Multidrug Efflux Pumps Over-Expression and its Association with Porin Down-Regulation and β-lactamase Production among Nosocomial *P. aeruginosa* Isolates from University of Malaya Medical Center, Malaysia

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Abstract*—Pseudomonas aeruginosa* has multiple resistance mechanisms against standard antibiotics. Of all, efflux pump overexpression has become one of the most important mechanisms that contributed to the increasing antibiotic resistances. We have investigated the expression of multidrug resistance mechanisms among the local isolates in relation to their antibiotic susceptibility patterns against multiple antibiotics. The gene expression analysis of 88 *P. aeruginosa* isolates showed overexpression of efflux pump genes *mexB, mexY, mexCD* and *mexEF* were detected in 73%, 82%, 48% and 68% of the isolates respectively. Additionally, the β-lactamase gene *ampC* was overexpressed in 65% of the isolates. In contrast, 97% of the isolates had decreased *oprD* expression. All imipenem non-susceptible isolates also had *oprD* down-regulation. This was observed to be coupled with down-regulation of penicillin binding protein genes *PBP2* (77.0%) and *PBP3* (84.0%). Among the 65 imipenem and meropenem resistant, 41 isolates were metallo-β-lactamase (MBL) producers and these genes bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{NDM} and bla_{SIM} were detected in 20, 14, 4, 2, and in 1 isolates respectively. Thirty-three of these 65 isolates tested positive for 3 extended spectrum β-lactamases (ESBL) genes out of which PCR positive isolates were present in 25 isolates for the bla_{VEB} gene, 5 isolates for the bla_{TEM} gene and 3 isolates for the bla_{CTX-M} gene respectively. The multidrug resistance phenotypes in these clinical isolates were caused by the interaction of several different resistance mechanisms occurring within the same strain**.**

***Keywords*—**antibiotic resistance, *me*xXY, Multidrug efflux pumps, *Pseudomonas aeruginosa*, Real-time polymerase chain reaction**.**

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I. Introduction

PSEUDOMONAS AERUGINOSA is one of the most common causes of nosocomial infections worldwide [1].

Previous epidemiological studies revealed that significant rise in morbidity and mortality was due to infections caused by drug-resistant *P. aeruginosa* [2]-[4]. This organism is known to be resistant to many antimicrobial agents and is able to develop resistance to other antimicrobial agents in the course of antimicrobial chemotherapy [5]-[7]. *P. aeruginosa* is considered multidrug resistant if the isolate is resistant to at least three of the following eight antibiotics: piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem and colistin [8], [9]. Interestingly, virtually all known mechanisms of antimicrobial resistance can be found in *P. aeruginosa*. This includes derepression of chromosomal AmpC cephalosporinase, diminished outer membrane permeability (loss of OprD proteins) and overexpression of active efflux systems with wide substrate profiles [1]. Bacterial multidrug efflux systems capable of ejecting antimicrobials are mostly encoded by chromosomal genes and are generally grouped into five superfamilies based upon the amino acid sequence homology. These include the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the small multi-drug resistance (SMR) family, the multi-drug and toxic compound extrusion (MATE) family and the resistance-nodulation-division (RND) family [7]. The RND chromosomal systems are encoded by operons and are typically formed by three proteins located in the inner membrane, periplasm, and outer membrane of the bacterial cell [10]. There are 12 RND-type efflux systems found in *P. aeruginosa*, however, only four genetically different three-part efflux systems confer to the improvement of manifold resistances to all antipseudomonal agents [11], [12] : MexX-MexY, MexA-MexB-OprM, MexE-MexF-OprN and MexC-MexD-OprJ [13]-[15]. The inducible chromosome-encoded AmpC β–lactamase belongs to the molecular class C that confers resistance to β-lactams (cephalosporins, penicillins). The up-regulation of efflux pumps coupled with overexpression of β-lactamase in addition to porin loss, may increase the pump rate and co-resistance

phenotypes that could critically influence antimicrobial therapy in clinical settings [7], [16]. Few studies have concentrated on the recognized role of alterations of penicillin-binding proteins in relation to the carbapenem resistance in Gram-negative bacteria [17], [18]. One study has been done on other Gram-negative bacilli, as well as on the clinical isolates of *Acinetobacter baumannii*, which showed down-regulation of *PBP-2* associating well with reduced susceptibility to the carbapenems [19]. Neuwrith *et al*. (1995) found that an imipenem-resistant *Proteus mirabilis* isolate would carry *PBP-2* with reduced affinity to imipenem [20]. Besides this, a *PBP-2* mutant was found in *E. coli*, with increased imipenem MIC, however meropenem MIC was not affected, and this suggested that meropenem also binds to PBP-3 [21]. In addition, Ayala *et al*. (2005) demonstrated that alterations of *PBP-3* might possibly reduce susceptibility to imipenem in *Bacteroides fragilis* [17]. The expression level of proteins involved in various mechanisms of drug resistances in *P. aeruginosa* were commonly evaluated using the conventional method western blotting, which is complex and time consuming [7], [16], [22]-[24]. Recently, real-time PCR was introduced to quantify mRNA expression of various target genes [22].

