MOLECULAR CHARACTERISATION OF EXTENDED-SPECTRUM BETA-LACTAMASE- AND CARBAPENEMASE- PRODUCING *Pseudomonas aeruginosa* FROM A TERTIARY HOSPITAL IN MALAYSIA

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MOLECULAR CHARACTERISATION OF EXTENDED-SPECTRUM BETA-LACTAMASE- AND CARBAPENEMASE- PRODUCING *Pseudomonas aeruginosa* FROM A TERTIARY HOSPITAL IN MALAYSIA

ABSTRACT

Pseudomonas aeruginosa infections are responsible for high morbidity and mortality rates globally. Increasing resistance towards β-lactams, especially carbapenems pose a serious therapeutic challenge. However, the multilocus sequence typing (MLST) of extended-spectrum beta lactamase (ESBL)- and carbapenemase-producing clinical *P. aeruginosa* have not been reported in Malaysia. In addition, few studies in Malaysia reported characterisation of *P. aeruginosa* from hospital environmental sources. The objectives of this study were to determine the antibiotic susceptibility profiles, resistance genes, pulsotypes and sequence types of clinical and environmental *P. aeruginosa* from a tertiary hospital in Malaysia. These characteristics were analysed by disk diffusion, minimum inhibitory concentration, PCR, pulsed-field gel electrophoresis (PFGE), and MLST for 199 non-replicate clinical strains and 29 environmental strains. The 29 environmental strains were isolated from a total of 358 swab and fluid samples from healthcare workers' hands, frequently touched surfaces, medical equipment, patients' immediate surroundings, ward sinks and toilets and solutions or fluids of 12 selected wards. Less than 90% of the 199 clinical strains were susceptible towards the carbapenems and piperacillin-tazobactam, whilst ≥ 90% of the strains remained susceptible to all other classes of antimicrobial agents tested. All 29 environmental strains were susceptible to antibiotics tested. The 12 multidrug resistant strains displayed high level resistance to cephalosporins (48 to ≥ 256mg/L), and carbapenems (4 to 32 mg/L). Eleven clinical strains harboured the class 1 integron containing $bla_{\text{GES-13}}$, $bla_{\text{VIM-2}}$, $bla_{\text{VIM-6}}$, $bla_{\text{OXA-10}}$, *aacA(6')-Ib, aacA(6')-II, aadA6* and *gcuD* gene cassettes. The extra-integron

genes, *bla*$_{\text{GES-20}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{VIM-2}}$, and *bla*$_{\text{VIM-11}}$ were also found. The top three sources of clinical strains were sputum (25.6%), wound swab (16.6%), and tracheal aspirate (15.6%); the majority of which were isolated from patients in the medical wards (30.2%), surgical wards (16.6%), and the ICU (13.6%). Distribution of the environmental *P. aeruginosa* were mostly from moist and semi aqueous environments of handwashing sinks and toilets. The PFGE method subtyped the strains from the NICU into two major clusters and this finding agreed with the MLST data. This implies that there was an undetected outbreak of antibiotic susceptible *P. aeruginosa* clones at the time of sampling. Based on the PFGE analysis, strains were shown to be genetically heterogeneous with multiple subtypes of *P. aeruginosa* persisting in the different locations or wards in the hospital. The investigation of the genetic linkage for drug resistant international lineages is better mapped via MLST. The STs 235, 809, and 1076 clonal clusters consisted of MDRPA clinical strains. *P. aeruginosa* ST111 and ST235 strains were previously reported to be multidrug or extensively-drug resistant high-risk international clones found in France, Germany, Japan, Spain and Belgium. Overall, the Maximum Likelihood (ML) tree showed concordance in the clustering of clinical and environmental strains having the same STs, and PFGE clusters implying that both subtyping methods are useful for the investigation of the genetic relatedness *P. aeruginosa* lineages. This is the first report of the *bla*$_{\text{GES-13}}$ and *bla*$_{\text{GES-20}}$ ESBL-encoding gene variants and novel sequence types (STs 2329, 2335, 2337, 2338, 2339, 2340, and 2341) of *P. aeruginosa* in Malaysia.

Keywords: Antimicrobial resistance, MLST, PFGE, *Pseudomonas aeruginosa*, resistance genes.

PENCIRIAN MOLEKULAR STRAIN-STRAIN *Pseudomonas aeruginosa* YANG MENGHASILKAN EXTENDED-SPECTRUM BETA-LACTAMASE DAN CARBAPENEMASE DARIPADA SEBUAH HOSPITAL TERTIER DI MALAYSIA

ABSTRAK

Jangkitan *Pseudomonas aeruginosa* bertanggungjawab untuk kadar morbiditi dan kematian yang tinggi di seluruh dunia. Peningkatan kerintangan terhadap *β-lactam*, terutamanya *carbapenem* menimbulkan cabaran rawatan yang serius. Walau bagaimanapun, *multilocus sequence typing* (MLST) *P. aeruginosa* klinikal yang menghasilkan *extended-spectrum beta-lactamase* (ESBL) dan *carbapenemase* belum pernah dilaporkan di Malaysia. Selain itu, kajian dan pencirian *P. aeruginosa* dari sumber persekitaran dalam hospital di Malaysia jarang dilaporkan sebelum ini. Objektif kajian ini adalah untuk menentukan profil kerintangan antibiotik, gen rintangan, pulsotip dan *sequence type P. aeruginosa* klinikal dan persekitaran dari sebuah hospital tertier di Malaysia. Ciri-ciri ini dianalisa melalui *disk diffusion, minimum inhibitory concentration,* PCR, *pulsed-field gel electrophoresis* (PFGE), dan MLST untuk 199 strain klinikal dan 29 strain persekitaran. Strain persekitaran (n = 29) telah diasingkan daripada sejumlah 358 sampel swab dan cecair dari tangan kakitangan perubatan, permukaan wad yang kerap disentuh, peralatan perubatan, persekitaran sekeliling pesakit, sinki dan tandas wad, serta cecair dari 12 wad yang terpilih. Kurang daripada 90% daripada strain klinikal adalah sensitif kepada *carbapenem* dan *piperacillin-tazobactam*, manakala ≥ 90% daripada strain sensitif kepada semua kelas antibiotik yang diuji. Semua 29 strain persekitaran sensitif kepada antibiotik yang diuji. Strain *multidrug* (n = 12) MDRPA menunjukkan rintangan tahap tinggi kepada *cephalosporins* (48 hingga ≥ 256mg/L), dan *carbapenem* (4 hingga 32 mg/L). Sebelas strain klinikal membawa integron kelas 1 yang mengandungi kaset gen-gen *bla*$_{\text{GES-13}}$, *bla*$_{\text{VIM-2}}$, *bla*$_{\text{VIM-6}}$, *bla*$_{\text{OXA-10}}$, *aacA(6')-Ib, aacA(6')-*

II, aadA6 dan *gcuD*. Gen luaran integron, *bla*$_{\text{GES-20}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{VIM-2}}$, dan *bla*$_{\text{VIM-11}}$ juga dijumpai. Tiga sumber utama strain klinikal adalah sputum (25.6%), swab luka (16.6%), dan aspirat trakea (15.6%); kebanyakannya diasingkan daripada pesakit di wad perubatan (30.2%), wad pembedahan (16.6%), dan ICU (13.6%). Pengagihan *P. aeruginosa* persekitaran didapati dari permukaan yang lembap dan separa berair seperti sinki dan tandas. Kaedah PFGE *subtype* strain dari NICU ke dalam dua kluster utama dan hasil ini dipersetujui dengan data MLST. Ini menunjukkan bahawa terdapat wabak *P. aeruginosa* yang sensitif antibiotik, yang tidak terkesan pada masa pensampelan. Berdasarkan analisis PFGE, terdapat pelbagai *subtip P. aeruginosa* yang *heterogeneous* secara genetik yang dipencilkan dari lokasi atau wad berlainan. Siasatan perkaitan genetik bagi keturunan strain antarabangsa yang rintang antibiotik lebih jelas dicoretkan melalui MLST. ST 235, 809, dan 1076 terdiri daripada strain klinikal MDRPA. *P. aeruginosa* ST111 dan ST235 sebelum ini dilaporkan sebagai klon antarabangsa yang berisiko tinggi dan rintang antibiotik di Perancis, Jerman, Jepun, Sepanyol dan Belgium. Secara keseluruhannya, pokok *Maximum Likelihood* (ML) menunjukkan keserasian antara klustering strain klinikal dan persekitaran yang mempunyai ST yang sama dengan kluster PFGE. Ini menunjukkan bahawa kedua-dua kaedah *subtyping* berguna untuk penyiasatan perkaitan genetik *P. aeruginosa*. Ini adalah laporan pertama varian ESBL *bla*$_{\text{GES-13}}$ dan *bla*$_{\text{GES-20}}$ dan ST baru (ST 2329, 2335, 2337, 2338, 2339, 2340, dan 2341) *P. aeruginosa* di Malaysia.

Kata kunci: Kerintangan antibiotik; MLST; PFGE; *Pseudomonas aeruginosa*; gen rintangan

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LIST OF SYMBOLS AND ABBREVIATIONS

3'-CS	:	3' conserved segment
5'-CS	:	5' conserved segment
≥	:	Equivalent or greater than
<	:	Less than
%	:	Percent
AST	:	Antibiotic susceptibility test/testing
bp	:	base pairs
BURST	:	Based upon related sequence types
CAO	:	CHROMagar Orientation
CF	:	Cystic fibrosis
CHEF	:	Contour-clamped homogenous fields
DNA	:	Deoxyribonucleic acid
ESBL	:	Extended-spectrum beta-lactam/lactamase
et al.	:	et alibi
MDRPA	:	Multidrug resistant *Pseudomonas aeruginosa*
MIC	:	Minimum inhibitory concentration
ML	:	Maximum likelihood
MLST	:	Multilocus sequence typing
P. aeruginosa	:	*Pseudomonas aeruginosa*
PCR	:	Polymerase chain reaction
PFGE	:	Pulsed-field gel electrophoresis
ST	:	Sequence type
USA	:	United States of America

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Pseudomonas aeruginosa, an ubiquitous aerobic Gram negative bacillus, is inherently resistant to many antimicrobial agents and tolerant to disinfectants (Deplano et al., 2005). It has been implicated in severe nosocomial infections such as bacteraemia, pneumonia, post-surgical infections and urinary tract infections among immunocompromised patients (United States of America, Centers for Disease Control and Prevention; Lister et al., 2009). The extraordinary ability of the pathogen to acquire resistance to multiple classes of antimicrobial agents through mobile genetic elements or chromosomal mutations poses a serious therapeutic challenge in treating both community and nosocomial acquired infections (Glupczynski et al., 2010; Livermore, 2002). Infections associated with the drug resistant *P. aeruginosa* significantly increase surgical intervention rates, duration of hospitalization, treatment costs, and mortality and morbidity rates, thus adversely affecting overall patient outcomes (Lister et al., 2009).

In the USA, an estimated 51,000 *P. aeruginosa* infections were reported every year and 13% of these were caused by multidrug resistant (MDRPA) strains (United States of America, Centers for Disease Control and Prevention). MDRPA are resistant to ≥ 1 antimicrobial agent from ≥ 3 antimicrobial classes according to Magiorakos et al. (2012) and classified as a serious threat by the United States of America, Centers for Disease Control and Prevention. In Malaysia, the antimicrobial resistance rates and emergence of MDRPA-associated clinical infections were previously reported. Raja and Singh (2007) reported the resistance rates of *P. aeruginosa* to ceftazidime, cefepime, imipenem, and meropenem as 10.9%, 38.9%, 9.9 %, and 36.8%, respectively while Pathmanathan et al. (2009) reported 19.6%, 19.6%, 20.6%, and 22.7% resistance to the same antimicrobials. Incidences of MDRPA in Malaysian were varied at 5.7% in 2006, 69% in 2008, and 19.6% in 2009 based on studies by Raja et al. (2007), Lim et al. (2009) and Pathmanathan

et al. (2009), respectively. Since then, there has been no update on the MDRPA incidences in Malaysia.

Genotypic characterisation of Malaysian *P. aeruginosa* strains has also been previously reported. Lim et al. (2009) showed that the clinical strains from six public hospitals in six different states in Malaysia were diverse and heterogeneous. Besides that, high levels of broad spectrum antimicrobial resistance conferred by metallo-beta-lactamase-encoding genes such as $bla_{\text{IMP-7}}$, $bla_{\text{IMP-4}}$, $bla_{\text{VIM-2}}$, and $bla_{\text{VIM-11}}$ as well as gene cassette-bearing class 1 and class 2 integrons were detected among Malaysian strains.(Ho et al., 2002; Khosravi et al., 2010; Khosravi et al., 2011; Lim et al., 2009). However, the multilocus sequence typing (MLST) of extended-spectrum beta-lactamase (ESBL)- and carbapenemase-producing clinical *P. aeruginosa* has not been reported in Malaysia. The data generated would be useful to elucidate the genotypes of the Malaysian drug resistant *P. aeruginosa* strains in relation to previously reported international drug resistant STs.

The majority of reports from Malaysia were focused on clinical strains but there are few reports on environmental strains from hospital sources. It is important to know the exogenous sources of *P. aeruginosa* in the hospital and to characterize those isolated strains for better understanding of their antibiotic susceptibility profiles and genotypes. The combined data from the clinical and environmental strains would be useful to the hospital infection control teams in their on-going surveillance measures.

1.1 Research objectives

The aims of this study were:

i. To determine the distribution and prevalence of clinical and environmental *P. aeruginosa* in a Malaysian tertiary care hospital.
ii. To determine the antibiotic susceptibility profiles of *P. aeruginosa* from clinical specimens and environmental sources.

iii. To determine the presence of ESBL- and carbapenemase- encoding genes harboured by drug resistant *P. aeruginosa.*

iv. To determine the genetic diversity of the clinical and environmental *P. aeruginosa* in the tertiary hospital by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

CHAPTER 2: LITERATURE REVIEW

2.1 Clinical significance of *Pseudomonas aeruginosa*

P. aeruginosa has long been associated with infectious diseases in humans. The organism was first described by C. E. Sédillot who observed the bluish-green colour of surgical dressings, and was later described with greater detail by Fordos, Lucke, and Gessard in the 1800's (Lister et al., 2009). The bacteria is a Gram negative, rod shaped, obligate aerobe, found in humans, animals, plants, soil, water and the inanimate objects. The ubiquitous and versatile nature of the bacteria is enabled by its auxotrophic characteristic and well-coordinated gene regulation and expression, requiring minimal nutrients for survival (Gooderham & Hancock, 2009; Kelsey, 2013).

P. aeruginosa is responsible for a wide array of clinical manifestations in humans, ranging from asymptomatic carriage in the respiratory tract, urinary tract, and skin to fatal disease (Hauser & Rello, 2003). The bacterium is the main cause of chronic pulmonary infections and has been implicated in various infections such as bacteremia, ventilator-associated pneumonia, urinary tract infections, burn wound infections, catheter-related infections and post-surgery wound infections (United States of America, Centers for Disease Control and Prevention; Keen et al., 2010). The pathogen is also frequently associated with cystic fibrosis (CF) lung disease and causes irreversible damage to the tissues in the lung of patients (Mathee et al., 1999). Moreover, the immunocompromised status of patients make them extremely susceptible to *P. aeruginosa* infections. (Gooderham & Hancock, 2009). The severity of the infection is determined by the type and condition of the infected or colonized host, the route of infection, and the presence of indwelling medical devices in a host (Hauser & Rello, 2003).

The incidence of *P. aeruginosa* infections globally have been reported. A population based study conducted by Parkins et al. (2010) in a large Calgary health region showed

that the overall mortality rate associated with *P. aeruginosa* infection was 29.0% (n = 284). Of that total, 45.0% were nosocomial acquisitions, 34.0% were healthcare-associated community onsets, and 21.0% were community acquisitions (Parkins et al., 2010). An estimated 400 deaths and 51,000 healthcare acquired infections attributed to *P. aeruginosa* were reported by the United States of America, Centers for Disease Control and Prevention (2013). A study conducted at a university hospital in Northern Thailand showed that the rate of ventilator-associated pneumonia caused by the pathogen was 16.7% while the mortality rate was 16.0% (Chittawatanarat et al., 2014). Abu et al. (2016) reported 2.7% of blood cultures which were positive for *P. aeruginosa* among community-acquired bacteraemia in paediatric patients from a tertiary hospital in Malaysia. There is a lack of targeted studies to determine the mortality and morbidity rates due to *P. aeruginosa* infections from Malaysia which made it difficult to assess the true situation in this country.

