UNIVERSITI PUTRA MALAYSIA

In Vitro STUDY OF EFFECTS OF ANTIBIOTICS ON Pseudomonas aeruginosa BIOFILM FORMATION

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

In Vitro STUDY OF EFFECTS OF ANTIBIOTICS ON *Pseudomonas aeruginosa* BIOFILM FORMATION

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February 2017

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Pseudomonas aeruginosa has gained increasing significance as opportunistic pathogens in hospitalised patients and reported to rapidly develop resistance to numerous antibiotic classes. Furthermore, the biofilm forming ability contributes to the high resistance of *P. aeruginosa* to antibiotics, making the treatment of biofilm infections more difficult. This study investigated the *in vitro* antiplanktonic and antibiofilm activities of potential classes of antimicrobial agents includes beta-lactam-beta-lactamase inhibitor combinations, cephalosporins, carbapenems, aminoglycosides, quinolone against established *P. aeruginosa* biofilms.

Henceforth, a total of seventy-six *P. aeruginosa* isolates were obtained from inpatients of Kuala Lumpur General Hospital. The antibiotic susceptibility profiles were determined by the minimum inhibitory concentration (MIC) using the broth microdilution method. Furthermore, biofilm forming ability was evaluated through crystal violet (CV) assay and Congo red agar (CRA). Besides, minimum biofilm inhibitory concentration (MBIC) were also assessed. A strong biofilm producing *P. aeruginosa* (PA-23) possessing biofilm-associated genes (*pelA*, *pslA*, *algD* and *cupA*) were chosen. This PA-23 isolate was used to observe the morphological effect of antibiotics on the biofilms using scanning electron microscopy (SEM) and determining the expression profiles of the aforementioned genes when treated with antibiotics.

Among the 76 *P. aeruginosa* isolates, 27 isolates (35.5%) were reported as strong biofilm producer and harboured all the biofilm-associated genes. In addition, the biofilm cells showed higher resistance than planktonic cells to different antibiotics and the ratio to MBIC to MIC were found to be highest for ceftazidime and gentamicin (32-64) folds. SEM results of antibiotics treated PA-23 showed

morphological changes appeared swelled, ruptured and shape alteration compared to the control which appeared normal bacilli shaped with smooth cell surfaces. The expression levels of the genes upon exposure to antibiotics showed varying expression of *pelA*, *pslA*, *algD* and *cupA* genes.

In conclusion, all the above data confirm and extend the notion that ciprofloxacin, piperacillin/tazobactam and ticarcillin/clavulanic acid were potent antibiotics with clear activity against *P. aeruginosa* biofilms.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KAJIAN *In Vitro* KESAN ANTIBIOTIK DALAM PEMBENTUKAN BIOFILM *Pseudomonas aeruginosa* BIOFILM

Oleh

PRIADARSSINI A/P JEEVAJOTHI NATHAN

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Pseudomonas aeruginosa telah mendapat peningkatan signifikasi sebagai patogen oportunis pada pesakit dimasukkan ke hospital dan dilaporkan boleh menahan rintangan hampir semua terapi antimikrobial. Tambahan pula, pembentukan biofilm munyukarkan keadaan untuk antibiotik menembusi dan menyumbang kepada rintangan yang tinggi *P. aeruginosa* kepada antibiotik membuat rawatan jangkitan biofilm lebih sukar. Kajian ini menyiasat *in vitro* aktiviti antiplanktonic dan antibiofilm agen antimikrob dari kumpulan (beta-lactam-beta-lactamase inhibitor combinations, cephalosporins, carbapenems, aminoglycosides, quinolone) yang berpotensi terhadap ditubuhkan biofilms *P. aeruginosa*.

Sebanyak tujuh puluh enam *P. aeruginosa* isolat-isolat diperolehi daripada pesakit dalaman Hospital Besar Kuala Lumpur. Profil kerentanan antibiotik ditentukan oleh kepekatan perencatan minimum (MIC) menggunakan kaedah broth microdilution. Tambahan pula, biofilm membentuk keupayaan dinilai melalui kristal ungu assay (CV) dan Congo agar merah (CRA). Selain itu, minimum biofilm kepekatan perencatan (MBIC) turut dinilai. *P. aeruginosa* yang kuat menghasilkan biofilm (PA-23) yang mempunyai gen biofilm-berkaitan (*pelA*, *pslA*, *algD* dan *cupA*) dipilih. PA-23 isolat telah digunakan untuk memerhatikan kesan morfologi antibiotik pada biofilm menggunakan mikroskop elektron pengimbas (SEM) dan menentukan profil ekspresi gen yang dinyatakan di atas apabila dirawat dengan antibiotik.

