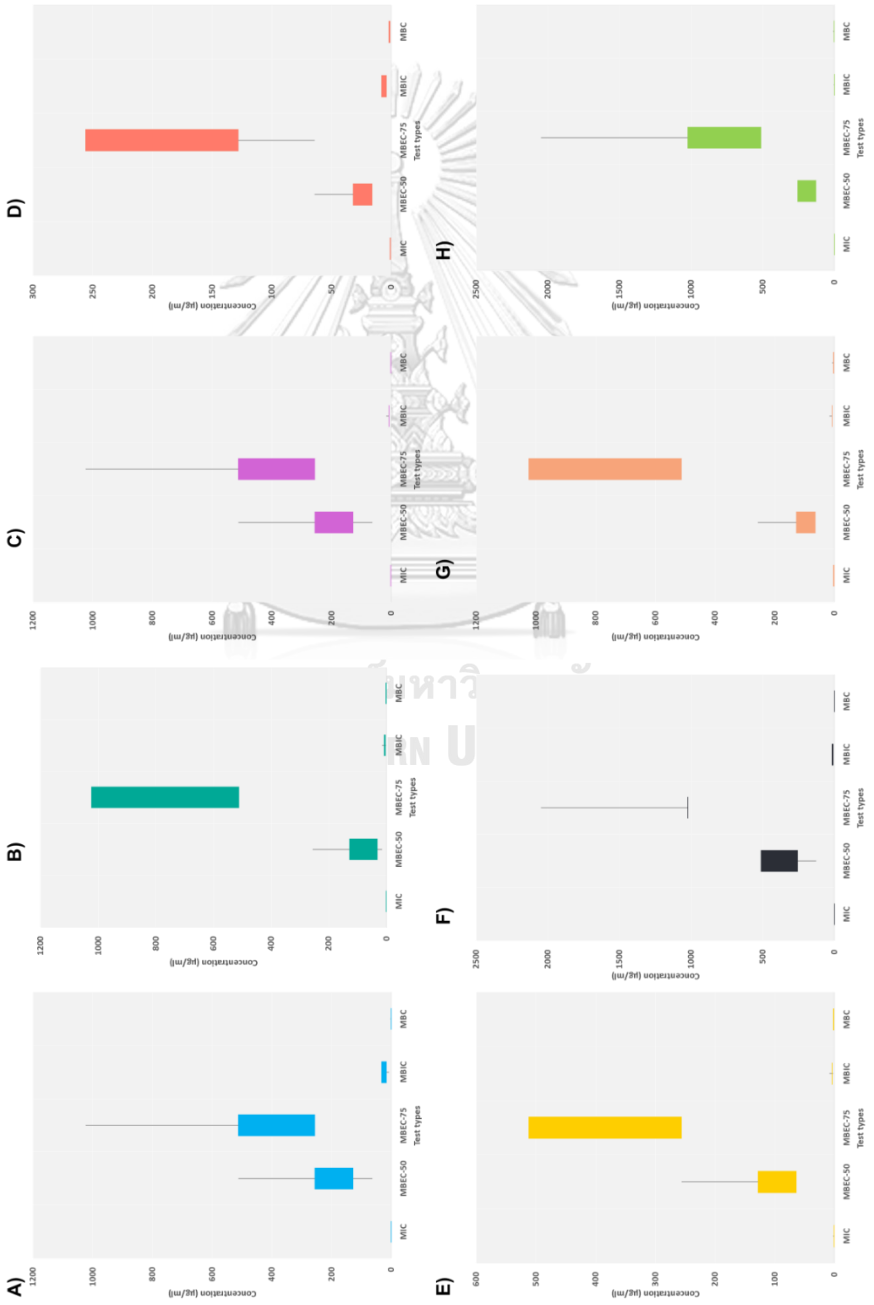


Figure 45 MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBEC = minimum biofilm eradication concentration; MBEC-75 = 75% non-viable cells; MBEC-50 = 50% non-viable cells; MBIC = minimum biofilm inhibitory concentration; of and *nontypeable* H. influenzae *CF isolates planktonic and biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics: nasopharyngeal ; A) Erythromycin B) Ciprofloxacin C) Ceftriaxone D) Sulfamethoxazole/Trimethoprim E) Levofloxacin F) Ampicillin G) Ceftazidime H) Aztreonam and middle ear effusion ; I) Erythromycin J) Ciprofloxacin K) Ceftriaxone l) Sulfamethoxazole/Trimethoprim M) Levofloxacin N) Ampicillin O) Ceftazidime P) Aztreonam*



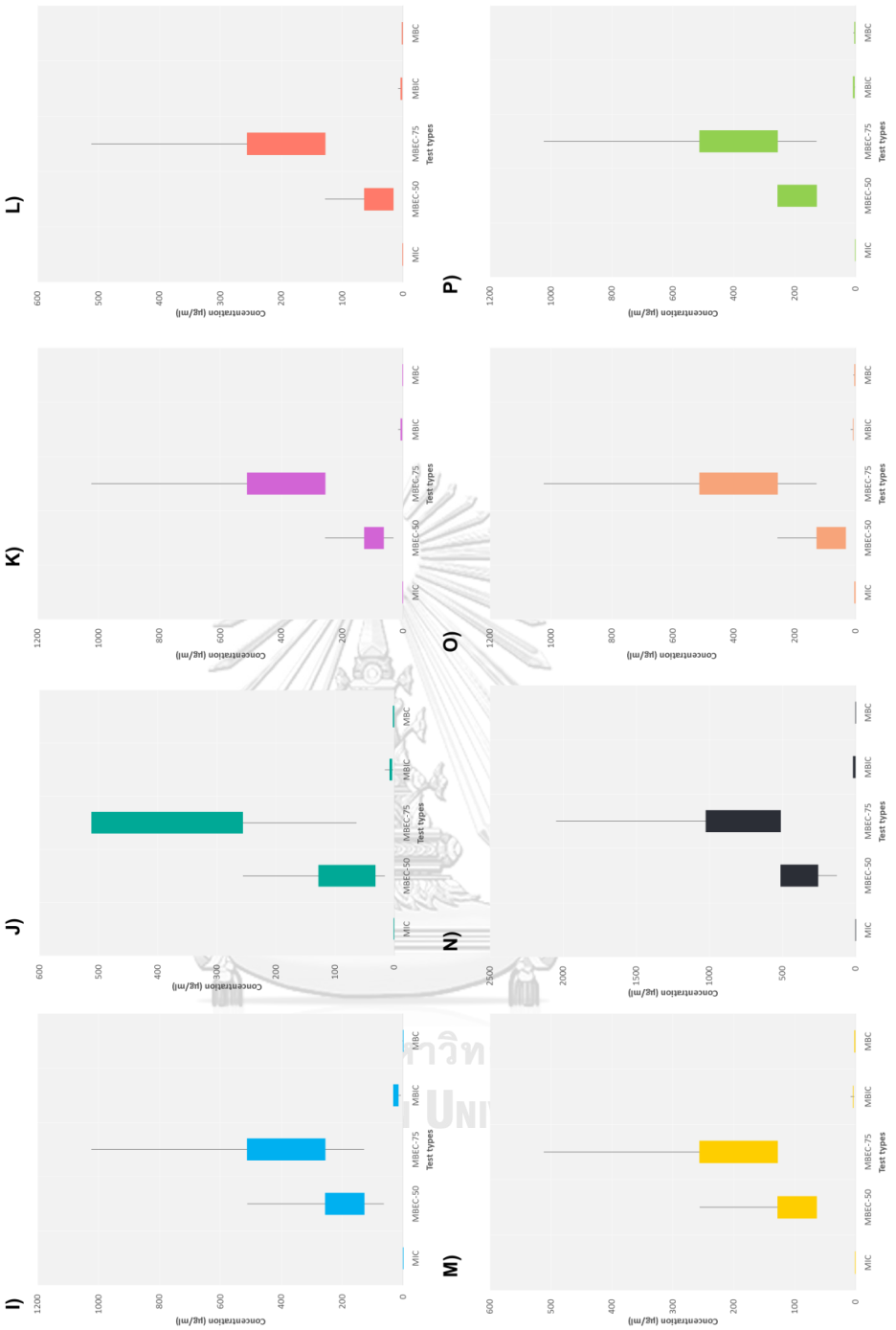

I)
J)
K)
L)
M)
N)
O)
P)
Concentration (µg/ml)
Test types
MIC
MBEC-50
MBEC-75
MBIC
MBC

Figure 46 MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBEC = minimum biofilm eradication concentration; MBEC-75 = 75% non-viable cells; MBEC-50 = 50% non-viable cells; MBIC = minimum biofilm inhibitory concentration; of P. aeruginosa *CF isolates planktonic and biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics with alone or in the presence of exo DNA, Dnase I: A)Tobramycin B) Ciprofloxacin C) Gentamicin D) Amikacin E) Levofloxacin F) Colistin G) Ceftazidime H) Aztreonam and glutathione reductase (GSH); : I)Tobramycin J) Ciprofloxacin K) Gentamicin l) Amikacin M) Levofloxacin N) Colistin O) Ceftazidime P) Aztreonam*

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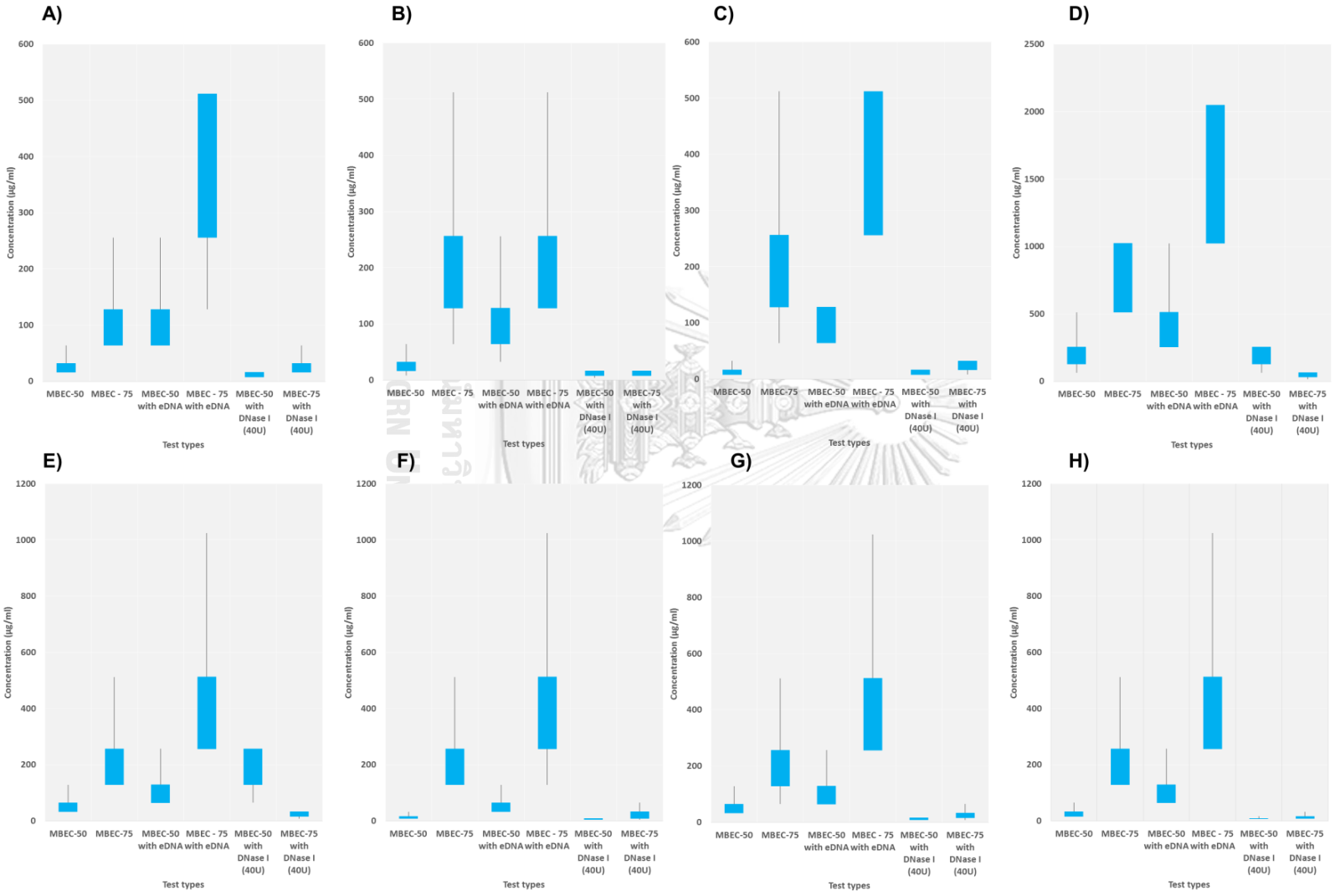

A)
B)
C)
D)
E)
F)
G)
H)
Concentration (µg/ml)
Test types
MBEC-50
MBEC - 75
MBEC-50 with eDNA
MBEC - 75 with eDNA
MBEC-50 with DNase I (40U)
MBEC-75 with DNase I (40U)

