UNIVERSITI PUTRA MALAYSIA

ANTIBIOTIC SUSCEPTIBILITY PATTERN, PIGMENT PRODUCTION AND MOLECULAR CHARACTERISATION OF Pseudomonas aeruginosa ISOLATES FROM CLINICAL AND SOIL SOURCES

SITI RUKHAIYAH BINTI SAFREN

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By

SITI RUKHAIYAH BINTI SAFREN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Master of Science

January 2015

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

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January 2015

Chairman : Mohd Nasir Bin Mohd Desa, PhD
Faculty : Medicine and Health Sciences

Pseudomonas aeruginosa (*P. aeruginosa*) is increasingly recognized as an emerging opportunistic pathogen of clinical relevance (Van Eldere, 2003) and well known for its adverse effects in the clinical setting. However, its adverse effects from the environmental source are still not much explored in comparison to those of clinical origin. This scope of research is also lacking in Malaysia, as there is the possibility of the environmental to be the initial source of infection. This study was conducted to isolate and analyse the difference of pigmentation production, antibiotic susceptibility patterns, virulence-related genes (*exoU, exoS, LasB, PlcH, AlgD* and *Nan1*) among 50 *P. aeruginosa* from diverse local clinical isolates (n = 30) obtained from a main Malaysian hospital and soil isolates (n =20) from random locations of Selangor state. *P. aeruginosa* isolates were further characterised for their DNA fingerprints using ERIC-PCR and BOX-PCR analysis. Only positive culture results for *P. aeruginosa* were included in this study. *P. aeruginosa* ATCC 27853 strain was also included as a reference strain (n = 51). Identification was done by conventional (different inducing media, selective media and differential media, gram staining, biochemical tests and antibiotic susceptibility test) and molecular approaches (PCR 16s rRNA *P. aeruginosa* specific primer, PCR virulence-related genes, ERIC-PCR and BOX-PCR). Based on the results obtained in this study, it showed that pyocyanin pigment were more common in the clinical isolates compared to soil isolates, and was known to contribute to resistance ability toward antibiotics. Pyocyanin pigment has been resolved to show cytotoxic properties and consequently help the multidrug resistance capability in clinical settings. Besides, it showed that high rate of antibiotic susceptible among 50 *P. aeruginosa* isolates from diverse local clinical and soil sources against antibiotic agents used, were in contrast with previous studies that showed high rate of antibiotic resistant in *P. aeruginosa* spp. Less than 30% of *P. aeruginosa* isolates from clinical sources were MDR compared to none from soil sources. Moreover, it indicated that the clinical isolates possess slightly higher virulence-associated genes compared to the soil isolates. *exoS, lasB* and *algD* genes grant high percentage of presence in both the soil and clinical isolates, meanwhile *nan1* gene grant the lowest percentage of presence in both the soil and clinical isolates. While only the

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clinical isolates grant high percentage of *plcH* gene possession, it contrasted to the soil isolates that grant low percentage of the same gene. In detailing with mutual relationship for genes (*exos* and*exoU*),the results showed that the soil isolates grant higher percentage of both *exoU* and *exoS*genes ($EXOS^+$ $EXOU^+$ or $EXOS^-$ $EXOU^-$) compared to the clinical isolates. In contrast, result with the clinical isolates grant a higher percentage of inverse relationship for both genes (*exos* and *exoU*) whereby one isolate that carried *exoU* does not carry *exoS* and vice versa ($EXOS^+$ $EXOU^-$ or $EXOS^-$ $EXOU^+$), but not both. In fact, this study also indicated that MDR isolates of *P. aeruginosa* from the clinical isolates have high possibility of *exoU* gene possession compared to other virulence-associated genes. By using genotypic approaches (BOX-PCR and ERIC-PCR), it showed comparison between the soil (environmental) and clinical isolates under BOX and ERIC DNA profiles. From the discriminatory indexes calculation in this study, it showed that the composite of BOX ERIC dendrogram highly discriminate *P. aeruginosa* isolates from the soil and clinical, and also ERIC dendrogram provides a more specific analysis in terms of distinctive clusters, which referred to the meaningful clusters based on pigmentation, antibiotic susceptibility and virulence-associated genes profiling results that were useful in clustering the heterogeneous group of *P. aeruginosa* isolates. Using both molecular approaches, all the 51 *P. aeruginosa* isolates from the soil and clinical sources (including positive control, *P. aeruginosa* ATCC 27853, clinical isolate) were able to be differentiated and it indicated diverse isolates. Besides, both methods are satisfactory for genetic analysis of local soil and clinical *P. aeruginosa* isolates. From testing both the molecular methods and based on the discriminatory index value, it showed that using only ERIC-PCR method is sufficient to discriminate and analyze local soil and clinical *P. aeruginosa* isolates, whereby this approach is more effective because low cost, shorter duration and less effort allocated.