In recent years, carbapenem resistance in clinical *P. aeruginosa* isolates has been also attributed to the production of metallo-β-lactamases (MBLs), which hydrolyze most β-lactams except aztreonam, and usually confer high-level resistance, metallo-β-lactamases can be classified, , into four groups A, B, C and D [25], [26]. Classes A, C and D perform through a serine-based mechanism, while class B or metallo-β-lactamases (MBLs) require zinc for their action. All these molecular classes of β-lactamases originated in *P. aeruginosa*, including extended spectrum β-lactamases (ESBLs) of classes A, B and D [27]. The current study sought to determine the gene expression profiles of the Malaysian isolates using real-time PCR to evaluate the resistance mechanisms responsible for antibiotic resistances among the *P. aeruginosa* clinical isolates. The expression levels of genes coding for AmpC cephalosporinase, the multidrug efflux pumps, the OprD outer membrane porin and the penicillin binding protein (PBP2, PBP3) and the presence of MBL producing genes was investigated.

II. Materials And Methods

A. Bacterial isolates

A total of 88 *P. aeruginosa* isolates isolated from various clinical samples from April 2009 to March 2010 were collected from different wards at the University of Malaya Medical Center, Kuala Lumpur, Malaysia. Samples were collected from patients hospitalized in the surgical (35%), medical (23%), orthopaedic (15%), paediatric (8%), neurosurgery (8%), intensive care unit (6%), otorhinolaryngology (3%), and gynaecology (2%) wards. Of these, 53% were from urine, 22%, wound, 11%, the indwelling medical devices, 6 %, blood, 6%, sputum and tissues 2%. Only a single bacterial isolate per patient was evaluated.

P. aeruginosa colonies were identified based on morphology, gram staining, pyocyanin production and biochemical tests by API20NE test (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions using standard laboratory procedures [28]. The protocol of this study (Reference Number: 902.5) has been approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur, Malaysia.

B. Extraction of total rna and synthesis of cDNA:

Bacterial isolates were subcultured onto nutrient agar for 24 hours at 37°C and a single colony of pure culture was cultured on LB (Luria Bertani) broth at 37°C for 16 to 18 hours and the cells were diluted in fresh LB broth (1:100), then grown to logarithmic phase (0.5 at OD_{600}) and the cell pellet was harvested by centrifugation at 4000 g for 10 min at 4°C. The supernatant was discarded and the cells pellet was resuspended. A 0.25 ml aliquot of the suspended culture, equivalent to 5×10^8 cfu/ml, was added to 0.5 ml of RNeasy bacteria protect solution (Qiagen, Hilden, Germany) and then disrupted with lysozyme (15 mg/ml) (Sigma Aldrich, Germany). Total RNA was then extracted using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Residual DNA was treated with DNase (1U/µl). RNA concentration was measured using NanoPhotometer™ (Implen, Germany).

The extracted RNA was kept at −80°C until use. For the cDNA synthesis, a total of 500 ng of RNA from all strains was reverse transcribed using QuantiTect Rev. Transcription Kit (Qiagen, Germany) according to manufacturer's instructions. Controls were run without reverse transcriptase, which eliminates the possibility of contaminating DNA in any of the samples. The cDNA was kept at –20°C until used.

C. Real-time PCR analysis

Transcriptional levels of *mexB*, *mexY*, *mexCD*, *mexEF*, *ampC*, *PBP2*, *PBP3* and *oprD* were determined by real-time PCR using gene-specific primers [18], [22], [24], [29], [30] (Table 1) in CFX96™ PCR detection systems (BioRad, Hercules, CA). The amplification conditions for real-time PCR comprised of initial denaturation at 95ºC for 10 min, followed by 35 cycles of 95°C for 20 sec, annealing at 57.8 to 64°C for 20 sec (depending on the gene), extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. The melting curves of each amplicon was assessed and compared with the melting temperature (Tm) obtained when using the DNA template to ensure that specific amplification has occurred. The transcriptional levels of the target genes were normalized using a reference gene, *rpoD*. This gene was consistently expressed in *P. aeruginosa* according to [24] and experiments showed stable expression of *rpoD* in our isolates (data not shown). Normalized expression of each target gene was calibrated against corresponding mRNA expression in *P. aeruginosa* ATCC 27853. All published equations and available models for calculations of the relative expression ratio allow only for the determination of a single transcription difference between one control and one sample. Therefore, the relative gene expression results are presented as ratios between the target gene (target) and the reference gene (*rpoD)*, which were obtained based on the following equation:

TABLE I
PRIMERS USED IN THE RELATIVE GENE EXPRESSION BY RT-qPCR

Genes		5´- sequence- 3´	reference
mexB	F	5´- GTGTTCGGCTCGCAGTACTC - 3	[22]
	R	5´- AACCGTCGGGATTGACCTTG - 3´	
mexY	F	5´- CCGCTACAACGGCTATCCCT - 3´	[22]
	R	5´- AGCGGGATCGACCAGCTTTC - 3´	
mexCD	F	5´ -GGAGTTCGGCCAGGTAGTGCTG- 3´	[29]
	R	5´ -ACTGCATGTCCTCGGGGAAGAA- 3´	
mexEF	F	5´ -CGCCTGGTCACCGAGGAAGAGT- 3´	[18]
	R	5´ -TAGTCCATGGCTTGCGGGAAGC- 3´	
oprD	F	5´-CGACCTGCTGCTCCGCAACTA - 3´	[30]
	R	5´- TTGCATCTCGCCCCACTTCAG- 3´	
ampC	F	5´- AGATTCCCCTGCCTGTGC- 3´	[22]
	R	5´-GGCGGTGAAGGTCTTGCT- 3´	
PBP2	F	5´ -GCCCAACTACGACCACAAG - 3´	[30]
	R	5´ -CGCGAGGTCGTAGAA ATA G - 3´	
PBP3	F	5´ -TGATCAAGTCGAGCAACGTC - 3´	[30]
	R	5´ -TGCATGACCGAGTAGATGGA - 3´	
rpoD	F	5´-GGGCGAAGAAGGAAATGGTC- 3´	[24]
	R	5´ -CAGGTGGCGTAGGTGGAGAA- 3´	

