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OprD Expression and Imipenem Resistance in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa **is an opportunistic pathogen that produces highly resistant to antibiotic treatment. Here we show a presence of correlation between** ***oprD*** **expression and imipenem resistance. Minimal inhibitory concentration (MIC) for planktonic cells of** ***P. aeruginosa*** **was measured using E test. The results revealed the presence of** ***oprD*** **expression in 10 strains of** ***P. aeruginosa*** **isolates from patients with cystic fibrosis in order to evaluate their impact on imipenem susceptibility profiles. We investigated the relation between** ***oprD*** **gene expression and imipenem susceptibility profile showed isolates. Surprisingly, the imipenem-susceptible (P8) has low** ***oprD*** **expression that appeared in imipenem-susceptible, indicated a correlation of other mechanisms. This work forms a basis for future studies revealing the mechanisms of imipenem resistance in** ***P. aeruginosa*****.**

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic human pathogen that can cause life-threatening infections especially in cystic fibrosis (CF) patients and individuals with a compromised immune system. This environmental bacterium is willingly able to survive both as free swimming planktonic form and in surface-associated communities known as biofilms. Although there are several antimicrobials (carbapenems, cefepime, ceftazidime, tobramycin and amikacin) that continue to be effective against *P. aeruginosa,* in the last few years the bacterium's increasing resistance to many others has been reported (Sanchez-Romero *et al*, 2007; Ruiz-Martinez *et al*, 2011), Carbapenems, particularly imipenen, are suitable alternative in treating multi drug resistant *P. aeruginosa*, yet the emergence and spread of carbapenem resistant strains have compromised the progress of therapeutic and control efforts (Riera *et al*, 2011).

OprD porin of *Pseudomonas aeruginosa* facilitates the uptake across the outer membrane of basic amino acids, small peptides that contain these amino acids, and their structural analogue imipenem. Indeed, prolonged imipenem treatment of patients with *P. aeruginosa* infections leads to imipenem resistant mutants that either lack *OprD* due to an *oprD* gene mutation (Lynch *et al*., 1987) or have strongly reduced *OprD* levels due to an *nfxC*-type mutation (*mexT*) which suppresses *oprD* expression at the same time as upregulation of the *mexEF-oprN* multidrug efflux operon (Fukuda *et al*., 1995; Kohler *et al*., 1997). Inactivating mutations in *OprD* have been documented to confer resistance to imipenem and to a lesser extent to meropenem and doripenem (Sanbongi *et al*., 2009).

The pathway to *OprD*-mediated resistance can involve mechanisms that decrease the transcriptional expression of *oprD*, characterized mechanisms include (i) disruption of the *oprD* structural gene by insertion of large IS elements (Wolter *et al*., 2004; Evans and Segal, 2007; Wolter *et al*., 2008; Wolter *et al*., 2009). (ii) Mutations, insertions (Yoneyama and Nakae, 1993), and/or deletions creating frame shifts (Pirnay *et al*., 2002) and premature stop codons premature termination of *oprD* transcription, (Pirnay *et al*., 2002; El Amin *et al*., 2005). (iii) coregulation with mechanisms of trace metal resistance, (iv) salicylate-mediated reduction, and (v) decreased transcriptional expression through mechanisms of coregulation with the multidrug efflux pump encoded by mexEF-oprN (Yoneyama and Nakae, 1993; Köhler *et al*., 1999).

The goal of the present study was to analyze the expression of *OprD* in a number of clinical isolates of *P. aeruginosa* with different imipenem susceptibility profiles, ranging from susceptible to intermediately susceptible, and how much this *oprD* expression gene would affect the imipenem resistance.

MATERIALS AND METHODS

Strains and media. The strains used in this study were routinely cultured on lysogeny broth (LB) medium, which was solidified with 1.5% agar when necessary, bacterial strains and susceptibility testing *P. aeruginosa* were isolated from sputum samples of different cystic fibrosis patients.

Determination of Imipenem Minimal Inhibitory Concentration (MIC)

The E-test method was used for MIC determination according to the manufactures instructions. In brief, bacterial suspensions were prepared from fresh colonies, the concentration adjusted to 0.5 McFarland turbidity, each isolate was uniformly spread on the surface of a Mueller Hinton agar (MHA) plate, and an imipenem E-test strip (from 0.002 to 32 µg/mL; bioMérieux, France) placed on the surface of agar plate. After overnight incubation at 37°C, MIC was been determined and categorized as sensitive (≤ 2 µg/ml), intermediate (4 µg/ml) or resistant (≥8 µg/ml) according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). In select cases, we also determined the MIC by a microdilution assay in microtiter plates to confirm the E-test findings (Andrews, 2001; Wiegand et al, 2008). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains for E test and microdilution assay, respectively.

