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Investigation of OprD Porin Protein Levels in Carbapenem-Resistant *Pseudomonas aeruginosa* Isolates

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Abstract

Background: The *Pseudomonas aeruginosa* porin OprD is a substrate-specific porin that facilitates the diffusion of basic amino acids, small peptides, and carbapenems into the cell. OprD-mediated resistance occurs as a result of decreased transcriptional expression of *oprD* and/or loss of function mutations that disrupt protein activity.

Objectives: In this study, we examined the level of *oprD* expression in *P. aeruginosa* clinical isolates to determine the contribution of OprD porins in carbapenem resistance.

Materials and Methods: Included strains were divided into two groups, comprised of multidrug-resistant (MDR) and isolated carbapenem-resistant (ICR) strains. The transcription product level of oprD was identified using real-time polymerase chain reaction (qPCR).

Results: Of the 18 clinical isolates, a decrease in the *oprD* level was found to be significant in 13 isolates. Nine of eighteen isolates with a significant decrease were determined in the first group and comprised MDR isolates that showed a statistically significant difference compared with the ICR group ($P = 0.001$). In the ICR group, *oprD* levels were found to be significantly low in 4 isolates. Six different patterns were determined by comparing band profiles in AP-PCR.

Conclusions: Although the data support the idea that the basic mechanism of imipenem resistance could be via the loss of *oprD*, they do not fully explain the role of *oprD* and indicate that other mechanisms may play an important role. Additionally, the significant decrease in the *oprD* levels in MDR strains suggests that *oprD* also plays a role in the emergence of both carbapenem and non-carbapenem resistance.

Keywords: Pseudomonas aeruginosa, OprD Porin Protein, qPCR, AP-PCR, *oprD* Expression

1. Background

The outer membrane of Gram-negative bacteria constitutes a semipermeable barrier that slows the penetration of antibiotics. The permeability of this barrier is known to vary greatly among species, with the outer membrane of *Pseudomonas aeruginosa* only 8% as permeable as that of *Escherichia coli* (1). This decreased permeability severely restricts the uptake of nutrients and other important compounds into the cell, which consequently must be imported into cells using a collection of water-filled protein channels called porins.

The *P. aeruginosa* family of porin proteins, defined based on their apparent sequence homology within the *P. aeruginosa* genome, plays an important physiological role in the transport of substances required for metabolism. However, these proteins also exhibit an affinity for certain hydrophilic antibiotics, such as β-lactams, aminoglycosides, tetracyclins, and some fluoroquinolones, allowing these compounds to transverse the otherwise insoluble outer bacterial membrane (2, 3). Deletion of one or more porin proteins has been shown to reduce the susceptibility of *P. aeruginosa* to certain antibacterial agents (4).

The *P. aeruginosa* porin OprD is a substrate-specific porin that facilitates the diffusion of basic amino acids, small peptides, and carbapenems into the cell (5). OprD-mediated resistance occurs as a result of decreased transcriptional expression of *oprD* and/or loss of function mutations that disrupt protein activity. Specific mechanisms resulting in decreased transcriptional expression of *oprD* include (i) disruption of the *oprD* promoter, (ii) premature termination of *oprD* transcription, (iii) co-regulation with trace metal resistance mechanisms, (iv) salicylate-mediated reduction, and (v) decreased transcriptional expression via co-regulation with the multidrug efflux pump encoded by *mexEF-oprN* (6).

2. Objectives

In this study, we examined the level of *oprD* expression in *P. aeruginosa* clinical isolates to determine the contribution of OprD porins in carbapenem resistance. Clinical isolates were further examined using additional molecular methods to determine the degree of variability among isolates.

3. Materials and Methods

3.1. Bacterial Isolates and Antibiotic Susceptibility Testing

Pseudomonas aeruginosa isolates were obtained from clinical samples sent to our laboratory routinely. Species identification was performed using conventional methods. Antibiotic susceptibility testing was carried out using the Kirby-Bauer disc diffusion method (7). Isolates were divided into two groups according to their resistance status: multiple-drug resistant (MDR) and isolated carbapenem resistant (ICR).