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CORAK KECENDERUNGAN ANTIBIOTIK, PENGHASILAN PIGMENDAN PENCIRIAN MOLEKUL BAGI ISOLAT *Pseudomonas aeruginosa* DARIPADA SUMBER KLINIKAL DAN TANAH

Oleh

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Pseudomonas aeruginosa (*P. aeruginosa*) semakin dikenali sebagai satu patogen oportunis baru muncul yang dikaitkan dengan klinikal (Van Eldere, 2003) dan terkenal dengan kesan buruknya di persekitaran klinikal. Walau bagaimanapun kesan buruknya dari sumber alam sekitar masih tidak banyak diterokai berbanding yang berasal dari klinikal. Skop penyelidikan ini masih kurang di Malaysia, di mana alam sekitar mungkin juga boleh menjadi sumber awal jangkitan. Kajian ini dijalankan untuk mengasingkan dan menganalisa perbezaan dari segi pengeluaran pigmen, kadar kecenderungan antibiotik, menentukan gen yang dikaitkan merbahaya (*exoU, exoS, lasB, plcH, algD* dan *nan1*) di antara 50 *P. aeruginosa* daripada pelbagai isolat klinikal (n = 30) yang diperoleh daripada hospital utama di Malaysia dan isolat tanah (n = 20) tempatan daripada lokasi rawak di negeri Selangor. Isolat *P. aeruginosa* seterusnya telah dicirikan dengan cap jari DNA menggunakan analisis ERIC-PCR dan BOX-PCR. Hanya keputusan positif kultur bagi *P. aeruginosa* dimasukkan ke dalam kajian ini. Strain *P. aeruginosa* ATCC 27853 juga dimasukkan sebagai strain rujukan (n = 51). Pengenalpastian dilakukan melalui kaedah konvensional (berbeza media mendorong, media memilih dan media membezakan, pewarnaan gram, ujian biokimia dan ujian kecenderungan antibiotik) dan juga kaedah molekular (PCR 16s rRNA *P. aeruginosa* spesifik primer, PCR-berkaitan dengan gen yang merbahaya, ERIC-PCR dan BOX -PCR). Berdasarkan keputusan yang diperoleh dalam kajian ini, ia menunjukkan bahawa pigmen pyocyanin lebih lazim dalam isolat klinikal berbanding dengan isolat tanah, dan dikenali menyumbang kepada keupayaan menentang antibiotik. Pigmen pyocyanin menunjukkan sifat sitotoksik dan seterusnya membantu keupayaan rintangan terhadap pelbagai ubatan di persekitaran klinikal. Selain itu, ia menunjukkan bahawa kadar yang tinggi bagi kecenderungan antibiotik di antara 50 isolat *P. aeruginosa* daripada pelbagai sumber klinikal dan tanah menentang agen antibiotik yang digunakan, dimana keputusan ini bertentangan dengan kajian-kajian sebelum ini yang menunjukkan kadar rintangan tinggi terhadap antibiotik di kalangan *P. aeruginosa* spp. Kurang daripada 30% isolat *P. aeruginosa* dari sumber klinikal mempunyai MDR berbanding tiada langsung daripada sumber tanah. Selain itu, ia menunjukkan

