J Microbiol Immunol Infect.
2009;42:197-209

Original Article

Genetic fingerprinting and antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* hospital isolates in Malaysia

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Received: May 20, 2008 Revised: July 1, 2008 Accepted: July 23, 2008

Background and purpose: *Pseudomonas aeruginosa* is the third most common pathogen causing nosocomial infections. The objective of this study was to investigate the antimicrobial resistance profiles and genetic diversity of hospital isolates of *P. aeruginosa* and to investigate the presence of several resistance genes and integrons.

Methods: In this retrospective study, 48 clinical isolates of *P. aeruginosa* from 6 public hospitals in Malaysia were analyzed by antimicrobial susceptibility test and DNA fingerprinting techniques.

Results: Most of the *P. aeruginosa* isolates were resistant to tetracycline (73%) and chloramphenicol (60%) and, to a lesser extent, cefotaxime (40%), ceftriaxone (31%), cefoperazone (29%), ticarcillin (25%), piperacillin (23%), and imipenem (21%). Less than 20% of the isolates were resistant to ceftazidime, gentamicin, cefepime, ciprofloxacin, amikacin, piperacillin-tazobactam, and aztreonam (10%). Of the 48 isolates, 33 were multidrug resistant. Two isolates were extended-spectrum β-lactamase (ESBL) producers using the double-disk synergy test. However, polymerase chain reaction (PCR) failed to detect any common ESBL-encoding genes in all isolates, except for bla_{OXA-10} in PA7 that was found to be part of a class 1 integron-encoded *aacA4*-bla_{IMP-9}-*catB8*-bla_{OXA-10} gene cassette. Using PCR, class 1 integron-encoded integrases were detected in 19% of the *P. aeruginosa* isolates. Repetitive extragenic palindrome-PCR generated 40 different profiles (*F* = 0.50-1.0) and enterobacterial repetitive intergenic consensus-PCR produced 46 profiles (*F* = 0.51-1.0). Pulsed-field gel electrophoresis with *Spe*I-digested genomic DNA resulted in 45 different profiles (*F* = 0.50-1.00).

Conclusion: Aztreonam appeared to be the most effective agent against multidrug-resistant *P. aeruginosa* isolates. Sixty nine percent of the *P. aeruginosa* isolates analyzed were multidrug resistant and the isolates were genetically diverse.

Key words: DNA fingerprinting; Drug resistance, microbial; Drug resistance, multiple; *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is one of the most important causative bacterial pathogens of nosocomial infections, particularly in immunocompromised patients, leading to pneumonia, bacteremia, and urinary tract infections [1,2]. *P. aeruginosa* is also a major cause of chronic lung infection and death in children and adults with cystic fibrosis [3].

The prevalence of multidrug-resistant (MDR) isolates has been increasing worldwide and poses a serious problem in hospital settings. The spread of these

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organisms is often difficult to control as *P. aeruginosa* exhibits intrinsic resistance to several antimicrobial agents [4]. Furthermore, some of these resistance genes have been found as gene cassettes in integrons, which could play an important role in the transfer of these resistance genes to other bacteria [5,6].

Rapid and discriminative subtyping methods are essential for determining the epidemiology of pathogenic isolates and are useful in the design of rational pathogen control methods. Several methods are available and these include pulsed-field gel electrophoresis (PFGE), arbitrary primed polymerase chain reaction (PCR) ribotyping, amplified fragment length polymorphism, and other PCR-associated typing methods. PCR-based techniques can be considered as rapid methods for subtyping *P. aeruginosa,* and have been shown to be useful in epidemiological study of *P. aeruginosa* isolates [3].

PFGE is an established subtyping method, and is considered to be the 'gold standard' for the majority of nosocomial pathogens, because it is highly discriminatory and reproducible, and the resulting banding profiles are easily interpreted [7]. PFGE was successfully applied to investigate cases of *P. aeruginosa* infection in several hospitals in Malaysia to trace sources of nosocomial spread [8].

The objective of this study was to determine the antimicrobial resistance and extended-spectrum β-lactamase (ESBL) profiles of *P. aeruginosa* isolates in Malaysia and their genetic diversity using PCR-based fingerprinting techniques and PFGE. The presence of clinically important resistance genes, class 1 and 2 integrons, was also determined via PCR.

Methods

Bacterial isolates and culture conditions

In this retrospective study, 48 non-repeat *P. aeruginosa* strains isolated in 2004 from 48 patients were selected randomly from 6 public hospitals located in different parts of Malaysia. The participating hospitals were Kota Bharu Hospital (n = 22) in the East, Sultanah Aminah Hospital (n = 11) in the South, Kuala Lumpur Hospital (n = 7) and Ipoh Hospital (n = 1) in the central region, Tengku Ampuan Rahimah Hospital (n = 3) in the West, and Queen Elizabeth Hospital (n = 4) in the East. The organisms were isolated from tracheal aspirates (n = 21), urine (n = 2), blood (n = 1), body fluids (n = 1), tracheal secretions (n = 1), cerebrospinal fluid (n = 1), swabs (n = 6), and unknown cultures (n = 15). Identification of *P. aeruginosa* was based on colonial morphology on MacConkey agar, positive oxidase reaction, and their biochemical properties. All isolates were cultured in Luria-Bertani (LB) broth and grown at 37°C for genomic DNA isolation. The isolates were stored in cryovials containing LB broth with 50% glycerol (Invitrogen, Carlsbad, CA, USA) at –20°C. The purity of the strains was checked before commencement of analysis.

