ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND DISTRIBUTION OF *exoU* AND *exoS* IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* AT A MALAYSIAN HOSPITAL

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Abstract. This study was conducted to determine the antibiotic susceptibility pattern and distribution of *exoU* and *exoS* among 44 clinical isolates of *P. aeruginosa* collected from different patients over a 3-month period in 2010 at a major Malaysian hospital. Susceptibility data by disk diffusion method for cefepime (30 µg), ceftazidime (30 µg), gentamicin (10 µg), piperacillin-tazobactam (100/10 µg) and ciprofloxacin (5 µg) were available for 38 isolates. Resistance to ceftazidime and piperacillin-tazobactam was the most common (74%) with five isolates not susceptible to three or more different antibiotics. PCR detection of *exoU* and *exoS* of all 44 isolates showed the former gene to be present in 18 and *exoS* in 41. In analyzing the two genes together, 17 isolates were detected for *exoU* and *exoS* with only two being negative for both genes. Only one isolate was detected for *exoU* alone whereas 24 for *exoS* alone. Distribution of the genes in relation to antibiotic susceptibility was inapplicable due to the majority of the isolates having similar susceptibility patterns, but the tendency of *exoU*-carrying isolates to be present in male patients (83%) and respiratory sites (61%) was observed ($p < 0.050$). The finding warrants further investigation in a larger sample of isolates.

Key words: *Pseudomonas aeruginosa*, antibiotic susceptibility, *exoU*, *exoS*

INTRODUCTION

Pseudomonas aeruginosa is widespread in nature, inhabiting various environmental localities such as surface, soil and water. It has minimal nutritional requirements but its high growth capability explain why it can easily disseminate in the surroundings (Ryan and Ray, 2010). In hospital settings, it is one of the common nosocomial pathogens causing ventilator-associated pneumonia and, burn, catheter-related, urinary tract and blood infections (Martin and Yost, 2011). Unfortunately, infection by *P. aeruginosa* is frequently invasive and toxigenic due to the various pathogenic properties of

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this bacterium. ExoenzymeU (ExoU) and exoenzymeS (ExoS), cytotoxins that can cause damage and lysis of many types of host cells, are among the important virulent determinants of this organism, which have been shown to be responsible for bacterial persistence and severity of infection (Shaver and Hauser, 2004).

In addition, *P. aeruginosa* is notorious for being naturally resistant to many antibiotics due to its outer membrane structure that serves as a permeability barrier for in-coming drugs (Mohanasoundaram, 2011). The presence of antibiotic resistance plasmids, which can be transferred and acquired through bacterial transduction and conjugation, makes this organism efficient at becoming more resistant. Currently, many *P. aeruginosa* isolates are no longer susceptible to the commonly used antibiotics (Pathmanathan *et al*, 2009; Nwankwo and Shuaibu, 2010; Mohanasoundaram, 2011). Consequently, the increasing cases of *P. aeruginosa* infection, along with the increasing emergence of multidrug resistance, make management therapy more problematic.

Although the mechanisms underlying the antibiotic resistance and virulence of *P. aeruginosa* have been well understood, the epidemiological trend pertaining to the incidence of antibiotic resistance and pathogenic *P. aeruginosa*, as well as the distribution pattern of the related virulence determinants at the genetic level in the organism, need to be temporally monitored for potential variations that may be related with social changes and improved living conditions. These data will be useful in identifying emerging factors that may affect the resistance and infection trends of *P. aeruginosa*. In Malaysia, the prevalence of antibiotic resistant *P. aeruginosa* and its infection have been reported, but investigation on the distribution of the virulence genes has not been extensive (Raja and Singh, 2007; Pathmanathan *et al*, 2009). Thus, in addition to the antibiotic susceptibilities, this study was conducted to determine the distribution of virulence genes (*exoU* and *exoS*) in relation to demographic and clinical factors of *P. aeruginosa* clinical isolates at a Malaysian Hospital.

MATERIALS AND METHODS

Bacterial isolate and identification

P. aeruginosa isolates (n = 44) were collected between January and April 2010 at the Microbiology Laboratory, Kuala Lumpur Hospital and transported to the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for re-identification by standard methods (Ryan and Ray, 2010). The hospital is one of the major government referral hospital located in a highly populated city of Kuala Lumpur, Malaysia, and is used by Universiti Putra Malaysia as its medical teaching hospital. The acquisition of isolates and the data retrieval were in line with the research approval by both parties.