TABLE II
THE OLIGONUCLEOTIDE PCR PRIMERS USED FOR GENE IDENTIFICATION OF THE Metallo-β-Lactamase

Genes		5´- sequence- 3´	bp*	reference
bla_{SHV}	F	5´-ATGCGTTATATTCGCCTGTG-3´	747	[73]
	R	5´-TGCTTTGTTATTCGGGCCAA-3´		
bla_{TEM}	F	5´-TCGCCGCATACACTATTCTCAGAATGA - 3´	445	[34]
	R	5´-ACGCTCACCGGCTCCAGATTTAT -3´		
bla_{CTX-M}	F	5´-ATGTGCAGYACCAGTAARGTKATGGC -3´	593	[74]
	R	5´TGGGTRAARTARGTSACCAGAAYCAGCGG-3´		
bla_{VEB}	F	5´-CGACTTCCATTTCCCGATGC-3´	643	[75]
	R	5´-GGACTCTGCAACAAATACGC-3´		
bla_{PER}	F	5´-AATTTGGGCTTAGGGCAGAA-3´	925	[76]
	R	5´-ATGAATGTCATTATAAAAGC-3´		
bla_{IMP}	F	5´-GAA GGY GTT TAT GTT CAT AC-3´	587	[33]
	R	5´-GTA MGT TTC AAG AGT GAT GC-3´		
bla_{VIM}	F	5´-GTT TGG TCG CAT ATC GCA AC-3´	382	[33]
	R	5-AAT GCG CAG CAC CAG GAT AG-3´		
bla_{SIM}	F	5´-TACAAGGGATTCGGCATCG-3´	570	[77]
	R	5´-TAATGGCCTGTTCCCATGTG-3´		
bla_{SPM}	F	5´-AAAATCTGGGTACGCAAACG-3´	271	[77]
	R	5´-ACATTATCCGCTGGAACAGG-3´		
bla_{GIM}	F	5´-TCGACACACCTTGGTCTGAA-3´	477	[77]
	R	5´-AACTTCCAACTTTGCCATGC-3´		
bla_{NDM}	F	5´-GGTTTGGCGATCTGGTTTTC-3´	621	[77]
	R	5´-CGGAATGGCTCATCACGATC-3´		

Ratio = $(E_{target\ gene})^{\triangle ct\ target\ (ATCC\ 27853\text{-}sample)} / (E_{rpoD})^{\triangle ct\ rpoD\ (ATCC\ 27853\text{-}sample)}$ [69], where E is the real-time PCR efficiency for a given gene, △ unknown sample versus a control ($\triangle ct_{\ ATCC\ 27853\text{-}sample}$) and C_t the crossing point of the amplification curve with the threshold.

Transcription data was analyzed using Bio-Rad CFX Manager software. According to the previous studies [22], [24], [30]-[32], the efflux system genes *mexB, mexXY, mexCD, mexEF* were considered overexpressed when the transcription levels of *mexB, mexCD, mexEF* were at least two fold and four fold of *mexY* higher than the wild-type reference strain *P. aeruginosa* ATCC 27853. Overexpression of *ampC* and down-regulation of *oprD* was considered significant when their transcriptional levels were respectively, ≥ 10 and ≤ 70% -fold, compared to the reference strain wild type. The data were subjected to statistical analysis using SPSS version 11.5 (SPSS Inc., Chicago, US). A chi-square was done to show the mRNA expression in comparison to antibiotics resistance. A *p*-value of ≤ 0.05 was considered indicative of a statistically significant difference. Fisher's exact test is to be used if less than 50% of the cells are less than 5.

D. DNA extraction

P. aeruginosa clinical isolates were subcultured on nutrient agar for 24 hours at 37ºC and a single colony of pure culture was cultured on LB (Luria Bertani) broth at 37ºC for 18 hours and the cells were diluted in another fresh LB broth (1:100), then grown to logarithmic phase (0.5 at OD_{600}) and the cell pellet was collected by centrifugation at 4000 g for 10 min at 4 ºC. The supernatant removed and the cell pellet

resuspended then DNA extracted using DNeasy blood and tissue mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was stocked up at -20°C until used.

E. The metallo - β- lactamase (MBL) detection:

Sixty-five *P. aeruginosa* isolates that were resistant to imipenem and meropenem were tested for MBL production according to Pitout *et al*'s method [33]. The genes encoding for the following Metallo β-Lactamase class B (*IMP, VIM, SIM, SPM, GIM* and NDM) were determined from genomic DNA using gene-specific primers as in (Table 2) by PCR amplification assay.

The amplification was carried out using Master cycler Gradient PCR (Eppendroff, U.S). The top taq™ master mix kit 250 unit (Qiagen, Hilden, Germany) were used in a final volume reaction of 25 µl. The PCR program consisted of an initial incubation of 10 min at 37°C and an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1.5 min. After the last cycle, the products were stored at 4°C and then analyzed by electrophoresis with 2.0 % agarose gels in TAE buffer using sybeer green dye for 50 min at 100 V and visualized with UV light using gel doc XR system (BioRad, Hercules, CA). Positive control for *bla IMP, VIM, SIM, SPM* genes were included in the respective PCR assays.