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bahawa isolat klinikal memiliki lebih banyak gen yang mempunyai kaitan bahaya berbanding dengan isolat tanah. Gen *exoS, lasB* dan *algD* memberikan peratus yang tinggi di mana ia terdapat di dalam kedua-dua isolat tanah dan klinikal, manakala gen *nan1* memberikan peratus terendah di dalam kedua-dua isolat tanah dan klinikal. Gen *PlcH* hanya wujud dalam isolat klinikal dalam peratus yang tinggi, berbeza dengan isolat tanah yang memberikan peratus yang rendah bagi gen yang sama. Dalam pencirian dengan hubungan mutual di antara kedua-dua gen (*exoU* dan *exoS*), ia menunjukkan bahawa isolat tanah memberikan peratus yang tinggi memiliki kedua-dua gen *exoU* dan *exoS* ($EXOS^+$ $EXOU^+$ dan sebaliknya $EXOS^-$ $EXOU^-$), berbanding dengan isolat klinikal. Sebaliknya dengan keputusan isolat klinikal, di mana ia memberikan peratus yang tinggi bagi hubungan songsang untuk kedua-dua gen (*exoS* dan e*xoU*) di mana satu isolat yang membawa gen *exoU*, tidak akan membawa gen *exoS* dan sebaliknya ($exoU^+$ $exoS^-$/$exoU^-$ $exoS^+$), tetapi bukan kedua-duanya sekali. Malah, kajian ini juga menunjukkan bahawa isolat MDR bagi *P. aeruginosa* daripada isolat klinikal mempunyai kebarangkalian tinggi memiliki gen *exoU* berbanding dengan gen merbahaya yang lain. Dengan menggunakan kaedah molecular (BOX-PCR dan ERIC-PCR), ia menunjukkan perbandingan antara isolat tanah (alam sekitar) dan isolat klinikal melalui profil BOX dan ERIC DNA. Dari pengiraan indeks diskriminasi dalam kajian ini, ia menunjukkan bahawa dendrogram dari komposit BOX ERIC sangat mendiskriminasikan isolat *P. aeruginosa* daripada sumber tanah dan klinikal, dan juga dendrogram ERIC memberikan analisa yang lebih spesifik dari segi kelompok yang berbeza, di mana merujuk kepada kelompok yang bermakna berdasarkan keputusan daripada profil pigmentasi, kecenderungan antibiotik dan gen-dikaitkan merbahaya yang berguna dalam mengkelompokkan kumpulan heterogenus bagi isolat *P. aeruginosa*. Dengan menggunakan kedua-dua kaedah molekular, kesemua 51 isolat *P. aeruginosa* daripada sumber tanah dan klinikal (termasuk kawalan positif, *P. aeruginosa* ATCC 27853, isolat klinikal) dapat dibezakan dan juga menunjukkan ia terdiri daripada isolat yang pelbagai. Selain itu, kedua-dua kaedah ini adalah memuaskan untuk analisa genetik bagi isolat *P. aeruginosa* daripada sumber tanah dan klinikal tempatan. Dengan menguji kedua-dua kaedah molekular dan berdasarkan nilai indeks diskriminasi, ia menunjukkan bahawa dengan hanya menggunakan kaedah ERIC-PCR sudah memadai untuk membezakan dan menganalisa isolat *P. aeruginosa* daripada sumber tanah dan klinikal tempatan, di mana pendekatan ini lebih berkesan kerana kos yang rendah, masa yang singkat dan kurang usaha diperuntukkan.

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LIST OF ABBREVIATIONS

AlgD gene	GDP-mannose 6-dehydrogenase AlgD (alginate)-encoding
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BLAST	The Basic Local Alignment Search Tool
Bp	Base pairs
CA	Cetrimide Agar
DNA	Deoxyribonucleic acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
et al.	Others
ExoS	Exoenzyme S
ExoU	Exoenzyme U
G + C	Guanine and Cytosine
LasB	ElastaseLasB-encoding gene
MHA	Mueller Hinton Agar
MCA	Mac Conkey Agar
MDR	Multi Drug Resistance
NanI	Neuraminidase-encoding gene.
NCBI	National Center of Biotechnology Information
PCR	Polymerase Chain Reaction
P. aeruginosa	*Pseudomonas aeruginosa*
PAP	Pseudomonas agar P
PAF	Pseudomonas agar F
PlcH	Haemolytic phospholipase C precursor-encoding gene