Antimicrobial susceptibility and data retrieval

Antibiotic susceptibility data of the isolates was based on the routine disk diffusion tests performed at the Department of Pathology, Kuala Lumpur Hospital, and interpreted according to the Clinical and Laboratory Standard Institutes guideline (CLSI, 2009). Demographic information on age, ethnicity and gender of the patients, as well as site of isolation, were retrieved for epidemiological analysis in relation to the virulence determinant genes of the isolates.

Detection of *exoU* and *exoS* by polymerase chain reaction (PCR)

Primers, listed in Table 1, were used in PCR detection and amplification of *exoU*

Table1
Nucleotide sequences of primer used in this study.

Gene	Oligonucleotide sequence[a]	Product size (bp)
exoU-F	5′-GGG AAT ACT TTC CGG GAA GTT-3′	428
exoU-R	5′-CGA TCT CGC TGC TAA TGT GTT-3′	
exoS-F	5′-CTT GAA GGG ACT CGA CAA GG-3′	504
exoS-R	5′-TTC AGG TCC GCG TAG TGA AT-3′	

[a]Mitov *et al*, 2010. F, forward; R, reverse; bp, basepair

and *exoS*. Genomic DNA of the isolates was prepared by boiling method (Mitov *et al*, 2010). PCR was carried out in a 25 µl reaction volume using a Biometra T1 thermal cycler (LabRepCo, Horsham, PA). The reaction mixture contained 1.5 µl of template DNA, 0.1 µl of each primer (0.25 µM) and 8.0 µl of GoTaq® Green Master Mix (Promega, Madison, WI). The PCR thermo cycling parameters were as follows: 94ºC for 5 minutes; 30 cycles of 94ºC for 40 seconds, 60ºC for 1 minute and 72ºC for 1.5 minutes, and a final step of 7 minutes at 72ºC, followed by cooling at 4ºC. PCR amplicons were separated by 1% agarose gel-electrophoresis containing 1:10,000 v/v GelRed™ (Biotium, Hayward, CA) and visualized under UV light. Representative DNA bands were purified and sequenced (1st Base Lab, Malaysia) using the same PCR primers. Homology search was performed using BLAST (http://www.ncbi.nlm.nih.gov/blast).

Analysis and statistical methodology

Data from the disk diffusion tests were assigned to the following susceptibility category: resistant, susceptible and intermediate. For simplicity, other data were grouped according to race (Malay and non-Malay), gender (male and female), ages (< 54 and ≥ 54 years old) and site of isolation (respiratory and non-respiratory), and each was analyzed in relation to distribution patterns of *exoU* and *exoS*. Chi-square (χ^2) and Fisher's exact tests were used to determine association of the categorical variables in a 2 x 2 contingency table. Significant level is set at $p < 0.050$.

RESULTS

Forty-four isolates from different patients were available with data of patient's demography and sites of isolation (Table 2). The ages of patients ranged from 6 months to 85 years old with median age of 54 years. Patients were of various races comprising Malay ($n = 25$), Indian ($n = 11$), Chinese ($n = 7$), and Singh ($n = 1$), with a total of 25 males and 19 females. The sites of isolation comprised non-respiratory swabs ($n = 13$), tracheal aspirates ($n = 15$), tissues ($n = 7$), blood ($n = 3$), urine ($n = 3$), sputum ($n = 2$) and pleural fluid ($n = 1$). Respiratory sites referred to tracheal aspirate and sputum samples, while the rest was considered as non-respiratory sites.

Antibiotic susceptibility tests by disk difusion method were obtained for cefepime (FEP) (30 µg), ceftazidime (CAZ) (30 µg) and gentamicin (GEN) (10 µg) for 41 isolates, while that for piperacillin-tazobactam (TZP) (100/10 µg) and

Table 2
Distribution of *exoU* and *exoS* among 44 clinical isolates of *P. aeruginosa* in relation to gender, race, age and isolation site of patients.