Antimicrobial susceptibility testing and screening for extended-spectrum β-lactamase

The susceptibilities of the bacterial isolates to 15 antimicrobial agents (ticarcillin 10 μg, piperacillin 100 μg, piperacillin-tazobactam 100 μg/10 μg, ceftriaxone 30 μg, ceftazidime 30 μg, cefepime 30 μg, cefoperazone 75 μg, cefotazime 30 μg, aztreonam 30 μg, imipenem 10 μg, amikacin 30 μg, tetracycline 30 μg, chloramphenicol 30 μg, ciprofloxacin 5 μg, and gentamicin 10 μg; [Oxoid Ltd, Basingstoke, UK]) were determined by the disk diffusion method according to the criteria published by the Clinical and Laboratory Standards Institute (CLSI) [9].

Double-disk synergy tests were performed according to established protocols [2] with slight modifications. The double-disk synergy tests were carried out as a phenotypic test for the production of ESBLs in the bacterial isolates. Briefly of 30 μg each ceftazidime, ceftriaxone, aztreonam, and cefepime disks were placed at a distance of 20 mm (center-to-center) from an amoxicillin-clavulanate 20 μg/10 μg disk on Mueller-Hinton agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37°C for 16 h. The results were interpreted as described previously [2]. *Escherichia coli* American Type Culture Collection (ATCC) 25922 and ATCC 35218, *Klebsiella pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853 cultures were used as quality control isolates for susceptibility testing.

Detection of bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, bla_{DHA}, and bla_{VEB} genes

Detection of various β-lactamase genes were performed by PCR using reverse and forward primer pairs, as listed in Table 1. [10-16] DNA templates were prepared by the boiling method as described previously [17]. PCR amplification was performed in a final volume of 25 μL containing 0.3 μM of each primer (Operon Biotechnologies GmbH, Köln, Germany), 35 μM of each deoxynucleoside triphosphate (dNTP), 1.4 mM magnesium chloride ($MgCl_2$) and 0.5 U Taq

DNA polymerase (Promega, Madison, WI, USA). The cycling parameters used were as previously described [1,10-14], and are listed in Table 1. Clinical isolates of *E. coli* were used as the positive control for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} genes. However, no positive controls were available for the detection of bla_{VEB} and bla_{DHA} genes, therefore, negative results should be treated with caution. All amplified products obtained were sequenced to validate their identity.

Detection of class 1 and 2 integrons

Detection of class 1 and 2 integrons was performed by PCR using the reverse and forward primer pairs listed in Table 1. The IntI1-F/IntI1-R primer pair was used

Table 1. Primer sequences and polymerase chain reaction (PCR) conditions used to investigate the antimicrobial resistance profiles and genetic diversity of *Pseudomonas aeruginosa*.

Primer	Oligonucleotide sequence (5' to 3')	PCR conditions	Study
ERIC-2	AAGTAAGTACCTGGGGTGAGCG	1 cycle of 5 min at 94°C 30 cycles of 1 min at 94°C, 1 min at 53°C, 4 min at 72°C 1 cycle of 10 min at 72°C	Jiang et al, 2006 [1]
REP	GCGCCGICATGCGGCATT	1 cycle of 4 min at 94°C 35 cycles of 1 min at 94°C, 1 min at 42°C, 8 min at 68°C 1 cycle of 8 min at 72°C	Navia et al, 1999 [16]
208	ACGGCCGACC	5 cycles of 5 min at 94°C, 5 min at 36°C, 5 min at 72°C 30 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C 1 cycle of 10 min at 72°C	Mahenthiralingam et al, 1996 [15]
IntI1-F	GGTCAAGGATCTGGATTTGG	1 cycle of 12 min at 94°C	Machado et al, 2005 [14]
IntI1-R	ACATGCGTGTAAATCATCGTC	35 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C; 1 cycle of 10 min at 72°C	
5'CS	GGCATCCAAGCAGCAAG	1 cycle of 10 min at 94°C	Machado et al, 2005 [14]
3'CS	AAGCAGACTTGACCTGA	35 cycles of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C 1 cycle of 8 min at 72°C	
IntI2-F	CACGGATATGCGACAAAAAGGT	1 cycle of 12 min at 94°C	Machado et al, 2005 [14]
IntI2-R	GTAGCAAACGAGTGACGAAATG	35 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C 1 cycle of 10 min at 72°C	
TEM-F	ATGAGTATTCAACATTTCCG	1 cycle of 5 min at 96°C	Oliver et al, 2002 [11]
TEM-R	CTGACAGTTACCAATGCTTA	35 cycles of 1 min at 96°C, 1 min at 58°C, 1 min at 72°C 1 cycle of 10 min at 72°C	
SHV-F	GGTTATGCGTTATATTCGCC	1 cycle of 5 min at 96°C	Oliver et al, 2002 [11]
SHV-R	TTAGCGTTGCCAGTGCTC	35 cycles of 1 min at 96°C, 1 min at 60°C, 1 min at 72°C 1 cycle of 10 min at 72°C	
OXA1-F	ACACAATACATATCAACTTCGC	1 cycle of 5 min at 96°C	Oliver et al, 2002 [11]
OXA1-R	AGTGTGTTTAGAATGGTGATC	35 cycles of 1 min at 96°C, 1 min at 60°C, 2 min at 72°C 1 cycle of 10 min at 72°C	
OPR1	GTCTTTCGAGTACGGCATTA	1 cycle of 5 min at 95°C	Yan et al, 2006 [10]
OPR2	ATTTTCTTAGCGGCAACTTAC	30 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C 1 cycle of 5 min at 72°C	
OXA 2,3	GCCAAAGGCACGATAGTTGT	1 cycle of 5 min at 94°C	Yan et al, 2006 [10]
OXB 2,3	GCGTTCGAGTTGACTGCCGG	35 cycles of 1 min at 94°C, 1 min at 60°C, 2 min 30 sec at 72°C 1 cycle of 10 min at 72°C	
CTX-MU1	ATGTGCAGYACCAGTAARGT	1 cycle of 7 min at 94°C	Pagani et al, 2003 [12]
CTX-MU2	TGGGTRAARTARGTSACCAGA	35 cycles of 50 sec at 94°C, 40 sec at 50°C, 1 min at 72°C 1 cycle of 5 min at 72°C	
DHA-1U	CACACGGAAGGTTAATTCTGA	1 cycle of 5 min at 96°C	Pai et al, 2004 [13]
DHA-1L	CGGTTATACGGCTGAACCTG	30 cycles of 1 min at 96°C, 1 min at 55°C, 2 min at 72°C 1 cycle of 10 min at 72°C	
VEB-1A	CGACTTCCATTTCCCGATGC	1 cycle of 5 min at 96°C	Jiang et al, 2006 [1]
VEB-1B	GGACTCTGCAACAAATACGC	30 cycles of 1 min at 96°C, 1 min at 55°C, 2 min at 72°C 1 cycle of 10 min at 72°C	