Antara 76 *P. aeruginosa* mengasingkan, 27 isolat (35.5%) didapati pengeluar biofilm kuat dan memendam semua gen biofilm-berkaitan. Di samping itu, sel-sel biofilm menunjukkan rintangan yang lebih tinggi daripada sel-sel plankton kepada antibiotik yang berbeza dan nisbah kepada MBIC kepada MIC didapati tertinggi

bagi ceftazidime dan gentamicin (32-64) lipatan. Keputusan SEM antibiotik dirawat PA-23 menunjukkan perubahan morfologi seperti lebam dan garis-garis pecah apabila dibandingkan dengan kawalan yang muncul riba biasa berbentuk dengan permukaan sel lancar. Tahap ungkapan gen apabila terdedah kepada antibiotik menunjukkan ungkapan yang berbeza-beza *pelA*, *pslA*, *algD* dan *cupA* gen.

Semua data di atas mengesahkan dan melanjutkan tanggapan bahawa ciprofloxacin, piperacillin/tazobactam dan ticarcillin/asid clavulanik adalah antibiotik kuat dengan aktiviti yang jelas terhadap *P. aeruginosa* biofilm.

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I certify that a Thesis Examination Committee has met on 13 February 2017 to conduct the final examination of Priadarssini a/p Jeevajothi Nathan on her thesis entitled "*In Vitro* Study of Effects of Antibiotics on *Pseudomonas aeruginosa* Biofilm Formation" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Declaration by Members of Supervisory Committee

This is to confirm that:

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LIST OF ABBREVIATIONS

%	Percentage
μg	Microgram
μL	Microliter
μm	Micrometer
μM	Micromolar
$A_{260/280}$	Ratio of the absorbance at 260 and 280 nm
ADH	L-arginine
ADI	Adipic acid
AFLP	Amplified fragment length polymorphism
AHL	Acyl-homoserine lactone
ARA	L-arabinose
ATCC	American Type Culture Collection
bp	Basepair
CAMHB	Cation adjusted Mueller Hinton broth
CAP	Capric acid
cDNA	Complementary DNA
cfu	Colony forming unit
CIT	Trisodium citrate
CLSI	Clinical and Laboratory Standards Institute
CP	Crossing point
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
dUTP	Deoxy uridine triphosphate

E	Efficiency
EG	Ecthyma gangrenosum
ESC	Esculin ferric citrate
g	Gram
g	Gravity
GEL	Gelatin
GLU	D-glucose
GNT	Potassium gluconate
HCl	Hydrochloric acid
ICU	Intensive care unit
LPS	Lipopolysaccharide
MAL	D-maltose
MAN	D-mannitol
MDR	Multidrug-resistant
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MBIC	Minimum biofilm inhibitory concentration
mL	Milliliter
MLT	Malic acid
mm	Millimeter
MNE	D-mannose
n	Number
Na_2CO_3	Sodium carbonate

NAG	N-acetyl-glucosamine
NaOH	Sodium hydroxide
NF	Necrotising fasciitis
ng	Nanogram
nm	Nanometer
NO_3	Potassium nitrate
NTC	Non-template control
^oC	Degree Celsius
OD	Optical density
ODc	Optical density cut-off value
PAC	Phenylacetic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qPCR	Real-Time PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
S	Subunit
SD	Standard deviation
SE	Standard error
TAE	Tris-acetate-ethylenediaminetetraacetic acid
Taq	*Thermus aquaticus*
Tm	Melting temperature
TRP	L-tryptophan
TSA	Tryptic Soy agar

U	Unit
URE	Urea
V	Voltage
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

Pseudomonas eruginosa is an ubiquitous, Gram-negative, nosocomial pathogen that has emerged globally as one of the most problematic pathogen for health care organisations due to its increasing mortality rate (Gomes *et al*., 2011). The increase rate of mortality is due to poor antibiotics therapy, prolonged hospitalisation and prolonged usage of mechanical ventilation as well as invasive devices (Woodford *et al*., 2011; Jacquelyn, 2013). Moreover, it is an important opportunistic pathogen that causes life-threatening nosocomial infections especially in patients who are immunosuppressed, patients with cystic fibrosis, diabetes mellitus, as well as severe burns (Keen *et al*., 2010). This bacterium is a particular challenge where it is responsible for diverse range of infections include, pneumonia, bacteremia, urinary tract infection and wound infection (Eberl & Tummler, 2004; Ryan *et al*., 2011). In most cases, infections are acquired after exposure to *P. aeruginosa* that persists on contaminated hospital equipment or by contact with healthcare personnel that have been exposed to this bacterium through contact with colonised patients (Centers for Disease Control & Prevention, 2014). This resilient microorganism is notorious for its ability to withstand desiccation and disinfection leading to their persistence in the hospital environment. Contributing to the endurance of *P. aeruginosa* is its tendency to form biofilm on abiotic surfaces, including, ventilators, catheters and other medical devices which enhancing bacterial transmission (Keene *et al*., 2004; Centers for Disease Control & Prevention, 2014).