Source *n* = 44	*exoU*+[1] *n* = 18 (41%)	*exoS*+[2] *n* = 41 (93%)	Both+[3] *n* = 17 (39%)
Male *n* = 25 (57%)	**15 (83%)**	23 (56%)	**14 (82%)**
Female *n* = 19 (43%)	**3 (17%)**	18 (44%)	**3 (18%)**
Malay *n* = 25 (57%)	11 (61%)	23 (56%)	10 (59%)
Non-Malay *n* = 19 (43%)	7 (39%)	18 (44%)	7 (41%)
Ages < 54 *n* = 22 (50%)	10 (56%)	20 (49%)	9 (53%)
Ages ≥ 54 *n* = 22 (50%)	8 (44%)	21 (51%)	8 (47%)
Respiratory *n* = 17 (39%)	**11 (61%)**	17 (41%)	**11 (65%)**
Non-respiratory *n* = 27 (61%)	**7 (39%)**	24 (59%)	**6 (35%)**

[1]In comparison to isolates with *exoU*- (not shown).
[2]In comparison to isolates with *exoS*- (not shown).
[3]In comparison to isolates with *exoU*+ alone, *exoS*+ alone and both- (not shown).
Both+/- = *exoU* and *exoS* were detected/not detected in a single isolate.
Significant associations are marked in bold ($p < 0.050$).

ciprofloxacin (CIP) (5 µg) was available for 40 and 38 isolates, respectively. Subsequently, complete susceptibility results for the five antibiotics were available for 38 isolates. The susceptibility patterns are summarized in Table 3 with seven different patterns. Isolates with pattern 1 were the most common, *n* = 28 (74%) (one isolate from blood). Only five (13%) isolates had pattern 3 (one isolate from pleural fluid), while the rest of the patterns (showing resistance to three or more antibiotics) had one isolate.

In the PCR detection of the virulence determinant genes on the 44 isolates (Table 2), *exoU* were detected in 18 of them while *exoS* in 41. In analyzing the two genes together, 17 isolates were detected for *exoU* and *exoS* with only two being negative for both genes. Only one isolate was detected for *exoU* alone whereas those with *exoS* alone accounted for 24 isolates. Among 38 isolates tested for the five antibiotics, there were five isolates that are resistant to three or more of different antibiotics and three of them carried both *exoU* and *exoS* genes (Table 3). The presence of *exoU* and *exoS* were confirmed by sequencing with ≥ 98% homology to those of *P. aeruginosa* strains in GenBank (data

Table 3
Antimicrobial susceptibility pattern and distribution of *exoU* and *exoS* among clinical isolates of *P. aeruginosa.*

Pattern no. $n = 38$	Antibiotic					No. (%)	Gene			
	Beta-lactam			FQ	AG		*exoU*+ $n = 16$	*exoS*+ $n = 35$	Both+ $n = 15$	Both- $n = 2$
	FEP	CAZ	TZP	CIP	GEN					
1	S	R	R	S	S	28 (74%)	8	26	8	2
2	S	R	R	R	S	1 (3%)	-	1	-	-
3	S	R	S	S	S	5 (13%)	4	5	4	-
4	R	R	S	R	R	1 (2.5%)	1	1	1	-
5	R	R	R	S	S	1 (2.5%)	1	1	1	-
6	R	S	S	R	R	1 (2.5%)	1	1	1	-
7	S	S	R	R	R	1 (2.5%)	1	-	-	-

R, resistance; S, susceptible; FQ, fluoroquinolone; AG, aminoglycoside
Both+/- = *exoU* and *exoS* were detected/not detected in a single isolate.

not shown). PCR reactions were repeated in three independent experiments and showed consistent results with no band for negative control.

For all the 44 isolates, significant correlation ($p < 0.050$) is observed when comparing gender and site of isolations in relation to the distribution of the virulence determinant genes (Table 2). There is a high frequency of *exoU*+ isolates among male patients (83% *vs* 17%; $p = 0.003/0.005$: χ^2/Fisher's exact test), which is also seen when both genes are present (82% *vs* 18%; $p = 0.007/0.012$: χ^2/Fisher's exact test). There is a significant association of *exoU* as well as in combination with *exoS* with respiratory isolates (61% and 65%; $p = 0.011/0.015$ and $0.005/0.010$ respectively: χ^2/Fisher's exact test), whereas non-respiratory isolates show a high frequency of *exoU*- isolates (77%) and a lower frequency (35%) for the presence of both genes. The rest of the categorical variables show no association.