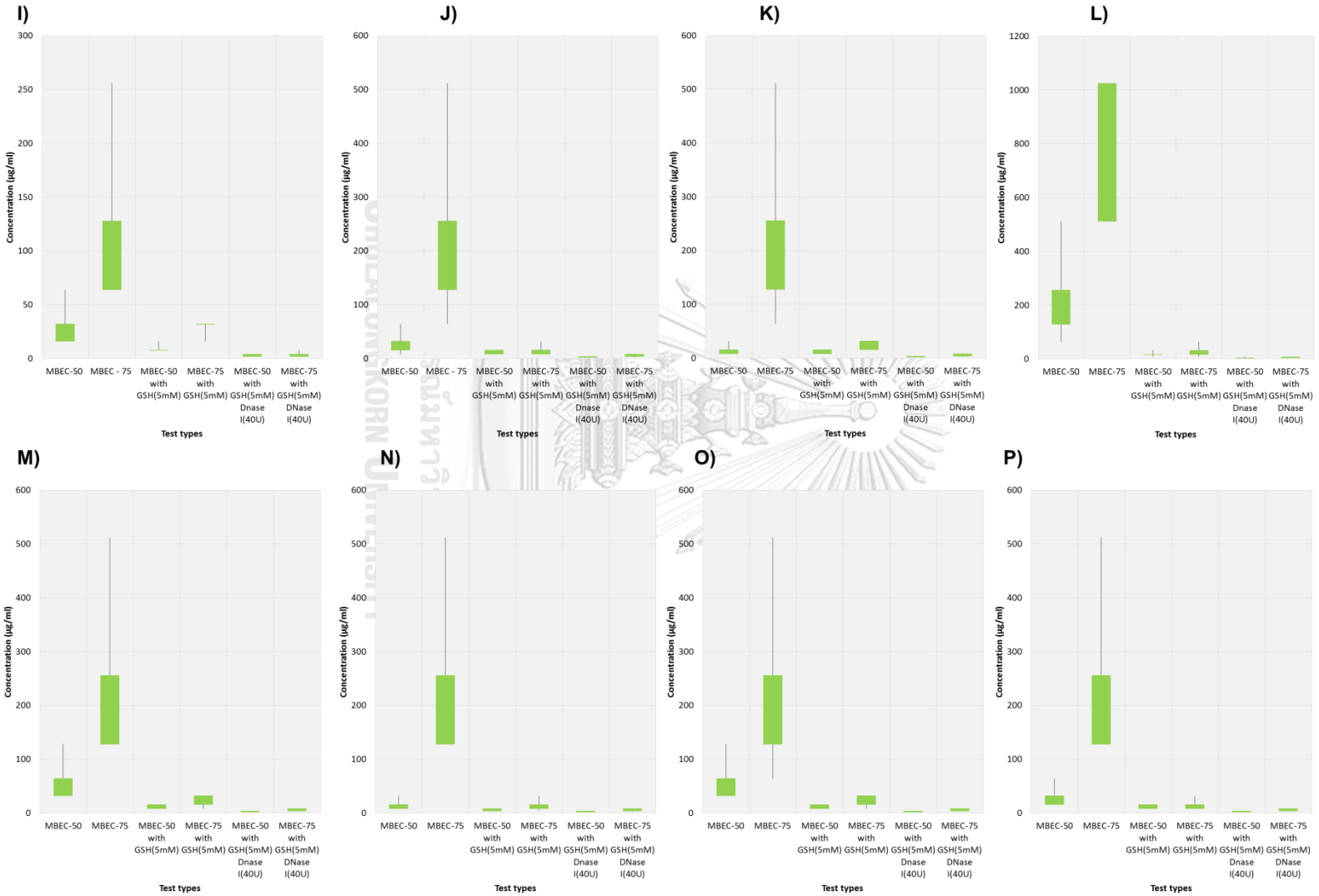
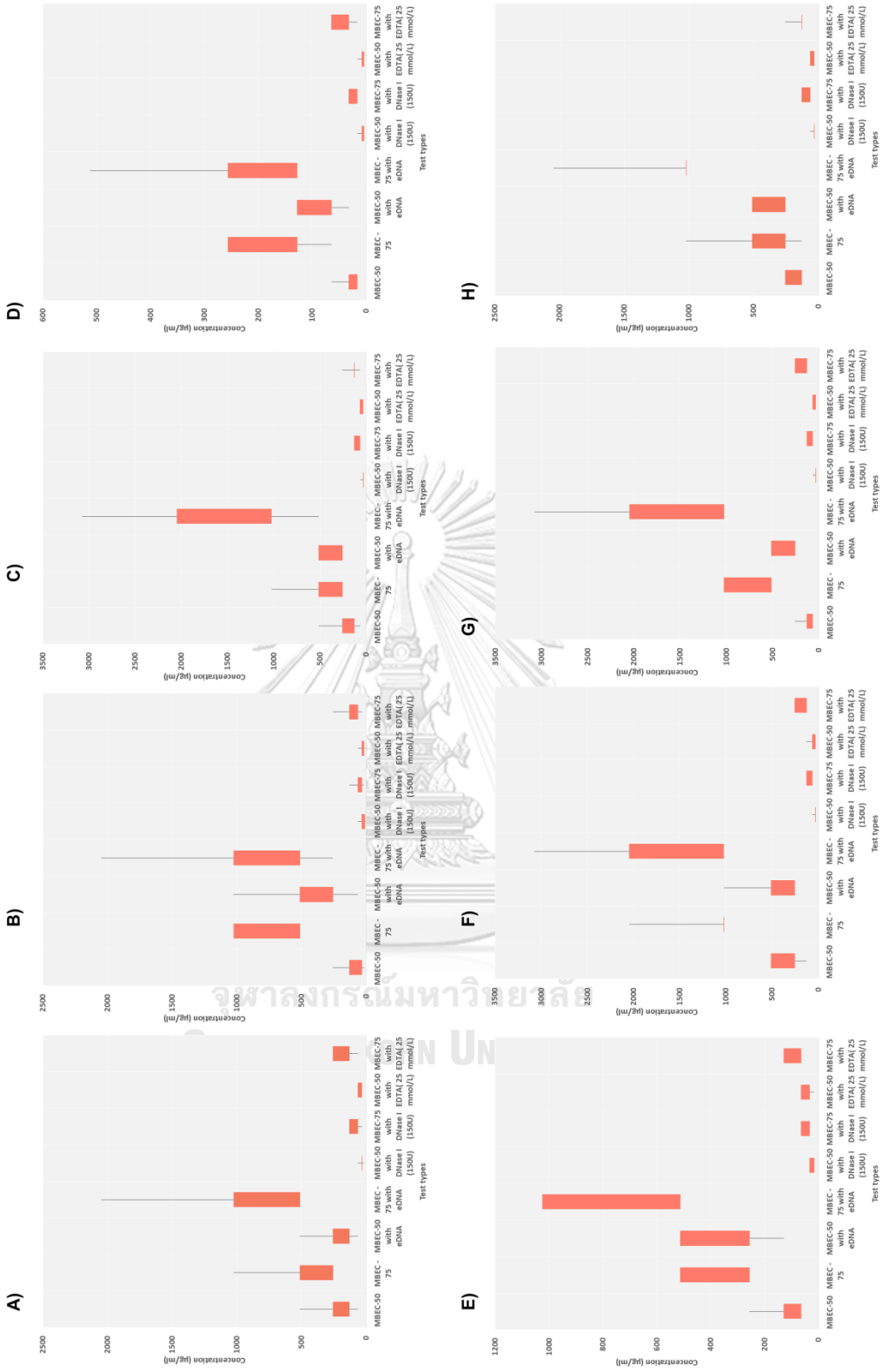

I)
J)
K)
L)
M)
N)
O)
P)
Concentration (µg/ml)
Test types
MBEC-50
MBEC - 75
MBEC-75
MBEC-50 with GSH(5mM)
MBEC-75 with GSH(5mM)
MBEC-50 with GSH(5mM) Dnase I(40U)
MBEC-75 with GSH(5mM) DNase I(40U)

Figure 47 MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MBEC = minimum biofilm eradication concentration; MBEC-75 = 75% non-viable cells; MBEC-50 = 50% non-viable cells; MBIC = minimum biofilm inhibitory concentration; of and *nontypeable* H. influenzae *CF isolates planktonic and biofilm were generated measuring bacteria cell viability as a percentage in the presence of antibiotics with alone or in the presence of exo DNA, Dnase I: and EDTA: nasopharyngeal ; A) Erythromycin B) Ciprofloxacin C) Ceftriaxone D) Sulfamethoxazole/Trimethoprim E) Levofloxacin F) Ampicilline G) Ceftazidime H) Aztreonam and middle ear effusion ; I) Erythromycin J) Ciprofloxacin K) Ceftriaxone l) Sulfamethoxazole/Trimethoprim M) Levofloxacin N) Ampicillin O) Ceftazidime P) Aztreonam*



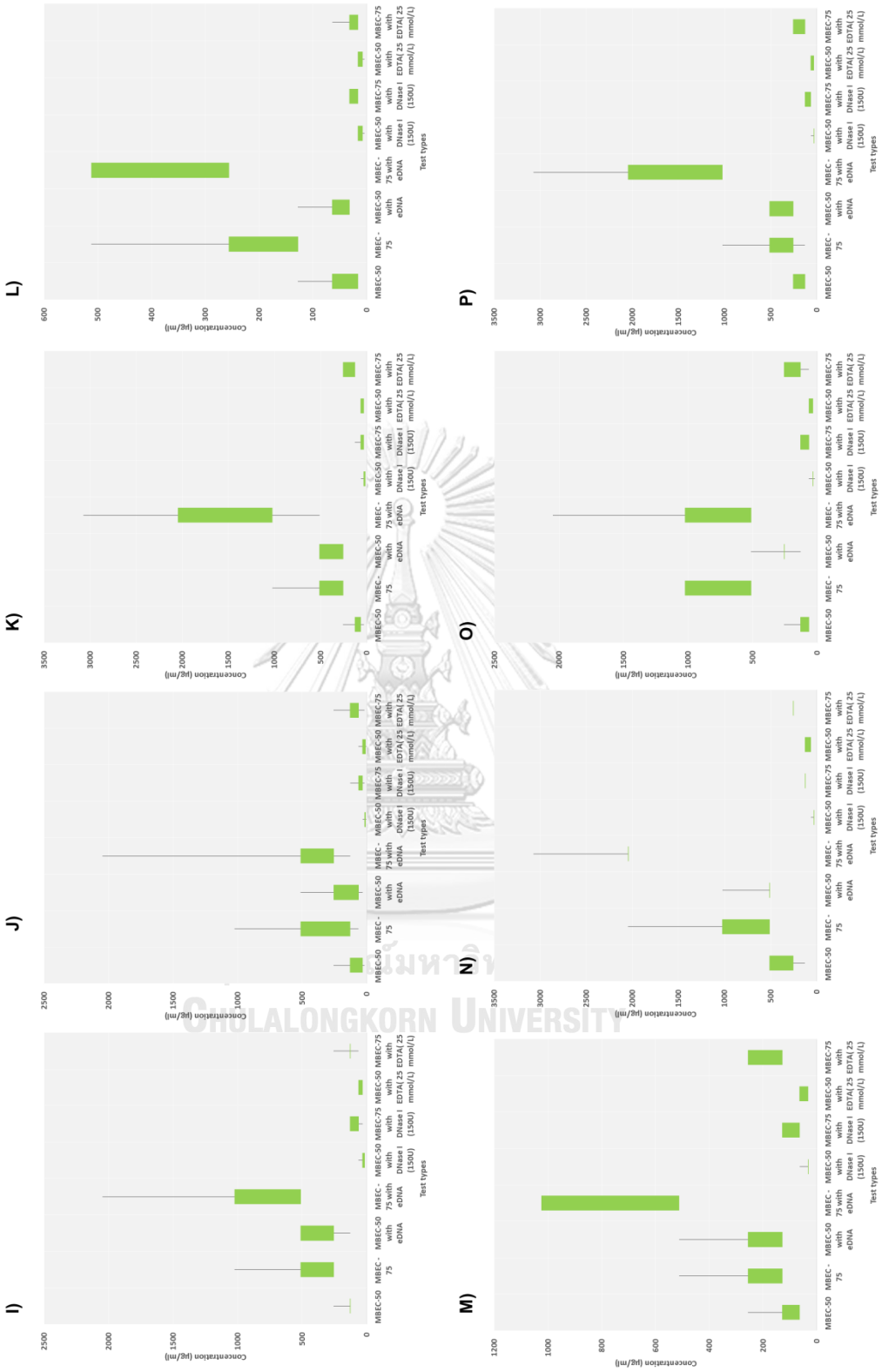


Figure 48 MBEC = minimum biofilm eradication concentration of P. aeruginosa *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides*

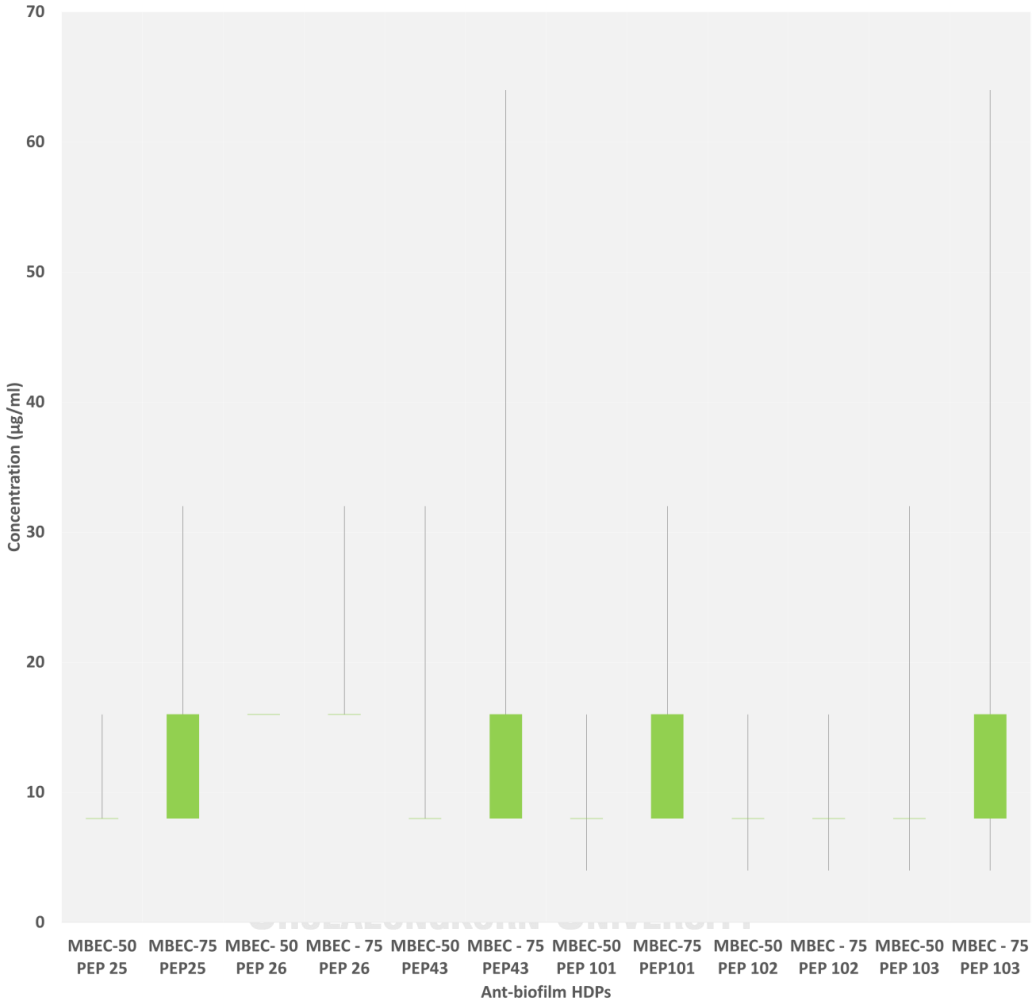


Figure 49 Correlation between the biofilm bio-volume and different concentration of anti-biofilm peptide PEP 102 and PEP 25 P. aeruginosa *CF isolates; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy*

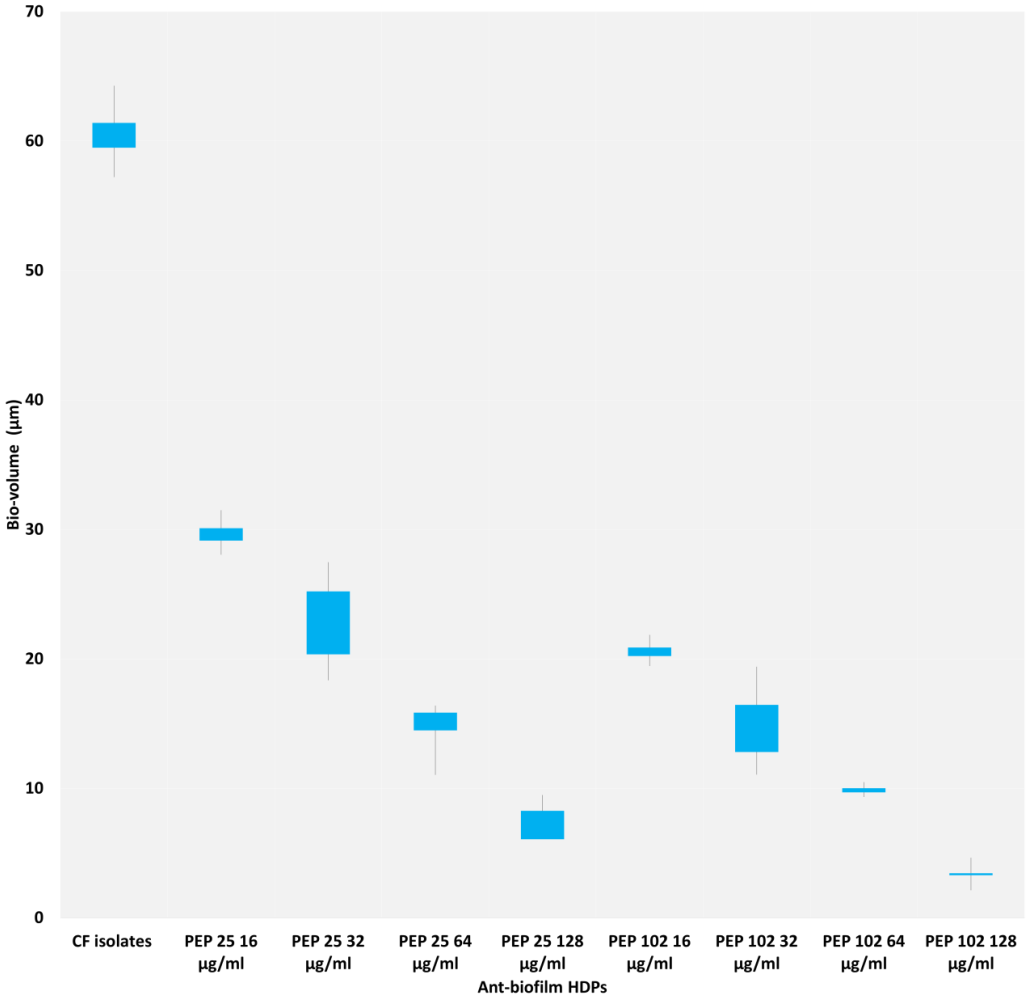


Figure 50 MBEC = minimum biofilm eradication concentration of P. aeruginosa *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides compare to the Tobramycin antibiotics with alone or in the presence of exo DNA, Dnase I and glutathione reductase (GSH).*