2.2 Environmental reservoirs of *P. aeruginosa*

P. aeruginosa is ubiquitous and found to inhabit natural and man-made environments. The presence of *P. aeruginosa* in hospitals is of particular concern because of the inherent and acquired antimicrobial resistance of the pathogen which adversely affect patients' outcomes. In addition, the presence of *P. aeruginosa* in intensive care units is a common and persistent problem in many different countries and one of the most frequently isolated pathogens in hospital outbreaks (Hauser & Rello, 2003). *P. aeruginosa* is successful in colonizing hospital environments because its nutritional requirements are very minimal and the large genome of the pathogen encoding for various virulence factors, resistance genes, and regulatory mechanisms is the key for survival in hospital environments (Gooderham & Hancock, 2009; Kipnis et al., 2006; Wolfgang et al., 2003). Previous reports showed that *P. aeruginosa* was cultured from the handwashing sinks of the ICU, mops, betadine, ventilators, indwelling catheters, aerosols, sink taps and water

distribution systems in the hospital (Hauser & Rello, 2003; Kelsey, 2013). Once the organism is established in biofilms in the hospital environment, complete eradication is difficult because of their highly robust and sturdy biophysical properties against chemical treatments (Kelsey, 2013; Lieleg et al., 2011).

The presence of *P. aeruginosa* on fomites and inanimate objects in the hospital indicates that there is a potential for contamination of sterile medical goods and cross infections among patients resulting in nosocomial infections. Suraiya et al. (2008) reported that *P. aeruginosa* isolated from fentanyl, morphine and water used for medication dilution was the causative agent of an outbreak in an ICU of a tertiary teaching hospital in Malaysia. Sekiguchi et al. (2007) reported outbreaks of a multidrug resistant *P. aeruginosa* clone from community hospitals in Japan which were linked to urinary catheters. An outbreak of *P. aeruginosa* among patients which were associated with flexible bronchoscopes was reported by Mackie et al. (2003). Hence, the role of the hospital environment as a reservoir for *P. aeruginosa* nosocomial infections cannot be downplayed and warrants further investigation to inform and guide hospital infection control management.

2.3 Emergence and development of *P. aeruginosa* antimicrobial resistance

P. aeruginosa is intrinsically resistant to ampicillin, ampicillin-sulbactam, amoxicillin-clavulanate, cefotaxime, ceftriaxone, ertapenem, tetracyclines, trimethoprim, trimethoprim-sulfamethoxazole, chloramphenicol and fosfomycin (Patel et al., 2014). The intrinsic resistance of the organism is attributed to the derepression of chromosomal AmpC β-lactamase, up-regulation of the MexAB-OprM efflux pump system, mutations to topoisomerases II and IV, and loss of OprD porins (Livermore, 2002). However, acquired antimicrobial resistance is of greater concern because of the rapid dissemination

of resistance genes borne on mobile genetic elements such as plasmids or integrons (Livermore, 2002).

An international expert group which proposed standardized definitions for acquired antimicrobial resistance defined multidrug resistance as an isolate which is non-susceptible to at least one agent in ≥ 3 antimicrobial categories (Magiorakos et al., 2012). The United States of America, Centers for Disease Control and Prevention reported that multidrug resistant *P. aeruginosa* (MDRPA) is a serious threat attributing to an estimated 6,700 infections a year in the US (United States of America, Centers for Disease Control and Prevention). The report further elaborates that about 13.0% of severe nosocomial infections were caused by MDRPA which means that many classes of antipseudomonal drugs of choice such as cephalosporins, aminoglycosides, fluoroquinolones and carbapenems are no longer effective.

The first mention of acquired resistance among *P. aeruginosa* was in a treatment failure using imipenem by Quinn et al. (1986). Since then, numerous international reports of acquired resistance were published. For example, various *P. aeruginosa* beta-lactamases classes and subtypes from Italy (Pagani et al., 2004), Korea (S. Lee et al., 2005), China (Jiang et al., 2006), France (Brasme et al., 2007), and the UK (Woodford et al., 2008) were reported. In addition, various *P. aeruginosa* carbapenemases classes and subtypes from France (Naas et al., 1999), Malaysia (Ho et al., 2002), Greece (Pournaras et al., 2003), Columbia (Crespo et al., 2004), and Korea (Lee et al., 2013) were reported. The rapid development of scientific technologies such as DNA sequencing of specific resistance genes, opened the way for further characterisation of the resistance genes in *P. aeruginosa*.

2.4 Mechanisms and genes of resistance

There are many mechanisms and resistance genes utilized by *P. aeruginosa* for adaptability and survivability. However, the extended-spectrum beta-lactamase (ESBL) and carbapenemase encoded genes were the main focus in this study as the enzymes produced by those genes inactivate the drugs of choice used for the treatment of invasive and severe *P. aeruginosa* infections in Malaysia (Pharmaceutical Services Division, 2014).

2.4.1 Extended-spectrum beta-lactamase

The extended-spectrum beta-lactamase (ESBL) has been widely reported among the Enterobacteriaceae group since the early 1980s but was only reported in *P. aeruginosa* since 1993 (Weldhagen et al., 2003). The majority of the ESBLs belong to the Ambler class A scheme and confer resistance to narrow-spectrum penicillins, extended-spectrum cephalosporins, and aztreonam (Weldhagen et al., 2003). The hydrolyzing actions of the class A ESBLs utilizes an active site serine for the formation of an acyl enzyme (Bush & Jacoby, 2010). The TEM, SHV, CTX-M, PER, PSE, and GES enzymes are some examples of the ESBLs which have been previously described (Bush & Jacoby, 2010).

In 1993, the first ESBL, PER-1, from *P. aeruginosa* isolated from a Turkish patient in France was characterized and reported (Naas et al., 1999; Weldhagen et al., 2003). Subsequently, the VEB (from France, Kuwait, India, China and Thailand), PER (from France, Turkey, Italy and Belgium), SHV (from France, Poland, Greece, and Thailand), TEM (from France), GES (from France and South Africa), and IBC (from Greece) were characterized and described (Weldhagen et al., 2003). This implies that there is a global dissemination of ESBLs-encoding genes in *P. aeruginosa*. However, to date, there was only one report of ESBL-producing *P. aeruginosa* (OXA-10) from Malaysia by Lim et al. (2009).

2.4.2 Carbapenemase

The carbapenemase enzymes, or also known as metalloenzymes, are classified as Ambler class B and hydrolyze beta-lactams by utilizing at least one active-site zinc ion (Bush & Jacoby, 2010). The members of the Amber class B isolated from *P. aeruginosa* include IMP, VIM, SPM, AIM, GIM, NDM, SIM and DIM (Bush & Jacoby, 2010; Poirel et al., 2011). These enzymes have poor hydrolytic active against monobactams, but are not inhibited by tazobactams and clavulanate (Bush & Jacoby, 2010).

There are various reports of carbapenemase subtypes such as IMP-4 (Khosravi et al., 2010), IMP-7 (Ho et al., 2002), IMP-9 (Lim et al., 2009), IMP-26 (Koh et al., 2010), VIM-1 (Giske et al., 2006), VIM-2 (Lee et al., 2002; Poirel et al., 2001; Pournaras et al., 2003), VIM-4 (Pournaras et al., 2003), VIM-8 (Crespo et al., 2004), VIM-11 (Khosravi et al., 2010). The reports originated from countries such as France, Columbia, Singapore, Korea, Greece, Hungary, Italy, Sweden and Malaysia, which suggests a global dissemination of these resistance genes on mobile genetic elements via horizontal gene transfer (Davies & Davies, 2010; Poirel et al., 2009).

2.4.3 Class 1 and 2 integrons

Integrons are genetic elements that exist in the majority of Gram negative bacteria, which are popularly known for their roles in antimicrobial resistance gene dispersion, acquisition and expression (Gillings, 2014). The structure of all integrons consists of an integron integrase-encoding gene (*IntI*), an integron-associated recombinant site (*attI*), and an integron-associated promoter (Pc) (Deng et al., 2015; Gillings, 2014). The function of the integron integrase (IntI), a member of the tyrosine recombinase family, is to catalyse the recombination of an in-coming gene cassette to the integron-associated recombinant site (*attI*) (Deng et al., 2015; Gillings, 2014). The integron-associated

promoter (Pc) then expresses the exogenously acquired genes as part of the gene cassettes (Gillings, 2014).

The most common mechanism of plasmid-mediated resistance gene transmission is the horizontal gene transfer mechanism (Davies & Davies, 2010). Integrons located on plasmids can be easily transferred via bacterial conjugation which result in rapid dispersion of the resistance genes among bacterial species (Davies & Davies, 2010). However, Stokes et al. (2012) recently reported that all the class 1 integron from clinical *P. aeruginosa* from two continents were located on chromosomes. Stokes et al. (2012) surmised that the chromosomally located integrons have the capability to be transmitted via horizontal gene transfer although the exact mechanism is still unclear. Therefore, the class 1 integron located on the chromosome in *P. aeruginosa* is the major form of the global spread of resistance genes rather than those located on the plasmids (Stokes et al., 2012; Wright et al., 2015).

The class 1 integron is the most commonly found and reported class of integron, followed by the class 2 integron among the Gram negative bacteria (Deng et al., 2015). Numerous studies described the class 1 integron in *P. aeruginosa* as gene cassettes which encoded for antimicrobial resistance. For example, Wright et al. (2015) reported 119 strains with metallo beta-lactamase-encoding, bla_{IMP} and bla_{VIM} genes, contained in the class 1 integrons. Simultaneous detection of the bla_{IMP} and bla_{VIM} genes located in gene cassettes on the class 1 integron was also described by Toval et al. (2015). Khosravi et al. (2011) were the first to report their findings of the class 2 integrons in *P. aeruginosa*. However, the class 2 integrons found in their study did not contain any genes of resistance while four and 10 of the class 1 integrons contained the bla_{IMP} and bla_{VIM} genes, respectively (Khosravi et al., 2011). In addition, Lim et al. (2009) described one strain possessing the class 1 integron which contained the *aacA4*-$bla_{\text{IMP-9}}$-*catB8*-$bla_{\text{OXA-10}}$ gene

cassette, where the OXA-10 is an ESBL subtype. Hence, there are varied combinations of resistance genes located within an integron with some simultaneously bearing metallo beta-lactamase- and ESBL-encoding genes.

2.5 Molecular subtyping of *P. aeruginosa*

The main aim of pathogen typing is to determine the genetic relatedness of a pathogen species isolated from a particular epidemiological event. This investigation is usually triggered by a sudden increase of infections involving a particular pathogen, a particular group of infected patients or when there are distinctive but unusual antimicrobial susceptibility patterns (Singh et al., 2006).

Subtyping is defined as characterisation below the species and sub-species levels and both phenotypic and genotypic methods are widely used on bacterial pathogens (Barrett et al., 2006). Commonly used phenotyping methods such as biotyping, serotyping, bacteriophage typing, bacteriocin typing, and antimicrobial resistance profiling have been used for comparison of phenotypic characteristics of strains in epidemiological investigations (Barrett et al., 2006; Singh et al., 2006).

Over the past three decades, DNA-based molecular methodologies began to replace the phenotypic approach in epidemiological investigations because the molecular approach was better at determining the genetic basis of strain interrelationships (Singh et al., 2006). Molecular techniques such as plasmid profiling, restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), enterobacterial repetitive intergenic consensus sequences (ERIC) PCR, repetitive extragenic palindromic sequences (REP) PCR, variable-number tandem repeat (VNTR) PCR, and so forth have been developed and utilized in many studies (Foxman & Riley, 2001; Sabat et al., 2013). For the purpose of this study, two standardized, highly

discriminative, and widely used molecular techniques, PFGE and MLST, were performed.

2.5.1 Pulsed-field gel electrophoresis

Of all the various types of DNA-based subtyping methods, PFGE was considered as the 'gold standard' for bacterial subtyping for several reasons. The macrorestriction analysis by PFGE was found have high discriminatory powers, broad applicability, high intra- and interlaboratory reproducibility, epidemiologic concordance, and the ability to separate large DNA fragments (1000 kbp) from small fragments (50 kbp) (Barrett et al., 2006; Hunter et al., 2005; Stemper et al., 2011). Developed by Schwartz and Cantor, the method was improved by utilizing the contour-clamped homogeneous electric field (CHEF) system that alternates the switching of electrical currents at 120° angles to separate the large DNA fragments from the smaller fragments (Stemper et al., 2011). The protocols for PFGE are standardized as a result of the PulseNet (the National Molecular Subtyping Network for Foodborne Disease Surveillance) programme coordinated by the US CDC since 1996, for the subtyping of foodborne pathogens (Hunter et al., 2005; Sabat et al., 2013; Stemper et al., 2011). Apart from the foodborne bacterial pathogens, the PFGE protocols have also been slightly modified and used by many researchers to subtype *P. aeruginosa* by using the *Spe*I restriction endonuclease (Glupczynski et al., 2010; Johnson et al., 2007; Kidd et al., 2011; Lim et al., 2009; Pournaras et al., 2003; Ranellou et al., 2012; Spencker et al., 2000; Suraiya et al., 2008).

However, there are several disadvantages of using the gel–based fingerprinting method such as Tris-dependent DNA degradation which can mislead into classifying strains as untypable (Evans et al., 1994; Koort et al., 2002; Römling & Tümmler, 2000). This problem is easily resolved by replacing the Tris electrophoresis buffer with 1X HEPES buffer or with the addition of 0.5μm Thiourea into the Tris buffer (Evans et al.,

1994; Koort et al., 2002; Römling & Tümmler, 2000). Another common problem with PFGE is the occurrence of incomplete DNA restriction resulting in artifacts or 'ghost bands' leading to false conclusions about pulsed-field profiles relationships (Barrett et al., 2006). To resolve this problem, optimisation of the methodologies should be carried out followed by repeated restriction and analysis of the strain (Barrett et al., 2006). The occurrence of point mutations, insertions and deletions which can add or lose fragments relative to the strains that did not undergo such genetic alterations, also lead to misinterpretation of the relationships between the pulse-field patterns (Barrett et al., 2006). To ensure that the right conclusions are obtained, the PFGE results must be interpreted within the proper context, taking into account epidemiologic and environmental investigations (Barrett et al., 2006).

2.5.2 Multilocus sequence typing

Multilocus sequence typing (MLST) was designed in 1998 primarily to overcome the problem of interlaboratory results portability which is faced by traditional molecular subtyping methods (Maiden et al., 1998). The MLST data is made freely available over the internet via the PubMLST database and other host databases to facilitate information sharing and standardized bacterial nomenclature (Maiden, 2006). Bacterial typing is performed by sequencing of seven housekeeping genes which are conserved and encode for proteins of fundamental metabolic functions (Maiden et al., 1998; Maiden, 2006). Arbitrary numbers are then assigned to the each of the MLST loci and the resulting allelic profile is assigned a sequence type number. For *P. aeruginosa*, the selected seven loci in the scheme are *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A, and *trp*E (Curran et al., 2004).

Since the development of the MLST scheme for *P. aeruginosa* by Curran et al. (2004), many researchers such as Giske et al. (2006), Johnson et al. (2007), Lim et al. (2009), Kidd et al. (2011), and Liakopoulos et al. (2013) have utilized this methodology for

pathogen subtyping in epidemiological investigations. Furthermore, Giske et al. (2006), Johnson et al. (2007) and Kidd et al. (2011) have simultaneously analysed their *P. aeruginosa* strains with PFGE and have concurred that both methods have high discriminatory ability, but do not share 100% concordance because the methods differ in their analysis of genetic variations. The superiority of MLST over PFGE, is the determination of *P. aeruginosa* international lineages and high risk drug resistant clones such as ST111, ST235, ST274, ST357, and ST654 which cannot achieved by using PFGE (Fernández-Olmos et al., 2013; Liakopoulos et al., 2013; Wright et al., 2015).

CHAPTER 3: METHODOLOGY

3.1 *Pseudomonas aeruginosa* strains collection

3.1.1 Clinical strains collection and identification

A retrospective study of clinical *P. aeruginosa* was conducted in a 562-bedded tertiary hospital in Selangor, Malaysia. From April to November 2014, one hundred and ninety-nine consecutive and non-replicate *P. aeruginosa* strains were obtained from the microbiology laboratory of the hospital. The strains were identified by basic biochemical tests and API20NE (bioMérieux, France). In addition, the information of the strains background such as specimen type, site of infection, locations or wards in the hospital, and the dates of culture and sensitivity testing were recorded (Appendix A). These strains were inoculated into Luria-Bertani agar stabs and labelled with a PAC prefix followed by consecutive numbering. The strains were then transported to the research laboratory in the University of Malaya, Kuala Lumpur, for further investigation and cryo-preserved at -80°C in 50% glycerol. All agar media, solutions and buffers used in this study are detailed in Appendix C.