DISCUSSION

The emergence of *P. aeruginosa* isolates to be resistant to more than one anti-pseudomonal drugs have been frequently reported worldwide (Nordmann *et al*, 2007; Pathmanathan *et al*, 2009; Nwankwo and Shuaibu, 2010; Mohanasoundaram, 2011). This is often referred to as multidrug resistance (MDR), but different definitions have been used to define the term due to different nature of tests and guidelines followed (Falagas *et al*, 2006). To avoid confusion, this study did not attempt to classify the isolates as so but used the susceptibility patterns against the battery of anti-pseudomonal agents tested. Analysis of the susceptibility patterns in the categorical variable-manner (*eg* MDR *vs* non-MDR) was also not performed as the majority of the isolates showed the same susceptibility pattern (pattern 1). Overall, the majority of the isolates that were collected over a 3-month period were

resistant to at least two of the beta-lactam group of antibiotics (CAZ, FEP and TZP), while the number of isolates resistant against the fluoroquinolone and aminoglycoside groups (CIP and GEN, respectively) was low (< 11%). This may be due to the use of members of the beta-lactam group as the first line of therapy regimen resulting in selective proliferation of isolates resistant to these antibiotics (MOH, 2001). A variable resistance prevalence rate was reported in different countries indicating different usage of the antibiotics (Van Eldere, 2003; Ogbolu *et al*, 2008; Anjum and Mir, 2010).

In Malaysia, although the incidence of antibiotic resistance in clinical isolates of *P. aeruginosa* has been reported (Raja and Singh, 2007; Pathmanathan *et al*, 2009), the relationship between demographics or clinical variables and distribution of virulence genes in *P. aeruginosa* isolates are still unclear. This study showed that, as far as *exoU* and *exoS* are concerned, there is a higher prevalence of the latter gene among the 44 clinical isolates. A similar distribution trend also has been observed in other studies with the majority of isolates carrying *exoS* (Feltman *et al*, 2001; Garey *et al*, 2008; Bradbury *et al*, 2010; Mitov *et al*, 2010).

A high *exoU* frequency has been reported to be associated with MDR isolates (Garey *et al*, 2008; Mitov *et al*, 2010), but such an association could not be ruled out in this study as the majority of the isolates has a similar antibiotic resistance pattern. However, the isolates with *exoU* in this study are significantly associated with males and respiratory sites. The higher prevalence of *exoU* among male patients in this study could be linked to different lifestyle or body metabolism, which could favor the isolates carrying the gene. This matter is very subjective and complex to be elucidated, but with regards to respiratory sites experimental evidence has been available for potential explanation. Shaver and Hauser (2004) showed that ExoU is sufficient for the bacterial persistence in lung. Thus, the *exoU*-carrying isolates may be selectively preferred to persist while those absent of the gene would be eventually eliminated from the respiratory sites. ExoS has been shown by Shaver and Hauser (2004) to also facilitate the bacterial dissemination in lung and the combination with ExoU would be an extra advantage for the isolates. Nevertheless, the mechanisms of the two cytotoxins and their potential synergistic effect in pathogenesis are still open for arguments (Finck-Barbancon *et al*, 1997; Bradbury *et al*, 2010). For an unclear reason, there has also been an inverse relationship reported for the two genes whereby one isolate that carried *exoU* does not carry *exoS* and vice versa (Feltman *et al*, 2001). Due to the low number of isolates with *exoU* alone in this study ($n = 1$), as well as considering the unresolved issues mentioned earlier, this study analyzed the distribution of the genes individually (absence *vs* presence of the respective genes, Table 2). Re-analysis was done on presence of the two genes together (both+) against the other patterns of the gene distribution (*exoU*+ alone, *exoS*+ alone and both-, Table 2), which also shows a significant correlation with male and respiratory sites.

In summary, this study has drawn some potential issues pertaining to *P. aeruginosa* infection. The shortcomings of this study are that the antibiotic susceptibility was determined by disk diffusion method and validation with other tests involving more antibiotics are needed. This study was also based on a single catchment center with a limited number of isolates, which may not represent the population

at large. Nevertheless, some consistencies in comparison to other studies were observed regarding the presence of *exoU* and *exoS*. The observed relationships with gender and site of isolation opens up further interesting questions for future investigations using a larger sample of isolates.

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