F. Extended spectrum β–lactamase (ESBL) detection

Sixty-five isolates of *P. aeruginosa* which showed resistance to meropenem and imipenem were evaluated for the ESBL production genes (bla *SHV, TEM CTX-M, VEB* and *PER* genes) by using the PCR amplification assay from genomic DNA using the specific primer as listed in (Table 2) and a top taq™ master mix kit 250 unit (Qiagen, Hilden, Germany) were used in a final volume reaction of 50 µl. The amplification was carried out using Master cycler Gradient PCR (Eppendroff, U.S). PCR amplification conditions included the first step of denaturation for 15 min at 95 °C; followed by 30 cycles of denaturation for 30 s at 94 °C, annealing temperature for 30 s at 50 °C for TEM, 56 °C for *Bla* SHV, 58 °C for CTX-M, 60 for VEB AND PER primers; then extension temperature for 2 min at 72 °C; and a final step extension for 10 min at 72 °C. Finally, cycles were ended at 4°C. Subsequently the PCR products (1/10 volume) were analyzed by electrophoresis with 2.0 % agarose gels in TAE buffer using sybeer green dye for 50 minute at 100 V and visualized with UV light using gel doc XR system (BioRad, Hercules, CA). *Klebsiella pneumoniae* strain that possess the SHV, TEM and CTX-M genes were kindly provided by Dr. Hans-Jürg Monstein [34], and were used as positive control strain. The representative amplified PCR products for each of the selective gene indicated were sent for sequencing.

III. RESULTS

The gene expression analysis of 88 *P. aeruginosa* isolates showed overexpression of efflux pump genes for *mexY* (82.0%, from 4.0 to 1731.0 fold), *mexB* (73.0%, from 4.0 to 50.0 fold), *mexEF* (68.0%, from 2.9 to 371) and *mexCD* (48.0%, from 2.0 to 522), while for *ampC* overexpression it was 65.0% (from 10.4 to 1806.0 fold). Down-regulation was noted for *oprD* (97.0%, from 0.2 to 0.7 fold), *PBP2* (77.0%, from 0.1 to 0.7 fold) and *PBP3* (84.0%, from 0.01 to 0.7 fold) as compared to expression of the respective genes in *P. aeruginosa* ATCC 27853 (Figure 1).

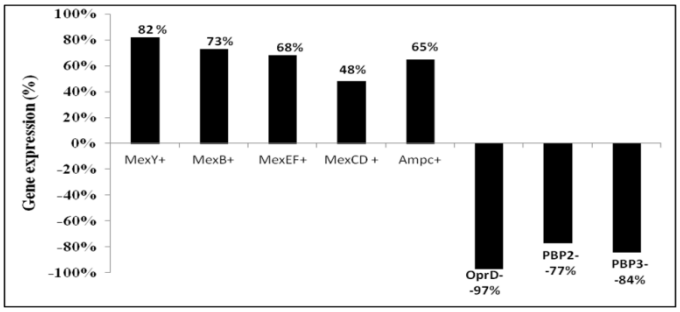

Fig.1 The percentage of gene expression level of *P. aeruginosa* clinical isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88). *The abbreviations, mexY*$^+$*, mexB*$^+$*, mexEF*$^+$*, mexCD*$^+$ *and ampC*$^+$ *designate,* MexXY, MexAB-OprM, MexEF, MexCD *and* AmpC *overexpression, respectively. oprD*$^-$ *denotes* OprD *porin down-regulation, PBP2*$^-$ *and PBP3*$^-$ *shows the penicillin binding protein down-regulation*.

Among the resistant isolates overexpressing *mexB*, the lowest mRNA expression was noted for isolates resistant to colistin (67%), whereas the highest mRNA expression was noted for isolates resistant to ciprofloxacin (78%). In relation to isolates that were resistant to meropenem, imipenem, gentamicin, ceftazidime, amikacin, aztreonam and piperacillin/tazobactam, the range of *mexB* mRNA expression ranged from 71% to 77% (Table 3). When *mexY* gene overexpression was examined, the lowest and highest mRNA expression were seen for isolates resistant to colistin (67%) and amikacin (90%) , whilst the mRNA expression of this gene for isolates resistant to gentamicin, ciprofloxacin, ceftazidime, aztreonam and piperacillin/tazobactam ranged from 76% to 86% as depicted in Table 3.

For the overexpression of *mexCD*, the lowest mRNA expression was seen with isolates resistant to gentamicin and piperacillin/tazobactam (49%). However, the highest overexpression was seen with isolates resistant to meropenem and aztreonam (55%). In relation to isolates resistant to other antibiotics, *mexCD* mRNA expression (up- regulation) ranged from 47% to 54% (Table 3).

For the overexpression of *mexEF*, the lowest mRNA expression was observed with isolates resistant to piperacillin/tazobactam (63%), and the highest mRNA expression was noted with isolates resistant to colistin (100 %). In relation to the isolates that were resistant to other antibiotics, mRNA expression of these isolates for *mexEF* gene up-regulation ranged from 65% to 70% (Table 3). For the overexpression of *ampC* gene, the lowest mRNA expression was seen with isolates resistant to colistin (50%) and the highest mRNA expression was noted with isolates resistant to piperacillin/tazobactam (75%). In relation to the resistant isolates to other antibiotics, mRNA expression of these isolates for *ampC* gene up-regulation ranged from 60% to 66.0% (Table 3). The *oprD*, mRNA expression of isolates resistant to meropenem, imipenem and colistin demonstrated 100% down-regulation and in relation to the resistant isolates to other antibiotics, mRNA expression of these isolates for

OprD gene down-regulation ranged from 94% to 98% (Table 3).