The strains were confirmed by PCR using primers PAL1, 5'-ATGGAAATGCTGAAATTCGGC-3' and PAL2, 5'-CTTCTTCAGCTCGA CGCGACG-3', which targeted the *oprL* gene (De Vos et al., 1997). Extracted DNA from a simple boiling method (Alexopoulou et al., 2006) was used for PCR. Additional confirmation was performed using primers PA SS-F, 5'-GGGGGATCTTCGGACCTCA-3' and PA SS-R, 5'- TCCTTAGAGTGCCCACCCG-3', which targeted the 16S rDNA variable regions 2 (V2) and regions 8 (V8) (Spilker et al., 2004) respectively. These primers were found to be more specific in identifying and differentiating *P. aeruginosa* from other *Pseudomonas* species. *P. aeruginosa* ATCC 27853 was used as the positive control while ultrapure H_2O was used as the negative control. The primers used in this study (Integrated DNA Technologies, Singapore) are

detailed in Appendix D while the reagents used for PCR are the Promega Go Taq® Green Master Mix (Promega, USA).

3.1.2 Environmental strains collection

A total of 358 environmental samples were collected from the tertiary hospital including the Intensive Care Unit (ICU), Neonatal Intensive Care Unit (NICU), surgical, medical, gynaecology, and haematology wards from January to February 2015. These locations were identified to be potential reservoirs of MDRPA strains. MDR is defined as resistant to ≥ 1 antibiotics from ≥ 3 antibiotic groups (Magiorakos et al., 2012). The samples were categorized into patients' immediate surroundings (e.g., bed rails, food table surface, mattress surface, curtains), medical equipment (e.g., defibrillator handles, infusion pump keys, medication trolley surface), healthcare workers' hands, sinks and drain surface from wards, toilets and pantry, frequently touched surfaces (e.g., laptop keys, light and fan switches, counter tops), and solutions or fluids (e.g., sterile NaCl 0.9% for drug dilution, povidone iodine, hand rub solutions). Samples taken from levelled and even surfaces were swabbed using Transwab with Amies Charcoal Medium (Medical Wire, UK), moistened with sterile 0.85% NaCl, at approximately 15 cm^2 at right angles for 1 minute (Lerner et al., 2013). Uneven or protruding surfaces (e.g., bedrails, tubing, respirators) were swabbed generously to obtain a large surface area. Approximately 5 to 10 ml of solutions or fluids were collected in 15 ml pre-sterilized centrifuge tubes (Axygen, USA) and sealed with parafilm. These were then labelled according to the sampling site and location (Appendix B) and placed in a chilled transport container for transportation to the research laboratory for immediate processing.

The swab samples were directly inoculated onto CHROMagar Orientation (CAO), (BD, USA), and incubated overnight at 37°C. Solutions or fluids were centrifuged at 10,000 X g for 10 min, then the pellets were inoculated on CAO and incubated overnight

at 37°C. Presumptive *P. aeruginosa* colonies (labelled with PPAE prefix), which are typically creamy, transparent greenish to yellowish rough-edged colonies, were isolated based on the CAO colour morphotypes differentiation guide (BD, USA) followed by the oxidase test to eliminate the oxidase negative isolates.

Oxidase positive isolates were identified using API 20NE, (bioMérieux, France) and confirmed using PCR as previously described for the clinical strains. *P. aeruginosa* ATCC 27853 was used as the positive control while *Escherichia coli* ATCC 35218 was the negative control for the API 20NE tests. The PAE prefix was used to relabel the confirmed *P. aeruginosa,* and these were cryo-preserved at -80ºC in 50% glycerol.

3.1.3 Antibiotic susceptibility testing

3.1.3.1 Kirby Bauer method

All *P. aeruginosa* strains were tested for susceptibility to amikacin (AMK), ceftazidime (CAZ), cefepime (FEP), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IPM), meropenem (MEM), polymyxin B (PMB), and piperacillin/tazobactam (TZP), (BBL, BD, USA) according to CLSI Performance Standards for Antimicrobial Susceptibility Testing (Patel et al., 2014). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 35218 were used as positive and negative controls, respectively.

3.1.3.2 Minimum inhibitory concentration method

The CAZ, FEP, IPM, and MEM minimum inhibitory concentration (MIC) E test strips (bioMérieux, France) were used to determine cephalosporin and carbapenem resistance levels among resistant strains. Performance and interpretation of the MIC results were also performed according to CLSI standards (Patel et al., 2014).

3.1.4 Detection of ESBL and carbapenem resistant genes

The detection of selected ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1}$ like, *bla*$_{CTXM}$ for phylogenetic group 1, 2 and 9, *bla*$_{CTXM-8/-25}$, *bla*$_{VEB}$, *bla*$_{PER}$, and *bla*$_{GES}$) and carbapenemase genes (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{AIM}$, *bla*$_{GIM}$, and *bla*$_{SIM}$) was performed on all strains using multiplex PCR with primers and conditions as previously described (Dallenne et al., 2010; Poirel et al., 2011). The amplicons were purified and submitted to a commercial company for sequencing to validate the results. The resulting nucleotide sequences were aligned to reference sequences in the NCBI BLAST-n database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The positive control strains, *Klebsiella pneumoniae* strain KPC06 and *K. pneumoniae* ATCC BAA-1705, were used as reference for *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTXM-1}$, and *bla*$_{KPC}$ respectively. PCR amplicons for *bla*$_{GES}$, *bla*$_{IMP}$, and *bla*$_{VIM}$ were sequenced to validate the products and the strains with these confirmed genes were used as positive controls in subsequent multiplex PCR tests. Ultrapure H_2O was used as the negative control for all multiplex PCR runs. Positive controls were unavailable for *bla*$_{OXA-1\ like}$, *bla*$_{CTXM}$ phylogenetic group 2 and 9, *bla*$_{CTXM-8/-25}$, *bla*$_{VEB}$, *bla*$_{PER}$, *bla*$_{SPM}$, *bla*$_{NDM}$, *bla*$_{AIM}$, *bla*$_{GIM}$, and *bla*$_{SIM}$. Therefore, negative results were treated with caution.

3.1.5 Class 1 and 2 integron detection and gene cassette determination

Strains which were resistant to any class of antimicrobial agents were subjected to PCR detection of class 1 and 2 integron-encoded integrases, *intI1* and *intI2,* according to established protocols (Machado et al., 2005). The 5'CS/3'CS and attI2-orfX region primer pairs were used to amplify the integron variable region of the class 1 and class 2 integrons, respectively (Machado et al., 2005). Subsequent sequencing of the integron variable region to confirm the presence and content of gene cassette insertions was also performed.

3.1.6 PFGE

PFGE was conducted on all strains according to established protocols (Lim et al., 2009) with minor modifications using CHEF MAPPER (Bio Rad, Hercules, CA). Briefly, *Spe*I-restricted DNA plugs were electrophoresed for 23 hours with pulse times of 1 s and 40 s, at 6 Vcm^{-1}. *Xba*I-digested *Salmonella* serotype Braenderup H9812 was used as the DNA size marker (Hunter et al., 2005). Both restriction enzymes used were from Promega, USA. For strains that could not be typed because of Tris-dependent DNA degradation, the electrophoresis was repeated with 1 X HEPES as the electrophoresis buffer (Koort et al., 2002). The gels were visualized in Gel Doc XR (Bio-Rad Laboratories, CA, USA) after staining with GelRed (Biotium, CA, USA). The reagents used for PFGE are detailed in Appendix C. The similarity indices of the PFGE fingerprints were calculated with Dice co-efficient at 1.0% optimization and 1.5% tolerance while clustering was done using unweighted pair group method with arithmetic mean (UPGMA) algorithm in the BioNumerics 7 software (Applied Maths, bioMérieux, Belgium).

3.1.7 MLST

Thirty-two clinical and six environmental strains were selected for MLST, based on the PFGE analyses: (i) indistinguishable pulsotypes (n = 10), (ii) pulsotypes with ≥85% genetic similarity (n = 6), (iii) pulsotypes with ≤85% genetic similarity (n = 4) and (iv) the MDRPA strains (n = 9). The primers and cycling conditions used were obtained from the MLST webpage (http://pubmlst.org/paeruginosa/) and published procedures (Curran et al., 2004).

eBURST v3 (http://eburst.mlst.net/) analysis using the most stringent definition, where the sequence types (STs) were identical or shared at least 6/7 alleles, were used to detect clonal complexes or BURST groups (BG) among the STs in this study (n = 29) and

the *P. aeruginosa* PubMLST database (n = 5265, http://pubmlst.org/paeruginosa/) (Feil et al, 2004). The STs were then classified as BG founders, single locus variants (SLVs), double locus variants (DLVs), or singletons (Spratt et al., 2004)

The nucleotide sequences of the STs were aligned to reference sequences from the *P. aeruginosa* PubMLST database using MUSCLE (MEGA7) (Kumar et al., 2016) and concatenated with Sequence Matrix 1.8. In order to estimate the distances between the sequences, the Maximum Likelihood (ML) method based on the Tamura-Nei model was utilized (Tamura & Nei, 1993). The tree topology was determined by neighbour-joining method and the final phylogenetic tree was rooted using *Acinetobacter* spp. as the outgroup (Hall & Barlow, 2006).

The BGs and ML tree were assessed using 2000 bootstrap replicates and bootstrap percentages ≥70% were considered to be reliable (Hall, 2013).

CHAPTER 4: RESULTS

4.1 Strains identification

4.1.1 Biochemical testing results

Biochemical identification using API 20NE identified all 199 clinical strains as *P. aeruginosa* (Figure 4.1). Out of the 358 environmental samples, 29 *P. aeruginosa* strains from 23 samples were also isolated and identified. Examples of the environmental samples cultured on CHROMagar Orientation media are shown in Figure 4.2.

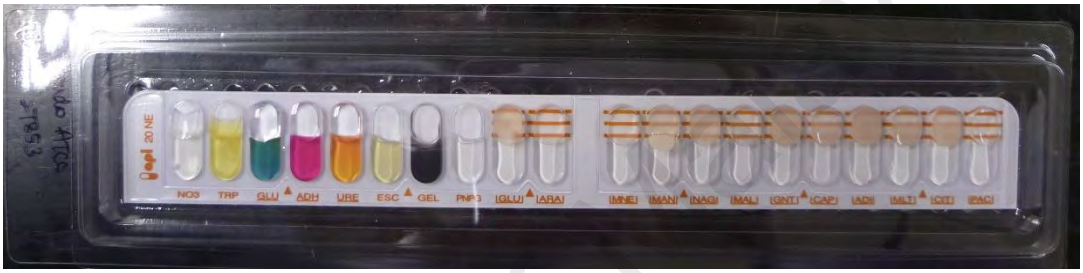


Figure 4.1: A representative photo of biochemical reactions for *P. aeruginosa* identification using an API 20NE test kit. Positive biochemical reactions are indicated with colour changes for the ADH (pink), URE (orange), and GEL (black) tests while the positive results for assimilation of substrates such as GLU, MAN, NAG, GNT, CAP, ADI, MLT, and CIT are visualized as opaque or turbid.

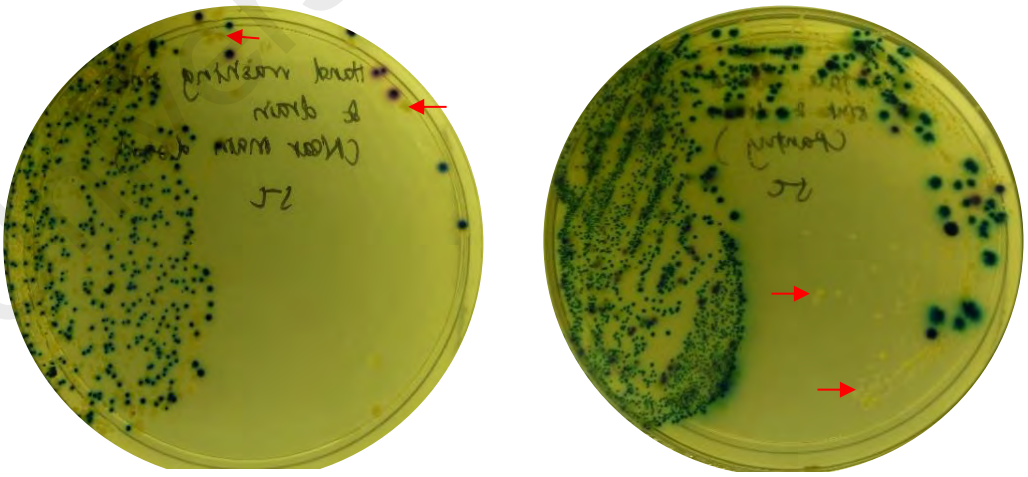


Figure 4.2: Environmental isolates cultured on CHROMagar Orientation plates. Presumptive *P. aeruginosa* morphotypes are transparent, yellowish to greenish rough-edged colonies (indicated by the red arrows). The other presumptive morphotypes such as *Enterobacter* spp. (deep blue with violet halos), *Enterococcus* spp. (small, blue-green to blue), and *Escherichia coli* (large, dark rose to pink) colonies are also seen.

4.1.2 Confirmation of strain identity by PCR

All 199 clinical strains were confirmed to be *P. aeruginosa* by PCR using the PAL and PA SS primer pairs which yielded 504 bp and 956 bp products, respectively (Figure 4.3). Of the 170 oxidase positive environmental strains, 40 were positive for the *oprL* gene and only 29 strains were positive for 16S rDNA variable regions 2 and 8 genes; implying that only 29 strains were confirmed to be *P. aeruginosa* while the rest were *Pseudomonas* spp. The results obtained using the PA SS primer pairs were concordant with the results from the API 20NE biochemical tests. Therefore, the final confirmation of the strains identities were based on the PCR using the PA SS primers which were more specific in the detection of true *P. aeruginosa* (Figure 4.4).

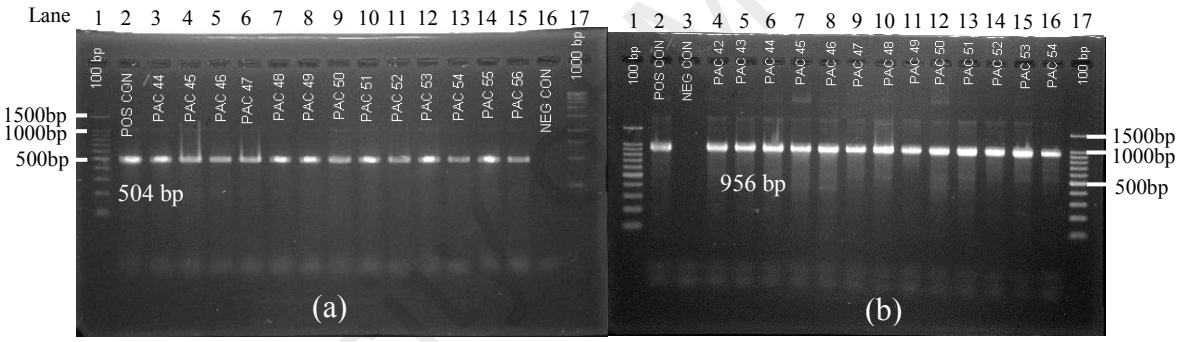


Figure 4.3: Representative agarose gel photos of clinical *P. aeruginosa* confirmed by PCR using the (a) PAL primers and (b) PA SS primers. (a) Lane 1: 100bp DNA ladder (Promega). Lane 2: Positive control. Lanes 3 to 15: *P. aeruginosa* strains (strains ID are indicated in the photos). Lane 16: Negative control. Lane 17: 1000bp DNA ladder (Promega). (b) Lanes 1 and 17: 100bp DNA ladder (Promega). Lane 2: Positive control. Lane 3: Negative control. Lanes 4 to 15: *P. aeruginosa* strains (strains ID are indicated in the photos).