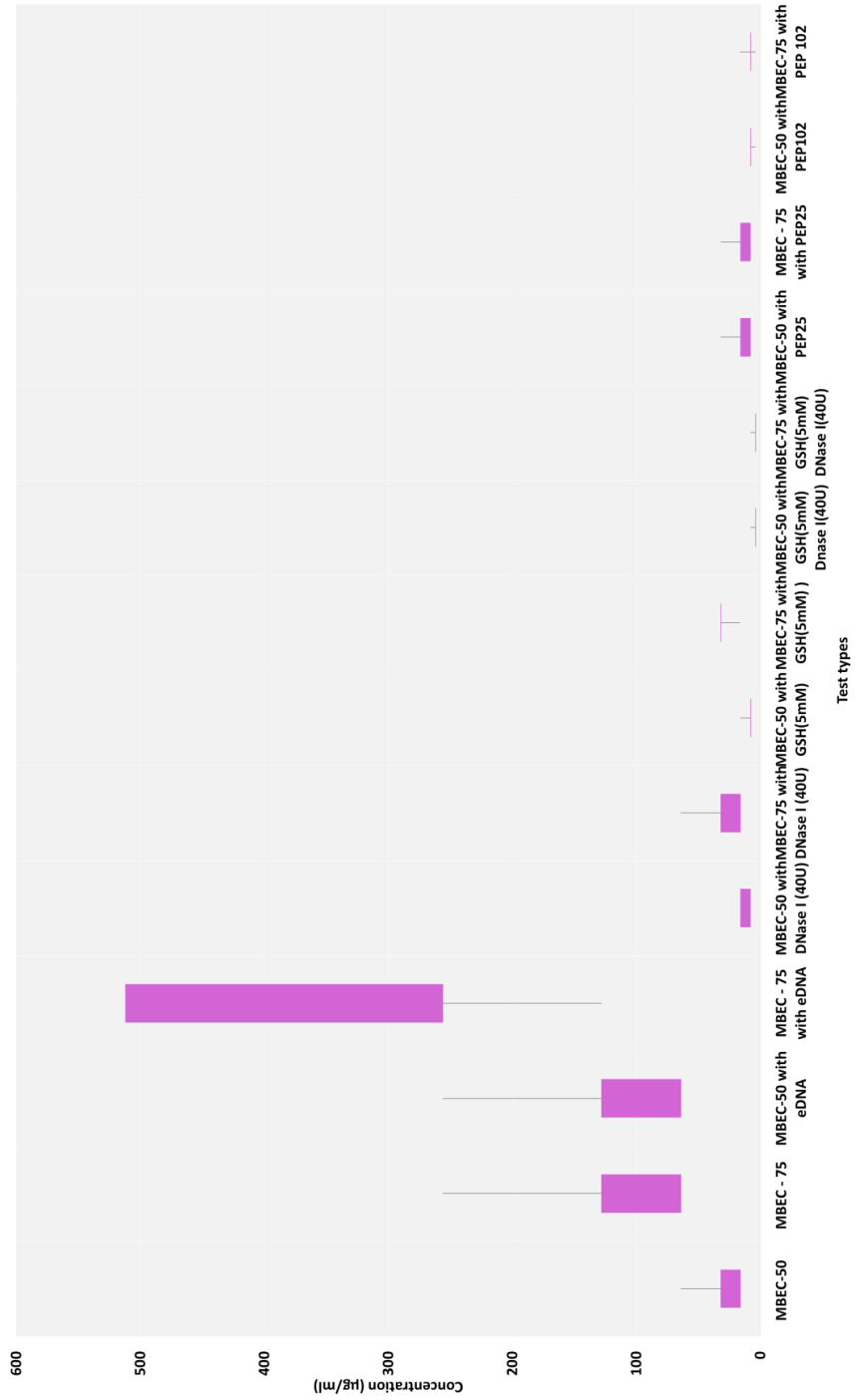


Figure 51 MBEC = minimum biofilm eradication concentration of nasopharyngeal nontypeable H. influenzae *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides*

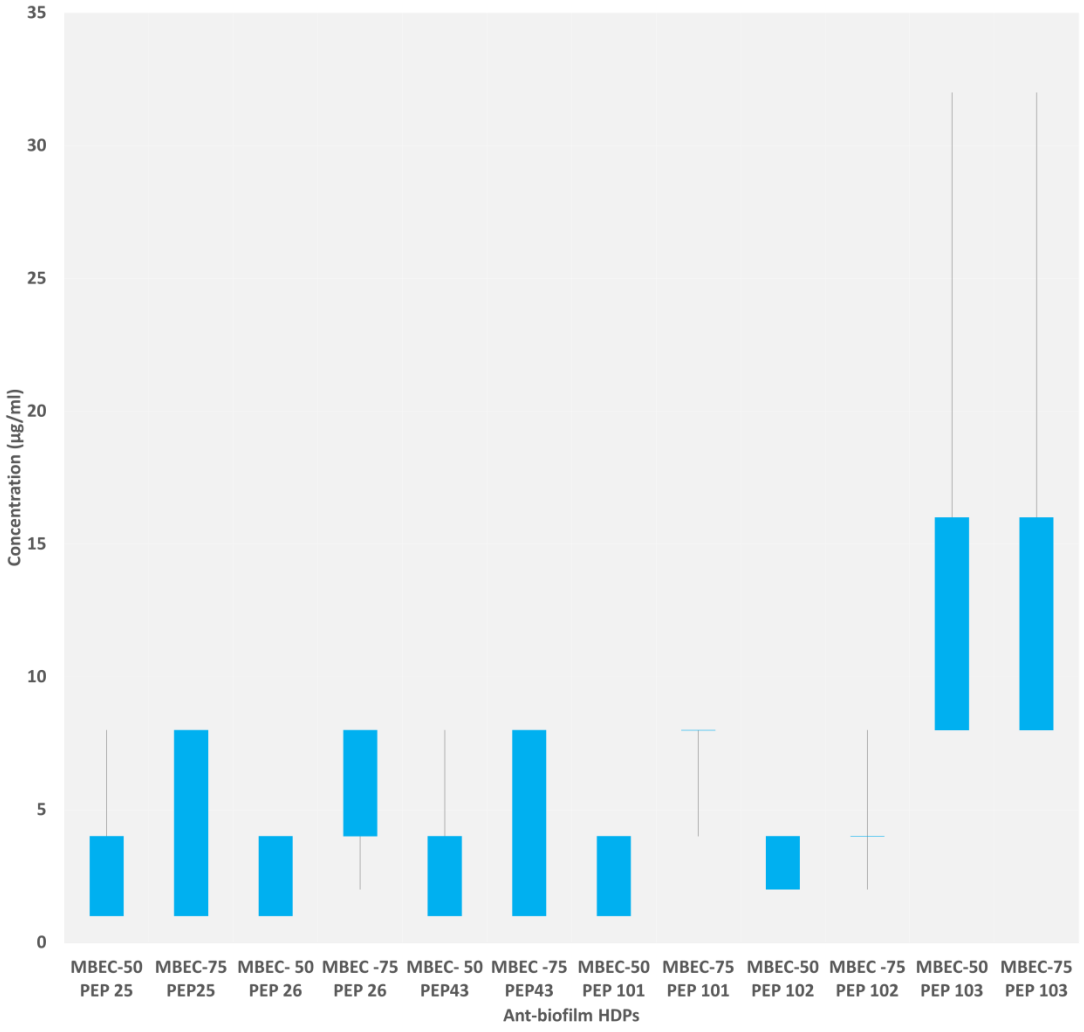


Figure 52 Correlation between the biofilm bio-volume and different concentration of anti-biofilm peptide PEP 102 and PEP 25 nasopharyngeal nontypeable H. influenzae *CF isolates; measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy*

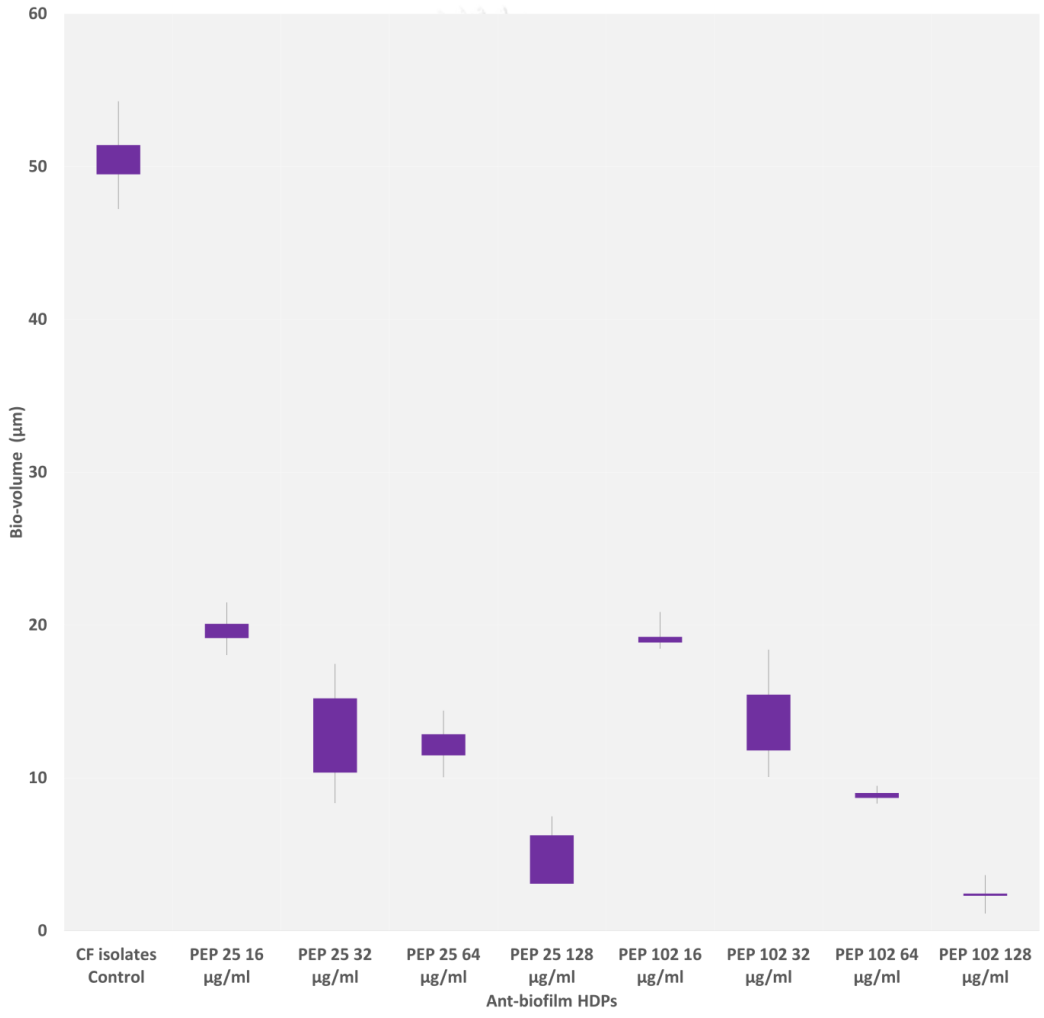


Figure 53 MBEC = minimum biofilm eradication concentration of nasopharyngeal nontypeable H. influenzae *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides compare to the Ciprofloxacin antibiotics with alone or in the presence of exo DNA, Dnase I and EDTA).*

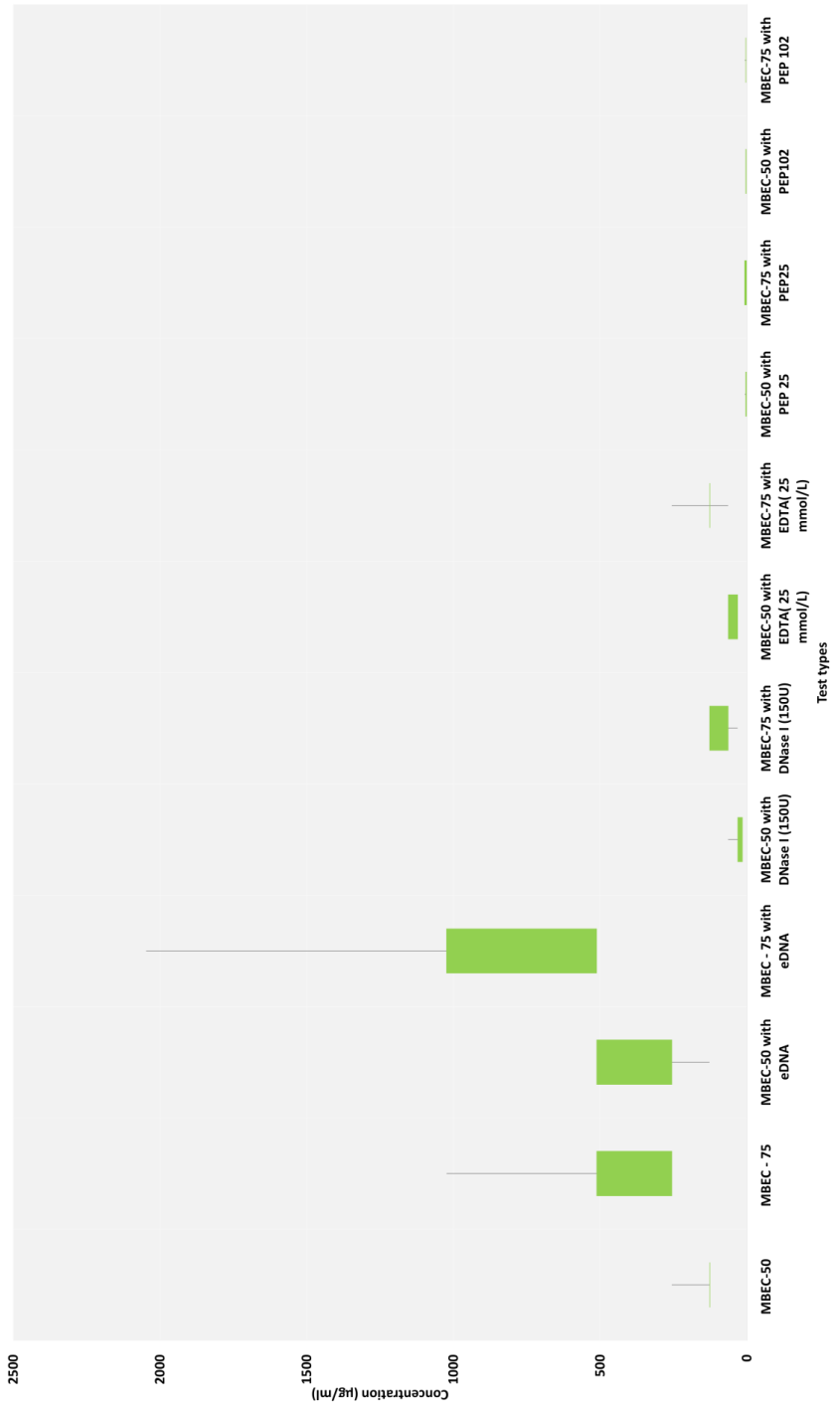


Figure 54 MBEC = minimum biofilm eradication concentration of middle ear effusion nontypeable H. influenzae *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides*