For *PBP2* gene, isolates resistant to colistin had the lowest mRNA expression (50%), while the highest mRNA expression value was 80% for isolates resistant to meropenem and imipenem. On the other hand, for the *PBP3* gene, the mRNA expression was down-regulated and showed the lowest and highest mRNA expression for isolates resistant to colistin (50%) and imipenem (91%) respectively. However, in relation to the resistant isolates to other antibiotics investigated, these isolates showed low mRNA expression for *PBP2* and *PBP3* which varied from 75% to 86% (Table3). With regard to the significance of the above results, the overexpression of *MexY* gene were significantly different in isolates resistant to amikacin (90%), gentamicin (86%) ($p < 0.01$) and ciprofloxacin (84%) as compared to other antibiotics ($p < 0.037$) as shown in Table 4. On the other hand, for *AmpC* gene, mRNA expression in *P. aeruginosa* isolates demonstrated high significant differences towards piperacillin/tazobactam antibiotics as compared to otherantibiotics ($p < 0.05$) (Table 3). All *P. aeruginosa* clinical isolates that were resistant to imipenem showed significantly decreased *OprD* gene expression ($p = 0.016$). The down-regulation of mRNA expression of *PBP3* was observed in isolates resistant imipenem (91%) ($p = 0.04$). Likewise, the down-regulation of penicillin binding protein (*PBP3*) demonstrated a significance in isolates that were resistance to piperacillin/tazobactam (84%) ($p = 0.047$) as compared to *P. aeruginosa* ATCC 27853 (Table 4).

Sixty-five of the clinical *P. aeruginosa* isolates that were resistant to imipenem and meropenem were evaluated for presence of metallo-beta-lactamase (MBL) genes and extended

TABLE III
PERCENTAGE OF THE MRNA TRANSCRIPTION LEVELS OF GENE EXPRESSION OF MULTIDRUG RESISTANT ISOLATE IN COMPARISON TO P. AERUGINOSA ATCC 27583 (N=88)

Antibiotic	% resistance	Modal MIC[a] (µg/ml)	mRNA expression[b]							
			Efflux pump				β-Lactamase	Outer membrane protein	Penicillin binding Protein	
			mexB$^+$	*mexY*$^+$	*mexCD*$^+$	*mexEF*$^+$	*ampC*$^+$	*oprD*$^-$	*PBP2*$^-$	*PBP3*$^-$
Gentamicin	94.0	24 - 1024	77.0	86.0*	49.0	69.0	66.0	97.0	77.0	86.0
Ceftazidime	92.0	32 - 256	77.0	82.0	48.0	67.0	63.0	98.0*	78.0	85.0
Ciprofloxacin	92.0	4 - 125	78.0	84.0*	48.0	68.0	65.0	96.0	78.0	85.0
Imipenem	74.0	32	74.0	80.0	54.0*	68.0	66.0	100 *	80.0	91.0*
Amikacin	66.0	32 - 256	72.0	90.0*	47.0	67.0	60.0	98.0	79.0	83.0
Piperacillin/ Tazobactam	58.0	128 - 256	77.0	86.0	49.0	63.0	75.0*	94.0	75.0	84.0*
Aztreonam	56.0	32 - 256	74.0	76.0	55.0	65.0	63.0	98.0	76.0	86.0
Colistin	7.0	6 - 48	67.0	67.0	50.0	100	50.0	100	50.0	50.0

All values are in percentage (%)

*Significantly different from other groups (p<.05).

The abbreviations *mexB*$^+$, *mexY*$^+$, *mexCD*$^+$, *mexEF*$^+$ and *ampC*$^+$ denote MexAB-OprM, MexXY, MexCD, MexEF and AmpC overexpression, respectively; *oprD*$^-$, OprD porin down-regulation; *PBP2*$^-$ and *PBP3*$^-$denote PBP2 and PBP3 down-regulation, respectively.

[a]Modal MIC is defined as the antimicrobial MICs that were more frequently observed at each association of resistance mechanisms.

[b]Value denotes percentage of isolates among the respective antibiotic resistant isolates showing upregulation/Down-regulation for the respective gene. $^+$ coding for Overexpression coding for Down-regulation.

[a] *Number and percentage of the isolates that were* resistant to *antibiotics.*

Up: up- regulation, down –regulation

TABLE IV
THE SIGNIFICANT ASSOCIATION BETWEEN RESISTANT ISOLATES TO DIFFERENT ANTIBIOTICS AND GENE EXPRESSION (N=88)

Antibiotics	Gene	No (%) Resistant [a]	Up	Down	χ^2 p-value
Gentamicin		83 (94%)	71	12	0.001*
	mexY				
Ceftazidime		81 (92%)	2	79	0.001*
	oprD				
Ciprofloxacin		81 (92%)	68	13	0.037*
	mexY				
	oprD		0	65	0.016*
Imipenem	*PBP3*	65 (74%)	7	58	0.04
		58 (66%)	52	6	0.012
Amikacin	*mexY*				0.05
Piperacillin/ Tazobactam	*ampC*	51(58%)	38	13	
	PBP3		8	43	0.047

[a] *Number and percentage of the isolates that were* resistant to *antibiotics.*
Up: up- regulation, down –regulation

spectrum beta-lactamase (ESBL) production genes using PCR. Thirty-three out of 65 of the imipenem and meropenem resistant *P. aeruginosa* isolates used in the study tested were positive for ESBL genes. Out of 33 ESBL, genes were amplified in 25 isolates for bla_{VEB}, in 5 isolates for bla_{TEM} and in 3 isolates for bla_{CTX-M}. No bla_{SHV} and bla_{PER} genes were detected for imipenem and meropenem resistant isolated in this study as illustrated in Table 5.