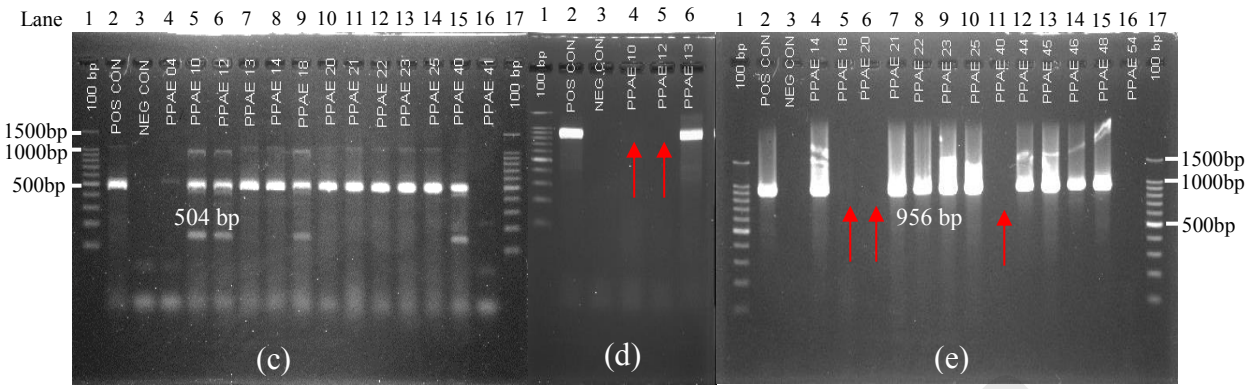


Figure 4.4: A montage of representative agarose gel photos of presumptive environmental *P. aeruginosa* analysed by PCR using the (c) PAL primers and confirmed using the (d & e) PA SS primers**.** Compared to the PAL primers, the PA SS primers were more specific in detecting true *P. aeruginosa* (missing bands are indicated by the red arrows in (d) and (e)). (c) Lanes 1 and 17: 100bp DNA ladder (Promega). Lane 2: Positive control. Lane 3: Negative control. Lanes 4 to 16: Presumptive *P. aeruginosa* strains (strains ID are indicated in the photos). (d) Lane 1: 100bp DNA ladder (Promega). Lane 2: Positive control. Lane 3: Negative control. Lanes 4 to 6: Presumptive *P. aeruginosa* strains (strains ID are indicated in the photos). (e) Lanes 1 and 17: 100bp DNA ladder (Promega). Lane 2: Positive control. Lane 3: Negative control. Lanes 4 to 16: Presumptive *P. aeruginosa* strains (strains ID are indicated in the photos).

4.2 Strain distribution and prevalence in the hospital

The 199 clinical *P. aeruginosa* strains were isolated from various sources obtained from different locations in the hospital (Table 4.1). The top three sources of clinical strains were sputum (25.6%), wound swab (16.6%), and tracheal aspirate (15.6%); the majority of which were isolated from patients in the medical wards (30.2%), surgical wards (16.6%), and the ICU (13.6%). Sixty-one percent (n = 122) and 39% (n = 77) of the clinical strains were from male and female patients, respectively. The detailed strain background information are found in Appendix A. Table 4.2 shows the sources of the 29 environmental *P. aeruginosa* strains from 10 different wards in the hospital. The majority of strains were isolated from sinks and drains located in the Surgical (31.0%), and Medical (24.1%) wards. The details of the environmental strains background are shown in Appendix B.

Table 4.1: Specimen types and sources of clinical *P. aeruginosa* strains studied (Phoon et al., 2018). Reprinted permission granted by Mary Ann Liebert, Inc.

Locations/wards	Specimen types												
	Abscess / Pus	Blood	Bone	BAL/ NPA[a]	Catheter tip	Eyes/ Cornea	Sputum	Throat swab	Tissue	Tracheal aspirate	Urine	Wound swab	Total
Intensive Care Units		3			1		4			18		1	27
Haematology	3	9		1			1		2	2	1	2	21
Emergency		1				1							2
Ophthalmology						1							1
Obstetrics & Gynaecology	1	2					1				1	3	8
Otorhinolaryngology	2												2
General Medicine	4	3					37	1		3	8	4	60
Paediatrics	1	11		1		7			1	4		1	26
Orthopaedics	2		1				1		3		2	10	19
General Surgery	3					1	7		4	4	2	12	33
Number of specimens	16	29	1	2	1	10	51	1	10	31	14	33	199

[a]BAL/NPA = Bronchoalveolar lavage / nasopharyngeal aspirate

Table 4.2: Environmental sources of *P. aeruginosa* strains studied.

Sources	Wards										
	Surgical 1	Surgical 2	Surgical 3	Gynaecology	Medical 1	Medical 2	Medical 3	Haematology 1	Haematology 2	Haematology 3	Number of specimens
Sink & drain	2	3	4	1	2	4	2	1		1	20
Counter top and around sink surfaces	1		1					1	4		7
Patient mattress surface					2						2
Total	3	3	5	1	4	4	2	2	4	1	29

4.3 Antibiotic susceptibility profiles

4.3.1 Kirby Bauer results

The susceptibility rates for clinical *P. aeruginosa* tested against all the antimicrobial agents in this study ranged from 88.4% (piperacillin/tazobactam and imipenem) to 100% (polymyxin B). All the strains tested were susceptible towards polymyxin B. The antimicrobial agents with the highest resistance rates were imipenem and meropenem (11.6%, n = 23), followed by piperacillin/tazobactam (8.0%, n = 16) and ceftazidime (7.0%, n = 14). All the environmental strains were susceptible to the antibiotics tested except for one strain, PAE22, which was resistant to imipenem and meropenem. This strain was subjected to MIC testing for confirmation of the carbapenem resistance. The antibiograms of all strains are listed in Appendix E and the representative antibiotic susceptibility plates are shown in Figure 4.5. The antibiotic susceptibility rates for the clinical strains are summarised in Table 4.3.

Among the 199 clinical strains, twelve MDRPA strains (6.0%) were isolated from ICU (n = 3), Surgical 1 (n = 1), Gynaecology (n = 2), Medical 1 (n = 3), Haematology 1 (n = 1), and Haematology 3 (n = 2) wards. The strains which were intermediate or resistant to ceftazidime (n = 20), cefepime (n = 14), imipenem (n = 26), and/or meropenem (n = 35) were further analysed by MIC testing.

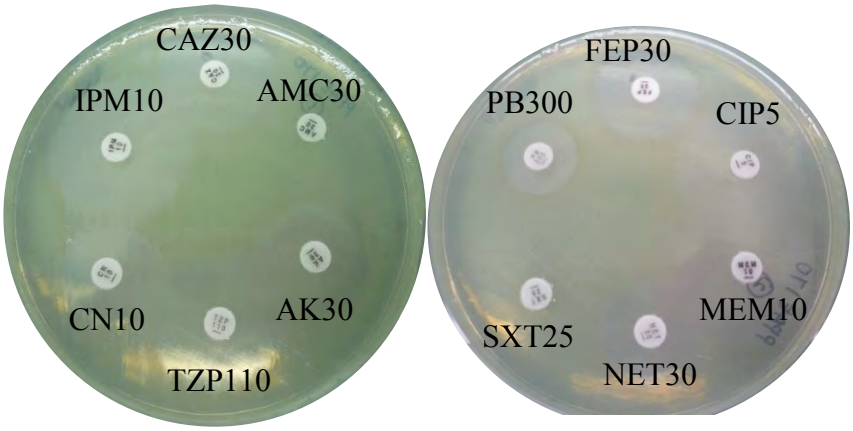


Figure 4.5: Representative photos of antibiotic susceptibility testing for *P. aeruginosa* on Mueller Hinton II plates using Kirby Bauer method.

Table 4.3 Antibiotic susceptibility rates of clinical *P. aeruginosa* by Kirby Bauer method (Phoon et al., 2018). Reprinted permission granted by Mary Ann Liebert, Inc.

Antimicrobial agents[a]	No. (%) of isolates		
	Susceptible	Intermediate	Resistant
CAZ	180 (90.5)	5 (2.5)	14 (7.0)
FEP	186 (93.5)	0 (0)	13 (6.5)
TZP	176 (88.4)	7 (3.5)	16 (8.0)
IPM	175 (88.4)	0 (0)	23 (11.6)
MEM	173 (86.9)	3 (1.5)	23 (11.6)
GEN	175 (93.1)	0 (0)	13 (6.9)
AMK	190 (95.5)	1 (0.5)	8 (4.0)
NET	187 (94.0)	0 (0)	12 (6.0)
CIP	191 (96.0)	0 (0)	8 (4.0)
PMB	197 (100)	0 (0)	0 (0)

[a]Ceftazidime (CAZ), Cefepime (FEP), Piperacillin/Tazobactam (TZP), Imipenem (IPM), Meropenem (MEM), Gentamicin (GEN), Amikacin (AMK), Netilmicin (NET), Ciprofloxacin (CIP), Polymyxin B (PMB)

4.3.2 Minimum inhibitory concentration results

Figure 4.6 shows the representative pictures of minimum inhibitory concentration (MIC) E test strips used in this study and Tables 4.4 and 4.5 summarize the MIC results for cephalosporins and carbapenems, respectively. Based on the MIC results, 13 of 20 (65.0%) clinical strains tested were confirmed to be resistant to ceftazidime, while 12 of

14 strains (85.7%) were confirmed to be resistant to cefepime. Twenty three of 26 (88.5%) clinical strains tested strains were confirmed to be resistant to imipenem and 21 of 35 strains (60%) were confirmed to be resistant to meropenem. However, the environmental strain, PAE22, which was resistant to imipenem and meropenem by disk diffusion had MIC values of 3.0 mg/L (intermediate) for imipenem and 2.0 mg/L (sensitive) for meropenem. Hence, strain PAE22 was considered as a non-resistant strain.

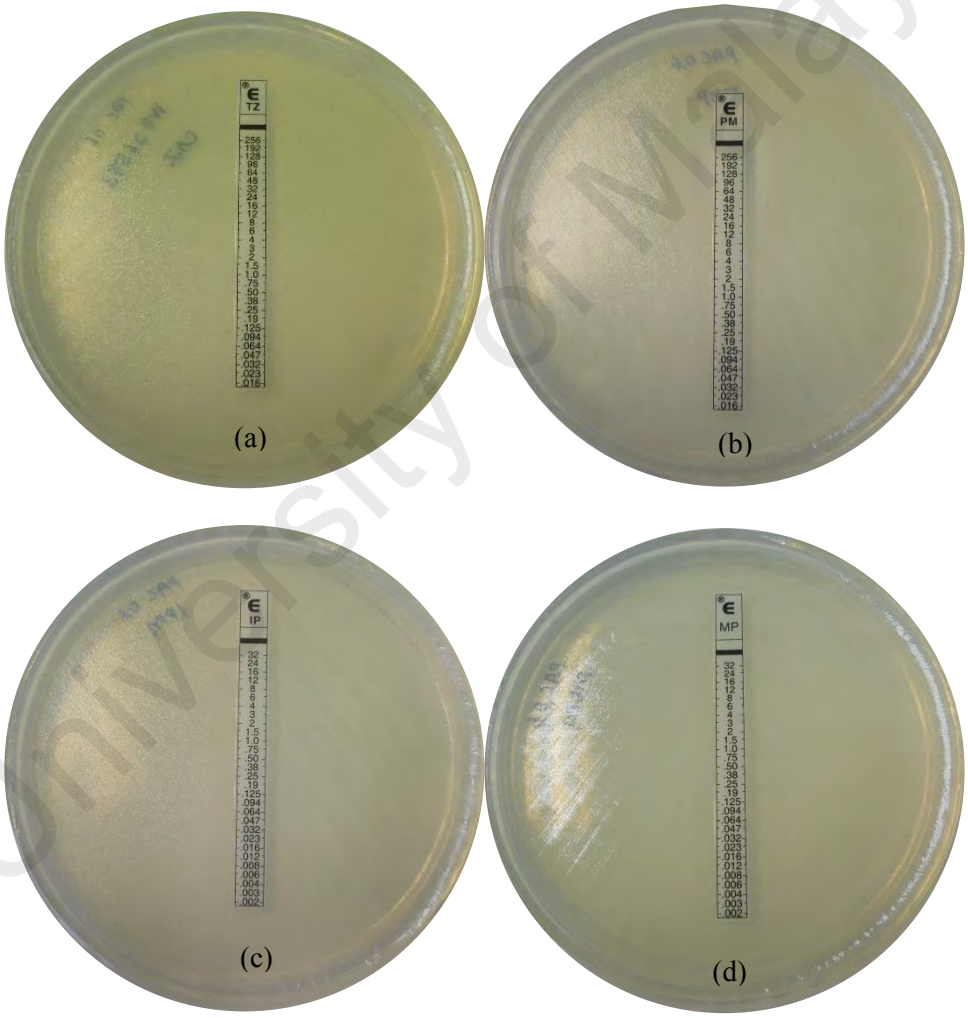


Figure 4.6: Representative photos of minimum inhibitory concentration testing for *P. aeruginosa* using (a) ceftazidime, (b) cefepime, (c) imipenem and, (d) meropenem E test strips on Mueller Hinton II agar.

Table 4.4: Minimum inhibitory concentration results for cephalosporins, ceftazidime (CAZ) and cefepime (FEP).

MIC (mg/L)[a]	CAZ		FEP	
	n	%	n	%
256	11		4	
128	1		3	
96	0	65.0	1	85.7
64	1		3	
48	0		1	
24	1		1	
16	4	25.0	0	7.1
8	1		1	
1.5	1	10.0	0	7.1
Total	20	100.0	14	100.0

[a]MIC interpretive criteria for cephalosporins based on CLSI standards (Patel et al., 2014); Susceptible: ≤8, Intermediate: 16, Resistant: ≥32.

Table 4.5: Minimum inhibitory concentration results for carbapenems, imipenem (IPM) and meropenem (MEM).

MIC (mg/L)[b]	IPM		MEM	
	n	%	n	%
32	21		13	
16	2	88.5	0	60.0
12	0		4	
8	0		4	
6	0	3.8	3	8.6
3	1		0	
2	0		2	
1.5	0		1	
1	1		2	
0.75	0	7.7	1	31.4
0.5	0		2	
0.38	1		1	
0.125	0		1	
0.094	0		1	
Total	26	100.0	35	100.0

[b]MIC interpretive criteria for carbapenems based on CLSI standards (Patel et al., 2014); Susceptible: ≤2, Intermediate: 4, Resistant: ≥8

4.4 Resistance genes, class 1 and class 2 integrons

4.4.1 ESBL and carbapenem resistance genes

Of the 199 clinical strains, only two strains possessed the extra-integron Ambler class A ESBL gene, *bla*$_{\text{GES-20}}$, while seven possessed the Ambler class B metallo-beta-lactamase (MBL)/carbapenemase genes, *bla*$_{\text{IMP-4}}$ (n = 3), *bla*$_{\text{VIM-2}}$ (n = 2), and *bla*$_{\text{VIM-11}}$ (n = 4). Two strains harboured both the *bla*$_{\text{VIM-2}}$ and *bla*$_{\text{IMP-4}}$. All these were MDRPA strains. The nucleotide sequences of the PCR amplicons were validated by DNA sequencing and the BLAST-N search results for nucleotide sequence data are shown in Appendix G to Appendix J. Table 4.6 summarises the genetic contents of the gene cassettes, extra-integron ESBL- and carbapenemase-encoding genes, the resistant phenotypes, and MICs for cephalosporins and carbapenems. Representative agarose gel photos of PCR amplified products of *bla*$_{\text{GES}}$, *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$ are shown in Figure 4.7 and 4.8.

Table 4.6: Gene cassette contents of class 1 integrons, extra-integron resistance genes and resistant phenotypes of *intI*1-bearing clinical *P. aeruginosa* from a Malaysian tertiary hospital (Phoon et al., 2018). Reprinted permission granted by Mary Ann Liebert, Inc.