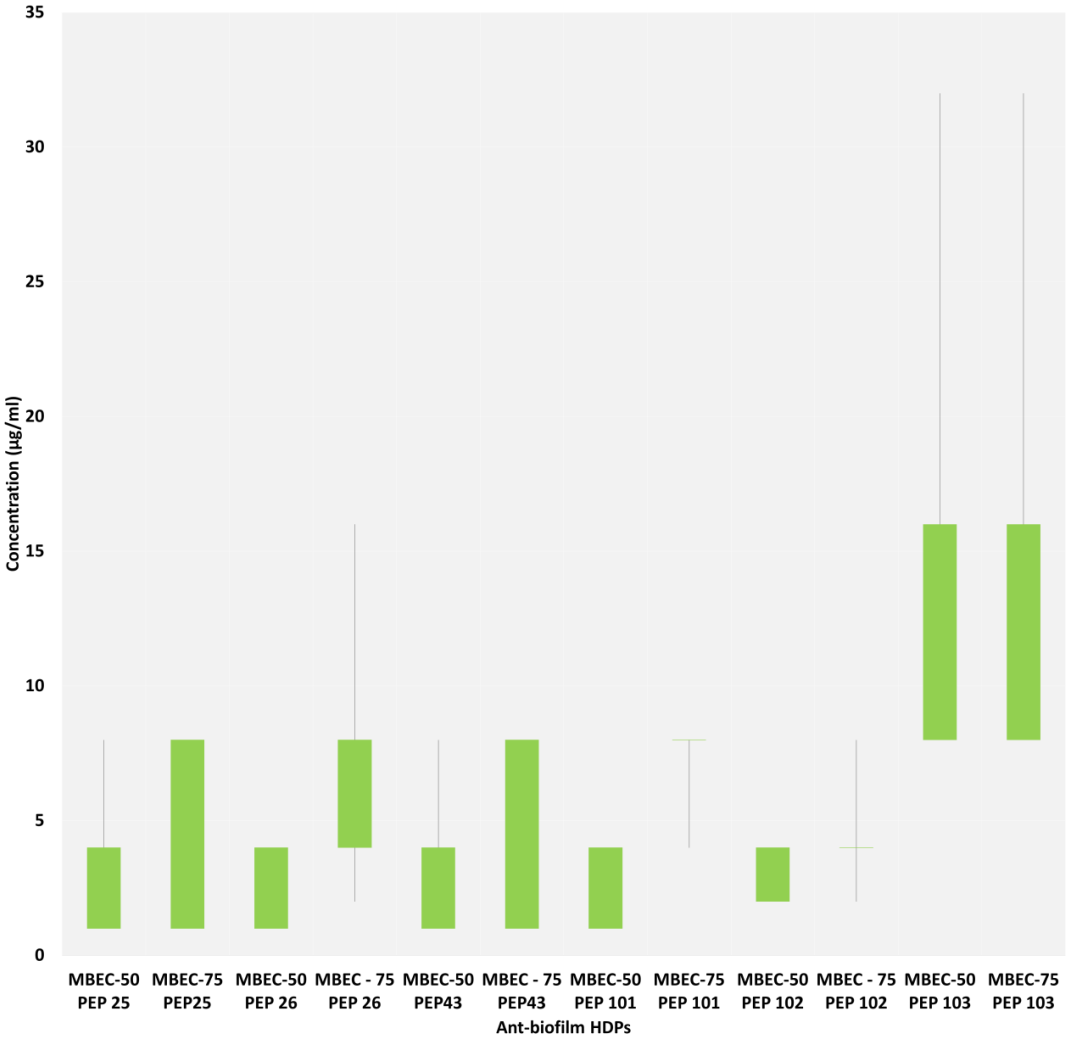


Figure 55 Correlation between the biofilm bio-volume and different concentration of anti-biofilm peptide PEP 102 and PEP 25 middle ear effusion nontypeable H. influenzae *CF isolates measured with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy*

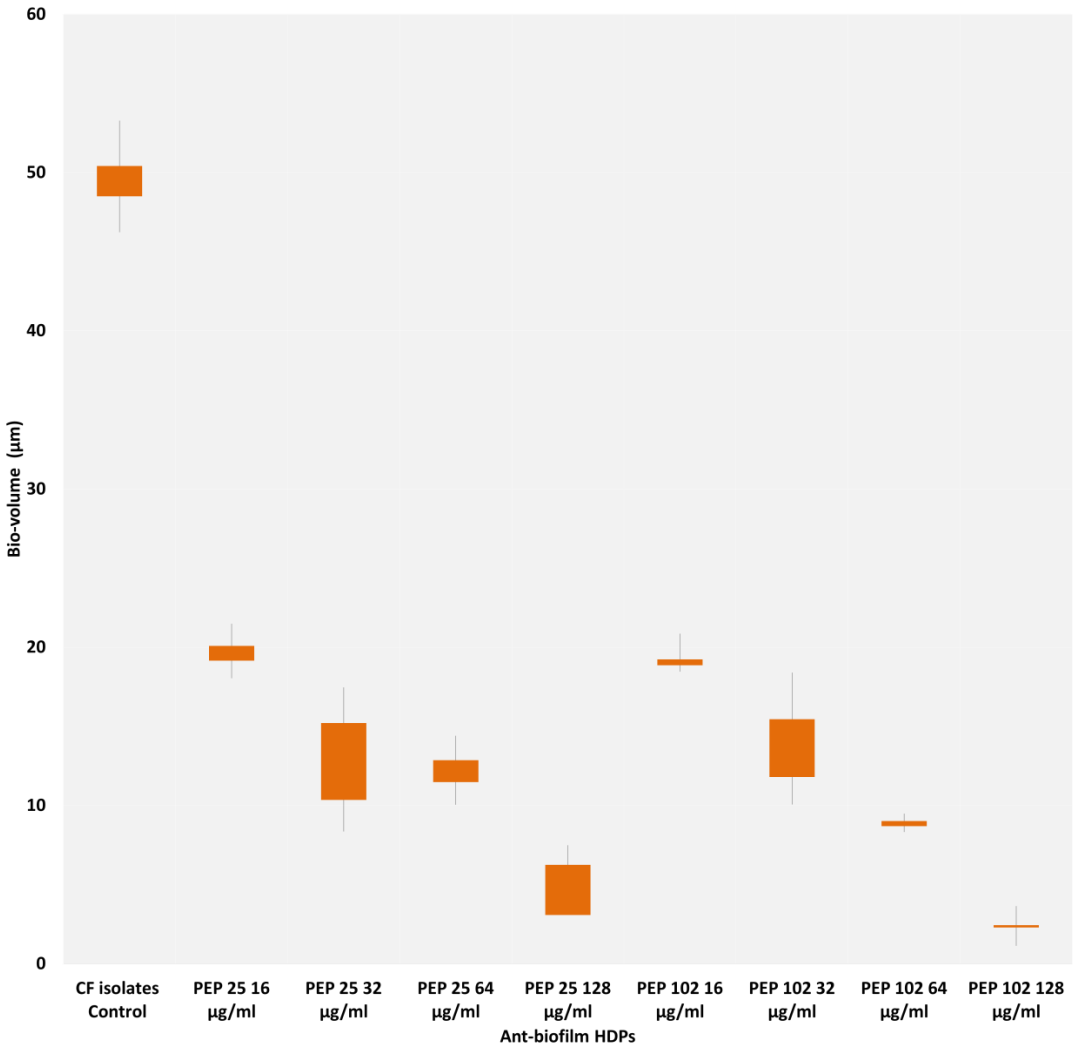


Figure 56 MBEC = minimum biofilm eradication concentration of middle ear effusion nontypeable H. influenzae *CF isolates biofilm were generated measuring bacteria cell viability as a percentage with 50% and 75% non-viable cells in the presence of anti-biofilm peptides compare to the Ciprofloxacin antibiotics with alone or in the presence of exo DNA, Dnase I and EDTA).*

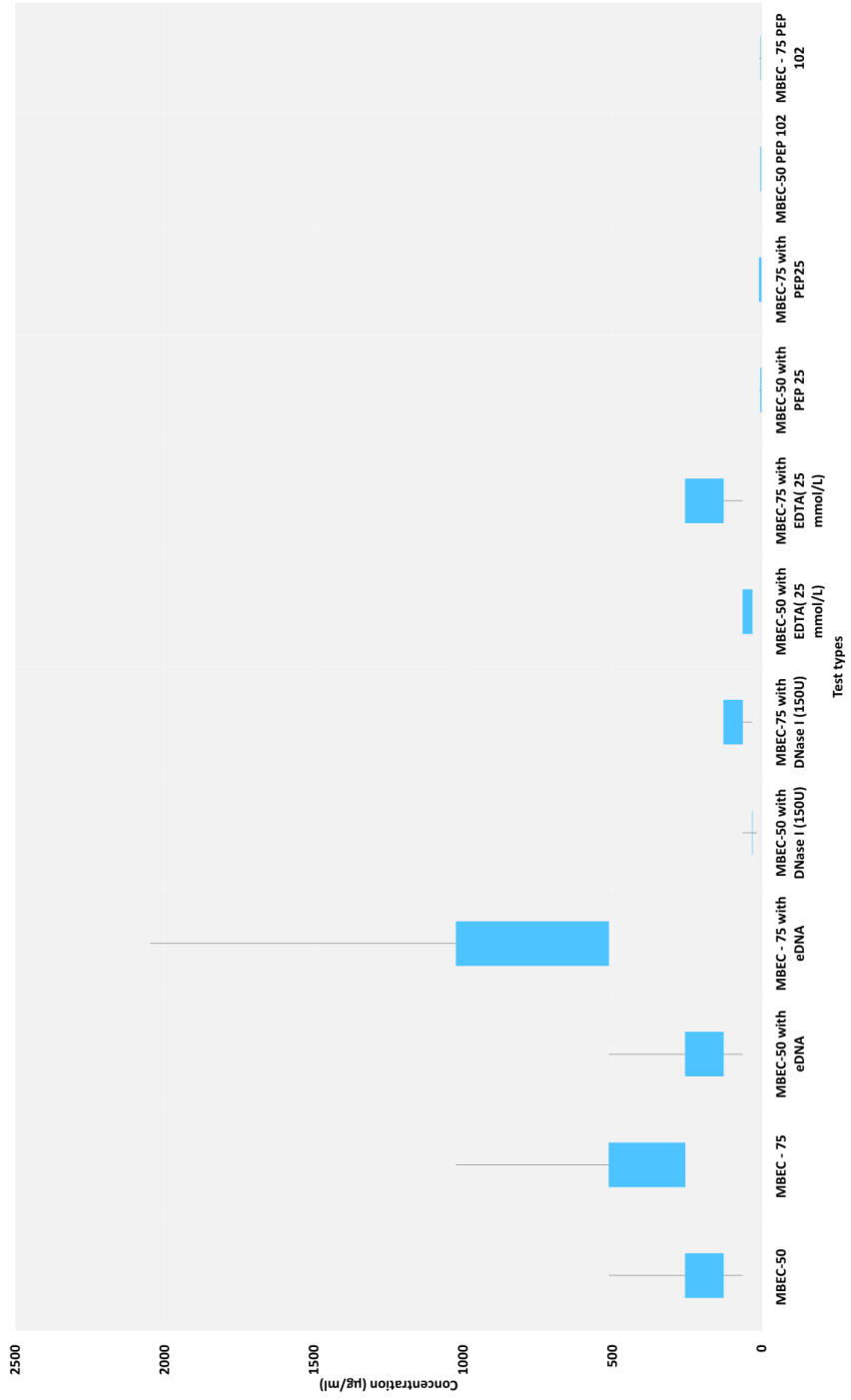
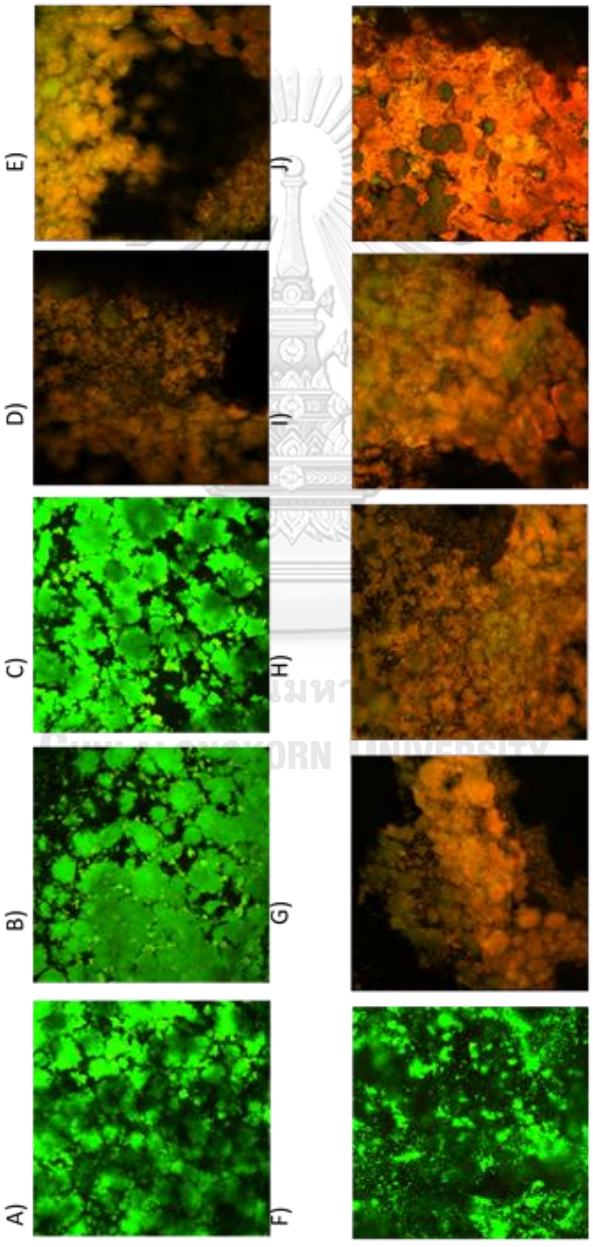


Figure 57 Effect of and anti-biofilm peptide PEP102 and tobramycin antibiotics responses (alone or in the presence of exo DNA, glutathione reductase (GSH) and Dnase I) nontypeable H. influenzae *CF isolates biofilms. Visualized with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy. A) Control B) Ciprofloxacin 2µg/ml C) Ciprofloxacin 2µg/ml + Dnase 150U) Ciprofloxacin 2µg/ml + EDTA 25mmol/L E) Ciprofloxacin 2µg/ml + EDTA 25mmol/L + Dnase 150U F) Control G) PEP 102 8µg/ml H) PEP 102 16µg/ml I) PEP 102 32µg/ml J) PEP 102 64µg/ml. K) 3D analysis of nontypeable* H. influenzae *CF isolates biofilms biofilm expose to PEP 102.*



K)