to detect the *intI1* integrase gene of class 1 integrons, whereas the 5'CS/3'CS primer pair was used to amplify the integron variable region, which contains the gene cassettes. For class 2 integrons, the primers used were the IntI2-F and IntI2-R pair to detect the *intI2* integrase gene. PCR amplifications were carried out in a final volume of 25 μL containing 0.3 μM of each primer pair (Operon Biotechnologies GmbH), 35 μM each of dNTP, 1.4 mM $MgCl_2$ and 0.5 U Taq DNA polymerase. The cycling parameters for the detection of the *intI1* and *intI2* genes were as previously described [14] and are listed in Table 1. Amplified products obtained were sequenced to corroborate their identities.

Polymerase chain reaction typing methods

Chromosomal DNA from the *P. aeruginosa* isolates was obtained using standard protocols [17]. Randomly amplified polymorphic DNA (RAPD) analysis was performed using primer 208 [15]. Enterobacterial repetitive intergenic consensus (ERIC) analyses were performed using the ERIC-2 primer (Operon Biotechnologies GmbH). Repetitive extragenic palindrome (REP) analysis was performed using the REP oligonucleotides (Operon Biotechnologies GmbH) as a primer. Each PCR reaction was carried out in a 25 μL volume using 1.5 U of Taq DNA polymerase in the reaction buffer provided by the manufacturer containing 2.5 mM $MgCl_2$, 50 μM each of dNTP, 0.3 μM of the selected primer, and 5 μL of DNA template. The cycling parameters used are listed in Table 1.

Following PCR amplification, 10 μL aliquots of each sample were subjected to electrophoresis on 1.4% agarose gels for RAPD and REP-PCR, and 1.6% agarose gels for ERIC-PCR.

Pulsed-field gel electrophoresis typing

PFGE was performed according to established protocols [18] with slight modifications. Briefly, equal volumes of 1% Seakem Gold agarose (Cambrex Bio Science Rockland, Inc, Rockland, ME, USA) were then mixed with 100 μL cell standardized suspension (OD_{610} = 1.4) to form plugs. The bacteria were lysed within the plugs with cell lysis buffer (50 mM Tris; 50 mM ethylenediaminetetraacetic acid [TE buffer; pH 8.0], 1% sacrosine, 1 mg/mL proteinase K) and incubated at 54°C for 3 h. The plugs were then washed thoroughly with sterile de-ionized water (twice) and TE buffer (6 times). Then, 1.5-mm plug slices were digested overnight with the restriction enzyme *SpeI* (Promega) at 37°C. The plugs were then loaded onto a 1.0% agarose gel (Sigma Type 1; Sigma Corp, St. Louis, MO, USA). PFGE was performed with the CHEF DR II (BioRad, Hercules, CA, USA) with the following conditions: 200 V for 26 h with pulse times of 5 to 60 sec. Gels were photographed under ultraviolet light after staining with ethidium bromide 0.5 μg/mL.

Fingerprint pattern analysis

The banding patterns generated by RAPD, ERIC-PCR, REP-PCR, and PFGE were analyzed using GelCompar II (Version 2.5; Applied Maths, Kortrijk, Belgium). All the PCR fingerprints and PFGE profiles were assigned arbitrary designation and analyzed by defining a similarity (Dice) coefficient $F = 2n_{xy}/(n_x + n_y)$ where, n_x = number of fragments for isolate X, n_y = number of fragments for isolate Y, and n_{xy} = number of shared fragments between isolates X and Y [19].

Gel pictures were converted into tagged image file format images prior to export into GelCompar II for cluster analysis which was carried out based on the unweighted pair-group method with arithmetic averaging using the position tolerance of 0.15 [20].