3.2. Minimum Inhibitory Concentration (MIC) Testing

Minimum inhibitory concentrations (MIC) of ceftazidime (CAZ), gentamicin (CN), piperacillin-tazobactam (TZP), ciprofloxacin (CIP), imipenem (IMP), and meropenem (MEM) were determined using the Vitek 2 system (bioMerieux, France). The Densi-Check 2 system (bioMérieux, France) was used to calibrate the turbidity of samples against the 0.5 McFarland standard. Susceptibility tests were performed on the Vitek 2 system using AST-N174 cards, according to the manufacturer's instructions. MIC values of ≥ 32 μg/mL for ceftazidime, ≥ 16 μg/mL for gentamicin, ≥ 128 μg/mL for piperacillin-tazobactam, ≥ 4 μg/mL for ciprofloxacin, ≥ 16 μg/mL for imipenem, and ≥ 16 μg/mL for meropenem were defined as resistance (7).

3.3. Quantitative Real-Time PCR

Transcript levels of *oprD* were analyzed by real-time polymerase chain reaction (qPCR) using a LightCycler instrument (Roche Diagnostics, Germany). Total RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics, Germany) and converted into cDNA for qPCR using the Transcriptor High-fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany). The quality and purity of the RNA obtained was evaluated spectrophotometrically (Maestrogen Nanodrop, USA). As a result of evaluation, required volume was calculated for 100 ng cDNA. Quantitative PCR was performed in capillary glass using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Germany) with primers specific for *oprD*, and *rpsL* (Table 1).

Control cDNA was obtained from *P. aeruginosa* strain PAO1. Amplification of triplicate cDNA samples from each isolate was performed under the following conditions: initial denaturation for 10 minutes at 95°C, followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing at 68°C for 10 seconds, and elongation at 72°C for 15 seconds. A final melting curve analysis was performed using a single read at 90°C.

3.4. Arbitrarily Primed PCR (AP-PCR)

To evaluate the similarities among strains, arbitrarily primed PCR (AP-PCR) was performed using an M13 primer of sequence 5'-GAGGGTGGCGGTTCT-3'. PCR was carried out under the following conditions: 2 cycles of 94°C for 5 minutes, 40°C for 5 minutes, and 72°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 40°C for 1 minute, and 72°C for 2 minutes. Amplification products were identified by agarose gel electrophoresis, and similarities among isolates were evaluated by comparison of the band profiles.

3.5. Evaluation of Gene Expression

Transcription data were analyzed using the LightCycler Relative Quantification software. Relative expression values (R) were determined using the ' $\Delta\ \Delta C_t$' method; the gene encoding ribosomal protein RpsL was used as a control (10). *P. aeruginosa* strain PAO1 was used as a standard for normalization of relative mRNA levels. Reduced *oprD* expression was defined as transcription levels $\leq 70\%$ of those of the PAO1 isolate (11).

Primer dimers and other artifacts were evaluated by melting curve analysis. To confirm that specific amplification had occurred, the melting curves of each amplicon were assessed and compared with Tm values obtained using PAO1 DNA as the template.

3.6. Statistical Analyses

All statistical analyses were carried out using SPSS statistical software (version 17.0). Comparisons among groups were performed using a one-way ANOVA test.

4. Results

4.1. Antibiotic Susceptibility Testing

Clinical isolates were divided into two groups based on their drug susceptibility profiles. MDR isolates were defined as those exhibiting resistance to ceftazidime (MIC ≥ 32), piperacillin (MIC ≥ 128), imipenem (MIC ≥ 16), and gentamicin (MIC ≥ 16) (12). All ICR isolates were resistant to imipenem, and an additional three isolates (33%) also exhibited resistance to meropenem. Antibiotic sensitivities and MIC data for each group are summarized in Table 2.

4.2. Gene Expression

Relative mRNA expression levels of *oprD* were determined by qPCR. Decreased *oprD* expression was observed in 16 of 18 *P. aeruginosa* clinical isolates, with significant decreases detected in 13 isolates (72%). Within the MDR group, *oprD* expression was significantly decreased in all 9 isolates. *oprD* levels were decreased in 7 ICR isolates, with significant decreases found in 4 of these isolates. Detailed expression data for each group are shown in Table 2. Due to the consistently low expression seen in the MDR group, differences in mRNA expression between the groups were statistically significant ($P = 0.001$).