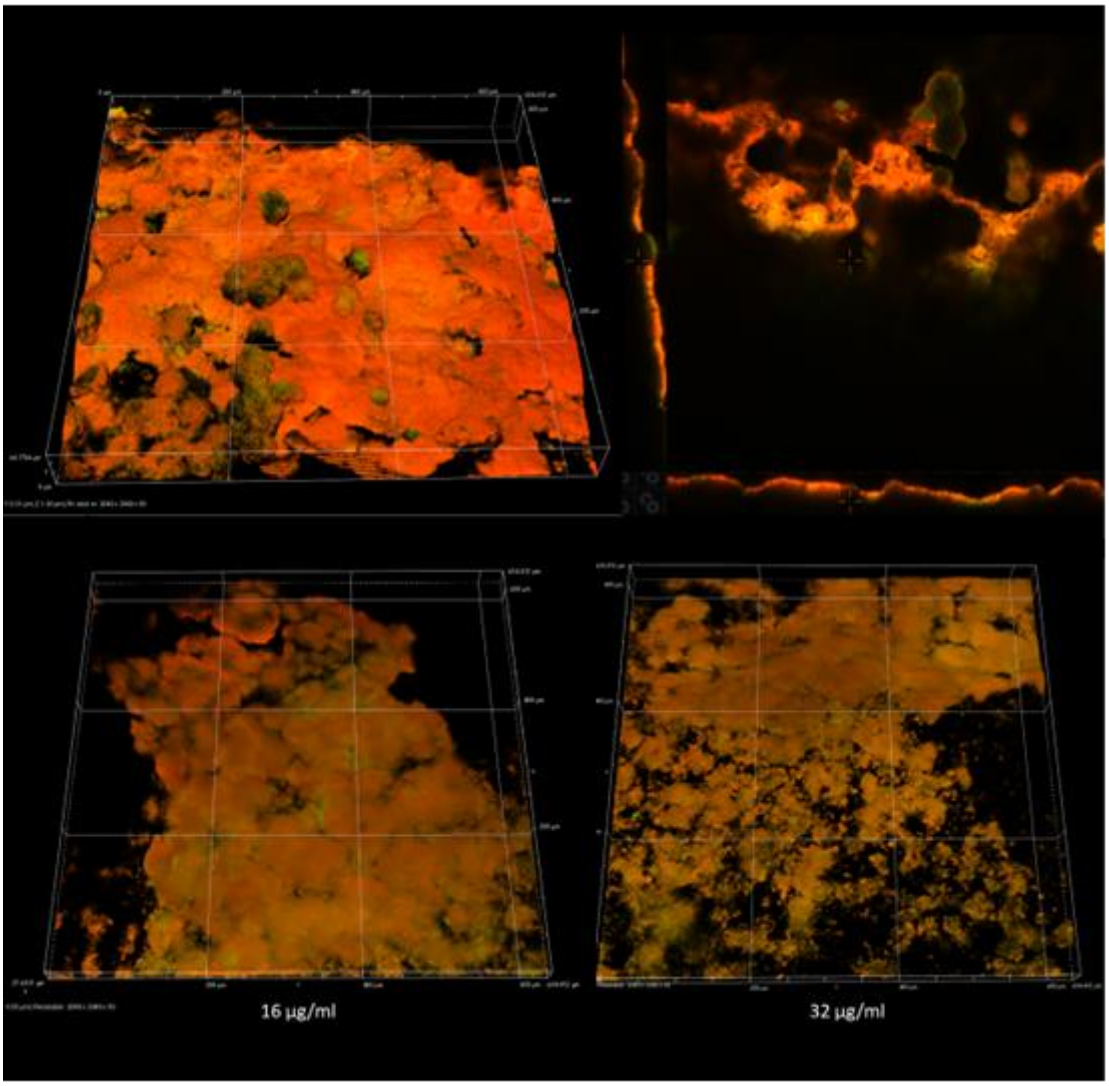
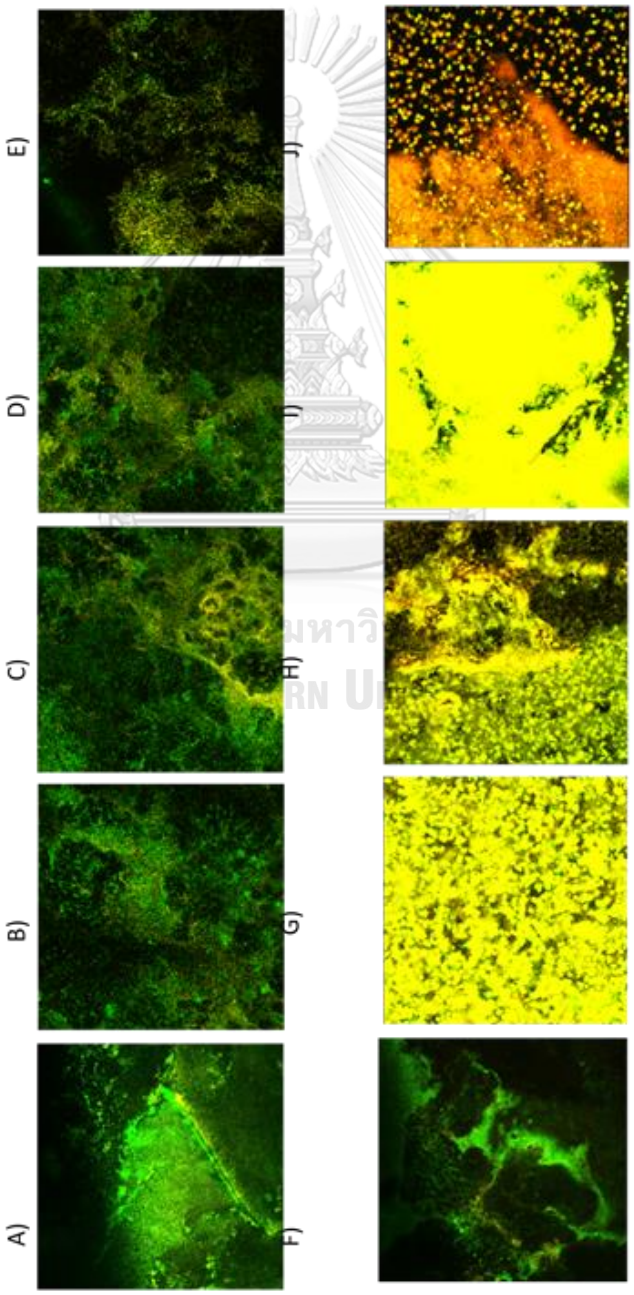
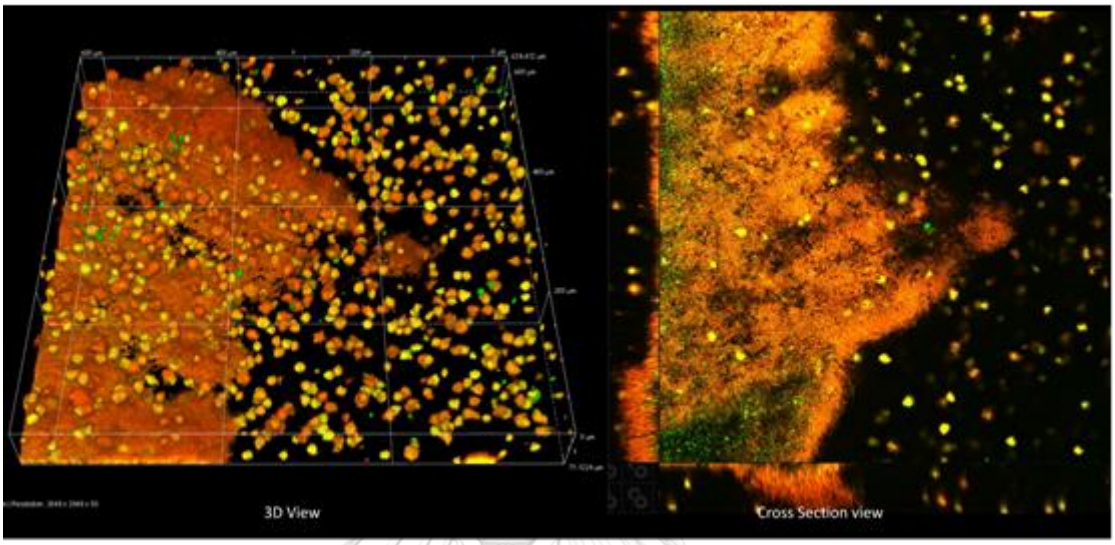


Figure 58 Effect of and anti-biofilm peptide PEP102 and tobramycin antibiotics responses (alone or in the presence of exo DNA, glutathione reductase (GSH) and Dnase I) P. aeruginosa *CF isolates biofilms. Visualized with LIVE/DEAD BacLight Bacterial Viability Kit, Green = Live cells/Red = Dead cells under the high resolution confocal scanning laser microscopy. A) Control B) Tobramycin 16µg/ml C) Tobramycin 16µg/ml + GSH 5mM D) Tobramycin 16µg/ml + Dnase 40U E) Tobramycin 16µg/ml + GSH 5mM + Dnase 40U F) Control G) PEP 102 16µg/ml H) PEP 102 32µg/ml I) PEP 102 64µg/ml J) PEP 102 128µg/ml K) 3D analysis of* P. aeruginosa *CF isolates biofilms biofilm expose to PEP 102 128µg/ml. L) 3D analysis of* P. aeruginosa *CF isolates micro colony biofilms expose to PEP 102 128µg/ml and 16 µg/ml.*



K)


3D View
Cross Section view

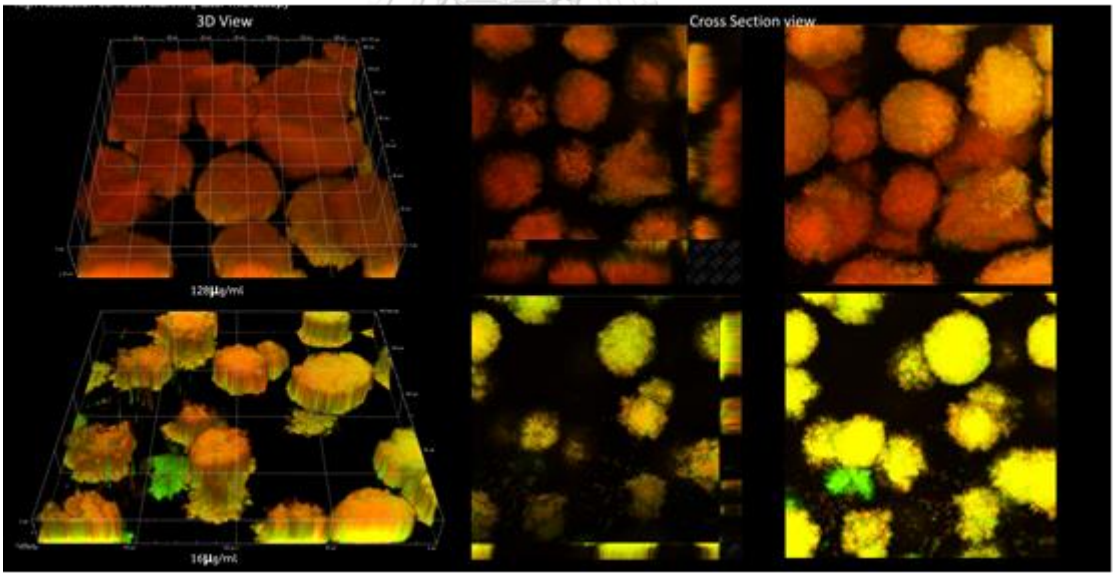

3D View
Cross Section view
128µg/ml
16µg/ml

Figure 59 Characterization anti-Biofilm activity of PEP 102 (124µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) and tobramycin antibiotics(16µg/ml) on P. aeruginosa *CF isolates biofilms bio volume in CF air way primary epithelial cell culture biofilm model measured by high resolution confocal scanning laser microscopy. CC- Control, S- Submerge A- aerosol TBM - Tobramycin*

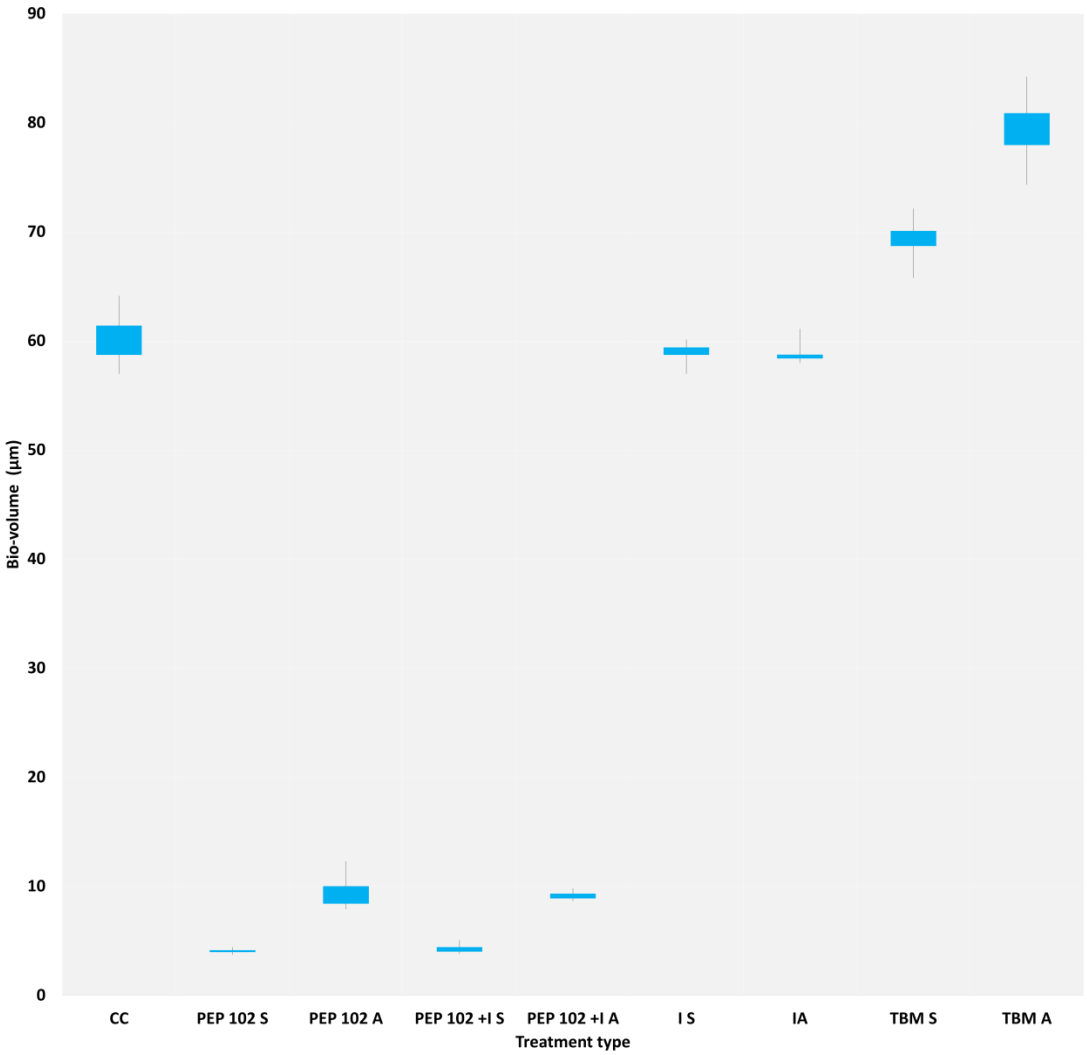


Figure 60 Characterization anti-Biofilm activity of PEP 102 (124µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) and ciprofloxacin antibiotics (2µg/ml) on nontypeable H. influenzae *CF isolates biofilms bio volume in CF air way primary epithelial cell culture biofilm model measured by high resolution confocal scanning laser microscopy. CC- Control, S- Submerge A- aerosol CP- Ciprofloxacin*

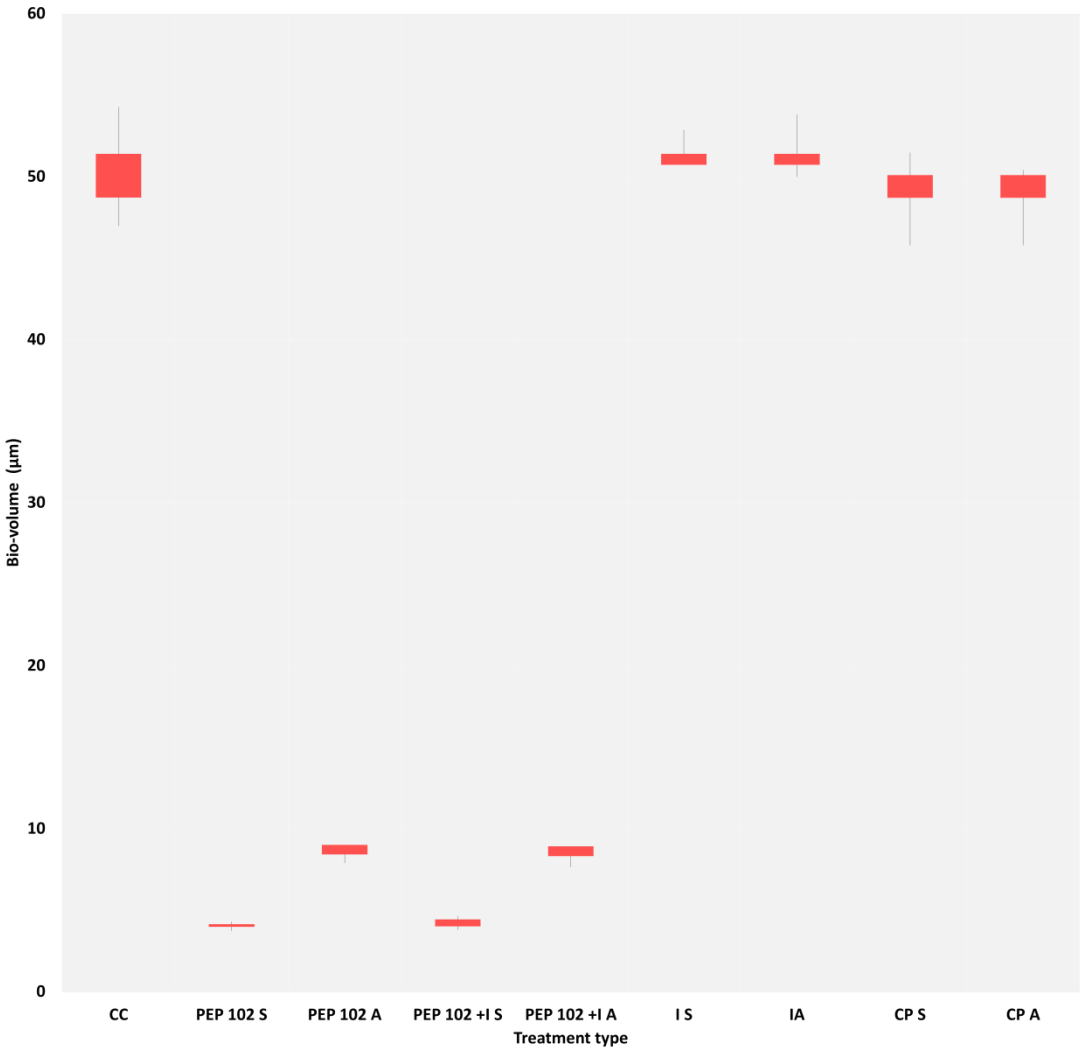


Figure 61 Trans epithelial electrical resistance of air way primary epithelium. Data represent the mean values for nontypeable H. influenzae *CF isolates biofilms* P. aeruginosa *CF isolates biofilms after treatment with PEP 102 (124µg/ml) alone or together with Inhibitory peptide –I.*