Integron locus/ Accession number	Gene cassette contents	Amplicon size (bp)	Extra-integron resistance genes	Locus	Resistant phenotype[a]	MIC (mg/L)	No. of strains (Strain ID)
GU169702	*bla*$_{GES-13}$	1200	*bla*$_{GES-20}$	CP022000	CAZ-FEP-TZP-IPM-MEM-GEN- NET-CIP	CAZ (>256), FEP (64,128), IPM (>32), MEM (>32)	2 (PAC06, PAC08)
KR337993 (5'CS)	*bla*$_{VIM-6}$	1200, 2600	*bla*$_{VIM-11}$	NG_050338	CAZ-FEP-TZP-IPM-MEM-GEN-AMK-NET	CAZ (>256), FEP (64,256), IPM (16,>32), MEM (6,8)	2 (PAC138, PAC148)
KU839731 (3'CS)	*bla*$_{OXA-10}$-*AAC(6')-Ib*						
KR337993 (5'CS)	*bla*$_{VIM-6}$	2600	*bla*$_{VIM-11}$	NG_050338	CAZ-FEP-TZP-IPM-MEM-GEN-AMK-NET-CIP	CAZ (>256), FEP (>256), IPM (>32), MEM (>32)	1 (PAC36)
KX241477 (3'CS)	*bla*$_{OXA-10}$-*aac(6')-II*						
MF168946	*bla*$_{VIM-2}$	1200, 2600	*bla*$_{VIM-11}$	NG_050338	CAZ-FEP-TZP-IPM-GEN-AMK- NET	CAZ (>256), FEP (48), IPM (>32), MEM (12)	1 (PAC200)
KY860572	*aadA6-gcuD*	1400	*bla*$_{IMP-4}$	KX711879	CAZ-FEP-TZP-IPM-MEM-GEN-AMK-NET-CIP	CAZ (>256), FEP (128,>256), IPM (>32), MEM (>32)	2 (PAC45, PAC51)
			bla$_{VIM-2}$	FR695889/ FR695890			
KY860572	*aadA6-gcuD*	1400	*bla*$_{IMP-4}$	KX711879	CAZ-FEP-IPM-MEM-GEN-NET-CIP	CAZ (>256), FEP (>256), IPM (>32), MEM (>32)	1 (PAC96)
KY860572	*aadA6-gcuD*	1400	Not detected	Not applicable	GEN-AMK-NET-CIP	N/T[b]	1 (PAC99)
KY860572	*aadA6-gcuD*	1400	Not detected	Not applicable	CAZ-FEP-TZP-IPM-MEM-GEN-AMK-NET-CIP	CAZ (>256), FEP (128), IPM (>32), MEM (>32)	1 (PAC17)

[a]Antimicrobial agents: ceftazidime (CAZ), cefepime (FEP), piperacillin-tazobactam (TZP), imipenem (IPM), meropenem (MEM), gentamicin (GEN), amikacin (AMK), netilmicin (NET), and ciprofloxacin (CIP).
[b]Not Tested

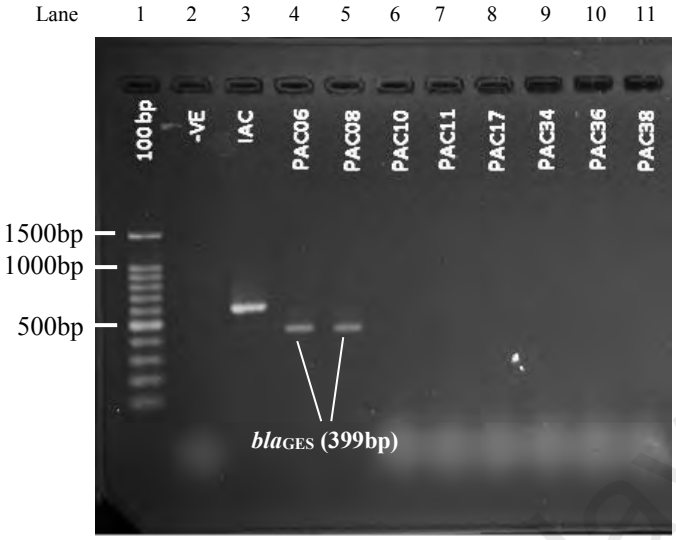


Figure 4.7: Representative agarose gel photo of PCR amplified *bla*$_{GES}$ products. Lane 1: 100bp DNA ladder (Promega). Lane 2: Negative control. Lane 3: Internal amplification control. Lanes 4 to 11: *P. aeruginosa* strains (strains ID are indicated in the photos).

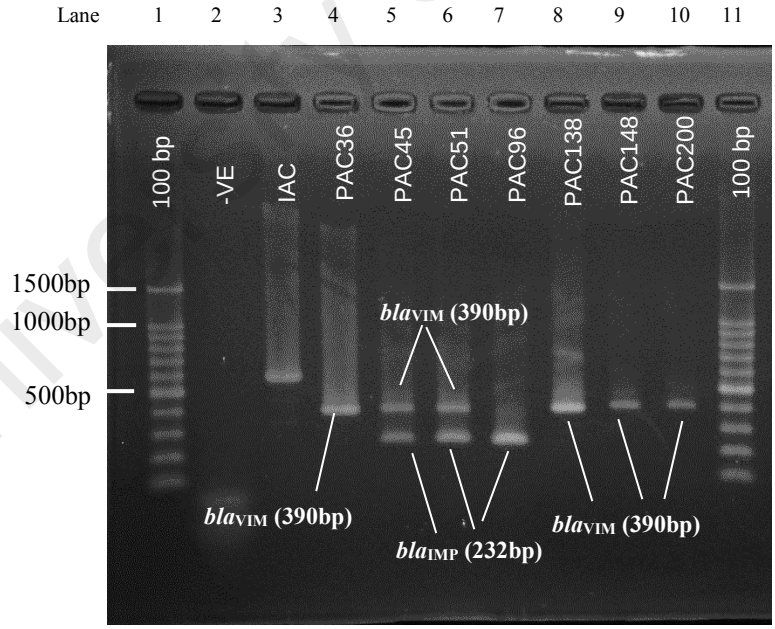


Figure 4.8: Representative agarose gel photo of PCR amplified *bla*$_{IMP}$ and *bla*$_{VIM}$ products. Lanes 1 and 11: 100bp DNA ladder (Promega). Lane 2: Negative control. Lane 3: Internal amplification control. Lanes 4 to 10: *P. aeruginosa* strains (strains ID are indicated in the photos).

4.4.2 Integron-borne gene cassettes

Eleven (10 MDRPA and 1 non-MDRPA) strains harboured the *intI1* integrase gene. However, none were positive for the *intI2* integrase gene. None of the tested resistance genes and integrons were present in two MDRPA strains; PAC95 and PAC167.

Further analysis of the sequenced 5'CS and 3'CS variable region of the class 1 integrons yielded eight different gene cassettes; $bla_{\text{GES-13}}$, $bla_{\text{VIM-2}}$, $bla_{\text{VIM-6}}$, $bla_{\text{OXA-10}}$, which confer resistance toward beta-lactams; *aacA(6')-Ib, aacA(6')-II* and *aadA6* which confer resistance against aminoglycosides; and *gcuD* which encodes for a hypothetical protein of unknown function. Strains with high MIC levels for CAZ, FEP, IPM and MEM possessed the corresponding resistance genes except for PAC17, which was resistant to beta-lactams and fluoroquinolone but only harboured the *aadA6-gcuD* in the class 1 integron gene cassette (Table 4.6).

The *intI1* and gene cassette BLAST-N analysis results of the nucleotide sequence data are shown in Appendix K and Appendix (L to P), respectively. Representative agarose gel photos of PCR amplified products of class 1 integron-encoded integrase, *intI1*, and the class 1 integrons are shown in Figures 4.9 and 4.10, respectively.

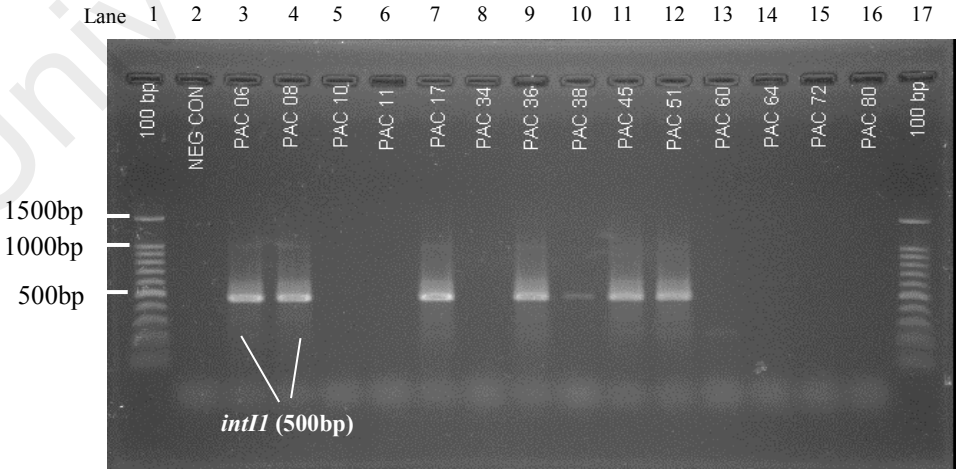


Figure 4.9: Representative gel agarose photo of PCR amplified products for class 1 integron-encoded integrase, *intI1*. Lanes 1 and 17: 100bp DNA ladder (Promega). Lane 2: Negative control. Lanes 3 to 16: *P. aeruginosa* strains (strains ID are indicated in the photos).

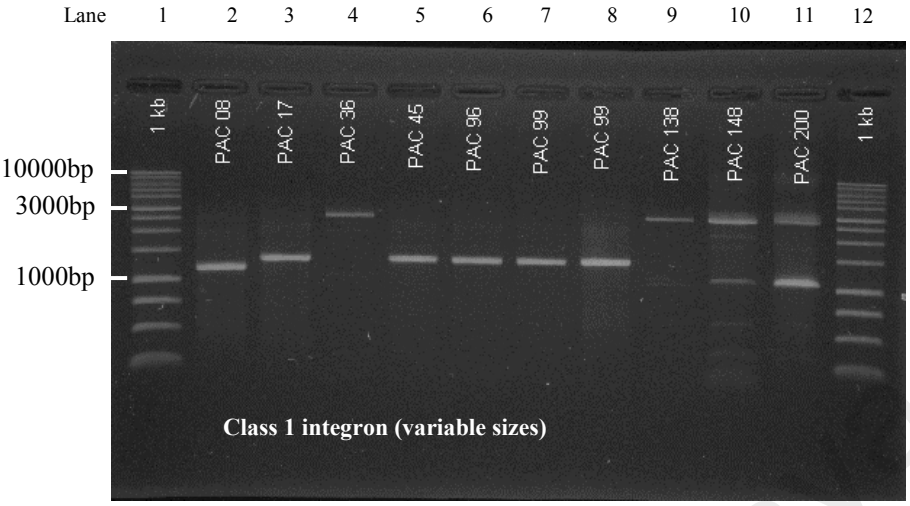


Figure 4.10: Representative gel agarose photo of PCR amplified products for class 1 integron. Lanes 1 and 12: 1000bp DNA ladder (Promega). Lanes 2 to 11: *P. aeruginosa* strains (strains ID are indicated in the photos).

4.5 Genetic diversity of *P. aeruginosa*

4.5.1 Genetic relatedness of clinical strains based on PFGE analyses

PFGE analysis of the 199 *Spe*I-digested chromosomal DNA yielded 163 reproducible pulsed-field profiles (PFPs; $F = 0.61 – 1.00$) with 10 to 30 restriction fragments (Figure 4.12) indicating that the strains were genetically diverse. Cluster analysis generated 52 clusters (C1 to C52) at ≥85% similarity or three-band difference and 27 singletons (UP1 to UP27). One untypeable strain (PAC 199) was excluded from the analysis. Three large clusters were observed in the hospital at ≥85% similarity. Two different clusters, C16 and C44, comprised of strains which originated from the NICU. Cluster C16, which was the largest cluster in the hospital, comprised of nine indistinguishable strains, while cluster C44 contained six indistinguishable strains. Clonal strains in C35 (n = 5) were isolated from Medical wards 1 and 3, and Haematology 3. A representative PFGE gel photo of *Spe*I-digested *P. aeruginosa* is shown in Figure 4.11.

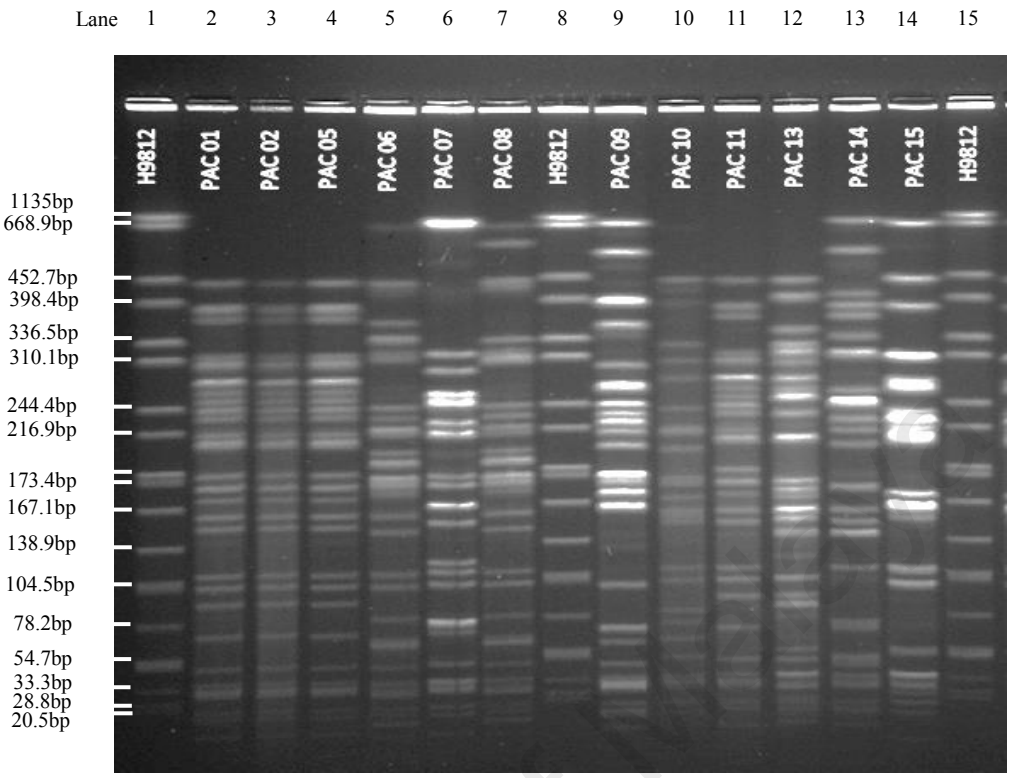


Figure 4.11: A representative PFGE gel photo for *Spe*I-digested *P. aeruginosa*. Lanes 1, 8 and 15: *Salmonella* serotype Braenderup H9812 DNA size marker. Lanes 2 to 7 and 9 to 14: *P. aeruginosa* strains (strains ID are indicated in the photos).