Results

Antibiograms

All 48 *P. aeruginosa* isolates were found to be resistant to amoxicillin-clavulanic acid, while the majority of the isolates were resistant to tetracycline (73%) and chloramphenicol (60%). The resistance rates for the other antibiotics tested using the disk diffusion method were as follows: cefotaxime (40%), ceftriaxone (31%), cefoperazone (29%), ticarcillin (25%), piperacillin (23%), imipenem (21%), ceftazidime and gentamicin (19% each), cefepime and ciprofloxacin (17% each), amikacin and piperacillin-tazobactam (15% each), and aztreonam (10%). MDR isolates resistant to 2 or more classes of antibiotics were found in 69% of the isolates tested (Table 2). All the imipenem-resistant isolates were also resistant to chloramphenicol and 80% of them were resistant to the cephalosporin group of antibiotics (cefoperazone, ceftazidime, cefotaxime, cefepime, and ceftriaxone). Among the cephalosporin-resistant group, all the ceftazidime-resistant isolates were also resistant to ceftriaxone and cefoperazone. A large number (75%) of the *P. aeruginosa* isolates that were resistant to ciprofloxacin were also resistant to tetracycline. However, 60% of carbapenem-resistant isolates were susceptible to aztreonam (monobactam). Resistance to third-generation cephalosporins was

Table 2. Characterization of the 48 *Pseudomonas aeruginosa* isolates, including antibiotypes, production of extended-spectrum β-lactamase (ESBL), integron content, size of gene cassettes, polymerase chain reaction (PCR), and pulsed-field gel electrophoresis (PFGE) profiles.

Isolate no.	Antibiotic resistance	ESBL producer[a]	Presence of class 1 integron-encoded *intI1*	Size of gene cassettes detected (kb)	Enterobacterial repetitive intergenic consensus profile	Repetitive extragenic palindrome-PCR profile	PFGE *SpeI* profile	Combined profile
PA1	Tetracycline, chloramphenicol	–	–		E1	R1	S1	E01R01S01
PA2	Tetracycline, chloramphenicol	–	–		E2	R2	S2	E02R02S02
PA3	Tetracycline	–	–		E3	R3	S3	E03R03S03
PA4	Tetracycline, chloramphenicol	–	–		E4	R4	S4	E04R04S04
PA5	Piperacillin, cefepime, cefoperazone, ceftriaxone, imipenem, gentamicin, amikacin, tetracycline, ciprofloxacin, chloramphenicol, piperacillin-tazobactam, ticarcillin, cefotaxime	–	+	1.4	E5	R5	S5	E05R05S05
PA6	Piperacillin, cefoperazone, ceftriaxone, imipenem, gentamicin, amikacin, tetracycline, ciprofloxacin, chloramphenicol, piperacillin-tazobactam, ticarcillin, cefotaxime	–	+	1.4	E5	R6	S5	E05R06S05
PA7	Piperacillin, ceftazidime, cefepime, cefoperazone, ceftriaxone, aztreonam, gentamicin, amikacin, tetracycline, cefotaxime, ticarcillin	–	+	1.4 2.0	E6	R7	S6	E06R07S06
PA8	Tetracycline, chloramphenicol	–	–		E7	R8	S7	E07R08S07
PA9	Tetracycline	–	–		E8	R9	S8	E08R09S08
PA10	Piperacillin, ceftazidime, cefepime, cefoperazone, ceftriaxone, imipenem, gentamicin, amikacin, ciprofloxacin, cefotaxime, ticarcillin, chloramphenicol	–	+	1.4	E9	R10	S9	E09R10S09
PA11	Piperacillin, cefoperazone, ceftriaxone, imipenem, gentamicin, amikacin, tetracycline, ciprofloxacin, chloramphenicol, ticarcillin, cefotaxime	–	+	1.4	E10	R10	S10	E10R10S10
PA12	Tetracycline	–	–		E11	R11	S11	E11R01S11
PA13	Tetracycline	–	–		E12	R12	S12	E12R12S12
PA14	Tetracycline, ciprofloxacin, chloramphenicol	–	–		E13	R13	S13	E13R13S13
PA15	Piperacillin-tazobactam, cefotaxime, cefepime	–	–		E14	R14	S14	E14R14S14

(Table continued on page 202)

(Table continued from page 201)

PA16	Piperacillin, ceftazidime, cefoperazone, ceftriaxone, imipenem, gentamicin, amikacin, tetracycline, chloramphenicol, ticarcillin, cefotaxime	–	+	1.4	E15	R15	S15	E15R15S15
PA17	Sensitive or intermediate resistance	–	–		E16	R16	S16	E16R16S16
PA18	Tetracycline, chloramphenicol, piperacillin-tazobactam, ticarcillin, cefotaxime	–	–		E17	R17	S17	E17R17S17
PA19	Tetracycline, ciprofloxacin	–	–		E18	R18	S18	E18R18S18
PA20	Tetracycline, chloramphenicol	–	–		E19	R19	S19	E19R19S19
PA21	Ceftazidime, cefepime, cefoperazone, ceftriaxone, aztreonam, gentamicin, amikacin, tetracycline, ciprofloxacin, chloramphenicol, ticarcillin, cefotaxime	+	+	1.4	E20	R20	S20	E20R20S20
PA22	Tetracycline, chloramphenicol	–	–		E21	R21	S21	E21R21S21
PA23	Tetracycline, cefotaxime	–	–		E22	R22	S22	E22R22S22
PA24	Imipenem, chloramphenicol	–	–		E23	R23	S23	E23R23S23
PA25	Sensitive or intermediate resistance	–	–		E24	R24	S24	E24R24S24
PA26	Cefepime, cefoperazone, tetracycline, chloramphenicol	–	–		E25	R25	S25	E25R25S25
PA27	Tetracycline, chloramphenicol	–	–		E26	R26	S26	E26R26S26
PA28	Tetracycline	–	–		E27	R27	S27	E27R27S27
PA29	Sensitive or intermediate resistance	–	–		E28	R28	S28	E28R28S28
PA30	Ceftriaxone, tetracycline, chloramphenicol, cefotaxime	–	–		E29	R29	S29	E29R29S29
PA31	Cefotaxime, tetracycline, ceftriaxone, cefoperazone	–	–		E30	R30	S30	E30R30S30
PA32	Tetracycline	–	–		E31	R31	S31	E31R31S31
PA33	Piperacillin, ceftazidime, cefoperazone, ceftriaxone, imipenem, chloramphenicol, cefotaxime	–	–		E32	R32	S32	E32R32S32
PA34	Piperacillin, ceftazidime, cefepime, cefoperazone, ceftriaxone, imipenem, aztreonam, tetracycline, chloramphenicol, piperacillin-tazobactam, ticarcillin, cefotaxime	–	+	1.4	E33	R32	S33	E33R32S33
PA35	Piperacillin, ceftazidime, cefoperazone, ceftriaxone, tetracycline, chloramphenicol, cefotaxime, ticarcillin	–	–		E34	R32	S34	E34R32S34
PA36	Imipenem, tetracycline, chloramphenicol	–	–		E35	R33	S35	E35R33S35