Among the 65 isolates of *P. aeruginosa* resistant to imipenem and meropenem, 41 isolates were MBL producers. Out of 41, MBL genes were amplified in 20 isolates for bla_{IMP}, in 14 isolates for bla_{VIM}, in 4 isolates for bla_{GIM}, in 2 isolates for bla_{NDM} and only in one isolate for bla_{SIM} as shown in Table 5. No bla_{SPM} gene was amplified in this isolates.

TABLE V
PREVALENCE OF ESBL AND MBL GENES AMONG *P. AERUGINOSA* ISOLATES (n =65)

Ambler class	Type of genes	Positive No.
ESBL gene positive (n = 33)	bla_{VEB}	25
	bla_{TEM}	5
	bla_{CTX-M}	3
MBL gene positive (n = 41)	bla_{IMP}	20
	bla_{VIM}	14
	bla_{GIM}	4
	bla_{NDM1}	2
	bla_{SIM}	1

IV. DISCUSSION

P. aeruginosa is an important pathogen associated with serious healthcare-associated infections that is an important cause of morbidity and mortality among hospital patients [35]. *P. aeruginosa* isolates in our society exhibited high resistance to most antimicrobial agents examined, except colistin, which showed low levels of resistance [36]. Therefore, the present study aimed to evaluate the chromosomally encoded resistance mechanisms that are responsible for the causes of resistance among 88 *P. aeruginosa* clinical isolates by observing the expression levels of the multidrug efflux pumps, AmpC cephalosporinase, the OprD outer membrane porin and the penicillin binding protein (PBP2, PBP3), that contribute to both intrinsic and acquired resistance and to evaluate the different mechanisms associated with reduced susceptibility to imipenem and meropenem.

In the present study, *mexB* mRNA expression of 88 *P. aeruginosa* clinical isolates as a marker for MexAB-OprM expression showed overexpression in up to 78% in the isolates that were resistant to piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, meropenem and imipenem drugs as shown in Table 3. This number is much higher than those obtained previously by multicenter Spanish study for isolates resistant to all first line agents (all β-lactams and aminoglycosides) that showed overexpression of *mexB* in only 25% of isolates [37]. Another study conducted at a tertiary teaching hospital located in São Paulo, Brazil showed overexpression of *mexB* gene in 27.1% of *P. aeruginosa* isolates that were resistant to antimicrobials and demonstrated overexpression of *mexB* in 62.5% of the isolates that were meropenem resistant [38]. This study confirmed the contributions of MexAB-OprM overexpression to intrinsic resistance to a number of antimicrobials including piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, and carbapenems antibiotics.

MexXY-OprM is the second efflux pump system that contributes to intrinsic and acquired resistance to antimicrobials, and is capable of pumping out amikacin, ciprofloxacin, gentamicin, meropenem, cefepime and erythromycin [31]. In the current study, mRNA expression analysis in 88 *P. aeruginosa* clinical isolates demonstrated that more than 80% showed up-regulation of *mexXY-oprM* among the isolates that were resistant to gentamicin, amikacin, ciprofloxacin, ceftazidime, piperacillin/tazobactam, meropenem and imipenem as shown in Table 3. These findings are in agreement with a previous study carried out by Hocquet *et al.* (2006) who found overexpression of *mexXY-oprM* in 84% of the isolates that were resistant to aminoglycosides, fluoroquinolones, ceftazidime, piperacillin/tazobactam and carbapenems [23]. This was also shown in many other studies where reduced susceptibility to various antibiotics known to be substrates for the MexXY-OprM pump were demonstrated [13], [23], [37]-[41]. In the results of this study, the mRNA expression of *P. aeruginosa* clinical isolates showed 90%, 86% and 84% overexpression of *mexY* gene in the isolates that were resistant to amikacin, gentamicin and ciprofloxacin respectively. Of those, more than 96% also showed reduced *oprD* transcription (Table 3). These results are in agreement with Xavier *et al.* (2010), who reported that 86.7% of the isolates that were resistant to amikacin, gentamicin and ciprofloxacin expressed *mexY* and of those, 79.2% also showed reduced *oprD* transcription [38]. In addition, the up-regulation of MexXY-OprM was highly

significant isolates that were resistance to aminoglycoside drugs, particularly amikacin and gentamicin (Table 3). These findings were also in concordance with previous studies by Poole (2007), Vila and Martines (2008) and Toma *et al.* (2010). They reported that most of the aminoglycoside drugs were significant substrates for MexXY-OprM [7], [31], [42]. In addition, the current study was consistent with other studies, which suggested the up-regulation of *mexY* also conferred reduced susceptibility to aminoglycosides detected in 6 of the 7 clinical isolates and was responsible for the decreased antimicrobial efficacy of aminoglycoside among clinical isolates. This is due to the longer aminoglycoside treatment periods in a clinical setting where there is a higher risk of occurrence of resistance and possibly therapeutic failure [37], [39], [41], [43]. The highest prevalence of *mexY* overexpression (84%) was found among isolates that were resistance to ciprofloxacin. These incidences of isolates hyperproducing MexXY-OprM were influenced by the use of aminoglycosides and fluoroquinolones drugs [40], [41]. Besides that, the *mexY* overexpression (80%) was noted in isolates that were resistant to meropenem and imipenem (Table 3). These support the results of the previous studies, which emphasized the capability of *P. aeruginosa* to develop dual resistance to carbapenems and fluoroquinolones due to independent mechanisms of resistance of different classes of antibacterial agents and stress the requirement for the judicious utilization of treatment when dealing with *P. aeruginosa* to avoid development of multidrug resistance [39, 40].