Modified Hodge Test (MHT)

Modified Hodge test was adopted for the detection of carbapenemases following the procedure described by Anderson *et al*. (2007) and Noyal *et al*. (2009). In brief; 0.5 McFarland dilution of the *E. coli* ATCC 25922 was prepared in 5 ml of saline. 1:10 dilution was prepared by adding 0.5 ml of overnight culture to 4.5 ml of saline. Thereafter, a lawn of the 1:10 dilution of *E. coli* ATCC 25922 was streaked to a MHA. Afterward, 10 µg Imipenem disc was placed in the center of the test area. In a straight line, the test organism was streaked from the edge of the disc to the edge of the plate. Up to four organisms can be tested on the same plate with one drug, and then the plate was incubated overnight at 37ºC in ambient air for 16–24 hours. After 16–24 hours of incubation, the plate was examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disk. MHT positive test has a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disk diffusion zone. MHT negative test has no growth of the *E. coli* 25922 along the test organism growth streak within the disc diffusion.

qRT-PCR

Strains were diluted from LB-grown overnight cultures 1:100 into M63 minimal medium supplemented with glucose, $MgSO_4$, and CAA and grown to an optical density at 600 nm (OD_{600}) of 0.6. RNA was isolated, and cDNA was prepared as previously described (Kuchma *et al*., 2005). Quantitative reverse transcription-PCR (qRT-PCR) was performed using an

ABI 7500 Fast System and analyzed using ABI Fast System software version 1.4. Expression levels were quantified in picograms of input cDNA using a standard curve method for absolute quantification, and these values were normalized to rplU expression. Each experiment was done with three replicates per sample. The primers used are *oprD*-RT Forward CCGCAGGTAGCACTCAGTTCG and *oprD*-RT Reverse GTAGTTGCGGAGCAGCAGGTC.

Statistical analysis

Data are presented as mean ± standard deviation. ANOVA test, correlation coefficient (r) and $LSD_{0.05}$ were employed for data analysis using Microsoft EXCELL 2010 application

RESULT AND DISCUSSION

Imipenem susceptibility and carbapenemase detection

Among fifty eight *P. aeruginosa* isolates evaluated with E test, forty seven (81.03%) isolates were susceptible, two (3.45%) isolates were intermediate, and nine (15.52%) isolates were resistant to imipenem.

All these 9 isolates were previously isolated from patients with cystic fibrosis. No carbapenemase activity was found among the intermediate and resistant isolates, as it is confirmed by the Hodge test. A positive strain would develop a 'cloverleaf shaped' (figure 1) zone of inhibition due to carbapenemase production, while our strains showed negative results (undistorted zone of inhibition). These results suggested that the imipenem resistance is due to *oprD* malfunction rather than carbapenemase.

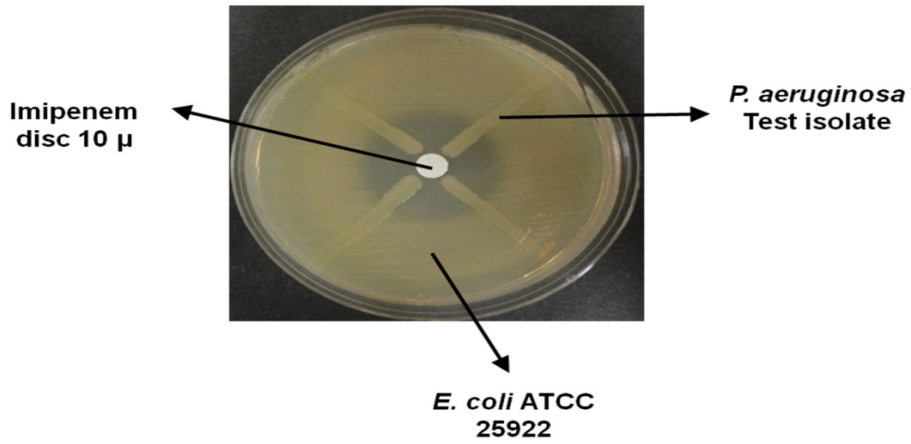

Figure 1: Modified Hodge test, the negative strain shows an undistorted zone of inhibition.