4.3. AP-PCR Analysis

The genetic similarity among isolates was determined using AP-PCR. Among the 18 clinical isolates tested, six distinct banding patterns were identified (Figure 1). Classifications of *P. aeruginosa* isolates based upon AP-PCR

analysis are shown in Table 2.

Table 1. PCR Primers Used in This Study

Gene	Primer	Sequence (5' – 3')	Product Size, bp	Reference
oprD			191	(8)
	oprD-F	TCCGCAGGTAGCACTCAGTTC		
	oprD -R	AAGCCGGATTCATAGGTGGTG		
rpsL			250	(9)
	rpsL-F	GCTGCAAAACTGCCCGCAACG		
	rpsL-R	ACCCGAGGTGTCCAGCGAACC		

Table 2. Antimicrobial Susceptibilities and Gene Transcription Levels of *P. aeruginosa* Isolates

Isolate	Group[a]	AP-PCR Category[b]	MIC, mg/L Levels						Relative Gene Expression[c] (*oprD*)
			CAZ	TZP	CN	IPM	MEM	CIP	
1	1	1	≥ 64	≥ 128	≥ 16	≥ 16	8	≥ 4	0.165
2	1	1	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0.285
3	1	1	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0
4	1	2	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0
5	1	2	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0.005
6	1	2	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0
7	1	3	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0.002
8	1	3	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0
9	1	3	≥ 64	≥ 128	≥ 16	≥ 16	≥ 16	≥ 4	0.001
10	2	4	2	8	≤ 1	≥ 16	4	≤ 0.25	0.029
11	2	4	2	8	≤ 1	≥ 16	8	≤ 0.25	0.034
12	2	4	4	32	≤ 1	≥ 16	≥ 16	≤ 0.25	0.04
13	2	5	4	8	≤ 1	≥ 16	4	≤ 0.25	0.509
14	2	5	4	16	≤ 1	≥ 16	≥ 16	≤ 0.25	0.28
15	2	5	2	8	≤ 1	≥ 16	4	≤ 0.25	2
16	2	6	2	≤ 4	≤ 1	≥ 16	≤ 0.25	≤ 0.25	0.736
17	2	6	4	≤ 4	≤ 1	≥ 16	4	≤ 0.25	14.256
18	2	6	4	64	2	≥ 16	≥ 16	≤ 0.25	0.875

[a]Group 1: Multiple drug resistant (MDR); Group 2: Isolated carbapenem resistant (ICR) *P. aeruginosa* isolates.
[b]AP-PCR category: Classification of *P.aeruginosa* isolates following AP-PCR analysis.
[c]Relative gene expression: expression levels were compared to fold PAO1.

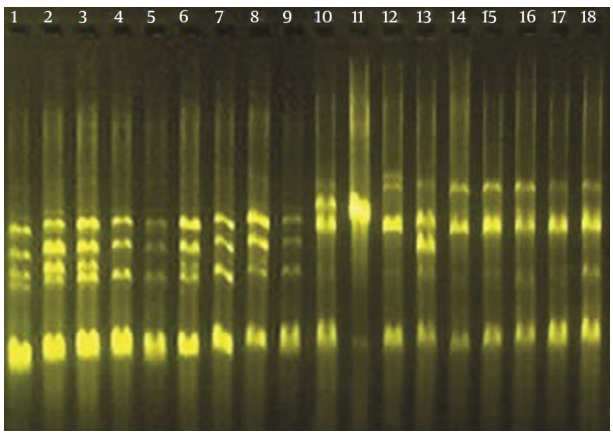

Figure 1. Agarose Gel Electrophoresis of Amplification Products Following AP-PCR

5. Discussion

Pseudomonas aeruginosa represents a phenomenon of bacterial resistance, and most of the known antimicrobial resistance mechanisms are displayed in this species, and multiple resistance mechanisms may be expressed simultaneously within the same isolate (4). Due to the increase in multi-drug resistant *P. aeruginosa* infections, a greater emphasis has been placed on identifying genetic characteristics underlying bacterial resistance and the clinical implications of these mutations.

Outer membrane protein OprD is considered the preferred portal of entry for carbapenems and similar drugs such as imipenem and meropenem also enter the cell via OprD (3, 13, 14). Any loss of *OprD* expression from the outer membrane significantly decreases the susceptibility of *P. aeruginosa* to carbapenems and has been shown to play

a major role in the acquired resistance to imipenem and, to a lesser extent, meropenem (15). One study showed that *OprD* expression was decreased in the vast majority of 29 multi-drug resistant *P. aeruginosa* isolates (97%) and played a significant role in their carbapenem resistance (16).