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rRNA	Ribosomal RNA
TBE	Tris-borate-EDTA
UPGMA	Unweighted Pair Group Mathematical Averaging
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CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa is a gram negative, aerobic, rod-shaped bacterium with unipolar motility, nutritionally versatile and exhibiting intrinsic resistance to several antimicrobial agents (Pagani *et al*., 2004; Ryan and Ray, 2004). It is widespread in nature and inhabiting various environmental localities, hence commonly found at surface, soil, water, plant, animal, human, and frequently isolated from the hospital environments and clinical specimens (Palleroni, 1992; Ryan and Ray, 2010). The remarkability of *P. aeruginosa* to adapt to a wide variety of surfaces is possibly due to its extensive genetic versatility that ensures its pathogenic potential (Finnan *et al*., 2004). The genomic diversity may allow this bacterium to expand its pathogenic potential (Head and Yu, 2004). Horizontal gene transfer and the highly conserved genome in *P. aeruginosa* are responsible in the adaption of the bacterium in various habitats (Mathee et al., 2008).

P. aeruginosa is a ubiquitous bacterium and an opportunistic human pathogen (Wunderink and Mendoza, 2007) which can cause severe effect or death to the person that have severe tissue damage or have compromised immune system (Van Delden and Iglewski, 1998). In hospital settings, *P. aeruginosa* is one of the common nosocomial pathogens causing ventilator-associated pneumonia, burn, catheter-related, urinary tract and blood infections (Martin and Yost, 2011). *P. aeruginosa*is highly responsible for nosocomial infection in patients (Taneja *et al*., 2009) and once the infection begins, it is frequently life threatening and often challenging to treat (Brewer *et al.,* 1996). *P. aeruginosa* nosocomial pneumonia is associated with crude mortality rate as high as 70% overall (Pennington, 1995).

P. aeruginosa from the environmental source might lead to various kinds of infection, especially in immunocompromised patients. In jacuzzis, existence of *P. aeruginosa* caused health effects such as folliculitis otitis externa, infections in the urinary system, respiratory tract and wounds; infections in cornea may also be linked to the use of spas. In pools, *P. aeruginosa*has been reported asrelated with the otitis externa, folliculitis dermatitis, conjunctivitis and pneumonitis (WHO, 2006).

P. aeruginosa have special mechanisms that provide them the resistance to most antimicrobial treatments such as efflux pumps system, reducing cell membrane permeability and inactivation of the antibiotics (Matsuo *et al*., 2004). Most of these mechanisms act together with the assistance of biofilm formation against antibiotic drugs. Other than intrinsic resistance, *P. aeruginosa* effortlessly form acquired resistance either by transformation in chromosomally-encoded genes or by the horizontal gene transfer of antibiotic resistance determinants (Spencer *et al*., 2003). Consequently, the antibiotic resistant *P. aeruginosa* is increasing due to multiple actions of various mechanisms (Van Eldere, 2003).

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P. aeruginosa has a variety of virulence factors including its component structures, extracellular toxin and enzymes secretion (Apodaca *et al*., 1995). Virulence factors can cause severe damage by different mechanisms such as exoenzyme S (*exoS*), exoenzyme U (*exoU*), elastase LasB (*lasB*), haemolytic phospholipase C precursor (*plcH*), GDP mannose 6-dehydrogenase (alginate) (*algD*) and neuraminidase (*nanl*). Exoenzyme S is encoded by the exoS gene, a protein with ADP-ribosyltransferase that is concealed by a type-III secretion system straight into the cytosol of epithelial cells, which then disrupts the normal organisation of eukaryotic cell's cytoskeleton (Shaver and Hauser, 2004; Sundin *et al*., 2004). Exo U is encoded by the exoU gene, a phospholipase that destroys the eukaryotic cell membrane (Tamura *et al*., 2004). LasB elastase is a zinc metalloprotease encoded by the lasB gene.It also has an elastolytic action on lung tissue and the phospholipids contained in pulmonary surfactants may be hydrolysed by phospholipase encoded by plcH (PLC-H) (Konig *et al*., 1997). The growth of mucoid colonies of *P. aeruginosa* consisted of alginates, involving algD genes, protects the bacteria from the host's immunologic response and from antibiotics,thus contributing to chronic pulmonic inflammation (Govan and Deretic, 1996). An extracellular neuraminidase, nan1 gene, is believed to play a crucial role in implantation of the bacteria (Davies *et al*., 1999).

Therefore, it is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics (Hanberger *et al*., 1999; Fluit *et al*., 2000), and to produce a variety of virulence factors (Mitov *et al*., 2010).