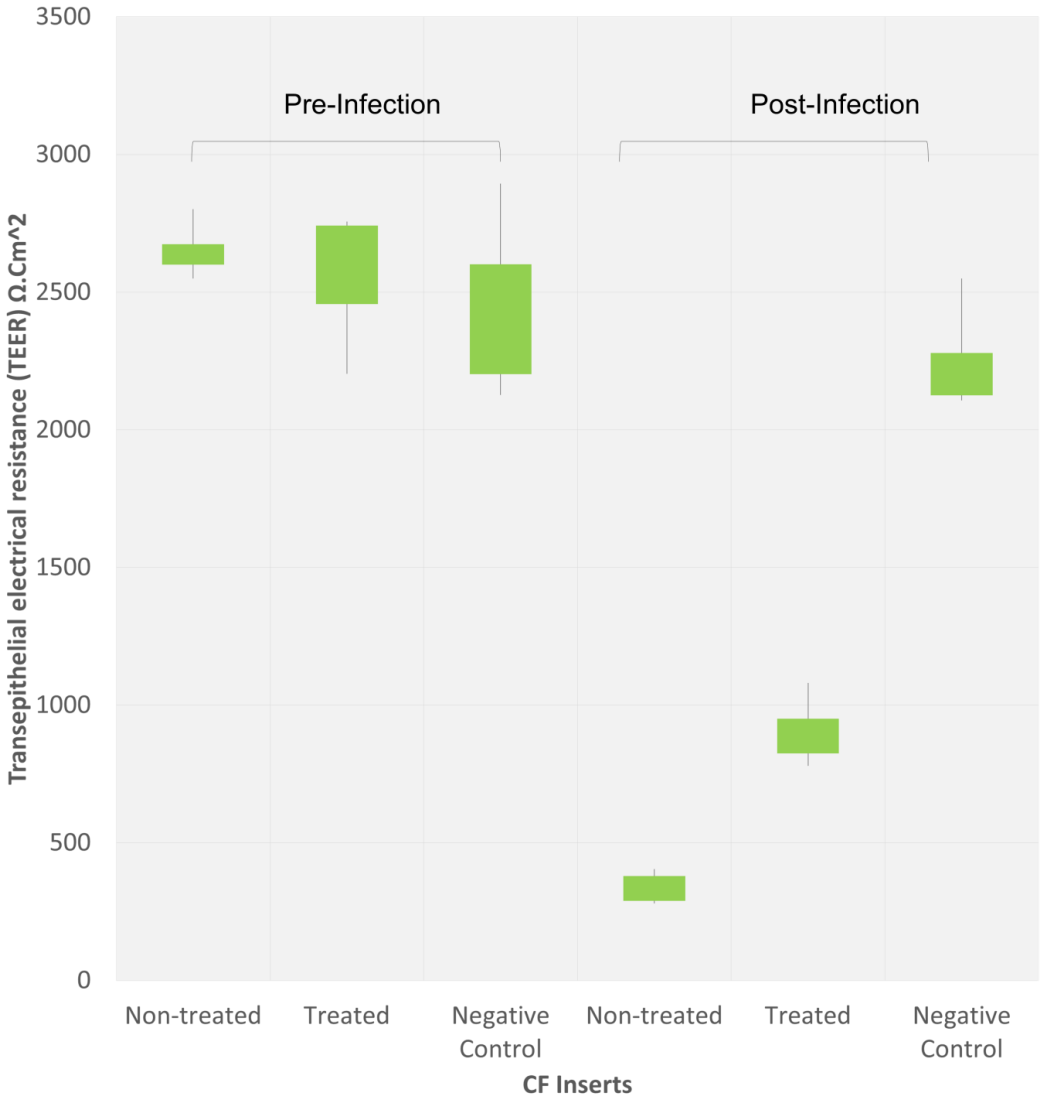


Figure 62 Characterization anti-Biofilm activity of PEP 102 (124µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) and tobramycin antibiotics(16µg/ml) on P. aeruginosa *CF isolates biofilms bio volume in CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. A) Cells with peptides only; B) Cells with Biofilm non treated; C) Treated with PEP 102 Submerge; D) Treated with PEP 102 Aerosol E) Treated with PEP 102 +I Submerge; F) Treated with PEP102 +I Aerosol; G) Treated with Tobramycin 16 µg/ml Submerge; H) Treated with Tobramycin 16µg/ml Aerosol*

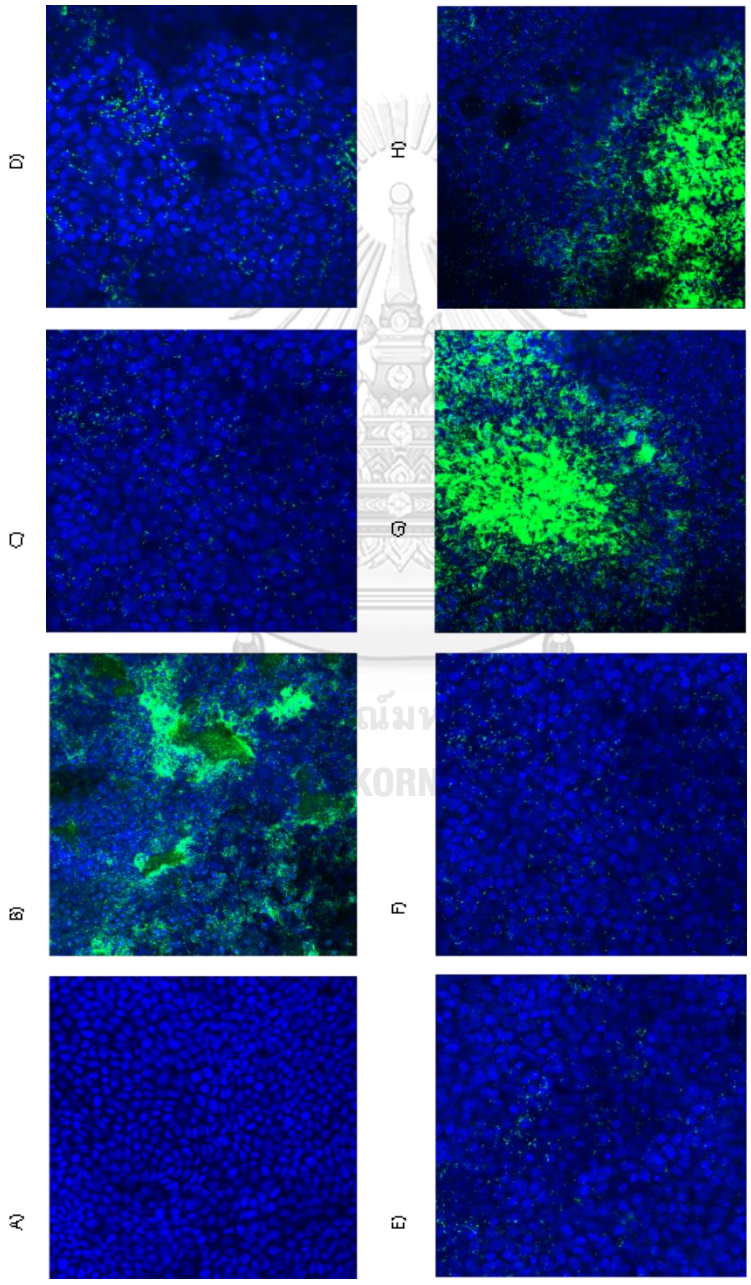


Figure 63 Characterization anti-Biofilm activity of PEP 102 (124µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) and tobramycin antibiotics(16µg/ml) on nontypeable H. influenzae *CF isolates biofilms bio volume in CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. A) Cells with peptides only ; B) Cells with Biofilm non treated; C) Treated with PEP 102 Submerge ; D) Treated with PEP 102 Aerosol E) Treated with PEP 102 +I Submerge; F) Treated with PEP102 +I Aerosol; G) Treated with Ciprofloxacin 2 µg/ml Submerge; H) Treated with Ciprofloxacin 2µg/ml Aerosol*

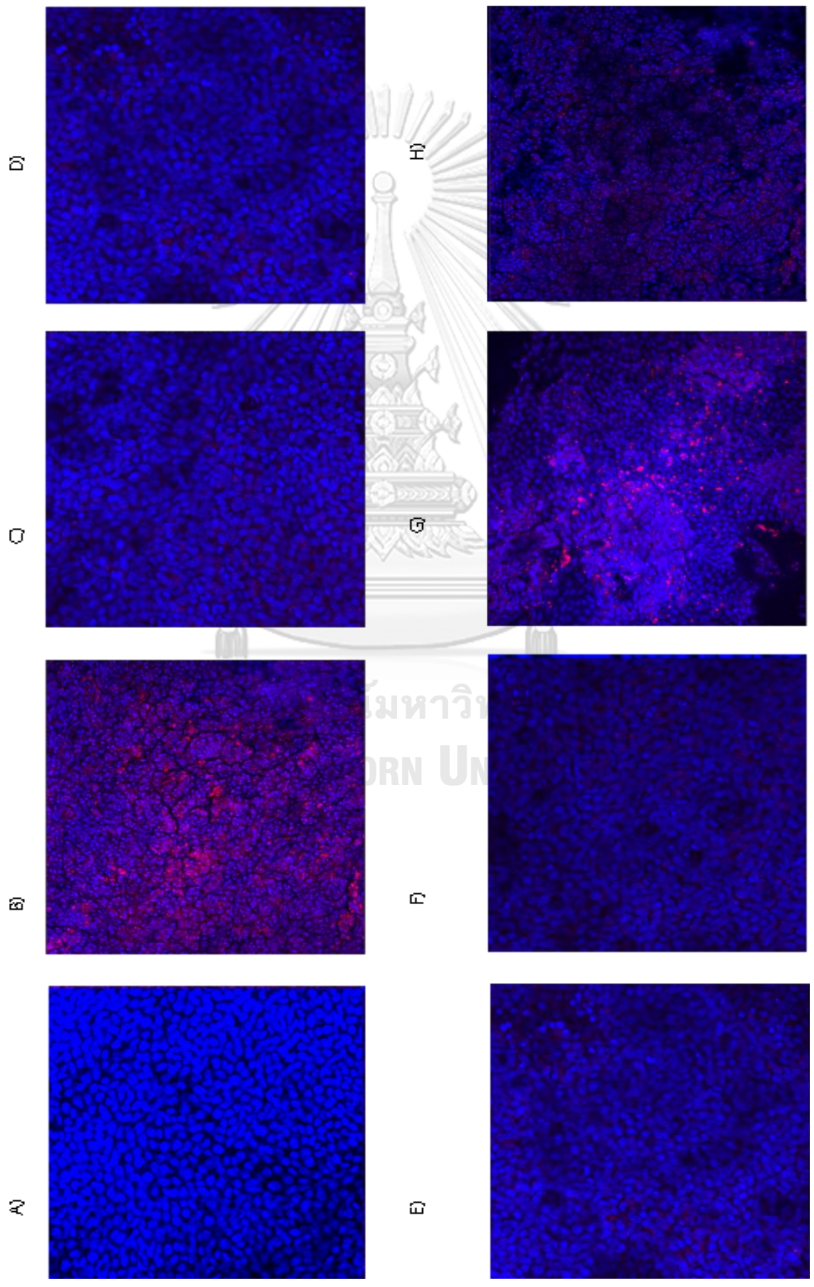


Figure 64 Characterization biofilm formation inhibitory activity of PEP 102 (16µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) on CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. low bacteria load of P. aeruginosa *CF isolates in A) Cells with non-treated; B) Cells with pre-treated PEP 102 Submerge; C) Cells with pre-treated PEP 102 Aerosol; D) Cells with pre-treated I Submerge E) Cells with pre-treated I Aerosol; F) Cells with pre-treated PEP 102 + I Submerge; G) Cells with pre-treated PEP 102 + I Aerosol; and nontypeable* H. influenzae *CF isolates H) Cells with non-treated; I) Cells with pre-treated PEP 102 Submerge; J) Cells with pre-treated PEP 102 Aerosol; K) Cells with pre-treated I Submerge L) Cells with pre-treated I Aerosol; M) Cells with pre-treated PEP 102 + I Submerge; N) Cells with pre-treated PEP 102 + I Aerosol;*

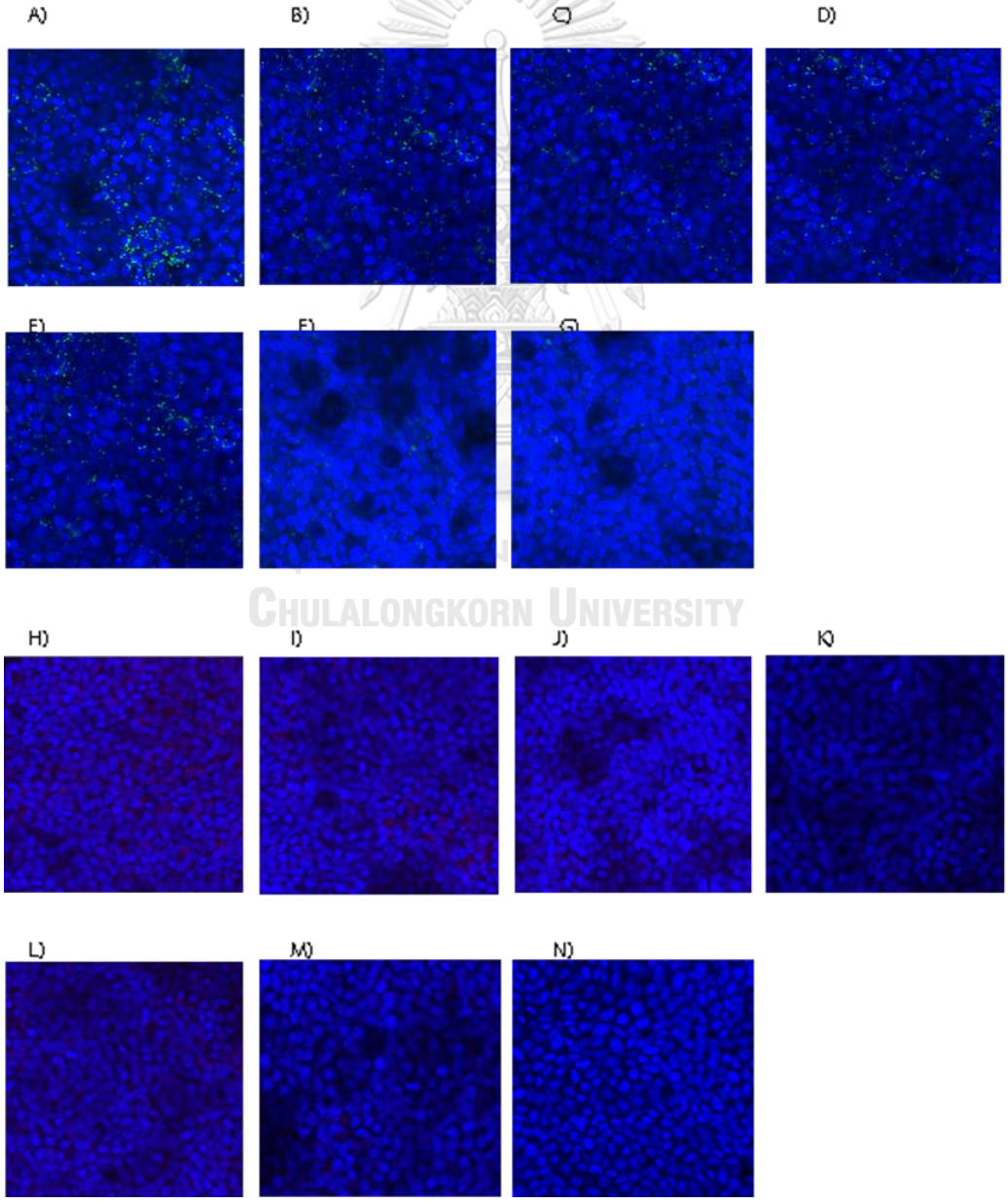


Figure 65 Characterization biofilm formation inhibitory activity of PEP 102 (16µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) on CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. S- Submerge A- aerosol