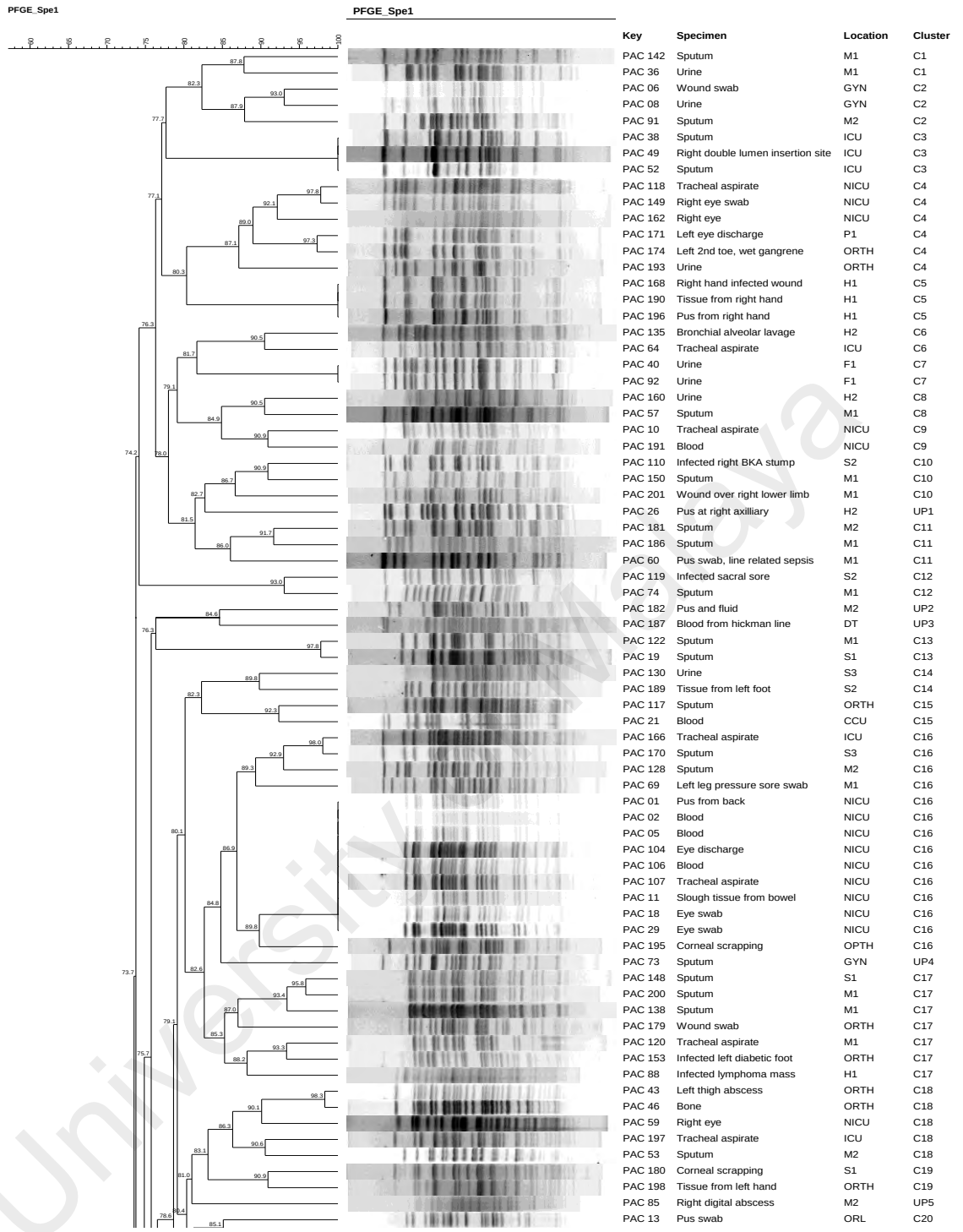
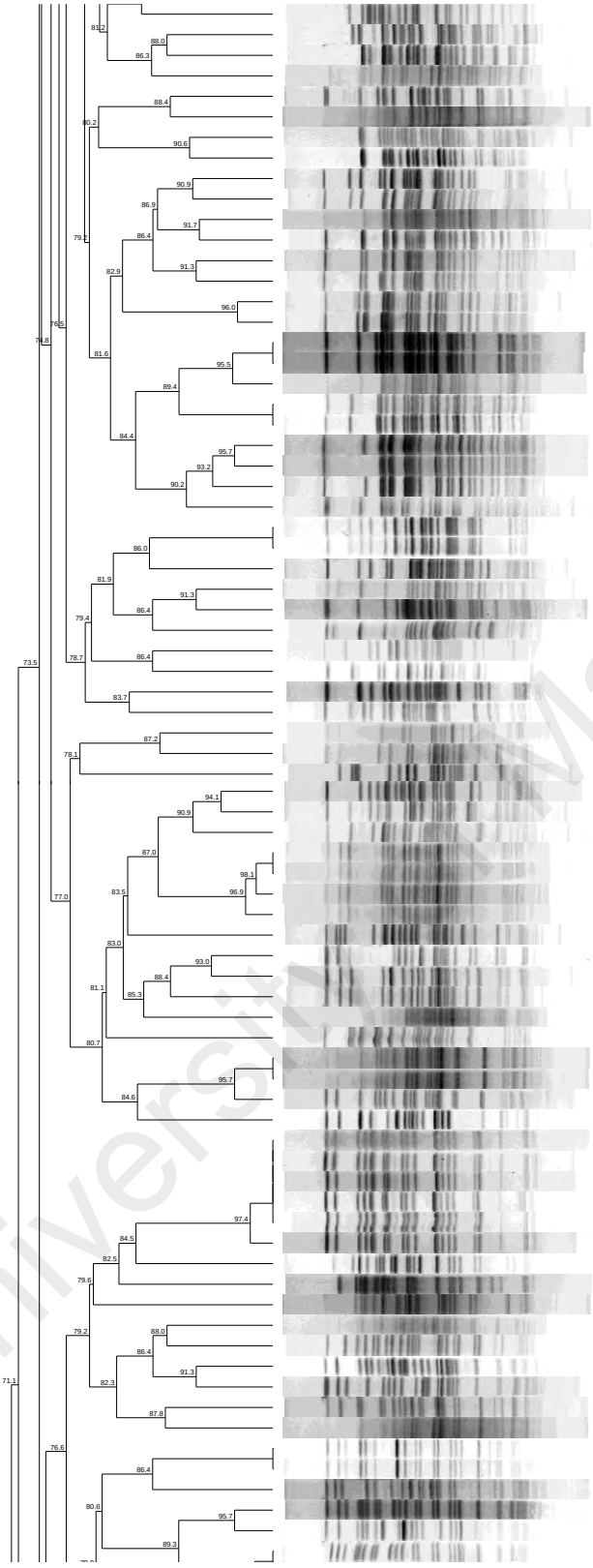
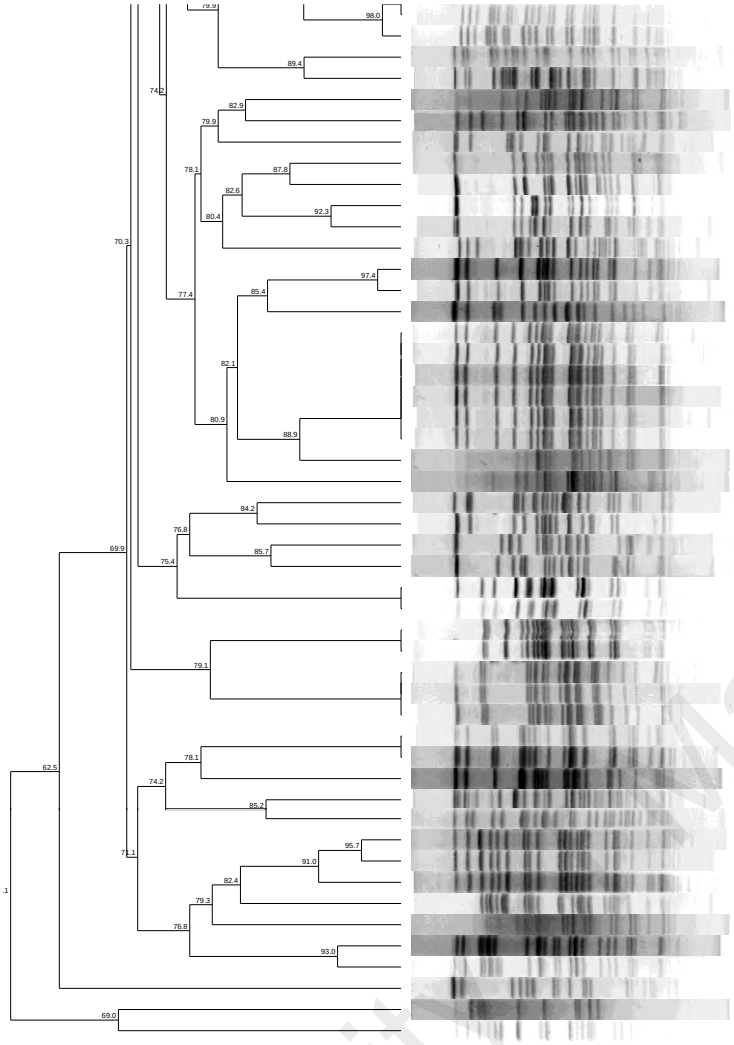


Figure 4.12: Dendrogram for clinical strains of *P. aeruginosa* based on *Spe*I digestion. Specimen type: Below knee amputation (BKA); Diabetic foot ulcer (DFU); Internal jugular catheter (IJC); Left eye (LE), Osteomyelitis (OM). Locations: Coronary care unit (CCU); Day transplant ward (DT); Emergency department (EMER); First class ward 1 (F1); First class ward 2 (F2); Gynaecology ward (GYN); Haematology 1 ward (H1); Haematology 2 ward (H2); Haematology 3 ward (H3); High dependency ward (HDW); Intensive care unit (ICU); Medical 1 ward (M1); Medical 2 ward (M2); Medical 3 ward (M3); Medical outpatient department (MOPD); Neonatal intensive care unit (NICU); Ophthalmology department (OPTH); Orthopaedic ward (ORTH); Otorhinolaryngology department (ORL); Paediatric 1 ward (P1); Surgical 1 ward (S1); Surgical 2 ward (S3); Surgical 3 ward (S3). PFGE type is categorized as cluster (C) or unique profile (UP).



PAC 79	Sputum	M1	C20
PAC 31	Tissue	S2	C21
PAC 32	Sputum	M2	C21
PAC 169	Right calf infected ulcer	ORTH	C21
PAC 101	Urine	F1	C22
PAC 152	Sputum	M1	C22
PAC 165	Peripheral blood	H1	C23
PAC 47	Urine	M1	C23
PAC 102	Sputum	M1	C24
PAC 72	Sputum	M2	C24
PAC 136	Tracheal aspirate	ICU	C24
PAC 34	Tracheal aspirate	M2	C24
PAC 194	Tracheal aspirate	M1	C24
PAC 62	Sputum	M2	C24
PAC 86	Blood	M2	C25
PAC 89	Tracheal aspirate	CCU	C25
PAC 54	Blood	M2	C26
PAC 55	Urine	M2	C26
PAC 161	Wound breakdown swab	GYN	C26
PAC 41	Tissue from right foot	ORTH	C26
PAC 44	Tissue from right foot	ORTH	C26
PAC 143	Wound breakdown	S3	C27
PAC 145	Pus swab	F2	C27
PAC 97	Wound break down	S3	C27
PAC 175	Wound swab	M2	C27
PAC 45	Tracheal aspirate	ICU	C28
PAC 51	Tracheal aspirate	ICU	C28
PAC 99	Thigh wound abscess	S2	C28
PAC 164	Urine	F1	C29
PAC 17	Blood	H1	C29
PAC 94	Blood, peripheral	DT	C29
PAC 12	Pus swab	M3	C30
PAC 20	Blood	CCU	C30
PAC 114	Post wound debridement	S2	UP6
PAC 28	Blood	GYN	UP7
PAC 04	Sputum	M3	C31
PAC 109	Infected right heel wound	ORTH	C31
PAC 68	Left leg ulcer swab	GYN	UP8
PAC 126	Right foot infected wound	ORTH	C32
PAC 96	Blood, red lumen	H3	C32
PAC 75	Abscess of left thumb	ORTH	C32
PAC 22	Blood, blue lumen	H3	C32
PAC 24	Blood	F2	C32
PAC 95	Tissue from back abscess	H3	C32
PAC 87	Blood, peripheral	H3	C32
PAC 84	Sputum	MOPD	UP9
PAC 42	Sputum	F2	C33
PAC 66	Sputum	M2	C33
PAC 81	Tissue from right hand	S2	C33
PAC 173	Sputum	M1	C33
PAC 77	Right breast cellulitis	S2	UP10
PAC 157	Tracheal aspirate	ICU	C34
PAC 158	Tip from right IJC	ICU	C34
PAC 167	Tracheal aspirate	ICU	C34
PAC 09	Pus swab	S2	UP11
PAC 123	Sputum	M1	C35
PAC 176	Sputum	M1	C35
PAC 192	Sputum	M1	C35
PAC 90	Sputum	M3	C35
PAC 98	Tracheal aspirate	H3	C35
PAC 183	Sputum	M1	C35
PAC 30	Sputum	M2	UP12
PAC 141	Post chest tube removal	M2	UP13
PAC 151	Sacral sore grade 3	S2	UP14
PAC 184	Tracheal aspirate	CCU	C36
PAC 78	Urine	M1	C36
PAC 03	Blood	H2	C36
PAC 129	Diabetic foot ulcer	ORTH	C36
PAC 139	Tracheal aspirate	CCU	C37
PAC 159	Right foot DFU	S2	C37
PAC 25	Blood	GYN	C38
PAC 27	Pus swab	GYN	C38
PAC 140	Right foot infected DFU	S2	C38
PAC 115	Left hand abscess	S2	C39
PAC 14	Sputum	F1	C39
PAC 112	Tracheal aspirate	ICU	C39

Figure 4.12, continued.



PAC 76	Wound of left elbow	P2	C39
PAC 111	Infected implant with OM changes	ORTH	C39
PAC 131	Crush injury of right foot	ORTH	C40
PAC 82	Urine	S2	C40
PAC 155	Tracheal aspirate	ICU	UP15
PAC 163	Sputum	M2	UP16
PAC 178	Sputum	M1	UP17
PAC 132	Left leg infected wound	S2	C41
PAC 93	Blood	EMER	C41
PAC 07	Nasopharangeal aspirate	P2	C42
PAC 67	Sputum	ICU	C42
PAC 83	Throat swab	M2	UP18
PAC 61	Sputum	S3	C43
PAC 71	Sputum	S3	C43
PAC 48	Sputum	M2	C43
PAC 105	Blood	NICU	C44
PAC 108	Blood	NICU	C44
PAC 113	Blood	NICU	C44
PAC 124	Blood	NICU	C44
PAC 70	Tracheal aspirate	NICU	C44
PAC 80	Blood	NICU	C44
PAC 146	Blood	ICU	C44
PAC 156	Tracheal aspirate	ICU	UP19
PAC 127	LE corneal ulcer	EMER	UP20
PAC 33	Tracheal aspirate	ICU	UP21
PAC 177	Sputum	M1	C45
PAC 185	Tracheal aspirate	HDW	C45
PAC 15	Tissue	S2	C46
PAC 16	Wound swab	S2	C46
PAC 39	Sputum	ICU	C47
PAC 50	Sputum	S3	C47
PAC 103	Sputum	M1	C48
PAC 121	Sputum	M1	C48
PAC 35	Sputum	M1	C48
PAC 37	Tracheal aspirate	S3	C49
PAC 63	Tracheal aspirate	S3	C49
PAC 56	Tracheal aspirate	S1	UP22
PAC 100	Left foot wound	S2	C50
PAC 147	Right ear auricular abscess	ORL	C50
PAC 144	Tracheal aspirate	S3	C51
PAC 188	Blood	P2	C51
PAC 116	Tracheal aspirate	ICU	C51
PAC 172	Blood	NICU	UP23
PAC 154	Urine	ORTH	UP24
PAC 58	Left knee, post wound debridement	ORTH	C52
PAC 65	Tracheal aspirate	ICU	C52
PAC 134	Sputum	M3	UP25
PAC 137	Tracheal aspirate	F2	UP26
PAC 23	Sputum	S3	UP27

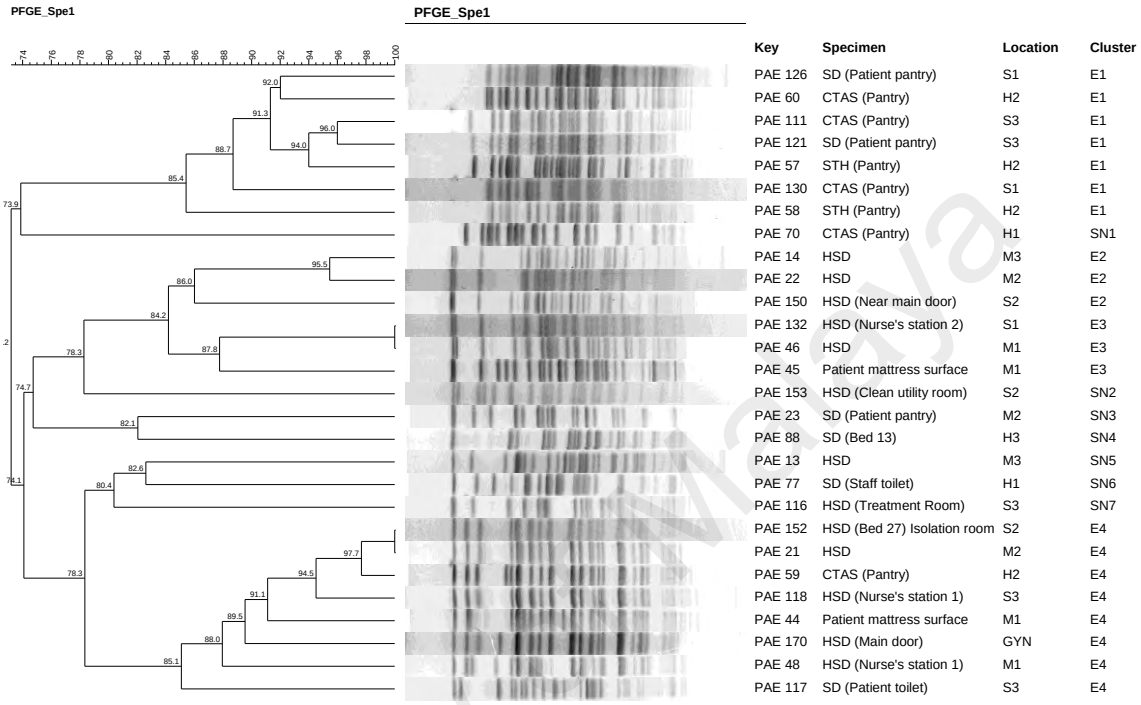
Figure 4.12, continued.

4.5.2 PFGE analyses for environmental strains

PFGE analysis of the 29 *Spe*I-digested chromosomal DNA yielded 19 to 28 restriction fragments with 26 reproducible pulsed-field profiles (PFPs; F = 0.72 – 1.00). These indicate that the strains were genetically diverse and the dendrogram is shown in Figure 4.13. Cluster analysis generated 4 clusters (E1 to E4) at ≥85% similarity and 7 singletons (SN1 to SN7).

Based on the PFGE dendrogram (≥85% similarity), all the strains (n = 7) from E1 originated from the sinks, sink tap handles and counter top surfaces of the pantries in the H2, S1, and S3 wards. However, the strains in E4 (n = 8) were isolated from various

surfaces of the GYN, H2, M1, M2, S2, and S3 wards. This implies that the genetically related environmental *P. aeruginosa* were distributed across the wards on various fomites, from the fifth to the seventh floors of the hospital.