MexEF-OprN is the third efflux system, which can export fluoroquinolones [44, 45]. Fluoroquinolones are the choice for mutant overproduction of multidrug efflux pumps *in vivo* and *in vitro*, including MexEF-OprN [46]. This supports our results, where mRNA expression analysis in isolates that were resistant to ciprofloxacin showed that 68% of them overexpressed MexEF-OprN (Table 3). The results of this study confirm a dual resistant phenotype in which the overexpression of MexEF-OprN was noted in 68% of the isolates that were resistant to both imipenem and ciprofloxacin drugs and in 70% of isolates that were resistant to meropenem as shown in Table 3. This dual resistance increases an anxiety concerning the promotion of carbapenem resistance during the utilization of fluoroquinolones. Therefore, the dual resistance to the carbapenems and fluoroquinolones remains a big problem because these drugs remain the main antimicrobials for treating infections due to multidrug-resistant *P. aeruginosa* and the development of resistance significantly compromises their efficacy. Previous studies reported that fluoroquinolones, β-lactams (e.g., meropenem) are substrates for the MexCD-OprJ system, although this efflux pump is not constitutively expressed under usual growth circumstances [14], [45]. This coincides with the results of this study, in which the overexpression *mexCD* was noted in 48% of the isolates that were resistant for ciprofloxacin and ceftazidime, in 55% of the isolates that were resistant for aztreonam and meropenem and in 54% of the isolates that were resistant to imipenem as is illustrated in Table 3. These numbers are higher than previously reported where only 31% of the carbapenem resistant isolates overexpressed the MexCD-OprJ efflux pump [47]. The mechanism leading to carbapenem resistance seem to be more complex and is very likely multifactorial, involving overproduction of AmpC or overexpression of the efflux pumps MexAB-OprM, MexXY-OprM and MexCD-OprJ [29]. In the Rodriguez-Martinez *et al*. (2009) study no isolate overexpressed all three efflux pumps [47], whereas our results demonstrated up-regulation for AmpC in >65% of the isolates that were resistant to carbapenems and more than 71% of these isolates also showed overexpression of *mexB* and *mexY*. In addition, up to 55% of the isolates those were resistant to carbapenems demonstrated over expression of *mexCD* as shown in Table 3.

AmpC overproductions play an important role in the multi-drug resistance phenotype among *P. aeruginosa*. The results of the current study demonstrated 65% of the isolates that were resistant to antibiotics examined exhibited up-regulated *ampC* gene expression as shown in Figure 1. This is considered much higher than the results obtained by Cabot *et al*. (2011) study that demonstrated only 24.2% of isolated exhibited *ampC* overexpression [37]. One factor possibly contributing to this difference is the resistance level for the different antibiotics used as our isolates showed higher rates of antimicrobial resistance. Another important issue to consider for an understanding of resistance dynamics is the interconnections between the different resistance mechanisms. The derepression of the chromosomal AmpC cephalosporinase and the up-regulation of the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux systems confer fluoroquinolone resistance, and resistance to some β-lactams and aminoglycosides [25]. This is clear with results of the current study which demonstrated that more than 60% of the isolates that were resistant to antimicrobial agents mentioned above showed up-regulation of an *ampC* and this was associated with more than 71%, 76%, 63% and 47% of these resistant isolates overexpressing *mexB*, *mexY*, *mexEF* and *mexCD* mRNA respectively, as is shown in Table 3. It has been reported that AmpC β-lactamase is able to cause very little activity alone against carbapenems but when combined with other resistance mechanisms can play a considerable role in reducing susceptibility to carbapenems [16], [48]. Additionally, the association of distinct mechanisms such as AmpC overproduction and porin down-regulation also play an important role in the carbapenem resistance phenotype among *P. aeruginosa* isolates [38]. In the present study, imipenem resistance was linked to 66% overexpression of AmpC. Of those, 100% showed decreased OprD expression. While, 52.2% carbapenems resistant was obtained by Xavier *et al*. (2010) study among these isolates, 20% overexpressed for AmpC, of those, 90.0% showed decreased OprD expression were considered lower than our findings. Theses is related to interplay of different resistant mechanisms that play an important role in the carbapenems resistance phenotype among *P. aeruginosa* clinical isolates studied as shown in Table 3. This study further confirms that chromosomal AmpC β-lactamase confers resistance to most cephalosporins and penicillins (piperacillin/tazobactam) in *P. aeruginosa* with high significance (Table 2). This finding is in line with other studies that reported that piperacillin/tazobactam resistance through chromosomal AmpC β-lactamase overexpression in *P. aeruginosa* clinical isolates [16], [38], [49].

The loss of OprD has been reported as the most consistent findings in carbapenem-resistant isolates [50]. In the current study, 100% of the isolates that were resistant to imipenem and meropenem showed down-regulation of *oprD* (Table 3). The results reported in this study are also consistent with pervious investigators [18], [37], [51], [52], who reported that the mechanism of resistance to imipenem in clinical strains is associated with low expression of *oprD* which increases the MIC level to these drugs.