1.1 Justification of this study

Pseudomonas aeruginosa is an opportunistic human pathogen (Wunderink and Mendoza, 2007) widespread throughout the environment (Hauser *et al*., 2002) that is responsible for a range of infections in individuals (Tummler *et al*., 1997). *P. aeruginosa* is increasingly recognized as an emerging opportunistic pathogen of clinical relevance (Van Eldere, 2003) and well known for its adverse effects in the clinical setting. However,the adverse effects of *P. aeruginosa*from environmental sourceswere still not much explored in comparison to those of clinical origin.This scope of research is also lacking in Malaysia, as there is a possibility that the environment can also be the initial source of infection.

This study was first started by assessing the conventional and molecular approaches in identifying *Pseudomonas aeruginosa* isolates from clinical and soil sources. So far, only clinical isolates have been studied extensively; the studies on isolation of *P. aeruginosa* from soil sources are much less extensive, especially in Malaysia. Hence, this study was further conducted to make data comparison between diverse local soil and clinical isolates based on pigmentation, distribution of antibiotic susceptibility patterns, virulence-related genes (*exoU, exoS, LasB, PlcH, AlgD* and *Nan1*) among local clinical isolates obtained from a major Malaysian hospital and soil isolates from random locations of Selangor state. *P. aeruginosa* isolates were further characterized

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for their DNA fingerprints using ERIC-PCR and BOX-PCR analysis.ERIC and BOX methods were chosen in this study for reasons such as specific primers for PCR amplification, which is a highly conserved central inverted repeat that is located in the extragenic regions of the bacterial genome unique to the enterobacteriae group (Sharples and Lloyd, 1990; C. Hulton *et al*., 1991; Zulkifli *et al*., 2009). By using specific primers, segments of DNA that were amplified are specific and known thus the results are more reliable. Furthermore, only 1-2 specific primers were used for PCR amplification, which gives the benefit of low expense. These methods are sensitive variant of the DNA amplification method, rapid, highly discriminatory and highly reproducible assay which proved to be a powerful surveillance tool for typing *Pseudomonas aeruginosa* isolates and useful for epidemiological studies of members of this genus; it can also determine the possibility of clonality in the bacteria (Gillings and Holley, 1997; Ran Janam *et al*., 2011; Wolska *et al*, 2011). However, other method such as RAPD (Random Amplification of Polymorphic DNA) was not chosen for few reasons, such as single short primer and none of the specific primers are required. Therefore, segments of DNA that are amplified are random, which means unknown segments of DNA will be amplified, showing DNA polymorphism. Furthermore, set of primers (> 3 primers) were used for PCR amplification, which contributes to higher expense compared to using BOX and ERIC methods. Besides, the results were not as sensitive as variant of the DNA amplification method and less discriminatory than using BOX and ERIC methods. It is interesting to know the inter species differentiation, their relatedness and to seek for any regularities or unique features among *P. aeruginosa* from clinical and soil source for better understanding of its molecular epidemiology. Knowing the pigmentation, drug resistance pattern, possession of virulence-associated genes and genotypic relatedness within these two sources (soil and clinical) provide useful information to determine the most efficacious treatment forpatients having *P. aeruginosa* infections and as a practical importance to controlling sources of infection. Therefore, this study can provide knowledges on difference of *P. aeruginosa* between the clinical and soil isolates.

1.2 Objective of this study

The hypothesis of this study is there is significant difference between clinical and soil isolates of *P. aeruginosa* with respect to pigmentation, antimicrobial susceptibility, virulence genes, phenotypic and genotypic features methods.

The objectives put forward are:

General Objective; to isolate and analyse the differences of *P. aeruginosa* from diverse local clinical and soil isolates using phenotypic and molecular approaches.

Specific Objectives;

1. To evaluate antibiotic susceptibility from diverse local clinical and soil isolates of *P. aeruginosa* by disk diffusion method.

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2. To assess pigment production of clinical and soil isolates using different inducing media.

3. To detect presence of important virulence genes of *P. aeruginosa* from the clinical and soil isolates by PCR.

4. To characterize the genomic patterns of the clinical and soil isolates of *P. aeruginosa* by ERIC-PCR and BOX-PCR methods.

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