(Table continued on page 203)

(Table continued from page 202)

PA37	Tetracycline	–	–		E36	R32	S36	E36R32S36
PA38	Ceftriaxone, tetracycline, chloramphenicol, cefotaxime	–	–		E37	R34	S37	E37R34S37
PA39	Sensitive or intermediate resistance	–	–		E38	R35	S38	E38R35S38
PA40	Tetracycline, chloramphenicol	–	–		E39	R36	S39	E39R36S39
PA41	Cefotaxime, tetracycline, chloramphenicol, piperacillin-tazobactam	–	–		E39	R36	S39	E39R36S39
PA42	Sensitive or intermediate resistance	–	–		E40	R37	S40	E40R37S40
PA43	Sensitive or intermediate resistance	–	–		E41	R36	S41	E41R36S41
PA44	Sensitive or intermediate resistance	–	–		E42	R32	S42	E42R32S42
PA45	Ciprofloxacin, chloramphenicol	–	–		E43	R37	S43	E43R37S43
PA46	Chloramphenicol	–	–		E44	R38	S44	E44R38S44
PA47	Piperacillin, ceftazidime, cefepime, cefoperazone, ceftriaxone, aztreonam, gentamicin, tetracycline, chloramphenicol, ticarcillin, cefotaxime	+	+	1.4	E45	R39	S45	E45R39S45
PA48	Piperacillin, ceftazidime, cefoperazone, ceftriaxone, imipenem, aztreonam, gentamicin, tetracycline, chloramphenicol, piperacillin-tazobactam, ticarcillin, cefotaxime	–	–		E46	R33	S38	E46R33S38

[a]Double-disk synergy test.

found in 19 of the isolates (40%) whereas 6 isolates (13%) were resistant to β-lactam inhibitors. Using the double-disk synergy test, ESBL-producing isolates were detected in only 2 of the isolates. Interestingly, both the ESBL-positive isolates were sensitive to the β-lactam inhibitor, but were resistant to the third-generation cephalosporins.

Detection of genes encoding extended-spectrum β-lactamases

PCR was carried out on the genomic DNA of the 48 *P. aeruginosa* isolates using primers specific for the following ESBL-encoding genes: bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-10}}$, $bla_{\text{CTX-M}}$, bla_{DHA}, and bla_{VEB}. Only 1 isolate, PA7 was found to harbor the $bla_{\text{OXA-10}}$ gene. The presence of the $bla_{\text{OXA-10}}$ gene was confirmed by direct sequencing of the class 1 integron gene cassette detected. However, PCR failed to detect any ESBL gene from the remaining 47 *P. aeruginosa* isolates, including 2 isolates that were indicated as putative ESBL-producers by the double-disk synergy test. Failure to detect any amplified products using primers specific for bla_{DHA} and bla_{VEB} should be treated with caution as no positive controls were available for these 2 ESBL-encoding genes.

Detection of integrons

Nine of the 48 (19%) *P. aeruginosa* isolates had the *intI1* gene encoding the integrase of class 1 integrons. However, none of the strains was positive for *intI2*-encoded integrase of class 2 integrons. All 9 isolates that were positive for the *intI1* gene were further analyzed for the presence of inserted gene cassettes within the integron variable region by PCR using the 5'CS/3'CS primer pair. Two different amplified products of 1.4 kb and 2.0 kb were obtained from the 9 isolates.

Direct sequencing of the 1.4 kb amplified product indicated the presence of the *aacA7-aadA6-orfD* gene cassette (accession number, DQ157702.1) within the integron variable region, while direct sequencing of

the 2.0 kb amplified product indicated the presence of the *aacA4-bla*$_{IMP-9}$-*catB8-bla*$_{OXA-10}$ gene cassette (accession number, AY033653). The *aacA7, aadA6,* and *aacA4* genes encode resistance to aminoglycosides, whereas *catB8* encodes resistance to chloramphenicol. Isolate PA7 harbored the *aacA4-bla*$_{IMP-9}$-*catB8-bla*$_{OXA-10}$ gene cassette and showed resistance to the aminoglycosides, amikacin and gentamicin, and intermediate resistance to chloramphenicol. However, streptomycin and spectinomycin were not used in this study, as both antibiotics are not recommended by the CLSI against *P. aeruginosa*. Both *bla*$_{IMP-9}$ and *bla*$_{OXA-10}$ encode for enzymes that have the ability to hydrolyze β-lactam antibiotics.