Expression of *oprD* was decreased significantly in 13 of the 18 imipenem-resistant *P. aeruginosa* clinical isolates examined in this study, including all 9 MDR isolates. The frequency of decreased *oprD* expression in MDR isolates was significantly higher than that of ICR isolates (P = 0.001), suggesting that *oprD* also plays an important role in the emergence of both carbapenem and non-carbapenem resistance.

The impact of OprD-mediated resistance on carbapenems can be quantified relative to its effect on the antibacterial potency of carbapenems (6). In a study evaluating isogenic wild-type and OprD-deficient mutant pairs, the loss of OprD decreased the susceptibility of *P. aeruginosa* to meropenem 4- to 32-fold, compared with 4- to 16-fold for imipenem and 8- to 32-fold for doripenem (14). Zeng et al. (17) investigated relative gene expression in 29 carbapenem-resistant and ceftazidime- and cefepime-sensitive *P. aeruginosa* clinical isolates and found that the loss of oprD was directly related to carbapenem resistance. In another study, Fournier et al. (18) detected loss of oprD, as a result of mutations or gene disruptions, in 94 of 109 (86.2%) imipenem-resistant *P. aeruginosa* isolates.

In this study, *oprD* mRNA levels were decreased in 7 of 9 ICR isolates evaluated, even though significance was observed in only 4 of these isolates, in contrast with prior studies. Although our data are consistent with the basic mechanism of imipenem resistance mediated by diminished OprD protein levels in the outer membrane, the poor correlation between *oprD* mRNA expression and carbapenem resistance suggests involvement of additional resistance mechanisms in these isolates.

Impermeability was long thought to be the driver of intrinsic resistance in *P. aeruginosa*; however, resistance has since been found to involve a more complex interplay between impermeability and multi-drug efflux pumps (19). The interaction of efflux pumps with meropenem differs from that with imipenem. While it is believed that both meropenem and imipenem are able to enter the cell via the OprD pathway, only meropenem is a substrate of the MexAB-OprM efflux pump (19). Furthermore this mechanism plays a role in the emergence of resistance to fluoroquinolones and other β-lactams, increasing the likelihood of cross-resistance (20). However, despite this additional mechanism, meropenem resistance is less likely to be acquired than imipenem resistance (77 vs. 68% sensitivities for meropenem and imipenem, respectively), as it requires both the loss of oprD expression and upregulation of MexAB-OprM (6, 21). In our study, all 18 *P. aeruginosa* clinical isolates were resistant to imipenem, compared with only 12 isolates exhibiting meropenem resistance, consistent with previously published reports.

The relationship between OprD deficiency and imipenem resistance has been well established; however, cases of discordant *OprD* expression and carbapenem susceptibility, due to genetic versatility and multiple resistance mechanisms displayed in this pathogen, have been reported (6). El Amin et al. (15) identified four imipenem-susceptible isolates with significant reductions in *oprD* mRNA levels caused by severe *oprD* mutations that resulted in frame shifts or premature termination. Furthermore, they reported that the *oprD* mRNA levels did not always correlate with imipenem resistance, and differences in imipenem susceptibility could not be explained by *oprD* mutations or efflux pump genes (15). In our study, increased *oprD* levels were detected in 2 of the 18 imipenem-resistant *P. aeruginosa* clinical isolates analyzed. Also we could not find any relation between genotype and resistance pattern in AP-PCR study. Further studies will be necessary to understand the mechanisms underlying this apparent discordance between *oprD* levels and imipenem resistance.

While OprD porin proteins play an important role in carbapenem resistance in *P. aeruginosa*, this resistance cannot be explained by OprD levels alone, and other important interactions may influence carbapenem susceptibility. Characterization of carbapenem resistance mechanisms could provide additional therapeutic targets or allow for alternative strategies to enhance the efficacy of carbapenems.

Footnotes

Authors' Contribution: All authors had equal contribution in preparing this article. IHC, CK and HAT drafted the first manuscript of this article. HAT and ARA carried out the identification studies and collecting data. HAT, ARA and IHC carried out the molecular studies. All authors read and approved the final manuscript.

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