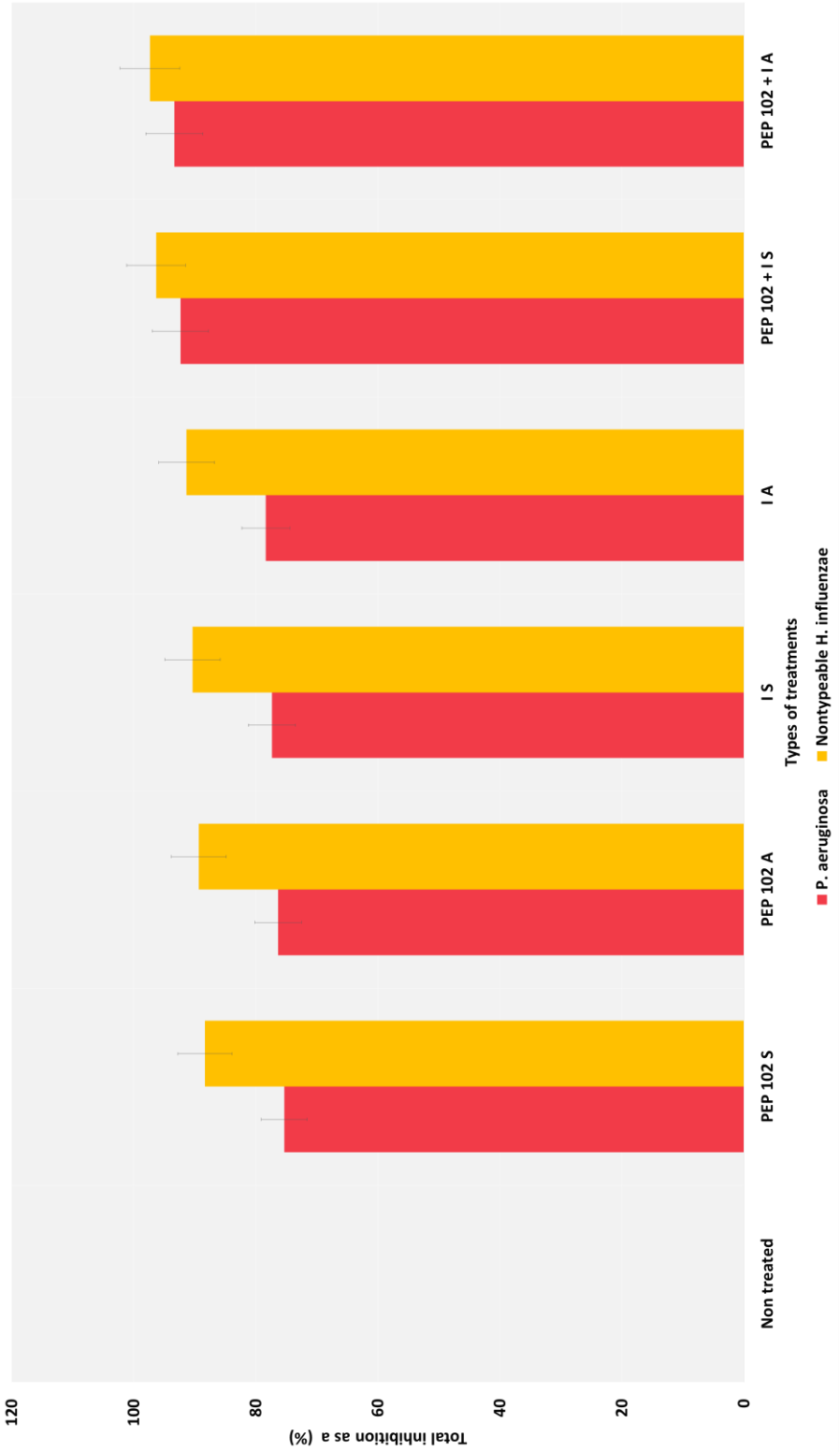


Figure 66 Characterization biofilm formation inhibitory activity of PEP 102 (16µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) on CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. P. aeruginosa *CF isolates with high bacteria load A) non-mucoid B) mucoid of* P. aeruginosa

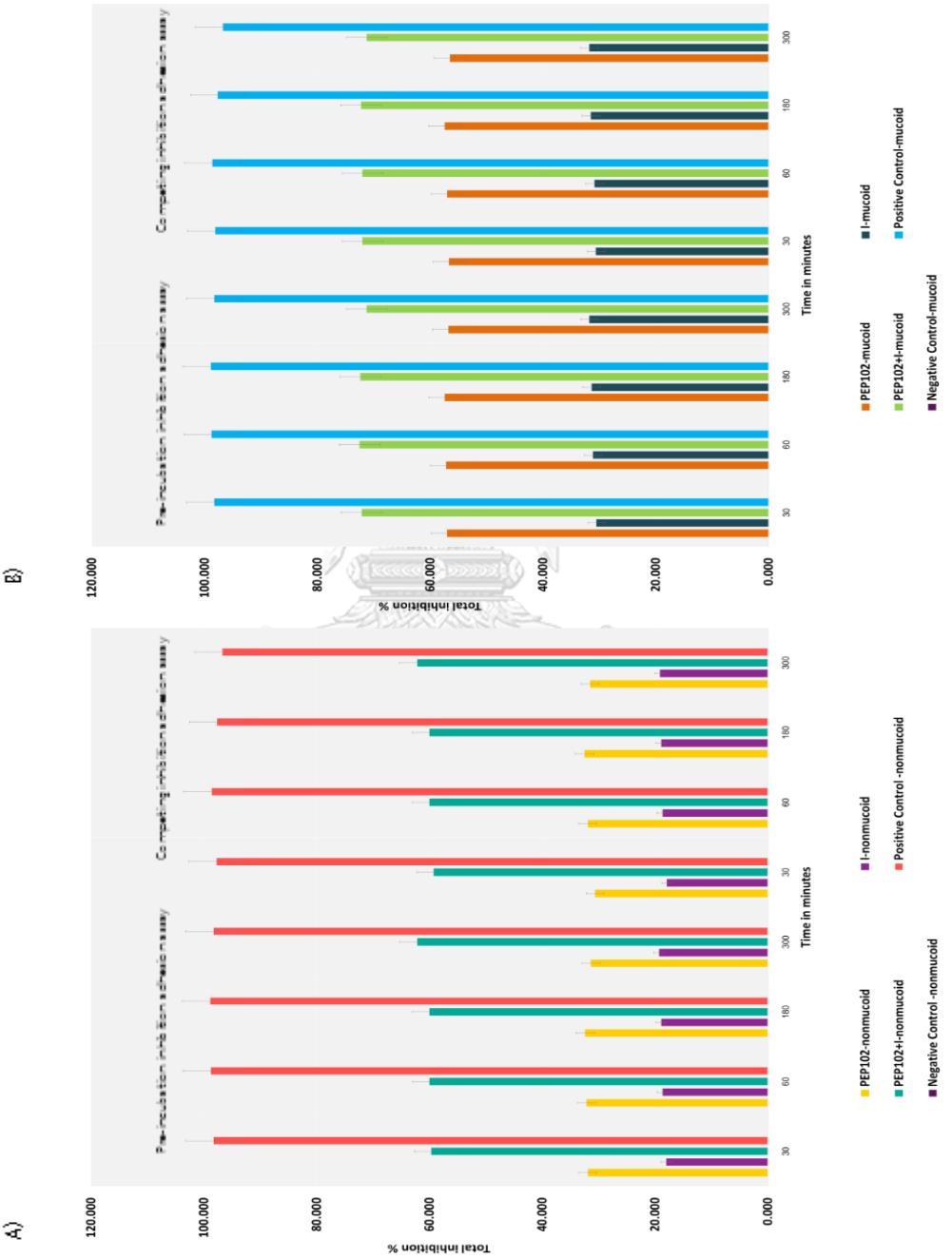
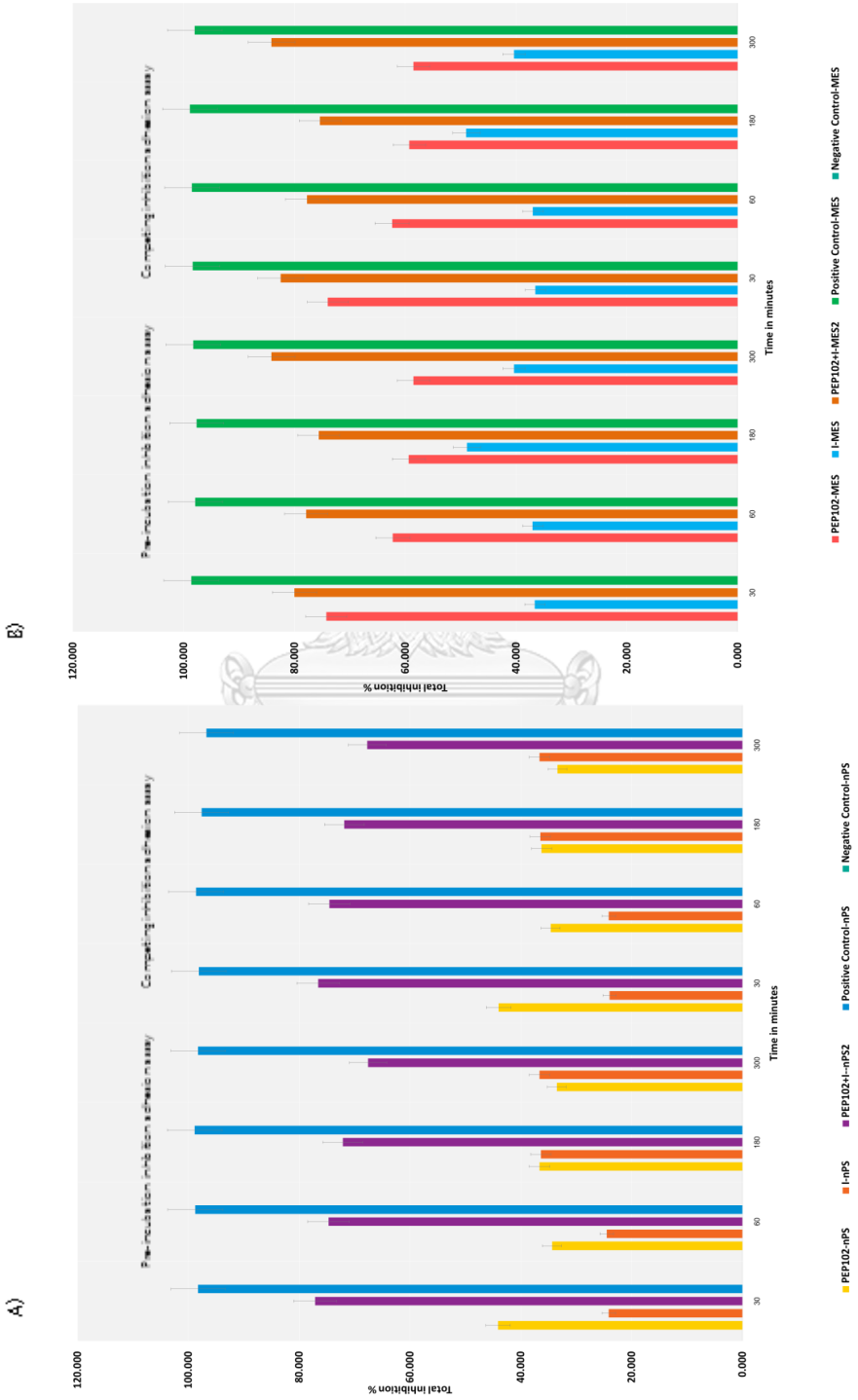
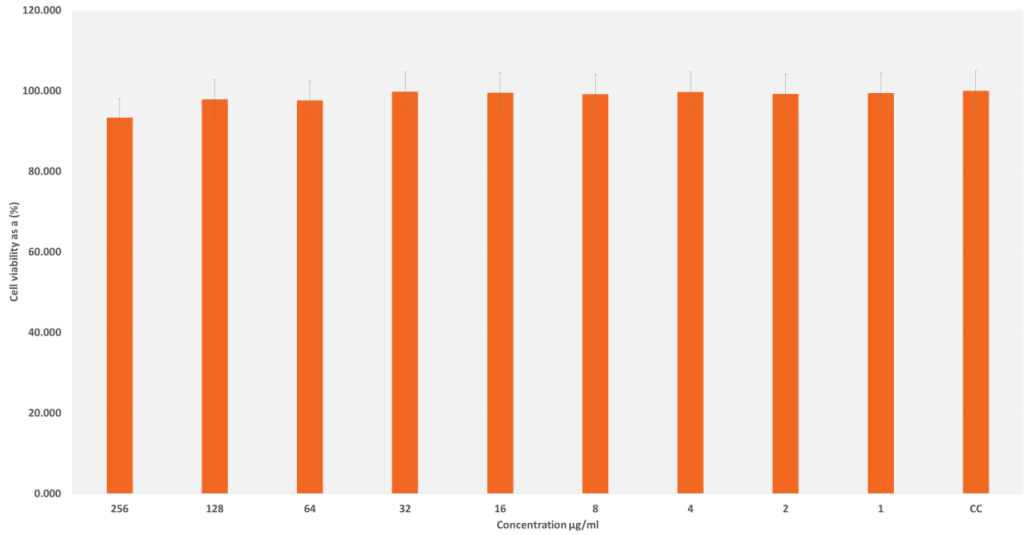