Both the ESBL-producing *P. aeruginosa* isolates (as determined by the double-disk synergy test) were positive for the *intI1* gene and harbored only the *aacA7-aadA6-orfD* gene cassette. All 9 isolates that harbored class 1 integrons were resistant to ceftriaxone and cefoperazone. However, isolates that were negative for the *intI1* gene were susceptible to amikacin.

Genetic diversity by DNA fingerprinting

Three PCR-based DNA fingerprinting methods were used to subtype the 48 *P. aeruginosa* clinical isolates. With ERIC-PCR, the 48 isolates were subtyped into 46 different profiles (F = 0.51-1.0). REP-PCR differentiated the isolates into 40 distinct profiles (F = 0.49-1.0), while RAPD-PCR distinguished each isolate with a unique profile resulting in 48 different fingerprints (F = 0.34-0.91). Both ERIC-PCR and REP-PCR were found to be reproducible as identical profiles were obtained in separate experiments using the same set of isolates. However, this was not the case for RAPD-PCR as differences were observed in the profiles that were obtained in 2 separate experiments. Thus, the results obtained from RAPD-PCR were not taken into account for the subsequent analysis. Two *P. aeruginosa* isolates, PA40 and PA41, were indistinguishable by ERIC-PCR and REP-PCR. Both these isolates were isolated from the same hospital and from tracheal aspirates but from different patients in different wards. Four of the integron-positive isolates (PA5, PA6, PA10, and PA11) were found to be clonally related, as determined by ERIC and REP-PCR. All 4 isolates were isolated from the same hospital but from different patients (3 were taken from tracheal aspirates and 1 from urine).

P. aeruginosa isolates PA39, PA46, PA40, and PA41 were grouped in the same cluster as their ERIC-PCR and REP-PCR profiles shared more than 85% similarity. These isolates were from 2 different hospitals (Kuala Lumpur Hospital [n = 3] and Queen Elizabeth Hospital [n = 1]) and were obtained from tracheal aspirates (n = 3) and swab samples (n = 1).

Genetic diversity by pulsed-field gel electrophoresis

Genomic DNA from the 48 *P. aeruginosa* isolates were digested with *SpeI* and separated by PFGE. This resulted in 45 distinct pulsed-field profiles (PFPs; F = 0.50-1.00) made up of 12 to 26 restriction fragments. All the profiles were reproducible when the PFGE analysis was repeated once. Interpretation and analysis of the profiles were easier than for PCR-based profiles as the bands were distinct. The 2 *P. aeruginosa* isolates that were indistinguishable by ERIC and REP-PCR (PA40 and PA41) were similarly indistinguishable in their PFPs. Another 2 isolates that showed identical PFPs (PA5 and PA6) were also found to be identical when subtyped with ERIC-PCR.

Combined analysis

A dendrogram depicting the clustering of the isolates based on the 3 typing methods, ERIC-PCR, REP-PCR, and PFGE, is shown in Fig. 1. All 48 *P. aeruginosa* isolates were differentiated into 47 combined subtypes. Four of the integron-positive isolates (PA5, PA6, PA10, and PA11) were grouped within the same cluster, sharing more than 85% similarity in their profiles. Isolates PA40 and PA41 were indistinguishable in the combined analysis and, along with PA43, were grouped within the same cluster, as all 3 isolates shared more than 85% similarity in their combined profiles. All 3 isolates were collected from the same hospital (Kuala Lumpur Hospital) and from the same type of sample (tracheal aspirates) but from different wards.

Isolates PA39 and PA48 were also grouped within the same cluster as they shared more than 90% similarity. These 2 isolates displayed identical PFGE profiles, but were differentiated by ERIC-PCR. Both isolates were cultured from tracheal aspirates but were obtained from 2 different patients from separate hospitals (PA48 from Kota Bharu Hospital and PA39 from Kuala Lumpur Hospital).

Discussion

Antimicrobial susceptibility tests were carried out on *P. aeruginosa* strains that were isolated from 6 public hospitals in Malaysia to determine the extent