Key	Specimen	Location	Cluster
PAE 126	SD (Patient pantry)	S1	E1
PAE 60	CTAS (Pantry)	H2	E1
PAE 111	CTAS (Pantry)	S3	E1
PAE 121	SD (Patient pantry)	S3	E1
PAE 57	STH (Pantry)	H2	E1
PAE 130	CTAS (Pantry)	S1	E1
PAE 58	STH (Pantry)	H2	E1
PAE 70	CTAS (Pantry)	H1	SN1
PAE 14	HSD	M3	E2
PAE 22	HSD	M2	E2
PAE 150	HSD (Near main door)	S2	E2
PAE 132	HSD (Nurse's station 2)	S1	E3
PAE 46	HSD	M1	E3
PAE 45	Patient mattress surface	M1	E3
PAE 153	HSD (Clean utility room)	S2	SN2
PAE 23	SD (Patient pantry)	M2	SN3
PAE 88	SD (Bed 13)	H3	SN4
PAE 13	HSD	M3	SN5
PAE 77	SD (Staff toilet)	H1	SN6
PAE 116	HSD (Treatment Room)	S3	SN7
PAE 152	HSD (Bed 27) Isolation room	S2	E4
PAE 21	HSD	M2	E4
PAE 59	CTAS (Pantry)	H2	E4
PAE 118	HSD (Nurse's station 1)	S3	E4
PAE 44	Patient mattress surface	M1	E4
PAE 170	HSD (Main door)	GYN	E4
PAE 48	HSD (Nurse's station 1)	M1	E4
PAE 117	SD (Patient toilet)	S3	E4

Figure 4.13: Dendrogram for environmental strains of *P. aeruginosa* based on *Spe*I digestion. Specimen source: Counter top and around sink (CTAS), Handwashing sink and drain (HSD), Sink and drain (SD), Sink tap handle (STH). Locations: Gynaecology ward (GYN), Haematology 1 ward (H1), Haematology 2 ward (H2), Haematology 3 ward (H3), Medical 1 ward (M1), Medical 2 ward (M2), Medical 3 ward (M3), Surgical 1 ward (S1), Surgical 2 ward (S2), Surgical 3 ward (S3). PFGE type is categorized as cluster (C) or unique profile (UP).

4.5.3 MLST

4.5.3.1 Genetic relatedness of *P. aeruginosa* based on BURST analyses

Twenty-seven sequence types were identified among the 39 *P. aeruginosa* (Table 4.7). Analysis by eBURST v3, detected 10 BURST groups or clonal complexes (Figure 4.14). These comprised of STs 1417 (n = 2), 553 (n = 2), 266 (n = 2), 167 (n = 2), 381 (n = 2), 708 (n = 4), 809 (n = 2), 235 (n = 2), 1076 (n = 2), 2338 and 2341. The other STs were singletons. From Figure 4.14, the lack of genetic relatedness among all STs is observed,

except for STs 2338 and 2341, which are SLVs of each other. Further BURST analysis of this dataset in comparison to the *P. aeruginosa* MLST database, revealed that the Malaysian *P. aeruginosa* strains with STs 111, 179, 207, 235, 274, 381, 446, 532, and 2033 (n = 9) were international predicted founders while ST309 (n = 1) was a subgroup founder (Figure 4.15). The STs 266, 708, 1076, 2329, 2338, and 2341 (n = 6) were SLVs while STs 167, 274, 553, 809, 823, 1400, 1417, 1734, 2335, 2337, 2339, 2340, 2341 (n = 13) were singletons. In addition, STs 2329, 2335, 2337, 2338, 2339, 2340, and 2341 (n = 7) were determined to be the novel allelic profile strains among the studied strains.

Table 4.7: Sequence types and allelic profiles of representative clinical and environmental *P. aeruginosa* strains

Strain Label	ST	Allelic profile						
		acsA	***aroE***	***guaA***	***mutL***	***nuoD***	***ppsA***	***trpE***
PAC08	1076	5	4	57	62	1	1	26
PAC10	1400	44	54	99	48	1	1	163
PAC11	708	11	3	11	3	1	4	60
PAC17	235	38	11	3	13	1	2	4
PAC28	111	17	5	5	4	4	4	3
PAC29	708	11	3	11	3	1	4	60
PAC30	2329	134	8	57	27	1	6	3
PAC35	266	16	5	11	72	44	7	52
PAC36	1076	5	4	57	62	1	1	26
PAC47	381	11	20	1	65	4	4	10
PAC51	235	38	11	3	13	1	2	4
PAC54	2335	9	4	11	3	8	7	8
PAC60	2337	30	202	11	4	4	4	7
PAC64	553	17	5	1	11	4	4	45
PAC70	2338	16	5	1	54	58	7	19
PAC90	1417	16	10	11	85	4	4	10
PAC93	2339	11	5	11	5	1	6	2
PAC95	2340	9	131	5	6	12	17	8
PAC96	235	38	11	3	13	1	2	4
PAC98	1417	16	10	11	85	4	4	10
PAC103	266	16	5	11	72	44	7	52
PAC106	708	11	3	11	3	1	4	60
PAC107	708	11	3	11	3	1	4	60
PAC108	2341	16	5	1	3	58	7	19
PAC135	553	17	5	1	11	4	4	45
PAC148	809	36	3	6	13	3	6	26

Table 4.7, continued.

Strain Label	ST	Allelic profile						
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
PAC165	381	11	20	1	65	4	4	10
PAC167	532	5	4	5	5	5	20	4
PAC172	207	47	4	5	33	1	6	40
PAC191	274	23	5	11	7	1	12	7
PAC199	2033	15	5	30	72	3	6	68
PAC200	809	36	3	6	13	3	6	26
PAE14	167	40	5	11	5	4	38	37
PAE22	167	40	5	11	5	4	38	37
PAE45	309	13	8	9	3	1	17	15
PAE48	179	36	27	28	3	4	13	7
PAE70	1734	11	10	6	11	4	4	7
PAE88	446	18	4	5	3	1	17	13
PAE117	823	32	13	24	13	1	6	25

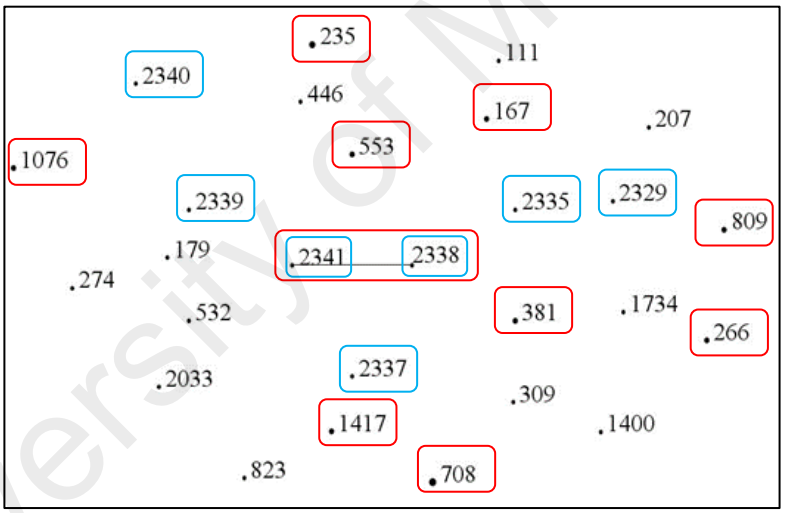


Figure 4.14: The eBURST v3 generated diagram of the Malaysian STs**.** The 10 BURST groups/clonal complexes are indicated in the red boxes. The novel allelic profiles are indicated in the blue boxes.

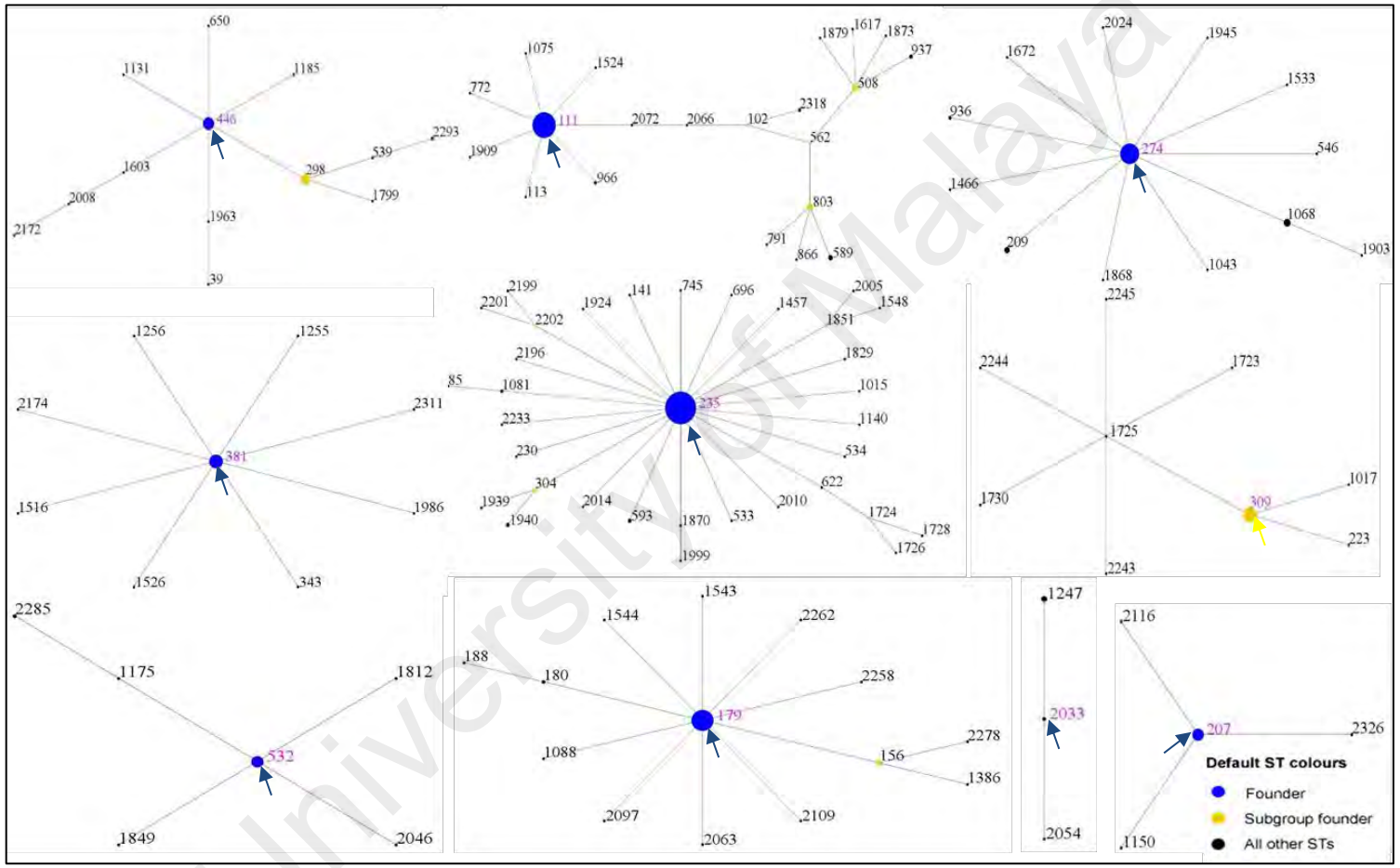


Figure 4.15: Composite image of the BURST groups displaying the lineages of Malaysian STs compared to the international STs from the *P. aeruginosa* PubMLST database. The BURST group founders are indicated by the blue arrows while the sub group founder is indicated by the yellow arrow.

4.5.3.2 Maximum likelihood analyses

Figure 4.16 and Figure 4.17 illustrate the ML rooted tree integrated with STs, PFGE clusters, resistance genes, source, and location of the clinical and environmental *P. aeruginosa* strains, respectively. The nucleotide changes per site are indicated by the 0 to 0.005 cladogram branch lengths (excluding the outgroup). From Figure 4.16, strains on the clades with ≥70% bootstrap probabilities had identical STs except ST381 (PAC47 and PAC165) which were located on distant branches. In contrast, single locus variants, STs 2338 and 2341 which did not share identical STs, were positioned side by side with 98% clade probability. From the ML analysis of the environmental strains (Figure 4.17), strains PAE14 and PAE22 had identical STs and shared 99% clade probabilities. However, strains PAE48 and PAE70 which did not have the same STs shared 78% clade probabilities.

4.5.3.3 Combined analyses for MLST, PFGE, and resistance genes genotypes

Overall, based on the Maximum Likelihood (ML) trees (Figure 4.16 and 4.17), concordance was observed between the clustering of strains having the same STs or single locus variants with the PFGE clusters. For example, ST553 with C6, ST1417 with C35, ST266 with C48, ST708 with C16, ST809 with C17, ST2338/ST2341 with C44, and ST167 with E2. However, ST235 and ST1076 were further subtyped by PFGE. Two antibiotic susceptible clones detected from the NICU in the integrated analysis, were ST708 and single locus variants, STs 2338 and 2341.

The MDRPA genotypes were observed among the strains of STs 235, 809, and 1076 clonal complexes, however, these drug resistant strains were of diverse backgrounds. In our study, the multidrug resistant ST235 strains showed high level MIC values for ceftazidime, cefepime, imipenem, and meropenem (>256, >128, >32, and 32 mg/L, respectively) due to the presence of the $bla_{\text{IMP-4}}$ and $bla_{\text{VIM-2}}$ extra integron

resistance genes. Besides that, the multidrug resistant characteristic for the strains of the ST809 could be attributed to the presence of the class 1 integron bearing the *bla*$_{\text{VIM-6}}$- *bla*$_{\text{OXA-10}}$- *AAC(6')-Ib* and the *bla*$_{\text{VIM-2}}$ gene cassette variants as well as the extra integron *bla*$_{\text{VIM-11}}$.

On the other hand, different types of class 1 integron gene cassettes and extra integron resistance genes were detected for ST1076 MDRPA strains. Strain PAC08 harboured the *bla*$_{\text{GES-13}}$ gene cassette and *bla*$_{\text{GES-20}}$ extra integron resistance gene while strain PAC36 harboured the gene cassette containing the *bla*$_{\text{VIM-6}}$, *bla*$_{\text{OXA-10}}$- *aac(6')-II* genes and the extra integron *bla*$_{\text{VIM-11}}$. The high level of MICs in these two strains (>64 mg/L for ceftazidime, and cefepime, and >32 for imipenem, and meropenem) could be due to the cumulative expression of their multiple resistance genes. However, there were no resistotypes among the environmental strains.