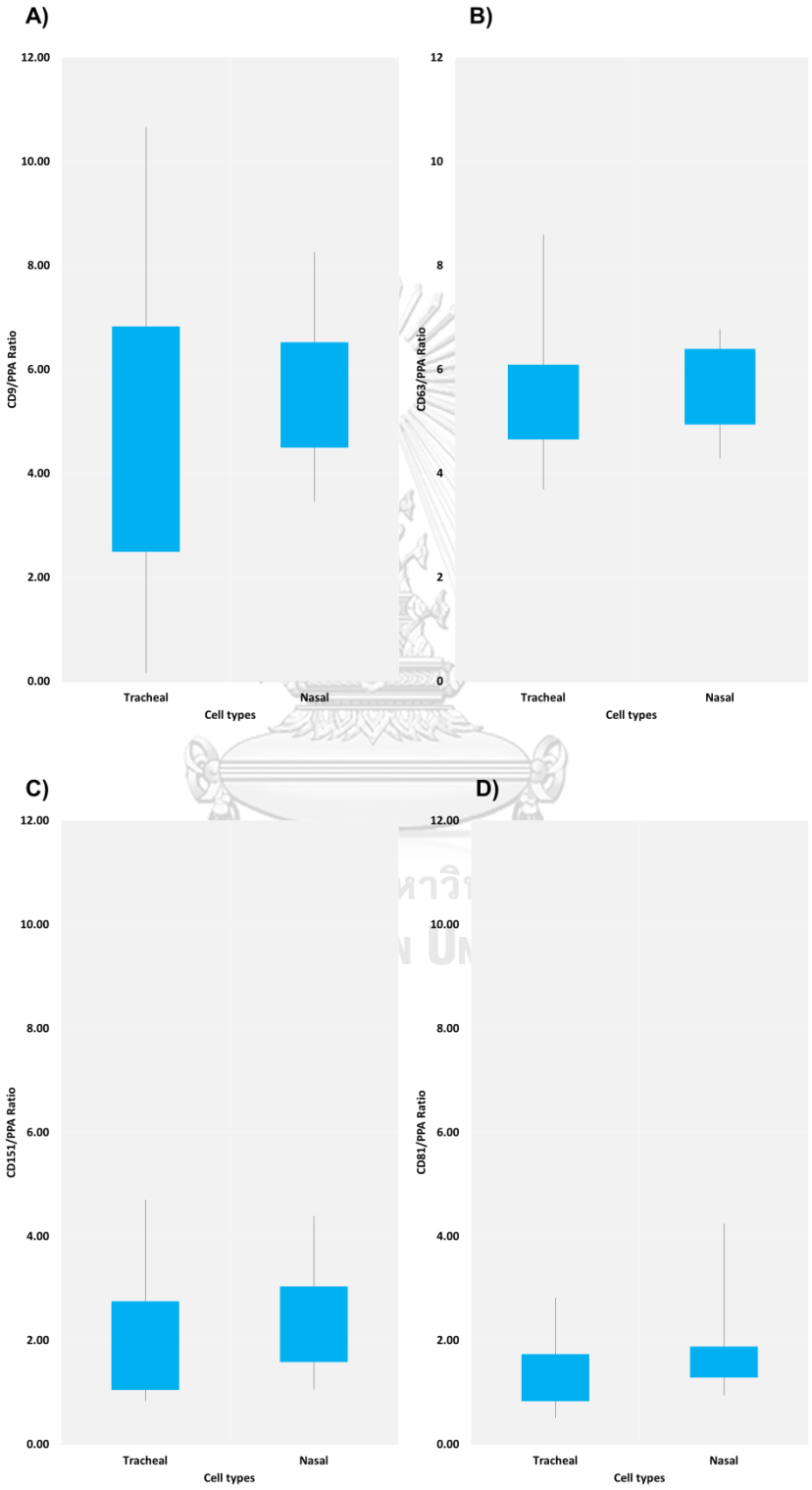
Figure 67 Characterization biofilm formation inhibitory activity of PEP 102 (16µg/ml) alone or together with Inhibitory peptide -I (200µg/ml) on CF air way primary epithelial cell culture biofilm model visualized by high resolution confocal scanning laser microscopy. nontypeable H. influenzae CF isolates with high bacteria load A) nasopharyngeal B) middle ear effusion nontypeable H. influenzae



Supplementary Figure 68 Characterization of cytotoxicity effect of Anti -Biofilm peptides PEP 102 on CF airway primary epithelial cells



Supplementary Figure 69 The expression levels of different tetraspanin receptors; CD9, CD63, CD81 and CD151 on CF airway epithelial cells.



Discussion

Quality of life in cystic fibrosis has been greatly improved by the use of antibiotics to treat lower airway disease and delay the progression from acute to chronic infection (10). However, significant challenges remain to be overcome due to biofilm infections. Once a patient becomes chronically infected by *P. aeruginosa* and nontypeable *H. influenzae*, the current treatment guidelines recommend long-term antibiotic therapy [11]. This has led to the development of antibiotic resistance due to selective pressure and sub-inhibitory local drug concentrations with in the biofilm [11]. In addition, the ability of *P. aeruginosa* and nontypeable *H. influenzae* to grow as a biofilm further increase its virulence and antibiotic tolerance [11]. Currently used antibiotics were unable to eradicate *P. aeruginosa* and nontypeable *H. influenzae* chronic biofilm infection, and the continuing amplified inflammatory response leads to declining lung function [11].

As stated above, the susceptibility of *P. aeruginosa* and nontypeable *H. influenzae* to antibiotic treatment can be greatly decreased in its sessile biofilm form. The biofilm inhibition and disruption activity of the currently used antibiotics were therefore investigated. Biofilms have been found to be resistant to most of them with higher concentrations, with many of them binding to the biofilm matrix components and deactivating them. Result in consistent with other clinical and *in vitro* studies that CF or respiratory isolates tend to form more biofilm matrix components and that significantly contributed for antibiotic tolerance [11, 13, 131, 132]. Furthermore, the treatment of

antibiotics together with GSH, DNase I, or EDTA able to eradicate the biofilm infections, allowing for targeted restoration of bactericidal activity and overcome the biofilm matrix components, similar with previous report.

However in those clinical isolates, some HDPs have been found to have significant anti-biofilm activity compare to antibiotic alone or together with GSH, DnaseI, or EDTA. HDPs alone that have similar activity to the penetrate biofilm structure and reduced the number viable bacterial cells less than 90%. Particularly, PEP 102 alone has been shown to inhibit the *P. aeruginosa* and nontypeable *H. influenzae* biofilm formation and significantly decreased the thickness of 24h biofilms pre-formed in CF air way epithelium. Also after treatment with PEP 102, the restoration of the epithelium integrity has improved significantly and previous studies unable to demonstrate such effects with either antibiotics or HDPs [11, 83, 89, 122, 132-134]. Similarly, when PEP 102 combine with tetraspanin CD 9 targeted inhibitory peptide have been shown to prevent biofilm formation >95% and disrupt those already established, with improved potency compared to other studies. The PEP 102 alone did not demonstrate significant activity against biofilm formation with relatively higher bacteria load. However, in combination with inhibitory peptide the biofilm formation prevention was in excess of 80% and the disruption of CD9 tetraspanin receptor may have likely contributed to this result. But, when presences of low bacteria lord both peptides able to achieved higher biofilm formation prevention alone or in combination for *P. aeruginosa* and

nontypeable *H. influenzae.* This emphasized the host pathogen interaction via CD 9 receptor play important role in biofilm formation [18, 93, 106]. Therefore, prevention of such interaction further enhanced the anti-biofilm therapy efficacy. Other mechanisms of inhibition, such as disruption of quorum sensing (QS) pathways, which have been described for LL-37 or other HDPs biofilm inhibition [89, 133] may also exist but were not investigated in this model. Given the nature of CF *P. aeruginosa* and nontypeable *H. influenzae* infections, the ideal PEP102 candidate would have significant activity against established biofilms or biofilm formation on respiratory epithelium. For our knowledge none of the previous studies abled to demonstrate such effects by HDPs using clinical isolates in CF primary cell models drive from patients with chronic infections [83, 99, 123-125, 133, 134].

In addition, the activity of PEP 102 to penetrate biofilm matrix has been shown under *in vitro* conditions that are representative of *in vivo* conditions in the CF lung, through the use of CF BAL fluid, mixed with mucin and minimum medium hydrogel (Mixture detail and technical details not shown due to IP restrictions). The concentration levels of PEP 102 to achieved maximum bacteria cell death (non-viable cells >90%) within the biofilm varied considerably in this study and the wide variation is consistent with what has been reported by other HDPs [83, 99, 123-125, 133, 134]. In addition, HDPs concentrations have also been varies between *P. aeruginosa* and nontypeable *H. influenzae*. These differences between in combination with the fact that the HDPs action was

depends on the diversity of the biofilm matrix components of particular bacteria strain, and demonstrate that needs to address when design the anti-biofilm therapies [4, 5, 135].

While CF BAL fluid, mixed with mucin and minimum medium hydrogel can be considered to be a close representation model of bronco alveoli micro environment in the CF lung. However, none of the previous studies have used such close environment to evaluate anti-biofilm activity of HDPs. In addition, both eDNA in biofilm matrix and mucins have been reported to increase the MIC values for synthetic HDPs such as HB43 [83, 99, 123-125, 133, 134](121). It has been previously observed that the bactericidal activity of LL-37 was inhibited by proteolytic degradation by neutrophil elastase and cathepsin D in CF BAL fluid (119) and that cationic HDPs bind to anionic components of BAL and CF sputum, such as F-actin, eDNA (77, 177), mucins (178), and glycosaminoglycans (119, 184). The use of close representation of bronco alveoli micro environment for testing in the present study, as evidenced by PEP 102 proteolytic stability in *P. aeruginosa* and nontypeable *H. influenzae* biofilm with significant potent activity after overnight incubation. In addition, used for CF derived primary cells with full differentiated epithelium demonstrated here for close represent in the CF lung environment, such as the excessive thick mucus secretion [11, 136]. The failure to achieve higher bacterial cell death within the biofilm in other tested short HDPs in the presence of CF BAL and mucins, may be related to the proteolytic stability or effect of eDNA in biofilm matrix and mucins [83, 99, 123-125, 133, 134]. In addition, when inhaled PEP 102 was able to preserve the

active against *P. aeruginosa* and nontypeable *H. influenzae* biofilm, and suitable HDP candidates for pulmonary delivery as inhalation to compatible with local lung environment [11]. For our knowledge none of the previous studies abled to demonstrate such effects by HDPs using clinically relevant models [83, 99, 123-125, 133, 134].

There is a need for novel alternative therapeutic agents that are highly active against CF biofilm infections and less vulnerable to development of resistance [11]. Moreover, the delivery of such molecules in high concentrations in the lower airway biofilm infections without the accompanying systemic effects and toxicity would be desirable [11]. The requirement for high concentrations can be met by the use of topical application such as inhaled or nebulized drug delivery [11]. Therefore, HDPs have the potential to meet the need for novel anti-biofilm therapy. Without novel therapy there is a risk that many of the patients with CF may be lost the lung function with increasing antimicrobial resistance [11]. Potential toxicity to host at high concentration, stability and water solubility are currently a limitation to the development of HDPs as anti-biofilm and antimicrobial agents. Here we demonstrate that used of computational prediction algorithm can successfully enhanced HDP anti-biofilm potency together with bactericidal activity and may reduce host cytotoxic effects.

Summary of the strengths and limitations

However, it must be noted that the microtiter plate method of biofilm analysis used here provides at most, a crude estimation of the anti-biofilm activity of HDPs as it does not accurately represent the conditions of the lung where biofilms form. However, the use of more sophisticated methods in our study such as CF BAL fluid, mixed with mucin and minimum medium hydrogel, CF primary epithelium, live/dead staining and super resolution confocal microscopy may provide a more accurate representation of the effects of HDPs on biofilms. That would also allow more detailed analyses of the biofilms such as thickness measurement, and 3D structural analysis compared to the previous studies. Ideally, before any HDP can be considered for biofilm infections it would have to demonstrate higher degree of activity against clinical isolates. The lack of appropriate clinical isolates to validate true efficacy of HDPs have been highlighted with most of the previous studies. While in PEP 102 the level of concentration in the biofilm can be further adjusted to low level, practically, this number is limited by epithelial biofilm infection model. The limited surface area of the airway primary epithelium further restricted the PEP 102 incubation time up to 6 hours and therefore the upper concentration limit (128 μg/ml) of the PEP 102 was selected. Therefore, the bactericidal activity and anti-biofilm of PEP 102, which can still have retained significant effect in a long time period with relatively low concentration. These results demonstrate that a high concentration may be desirable for the complete elimination of the biofilm infections as pulse

treatment. Alternatively, other approaches can be used for further reduction of PEP 102 concentration and enhanced the potency, such fold of linear peptide in to the different tertiary structures. In the present study, the PEP 102 was stable to human neutrophil elastase and bacteria elastase form *P. aeruginosa* and nontypeable *H. influenzae*, both of which are relevant to CF biofilms. It is possible that PEP 102 can be cleaved by other normal flora of host/bacterial enzymes and this may need to be determined using sophisticated *In vivo* models. If other off-target enzymes are identified that could potentially cleave PEP 102 in a disease model, they can be overcome through combing the PEP 102 with appropriate drug delivery molecules. It also necessary to further investigate the true effect of PEP 102 in term of bacteria gene expression to validate the possible anti-biofilm mechanism of PEP 102.

Conclusions

This HDP approach potentially provides the means to deliver effective bactericidal HDPs to the biofilm infections in clinically relevant concentrations, while limiting cytotoxicity distal to the host. HDP may provide novel alternative therapeutics in the prevention of chronic *P. aeruginosa* and nontypeable *H. influenzae* biofilm infections in respiratory tract.

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- FIGHTING PSEUDOMONAS AERUGINOSA AND NONTYPEABLE HAEMOPHILUS INFLUENZAE BIOFILMS WITH HOST DEFENCE PEPTIDE AS A NOVEL STEP FORWARD IN THE TREATMENT OF CHRONIC LUNG INFECTIONS. D.L. Wannigama, A. Kicic, C. Hurst, P.N. Monk, R.J. Storer, T. Chatsuwan, S.M. Stick, A. Cf -CHEST , Volume 155 , Issue 4 , 73A (2019).
- A SIMPLE ANTIBIOTIC SUSCEPTIBILITY ASSAY FOR PSEUDOMONAS AERUGINOSA AND ACINETOBACTER BAUMANNII BIOFILM COULD LEAD TO EFFECTIVE TREATMENT SELECTION FOR CHRONIC LUNG INFECTIONS. D.L. Wannigama, C. Hurst, L. Pearson, T. Saethang, U. Singkham-in, S. Luk-in, R.J. Storer, T. Chatsuwan, - CHEST , Volume 155 , Issue 4 , 77A (2019).
- HOST DEFENCE PEPTIDE AS AN INNOVATIVE NOVEL THERAPY FOR PSEUDOMONAS AERUGINOSA BIOFILM INFECTIONS IN THE CYSTIC FIBROSIS LUNG. Wannigama D, Kicic A, Hurst C, Monk P, Chatsuwan T, Stick S, AREST CF - (2019), Respirology, 24: 22-102. doi:10.1111/resp.13491 (2019).
- OVERCOMING BIOFILM MEDIATED RESPIRATORY INFECTIONS THROUGH EXPLOITATION OF PATHOGEN AND HOST-DIRECTED NOVEL PEPTIDES. Dhammika Leshan Wannigama, Cameron Hurst, Peter Monk, Anthony Kicic, Stephen Stick, Tanittha Chatsuwan - Respirology 23(S2): 67-68, (2018).
- Overcoming biofilm mediated respiratory infections through exploitation of pathogen and host-directed novel peptides. Dissertation - Dhammika Leshan Wannigama: 22410824; Outbound Research and Academic Exchange University of Western Australia (2018).

AWARD RECEIVED

- Outstanding Abstract and Research Award in ICIC & ISAAR 2019

Won the outstanding research and abstract award in ICIC (4th International Interscience Conference on Infection and Chemotherapy) & ISAAR (12th International Symposium on Antimicrobial Agents and Resistance) in Gyeongju, Korea from September 26 to 28, 2019

- Outstanding Post Graduate Student Award in Medicine 2018-2019

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- TSANZSRS 2019 Special Interest Group Best Research and Oral Presentation Award

Won the best research presentation award in Special Interest Group cell, Immunology & Molecular Biology of the Lung, The Annual Scientific Meeting for Leaders in Lung Health & Respiratory Science of The Thoracic Society of Australia and New Zealand (TSANZSRS 2019), Gold Coast Convention and Exhibition Centre, 29th March - 2 April 2019.

- Icomos2019 International Consortium of One Medicine One Science Travel Fellowship

Travel Fellowship to attend 4th International Conference on One Medicine

One Science, International Consortium of One Medicine One Science, Chiang Mai, Thailand - February, 2019

- 2018 APSR Young investigator Travel Awards - Asian Pacific Society of Respirology

Travel award to attend 23rd Congress of the Asian Pacific Society of Respirology, Taipei, Taiwan - November 2018

- Australian Awards postgraduate research exchange fellowship

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- 2018 Spring Outbound Doctoral Exchange Fellowship (Cross institutional Exchange)

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- Overseas Research Experience Scholarship for Graduate Student

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