P. aeruginosa clinical significance has been driven by its remarkable ability to acquire intrinsic antimicrobial resistance rapidly. Resistant towards the currently used antibiotics by *P. aeruginosa* have now been reported which limits treatment options and demonstrating an unanticipated event in health care settings that should be acted on immediately (Gomes *et al*., 2011; Samah *et al*., 2015). In addition, biofilm producing *P. aeruginosa* are able to survive in hostile environment because they are encased in extracellular polymeric substances (EPS) and they are well protected. This protective mode allows them to grow and colonise new niches under uncompromising environment (Hall-Stoodley *et al*., 2004; Ryder *et al*., 2007; Karatan & Watnick, 2009). Besides, this protective EPS matrix of *P. aeruginosa* resist the penetration of antibiotics (Drenkard & Ausubel, 2002).

P. aeruginosa has several proteins that associated in the formation biofilms, namely, polysaccharides encoded by *pslA*, *pelA*, *algD* and *cupA*. Polysaccharide synthesis locus (*Psl*) plays major role in biofilm formation by enhancing adhesion of *P. aeruginosa* on cell surface and intercellular which is important for initiation of biofilm and maintenance of it in a host (Ma *et al*., 2006; Byrd *et al*., 2009; Yang *et al*., 2011). It has a role in pathogenesis, protection against the immune system and as well as antibiotic resistance (Byrd *et al*., 2010; Yang *et al*., 2011). The first gene from the cluster which is *pslA*, shows strong similarities to that of UDP-glucose lipid

carriers. The expression from this particular gene comprising a coding region of 1,437 bp which restores the biofilm-forming phenotype, indicating that *pslA* is required for biofilm formation by *P. aeruginosa* (Overhage *et al*., 2005).

Pellicle polysaccharide (*Pel*) also has similar role as *psl* as it is required for formation of solid surface of biofilm, maintains cell to cell interaction in biofilm and enhance antibiotic resistance in biofilm (Friedman & Kolter, 2004; Colvin *et al*., 2010). *pelA* exhibits deacetylase activity, and that this activity is required for *pel*-dependent biofilm formation. This *pelA* gene is said to be expressed heavily (80%) amongst the biofilm producing isolates and those associated with the polysaccharide stage of biofilm development and maintenance (97%) (Sharma & Choudhury, 2015).

On the other hand, alginate protects the pathogen from harsh environments by forming extracellular matrix in biofilm in a host (Wozniak *et al*., 2003). This leads to persistence immune invasion (Leid *et al*., 2005). Besides, alginate also provides resistance to antibiotic and opsonophagocytosis (Simpson *et al*., 1993). *algD* is a tightly regulated gene encoding GDP-mannose dehydrogenase that is critical for *P. aeruginosa* alginate biosynthesis (Wozniak & Ohman, 1994).

As for chaperone-usher pathway-A (cupA) gene, it is required for biofilm formation on abiotic surfaces (Vallet *et al*., 2004) by encoding components and assembly factors of a putative fimbrial structure. Anaerobiosis is an inducer for the phase-variable *cupA* gene expression which raises the phase-variable expression of fimbrial genes that is important for biofilm formation by *P. aeruginosa* persisting in the largely anaerobic environment of the cystic fibrosis host lung (Vallet-Gely *et al*., 2007).

Previous studies found that *P. aeruginosa* infection especially involving biofilm producer is difficult to eradicate with the available antibiotics from the classes of beta-lactam-beta-lactamase inhibitor combinations, cephalosporins, carbapenems, aminoglycosides, and quinolone (Toussaint *et al*., 2015; Zeng *et al*., 2014; Teixeira *et al*., 2016). Studies involving simultaneous characterisation of planktonic and biofilms against available antibiotics is limited. In addition to these limitations, the roles played by *P. aeruginosa* biofilm associated virulence genes (*pslA*, *pelA*, *algD* and *cupA*) were also remains largely obscure. Hence, *in vitro* studies utilising *P. aeruginosa* planktonic and biofilm producers against available antibiotics as well as *P. aeruginosa* biofilm producer carrying aforementioned genes could shed some light in understanding these genes in antibiofilm resistance of currently available antibiotics.

Objectives of the study

General objective

To elucidate the expression of biofilm-associated genes (*pslA*, *pelA*, *algD* and *cupA*) and the resultant changes in the cellular morphology in strong biofilm producing *P. aeruginosa* when treated with antibiotics (pipercillin/tazobactam, ticarcillin/clavulanic acid, ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin and levofloxacin).

Specific objectives

1. To determine the *P. aeruginosa* isolates based on demographic data of the patients, source of isolates, antibiotic susceptibility patterns, ability to form biofilm and the presence of biofilm-associated genes.

2. To determine the minimum biofilm inhibitory concentration (MBIC) of antibiotics against strong biofilm producing *P. aeruginosa* isolates.

3. To determine a strain among the strong biofilm producing *P. aeruginosa* isolates that possesses biofilm-associated genes *pelA*, *pslA*, *algD* and *cupA*.

4. To examine the morphological changes of the selected *P. aeruginosa* (PA-23) strain when treated with antibiotics.

5. To determine the expression patterns of biofilm-associated genes (*pelA*, *pslA*, *algD* and *cupA*) when PA-23 treated with antibiotics.

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