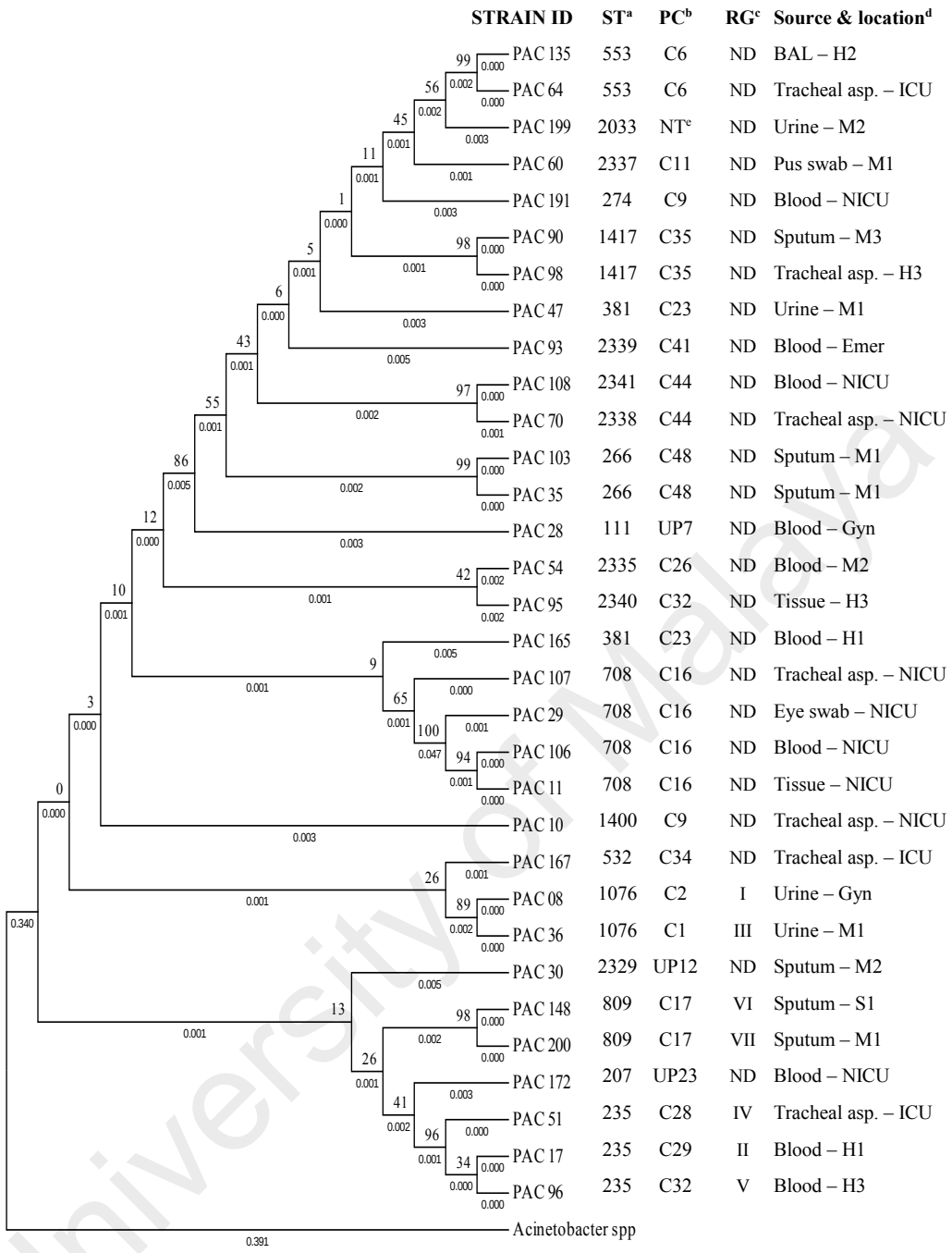


Figure 4.16: Bootstrap cladogram of clinical *P. aeruginosa* MLST sequence types (ST) based on Maximum Likelihood method (Tamura-Nei model) in comparison with PFGE clusters (PC). The bootstrap test (2000 replicates) percentage of associated taxa clustered together is shown next to the branches. [a]Sequence type; [b]PFGE cluster; [c]Resistance genes from class 1 integron gene cassettes (GC) and extra-integron (EI) genes detected by PCR and confirmed by nucleotide sequencing: (ND) Not Detected, (I) GC - $bla_{GES-13,}$ EI - bla_{GES-10}, (II) GC - *aadA6,* EI - Not detected, (III) GC - $bla_{VIM-6,}$ EI - $bla_{VIM-11,}$ (IV) GC - *aadA6-orfD,* EI - bla_{IMP-4} and bla_{VIM-2}, (V) GC - *aadA6-orfD,* EI - $bla_{IMP-4,}$ (VI) GC - bla_{VIM-6}- bla_{OXA-10}- *aacA4, EI* - $bla_{VIM-11,}$ and (VII) GC - $bla_{VIM-2,}$ EI - bla_{VIM-11}; [d] Source & location: Tracheal aspirate (Tracheal asp.), Braochoalveolar lavage (BAL), Intensive care unit (ICU), Neonatal intensive care unit (NICU), Emergency unit (Emer), Surgical 1 (S1), Surgical 3 (S3), Gynaecology (Gyn), Medical 1 (M1), Medical 2 (M2), Medical 3 (M3), Haematology 1 (H1), Haematology 2 (H2) and Haematology 3 (H3) (Phoon et al., 2018). Reprinted permission granted by Mary Ann Liebert, Inc.

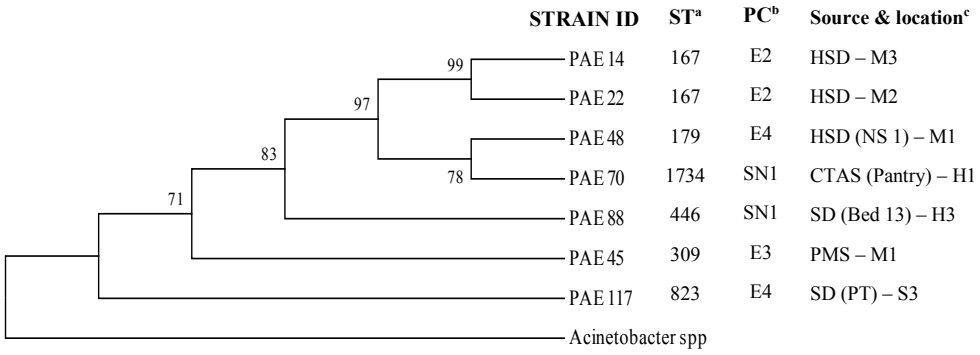


Figure 4.17: Bootstrap cladogram of environmental *P. aeruginosa* MLST sequence types (ST) based on Maximum Likelihood method (Tamura-Nei model) in comparison with PFGE clusters (PC). The bootstrap test (2000 replicates) percentage of associated taxa clustered together is shown next to the branches. [a]Sequence type; [b]PFGE cluster; [c]Source & location: Counter top and around sink surfaces (CTAS), Handwashing sink and drain (HSD), Patient's mattress surface (PMS), Sink and drain (SD), Nurses' station 1 (NS 1), Patients' toilet (PT), Surgical 3 ward (S3), Medical 1 ward (M1), Medical 2 ward (M2), Medical 3 ward (M3), Haematology 1 ward (H1), Haematology 2 ward (H2) and Haematology 3 ward (H3).

CHAPTER 5: DISCUSSION

The periodic investigation of antibiotic resistance profiles and the resistance genes among clinical *P. aeruginosa* strains Malaysia is useful to gauge the level of activity among commonly prescribed antipseudomonal drugs. In this hospital, the susceptibility of the strains towards carbapenems and piperacillin-tazobactam, were the lowest (≤ 90%), whilst ≥ 90% of the strains remained susceptible to all other classes of antimicrobial agents tested. This implies that therapeutic use of carbapenems and piperacillin/tazobactam could become limited. According to the hospital's 2014 Antibiotic Usage report (data not shown), piperacillin/tazobactam, meropenem, imipenem, cefepime, and ceftazidime were among the top 10 most frequently administered antibiotics in the wards. Antibiotic selection pressure resulted in the enhanced growth of antibiotic resistant variants (Davies & Davies, 2010). In a multicentre retrospective study by Micek et al. (2015), the initial inappropriate antibiotic prescription is significantly correlated ($p < 0.001$) to the incidence of MDRPA and *P. aeruginosa*-pneumonia amongst hospitalized patients. Hence, the hospital's continuous judicious antibiotic therapy and review of the annual antibiotic susceptibility profiles are fundamental to detect and prevent emerging MDRPA.

The environmental *P. aeruginosa* in this study were found to be susceptible to all antimicrobial agents tested. This implies that there were no environmental reservoirs of drug resistant *P. aeruginosa* found in various locations in the hospital at the time of sampling. A study conducted by Crespo et al. (2004), recovered imipenem-resistant strains from the sinks (n = 9) and stethoscope (n = 1) in the ICU and sinks (n = 3) in the NICU. Two studies reported recovering *P. aeruginosa* from bronchoscopes (Mackie et al., 2003) and from fentanyl, morphine and water for medication dilution (Suraiya et al., 2008) which were implicated in outbreaks among hospitalized patients. This study was

conducted randomly and therefore no assumption of links could be determined between the clinical and environmental strains. Future investigations with a more targeted approach is warranted.

In this study, the majority of the clinical *P. aeruginosa* strains were isolated from respiratory specimens from patients in the medical wards. Based on clinical reports (unpublished data), patients from these medical wards had underlying diseases such as diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD) and cancers which predisposed them to opportunistic *P. aeruginosa* infections. Results from an international study involving 12 hospitals from five countries showed that patients with DM or COPD co-morbidities were significantly ($p < 0.05$) more likely to have MDRPA infections (Micek et al., 2015). Our study concurred with these findings. The environmental *P. aeruginosa* strains in this study were mostly isolated from moist and semi aqueous environments such as handwashing sinks, tap handles, and pantry counter tops around the sink. This finding concurred with previous studies which showed that *P. aeruginosa* are commonly found in water systems and taps of healthcare facilities (Kelsey, 2013). The persistence of *P. aeruginosa* in the environment of the hospital is due to the biofilm forming ability of the bacteria which are highly robust and can withstand chemical treatments (Lieleg et al., 2011).

The high levels of resistance to beta-lactam antibiotics such as the cephalosporins and carbapenems were mediated by the *bla*$_{OXA}$, *bla*$_{VIM}$ and *bla*$_{IMP}$ genes detected in our study. Our data concurred with findings by other Malaysian studies (Khosravi et al., 2010, 2011; Lim et al., 2009) and previously published reports by Toval et al. (2015), Hansen et al. (2014), and Farshadzadeh et al. (2014). The IMP-4, VIM-2, and VIM-11 subtypes of *P. aeruginosa* identified in this study were similar to Khosravi et al.'s study (2010). However, to the best of our knowledge, this is the first report of the Ambler class A ESBL

gene, *bla*$_{GES}$ (*bla*$_{GES-13}$ and *bla*$_{GES-20}$ variants) detected in Malaysia. The *bla*$_{GES}$-bearing *P. aeruginosa* was first detected in Evgenidion General Hospital, Athens (2007-2008) and subsequently reported in studies from Turkey (2014) and Brazil (2012) (Iraz et al., 2014; Kotsakis et al., 2010; Polotto et al., 2012). No resistance genes were detected from two MDRPA strains (PAC95 and PAC167). Meanwhile, the PAC17 strain, which only harboured the *aadA6-gcuD* was resistant to beta-lactams and fluoroquinolone. Cephalosporin resistance may occur as a result of acquired beta-lactamases, total derepression of chromosomal AmpC or upregulation in efflux systems while carbapenem resistance is often due to loss of OprD porins, upregulation of efflux pump mechanisms or acquisition of beta-lactamases (Livermore, 2002). A cumulative combination of several other resistance mechanisms give rise to multidrug resistance. However, other mechanisms and genes of resistance were not exhaustively investigated in this study; hence future work on the MDRPA strains is warranted.

Two different subtyping tools were used to analyse the genetic relatedness of the strains in this study. The PFGE method subtyped the strains from the NICU into two major clusters and this finding agreed with the MLST data. This implies that there was an undetected outbreak of antibiotic susceptible *P. aeruginosa* clones at the time of sampling. PFGE subtyped the 199 clinical strains into 52 clusters (≥85% similarity) and 27 singletons and the 29 environmental strains into four clusters and seven singletons. This indicates that the strains were genetically heterogeneous and that there were multiple subtypes of *P. aeruginosa* circulating in the different locations or wards in the hospital. While there is a concordance between the strains clustered by PFGE and MLST, there were also exceptions. For example, ST235 and ST1076 which were further subtyped by PFGE. Johnson et al. (2007) reported that PFGE was more discriminatory than MLST (Simpson's *D* value, 0.999 and 0.975, respectively) for subtyping *P. aeruginosa*, which could explain the occurrence of the discordant results. In addition, another study reported

occasional overdiscrimination for some STs by PFGE and that the two typing methods are highly unlikely to have 100% concordance due to the basic differences in approach to molecular genotyping (Kidd et al., 2011).

PFGE genotyping is reliable, reproducible and advantageous for local outbreak investigations. However, it is limited because clustering of the strains cannot be linked to international lineages. The investigation of the genetic linkage for drug resistant international lineages is better mapped via MLST. *P. aeruginosa* ST111 and ST235 strains were previously reported to be multidrug or extensively-drug resistant high-risk international clones found in France, Germany, Japan, Spain and Belgium (Hansen et al., 2014; Hong et al., 2015). The ST235 strains in our study were also multidrug resistant with high MIC values for ceftazidime, cefepime, imipenem, and meropenem (>256, >128, >32, and 32 mg/L, respectively). However, the ST111 strain in this study was susceptible towards all antibiotics tested. ST235 strains harbouring *bla*$_{\text{VIM-2}}$, *bla*$_{\text{IMP-1}}$, and *bla*$_{\text{IMP-7}}$ were reported from a Singaporean study (Koh et al., 2010). Furthermore, *bla*$_{\text{VIM-2}}$-carrying-ST235 was widely distributed in a study involving six Asian countries while ST111 was not detected (Kim et al., 2013). Hence, the occurrence of the ST235 among Malaysian *P. aeruginosa* is indicative of a global dissemination of the drug resistant clone. A 2013 Spanish study showed that the ST809 strains isolated from cystic fibrosis patients were non-MDRPA (Fernández-Olmos et al., 2013). This report differs from our findings on MDRPA ST809 lineage which harboured metallo-beta-lactamase encoding genes. The ST1076 in this study is unique because one of the MDRPA strains possesses the ESBL-encoding *bla*$_{\text{GES}}$ while the other possesses the *bla*$_{\text{VIM-6}}$ MBL and *bla*$_{\text{OXA-10}}$ ESBL genes concurrently. These characteristics also differed from the recently reported ST1076 from South Korea which did not harbour any MBL-encoding genes (Lee et al., 2013).

The STs 179, 309 and 446 among the environmental *P. aeruginosa* in this study were also previously reported. In Spain, researchers at a university hospital reported characterizing ST179 as one of the 21 STs of persistent isolates from cystic fibrosis patients (Fernández-Olmos et al., 2013). ST309 and ST446 were genotyped by researchers from South Korea and were identified as metallo-β-lactamase-producing and multidrug resistant *P. aeruginosa*, respectively (Hong et al., 2015; Lee et al., 2013). However, in our study, these strains were all susceptible to all classes of antimicrobials tested. Future on-going surveillance of hospital environmental fomites and sources are warranted to monitor these STs to detect emergence of antimicrobial resistance which pose a serious nosocomial infection risk.

There are several limitations in this study. The sampling of clinical and environmental *P. aeruginosa* were not performed in parallel to establish the potential nosocomial infection link between the strains found in the hospital environment to the clinical strains isolated from patients. A more targeted study of nosocomial infections caused by exogenous sources of *P. aeruginosa* is warranted for future investigations. Furthermore, the study of the *P. aeruginosa* resistance mechanisms was not exhaustive and thus could not explain the multidrug resistance exhibited in two of the strains in this study, PAC95 and PAC167. Hence, further testing is required to determine other mechanisms of drug resistance in these strains.

CHAPTER 6: CONCLUSION

From this study, the highest numbers of isolated clinical *P. aeruginosa* were from respiratory specimens of patients with co-morbidities while the environmental *P. aeruginosa* were from handwashing sinks in the wards. The antimicrobial susceptibility profiles of all the *P. aeruginosa* strains in this tertiary hospital showed that the majority of the strains were susceptible to most of the antimicrobial agents tested. However, the presence of MDRPA strains is still a cause for concern because of the high level cephalosporin and carbapenem resistance mediated by the *bla*$_{\text{GES}}$, *bla*$_{OXA}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ resistance genes which can be easily disseminated via the class 1 integron. PFGE subtyping of all the strains showed that DNA fingerprints were heterogeneous and diverse. The two major clusters from the NICU subtyped by PFGE and MLST implied that there was an undetected outbreak of antibiotic susceptible *P. aeruginosa* clones within the sampling duration. By utilizing the MLST subtyping method, a high risk MDRPA clone was identified in this study. The clonal spread of the international high risk clone ST235 in Malaysia requires future close monitoring. To the best of our knowledge, this is the first report of the *bla*$_{\text{GES-13}}$ and *bla*$_{\text{GES-20}}$ ESBL-encoding gene variants and novel sequence types (STs 2329, 2335, 2337, 2338, 2339, 2340, and 2341) of *P. aeruginosa* in Malaysia.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications:

- **Phoon, H. Y. P.**, Hussin, H., Hussain, B. M., & Thong, K. L. (2018). Molecular characterization of extended-spectrum beta-lactamase- and carbapenemase-producing *Pseudomonas aeruginosa* strains from a Malaysian tertiary hospital. *Microbial Drug Resistance*. Advance online publication, doi: 10.1089/mdr. 2017.0258.

- **Phoon, H. Y. P.**, Hussin, H., Hussain, B. M., Lim, S. Y., Woon, J. J., Er, Y. X., & Thong, K. L. (2018). Distribution, genetic diversity and antibiotic resistance of clinically important bacteria from the environment of a tertiary hospital. *Journal of Global Antimicrobial Resistance, 14,* 132-140.

List of presentations:

- Phoon, H. Y. P., Hussin, H., & Thong, K. L. (2015, Apr). *A snapshot of antibiotic resistance of Pseudomonas aeruginosa in a major specialist hospital in the Klang Valley.* Poster presented at the Infection Disease & Microbial Genomics Conference, Putrajaya, Malaysia.

- Phoon H. Y. P., & Thong K. L. (2016, Dec). *Prevalence and distribution of potential nosocomial pathogens from exogenous sources in a tertiary care hospital in Malaysia.* Poster presented at the 21st Biological Science Graduate Congress, Kuala Lumpur, Malaysia.

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