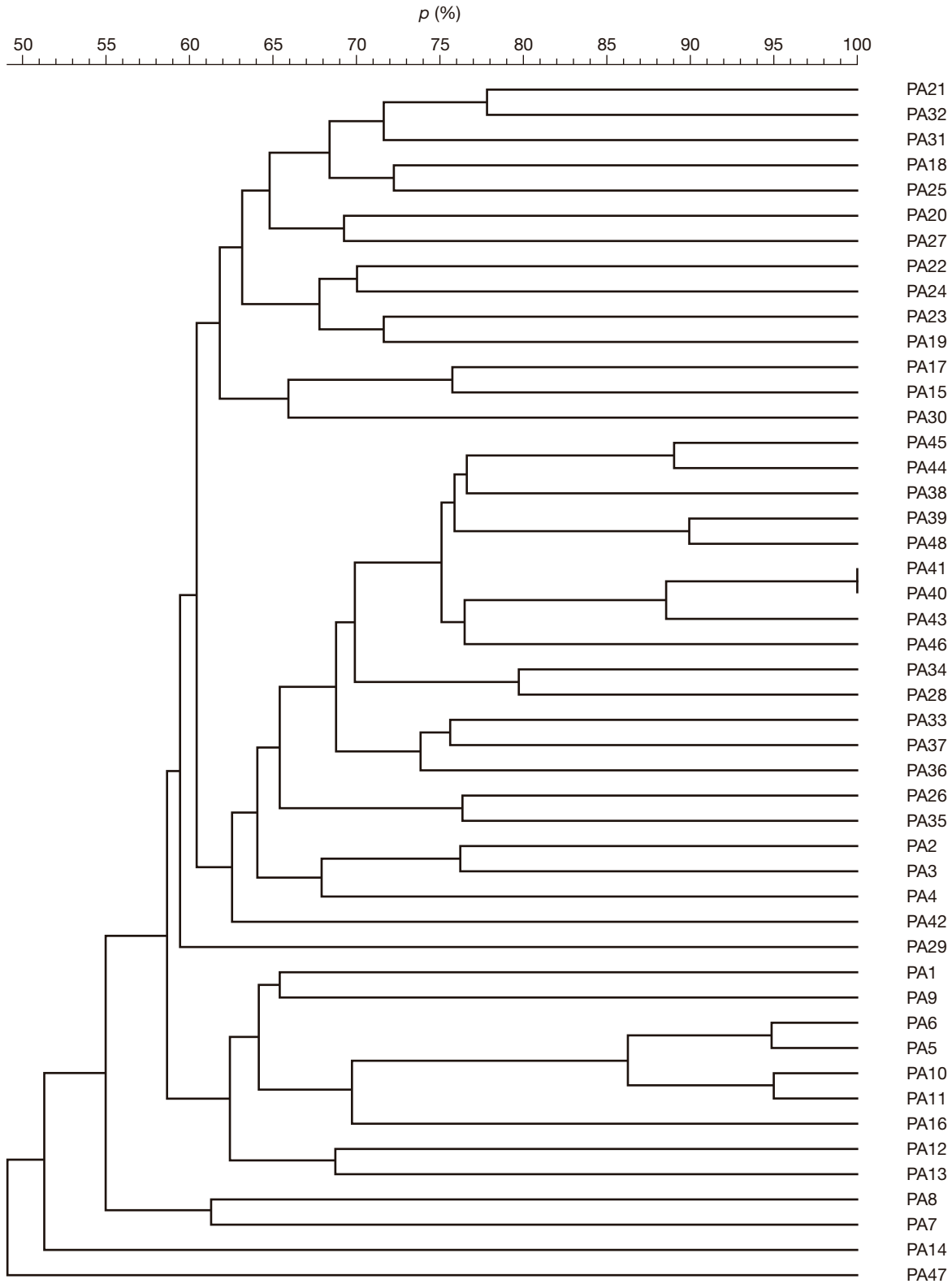

Fig. 1. Computed dendrogram derived from combined data analysis of enterobacterial repetitive intergenic consensus polymerase chain reaction, repetitive extragenic palindrome polymerase chain reation, and pulsed-field gel electrophoresis patterns for 48 *Pseudomonas aeruginosa* isolates.

of antimicrobial resistance and to compare the results with a previous study [21].

Results from the characterization of the 48 clinical isolates of *P. aeruginosa* obtained from various hospitals throughout Malaysia indicated that the isolates were most susceptible to aztreonam and, to a lesser extent, to amikacin and piperacillin-tazobactam. All 48 *P. aeruginosa* isolates were resistant to amoxicillin-clavulanic acid. This is in concordance with the results of Poirel et al, which showed that the minimal inhibitory concentration rate is more than 512 mg/mL, indicating that their *P. aeruginosa* isolates were resistant to amoxicillin-clavulanic acid [22]. High resistance rates were also found for tetracycline (73%) and chloramphenicol (60%). The only other published data on antimicrobial resistance rates among *P. aeruginosa* in Malaysia did not cover some of the antibiotics used in this study, such as tetracycline, chloramphenicol, and aztreonam [21]. However, a similar trend was observed for the other antibiotics that were characterized in both studies. Disturbingly, the resistance rates for these antibiotics were higher when compared with a study performed in 2005, with the exception of cefepime for which a resistance rate of 38.9% was reported [21]; in this study, the resistance rate was 17%. Cefoperazone showed the highest increase in resistance rate from 14.5% as previously reported [21] to 29%, as observed in this study. Other antibiotics such as amikacin, ceftazidime, imipenem, piperacillin, and gentamicin showed increases of between 6% and 13%. However, it should be noted that in the earlier study [21], the *P. aeruginosa* isolates were obtained from a single hospital, whereas the isolates were obtained from various hospitals throughout Malaysia in this study.

The resistance rate of *P. aeruginosa* isolates to ciprofloxacin in Malaysia is still relatively low when compared with other countries. For example, in a survey carried out at a teaching hospital in Istanbul, Turkey, it was reported that ciprofloxacin was the most active agent against *P. aeruginosa* despite a resistance rate of 25% [2]. A more recent survey carried out in a surgical intensive care unit of a hospital in Turkey showed a 59.2% rate of resistance against ciprofloxacin [23], which is higher than the usual reported range of 30% to 40% [24,25]. Although the ciprofloxacin resistance rate reported in this study was relatively low (17%), it has shown an increase from the 11.3% reported in 2007 [21]. Similarly, the resistance rate for gentamicin is reasonably low (19%) when compared with the 54.9% rate reported from Spain [26], 48.4% rate from Turkey [23], and 75% rate from Russia [27], but this has shown a slight increase from the rate of 12.9% in 2005 [21].

MDR isolates of *P. aeruginosa* have been reported in numerous countries with increasing frequency [1,4,28]. In this study, 69% of the 48 *P. aeruginosa* isolates that were investigated were MDR, a considerable increase from the approximately 6% reported in 2005 [21]. The high incidence rate of multidrug resistance, due perhaps to prolonged use of antibiotics, poses serious therapeutic problems in Malaysia and should be further investigated.