The results of the current study demonstrated that 80% of the *P. aeruginosa* clinical isolates that were resistant to imipenem and meropenem showed down-regulation of *PBP2*, while up to 91% of these isolates showed down-regulation of *PBP3* (Table 3). However, Farra *et al*. (2008) reported in their study, that decreased expression of *PBP2* and *PBP3* was noted in all strains in comparison to *PA018SR*, except for the clinical strain, which showed increased expression of both *PBPs*. Therefore, the increased expression of *PBP2* and *PBP3* genes is possibly not significant for resistance to carbapenems [53], previous studies reported that *PBP2* contributes significantly to β-lactam antibiotic resistance, and down-regulation of *PBP3* has effects on a number of genes in *Pseudomonas*, involved in antibiotic resistance [54], [55]. The present study shows that imipenem and meropenem resistance among clinical *P. aeruginosa* isolates not only occurred due to OprD down-regulation but was also linked with down-regulation of other genes such as *PBP2* and *PBP3*. This implies that down-regulation of the *oprD* gene significantly contributes to imipenem and meropenem resistance, which increases the MIC to these drugs. While *PBPs* can be one of the most likely mechanisms for carbapenem resistance among clinical *P. aeruginosa* isolates, the mechanisms of resistance to carbapenem (imipenem and meropenem) as a result of the interplay between diminished production of OprD, increased activity of AmpC, and several efflux systems has been illustrated to contribute high-level resistance to these agents [16], [25].

In the current study, we aimed to evaluate the different mechanisms associated with reduced susceptibility to imipenem and meropenem compared to other examined antibiotics. The mechanism of carbapenem (imipenem and meropenem) resistance is usually multifactorial, involving overexpression of efflux pumps, AmpC overproduction or decreased outer membrane OprD levels or by acquired β-lactamases, included extended spectrum types (ESBLs) and carbapenemases [56]-[58]. Therefore, this study was carried out to detect the prevalence of MBL producing *P. aeruginosa* in the University Malaya Medical Center (UMMC) in Kuala Lumpur, Malaysia, to discover the associated risk factors and probable treatment alternatives.

The most common and widespread acquired MBLs are those of the *IMP* and *VIM* types, which exhibit a worldwide distribution and for which several allelic variants are known [59], [60]. Previous studies pointed out that *IMP* and *VIM* types of MBLs are widespread in Asian countries [61]-[64]. This is in concordance with results of this study that most imipenem and meropenem resistant isolates harbor *IMP* and *VIM* types. Detection of two *NDM* positive isolates in this study suggested possibilities of spread via international tourists in this country.

The detection of bla_{IMP} and bla_{VIM} in this study is in agreement with a previous study that carried out at the University Malaya Medical Center (UMMC) in Kuala Lumpur, Malaysia [63]. It demonstrated that 32 out of 90 imipenem resistant *P. aeruginosa* clinical isolates tested positive for the presence of MBL genes including bla_{IMP} and bla_{VIM} and none of the isolates tested positive for other MBL genes [63]. However, another study also carried out in same medical centre showed that only one *P. aeruginosa* out of 50 imipenem resistant *P. aeruginosa* isolates was MBL gene PCR positive [65]. The results of this study are interesting in that this is the first study to show that imipenem and meropenem resistant *P. aeruginosa* isolates harbor bla_{SIM}, bla_{GIM} and bla_{NDM}. Recently, the New Delhi metallo-β-lactamase (*NDM*) has been reported from diverse locations such as the UK, the USA, Japan, India, Singapore, Australia and most recently the Middle East and this represents an emerging public health threat [66]-[68].

This study demonstrated that among the 65 carbapenem resistant *P. aeruginosa* isolates, 33 were ESBL producers, which is the first time ESBL genes in *P. aeruginosa* isolates in Malaysia is detected. The most frequently encountered ESBL gene in our study was bla_{VEB} (25 of 65), which is similar to (30 of 75) ESBL producing isolates of *P. aeruginosa* which was reported by Jiang et al [69].

The risk factors for the hospitalized patients towards infection or colonization by the ESBL-producing organisms are comparable to those acquired by healthcare-associated bacterial infections [70]. A long stay in a hospital or intensive care unit (ICU), inclusion of different kinds of indwelling catheters, greater severity of clinical status, performance of surgical interventions or invasive procedures, and mechanical ventilatory support or renal replacement therapy, have all been linked to the isolation of ESBL-producing organisms from hospitalized patients [71], [72].

Overall, *P. aeruginosa* clinical isolates in our society showed multiple resistance phenotypes leading to limited therapeutic choices. Therefore, infections caused by MDR *P. aeruginosa* have become a serious problem, especially in the healthcare-associated settings. This problem indicates the importance of performing antibiotic susceptibility testing before treatment.

Furthermore, infection with MDR *P. aeruginosa* is associated with risk factors including severity of illness, invasive devices, a bedridden state, and in hospitalized patients, lead to increased length of stay and increased therapeutic costs as well as significant morbidity and mortality. The continuous efforts of clinicians, microbiologists, pharmacists and communities should promote a greater understanding of this problem, with better hygiene and postoperative care and management. Most of the *P. aeruginosa* clinical isolates had a high level of resistance to examined antibiotics except colistin. Therefore, the only drug of choice in MDR isolates is the colistin despite its toxicity. In addition, the search for anti-pseudomonal agents with alternative mechanisms of action is very important in this situation. In addition, it is important to practice good antibiotic prescription policies and infection control practices to prevent the spread of genes responsible for these resistances, which could result in the return to the pre-antibiotic era.

V. CONCLUSIONS

In conclusion, the multidrug resistance phenotypes in these clinical isolates were caused by the interaction of several different resistance mechanisms occurring within the same strain such as overexpression of efflux pumps, AmpC overproduction, decreased outer membrane porin OprD, alteration of penicillin binding proteins. The clinical *P. aeruginosa* isolates that produce MBL and ESBL genes have the ability to increase the resistance to imipenem and meropenem among *P. aeruginosa*.

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