Carbapenems are not prone to inactivation by extended spectrum ß-lactamases and penetrate across the outer membrane of *P. aeruginosa* through a porin *OprD*, which allows selective penetration of basic amino acids, small peptides containing these amino acids, and carbapenems, their structural analogs. Prolonged treatment of *P. aeruginosa*-infected patients with imipenem has often allowed for the emergence of imipenem-resistant mutants. These resistant strains have either lost *OprD* or have strongly reduced *OprD* levels due to an nfxC type of quinolone-resistant mutation (mexT) which represses *oprD* expression and activates the mexEF-oprN multidrug efflux operon (Yoneyama and Nakae, 1993). Ochs *et al*. (1999) reported in their study that the possible mechanisms by which resistance to imipenem emerged in 17 imipenem-resistant *P. aeruginosa* clinical isolates, related to the loss of *OprD* was the predominant reason of imipenem resistance, *OprD* loss was caused by a chromosomal *oprD* mutation.

Analysis of *oprD* expression

The relationship between *oprD* expression and imipenem resistance were assessed in some imipenem resistant clinical strains. As it is illustrated in table 1, all resistant isolates showed low *oprD* expression values by comparison with the resistance isolates.

Table 1: *oprD* expression of *P. aeruginosa* isolates

Isolates	Imipenem susceptibility[a]	*oprD* expression values ± SD[b]
p1	Resistance	0.060 ± 0.034
P2		0.136 ± 0.007
P3		0.066 ± 0.003
P4		0.063 ± 0.028
P5		0.101 ± 0.033
P6		0.015 ± 0.004
P7	Sensitive	0.202 ± 0.094
P8		0.072 ± 0.007
P9		1.231 ± 0.227
P10	Intermediate	0.079 ± 0.014

[a]Sensitive (≤ 2 µg/ml), intermediate (4 µg/ml) or resistant (≥ 8 µg/ml).
[b]SD= standard deviation. P = 1.19×10^{-12}, LSD = 0.132

The results showed all the resistance isolates (P1, P2, P3, P4, P5, P6) have low *oprD* expression; whereas the sensitive isolates (P7, P9) developed high *oprD* expression. Interestingly, the low *oprD* expression that appeared in imipenem-susceptible (P8) and intermediate susceptible isolates (P10) indicated a correlation of other mechanisms. Less commonly, there is a mutation or deletions within *mexT* convert inactive MexT into an active form. Somehow, mutations occur in *mexS* located upstream of *mexT,* lead to accumulate various metabolites that serve as effectors molecules for MexT, which, in turn or in both cases, the expression of *mexEF-oprN* occurs at high level, alongside with a decline in the expression of *oprD* which is inadequate to elaborate quantities of *OprD* in the outer membrane sufficient for normal cellular function (Fukuda *et al*., 1995; Köhler *et al*., 1997).

Wolter *et al*. (2009) also demonstrated down-regulation in the production of the carbapenem channel *OprD* despite carbapenem hyper susceptibility. These isolates had decreased expression of the mexAB–oprM pump involved with intrinsic antibiotic resistance but over expressed the mexCD–oprJ and mexEF–oprN efflux systems normally associated with acquired resistance. Once again this might mean that there are other routes for carbapenems entrance.

We concluded that *oprD* expression correlated with imipenem resistance in these clinical isolates (r = 0.8). Loss of *oprD* is one of the most important mechanisms of resistance to imipenem in *P. aeruginosa*. Multiple studies have evaluated the importance of *oprD* mutation in clinical isolates of *P. aeruginosa* resistant to carbapenems. Always authors demonstrated a correlation between the levels of expression of *oprD* and the degrees of susceptibility to imipenem (Dib *et al*., 1995; Ocampo-Sosa *et al*., 2012; Lee *et al*., 2012). In this step, we aimed to gain an insight into the relationship between *oprD* expression and imipenem susceptibility profiles in imipenem-susceptible -intermediate and resistance clinical strains of *P. aeruginosa*. Our selection of these isolates was based on their imipenem susceptibility profiles, including organisms with a broad range of susceptibility: susceptible (MICs ≤ 2 µg/ml), intermediately susceptible (MIC = 4 µg/ml) or resistance (MICs ≥ 8 µg/ml) to imipenem.

In a conclusion, the results revealed a good correlation between *oprD* expression and imipenem resistance; however, there are other mechanisms that might be related to low *oprD* expression in sensitive isolates.

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