Both ESBL-producing isolates (PA21 and PA47) were detected by the double-disk synergy test, but PCR using primers specific for several ESBL-encoding genes failed to produce any amplified products with genomic DNA from these 2 isolates. PCR detection was only able to detect 1 $bla_{\text{OXA-10}}$ gene from isolate PA7 that was not characterized as an ESBL producer by the double-disk synergy test. Gençer et al [2] reported that the phenotypic laboratory detection of ESBL producers might be difficult due to the combined presence of an AmpC system and class D ESBLs as they confer resistance to β-lactam inhibitors. In this study, all the isolates were resistant to amoxicillin-clavulanic acid and this may be the reason why synergistic effects were not present in the majority of the isolates. Although many ESBL-encoding genes have been described in the literature, only a few of these (namely bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{\text{CTX-M}}$, bla_{DHA}, and bla_{VEB}) were tested by PCR using gene-specific primers in this study. The 2 ESBL-producing isolates may harbor other genes such as bla_{VIM}, bla_{IMP}, or bla_{PSE} that were not included in this study, but have been isolated from *P. aeruginosa* [5].

Oxacillin-hydrolyzing enzymes are frequently found in *P. aeruginosa* and OXA-10 derivatives are one of the most common OXA enzymes reported in *P. aeruginosa.* The presence of the OXA-10 derivative has been reported in *P. aeruginosa* isolates from Turkey, Taiwan, France, and China [1,11,29,30].

Only class 1-encoded *intI1* integrase was detected in 9 of the 48 *P. aeruginosa* isolates suggesting that these isolates harbor class 1 integrons. All the integron-positive isolates were resistant to β-lactams and cephalosporins, and were also MDR. Except for isolate PA34, all the integron-positive isolates were also resistant to amikacin and gentamicin. This is not surprising as genes encoding resistance to aminoglycosides can be present in the promoter region of the

integron leading to the expression of the inserted gene(s) [31,32].

Both ESBL producers were also found to be integron-positive and were resistant towards more than 5 classes of antimicrobial agents tested including β-lactams, cephalosporins, monobactams, tetracycline, and aminoglycosides.

The presence of class 1 integrons is a matter of great concern, as this is one of the most efficient genetic elements responsible for the spread of antibiotic resistance in the hospital environment [26]. Gene cassettes conferring resistance to several classes of antibiotics were identified through sequencing of the amplified product of the conserved segments (CS) region of the integron.

PCR fingerprinting (ERIC-PCR and REP-PCR) and PFGE showed that all 48 *P. aeruginosa* clinical isolates were genetically diverse and heterogeneous. PFGE and ERIC-PCR were more discriminatory than REP-PCR. Although both ERIC-PCR and REP-PCR analyses have been described as equally effective in characterizing clinical isolates of *P. aeruginosa* [3], this study found that ERIC-PCR was more discriminatory than REP-PCR, as ERIC-PCR was able to differentiate isolates that were not differentiated by REP-PCR. Therefore, ERIC-PCR may be a better PCR-based method for the subtyping of *P. aeruginosa* isolates. Nevertheless, PFGE is still considered the current gold standard for the subtyping of *P. aeruginosa* and other bacterial isolates. PFGE can confirm that all isolates were closely related [33]. Unfortunately, this method demands high-quality chromosomal DNA, hence the method is more laborious and time consuming compared with PCR-based methods.

Isolates PA40 and PA41 were indistinguishable by REP-PCR, ERIC-PCR, and PFGE. Both isolates were isolated from the same hospital and on the same day, but from different patients in separate wards. This indicated that the patients might have acquired the infection from that particular hospital. However, both isolates exhibited different antibiotypes, with PA41 showing resistance to cefotaxime, tetracycline, chloramphenicol, amoxicillin-clavulanic acid, and piperacillin-tazobactam, whereas PA40 was only resistant to tetracycline, chloramphenicol, and amoxicillin-clavulanic acid. It is therefore likely that PA41 was derived from PA40 by the acquisition of determinants that mediate cefotaxime and piperacillin-tazobactam resistance and that these isolates were resident in the hospital.

Interestingly, 4 of the integron-positive isolates (PA5, PA6, PA10, and PA11) were clonally related by the combined analysis. All 4 isolates were resistant to piperacillin, ticarcillin, cefoperazone, chloramphenicol, ceftriaxone, imipenem, gentamicin, amikacin, ciprofloxacin, and cefotaxime. In addition, isolates PA5 and PA6 were also resistant to piperacillin-tazobactam. All 4 organisms were isolated from the same hospital and within the same month, but from different patients and specimens (3 from tracheal aspirates and 1 from a urine sample) in different wards, suggesting a nosocomial spread of a resident clone in that hospital.

Combined analysis showed that all the integron-positive and MDR isolates were diverse and heterogeneous. In general, no direct correlation between DNA profiles and antibiotic susceptibility patterns was observed. Isolates with identical DNA profiles frequently belonged to different antibiotypes. The Malaysian MDR *P. aeruginosa* clinical isolates studied were genetically diverse and heterogeneous, suggesting that multiple subtypes of the species are involved in infection.

Acknowledgments

This work was partially funded by grants from the Malaysian Toray Science Foundation, Ministry of Science, Technology and Innovation (MOSTI), Malaysia, and the University of Malaya. We thank MOSTI for providing a National Science Foundation scholarship to Ms King-Ting Lim. Part of this study was presented as a poster at the Malaysian Science Technology Congress, Kuala Lumpur, Malaysia, in 2007.

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