*IS*Pa46, a novel insertion sequence in OprD porin of imipenem-resistant *Pseudomonas aeruginosa* isolate from a Cystic Fibrosis patient in Marseille, France

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INTRODUCTION

❖ Chronic colonization and pulmonary exacerbations due to *Pseudomonas aeruginosa* are associated with lung destruction and increased mortality in patients with Cystic Fibrosis (CF) **[1]**. Carbapenems can be used to treat *P. aeruginosa* infections in CF patients but emergence of carbapenem-resistant *P. aeruginosa* in CF is increasingly reported, especially in adults.

❖ Resistance to carbapenems in *P. aeruginosa* could be due to low permeability, multidrug efflux, or production of class B metallo-β-lactamase (MBL) including bla_{VIM}, bla_{IPM}, bla_{GIM}, bla_{SPM} **[2]**. However, the main reported mechanism of resistance to carbapenems involves the loss of OprD porin protein through mutations, deletions or insertions in the *oprD* gene.

❖ **Here, we report a novel insertion sequence that disrupts the *oprD* gene of *P. aeruginosa* clinical isolates from a CF patient who not received carbapenem therapy that lead to a carbapenem-resistant phenotype**.

❖ To the best of our knowledge, such mechanism of resistance has been reported only once in a CF patient from Nebraska, United States **[3]**.

MATERIALS AND METHODS

❖ The ***P. aeruginosa* strain_02** imipenem-resistant characterized here was isolated in May 2011 from a sputum sample of a 20-year-old female CF patient (ΔF508/ΔF508 mutation), followed up in Giens, Marseille and Nice, from 2005 to 2012 who did not receive carbepenem therapy during this period. Indeed, the patient was treated several times with associations of several anti-pyocyanic antibiotic cures mainly composed of ciprofloxacin or ceftazidime associated to tobramycin or colimycin but never received carbapenems.

❖ Retrospectively, **five** *P. aeruginosa* strains isolated in 2008-2009 from the same patient were also investigated.

❖ Strains identification was confirmed using MALDI-TOF MS; antibiotic susceptibility testing was conducted by E-test method and detection of metallo-β-lactamase activities was performed using the modified imipenem-ethylenediaminetetraacetic acid (IMI-EDTA) double disc synergy test. The presence of class B metallo-β-lactamase genes was checked by standard PCR and *oprD* gene was amplified and sequenced to investigate the gene functionality. And finally, a Multi-Locus Sequence Typing (MLST) analysis based on seven housekeeping genes was performed to investigate the clonality of these isolates.

RESULTS

❖ Antibiotic susceptibility testing :

❑ The antibiotic susceptibility test of the **Strain_02** isolated in May 2011 revealed a high resistance phenotype to β-lactams drugs but susceptible to others antibiotics family (**Table 1**).

❑ The IMI-EDTA double disk synergy test was negative for **Strain_02** (**Fig. 1**) and for all remaining five strains suggesting therefore no carbapenemase activities in these isolates

❑ Moreover, PCR investigation targeting bla_{VIM}, bla_{IPM}, bla_{GIM}, bla_{SPM}, and bla_{NDM-1} carbapenemase genes were all negative, suggesting that the imipenem resistance in these isolates was not caused by carbapenemase enzymes.

Table 1: Antibiotic susceptibilty of *P. aeruginosa* **Strain_02**

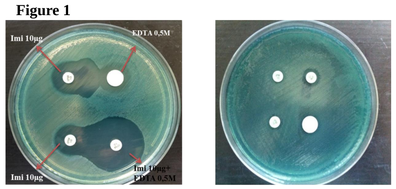
Antibiotics	MIC	Phenotype
Ticarcillin	> 32 µg/ml	Resistant
Ticarcillin/clavulanic acid	> 32 µg/ml	Resistant
Piperacillin/tazobactam	16 µg/ml	Resistant
Ceftazidim	> 8 µg/ml	Resistant
Cefepim	> 32 µg/ml	Resistant
Imipenem	32 µg/ml	Resistant
Meropenem	32 µg/ml	Resistant
Doripenem	128 µg/ml	Resistant
Rifampicin	16 µg/ml	Resistant
Gentamicin	< 4 µg/ml	Susceptible
Tobramycin	< 4 µg/ml	Susceptible
Ciprofloxacin	< 0.25 µg/ml	Susceptible
Fosfomycin	< 32 µg/ml	Susceptible
Colistin	< 1 µg/ml	Susceptible

❖ The investigation of the OprD porin functionality

❑ PCR amplification and sequencing of *oprD* performed on the strain isolated in May 2011 (**Strain_02**) gave PCR product of about 2.8-Kb instead of 1,468-bp as show with *P. aeruginosa* PAO1 strain (**Fig. 2**).

❑ Sequence analysis of the 2,805-bp reveals an insertion of a sequence of 1,337-bp of size at position 41 in *oprD* (**Fig. 3**). Within the sequence, an open-reading frame (ORF) of 1,227-bp was found and corresponded to an insertion sequence (IS) that had 92% identity with *IS*Pa1328 from *P. aeruginosa* (AY539833). This insertion sequence named *IS*Pa46, belongs to the *IS*256 family according to the ISfinder database nomenclature. Sequence comparison of oprD disrupted by IS in some *P. aeruginosa* is shown in **Figure 3.**

Figure 1



Positive control VIM-2 strain
Positive IMI-EDTA Synergy test

P. aeruginosa **strain_02**
Negative IMI-EDTA Synergy test

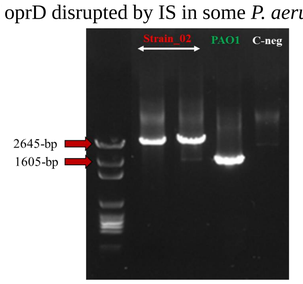


Figure 2: *oprD* gene PCR
Strain_02 : *P. aeruginosa* **imipenem-resistant**
PAO1 : *P. aeruginosa* **imipenem-sensitive**
C-neg : Control negative

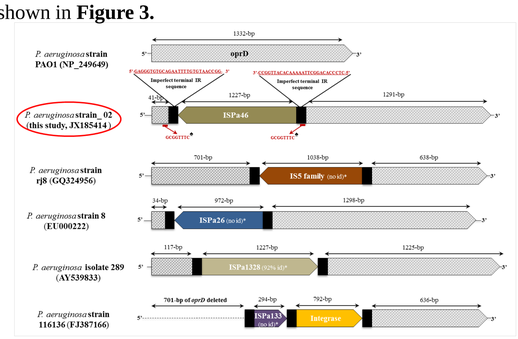


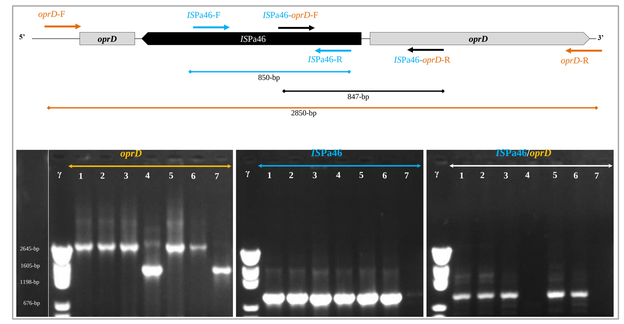
Figure 3: Sequence comparison of ***oprD* gene** of some *P. aeruginosa* strains

❖ Retrospective analysis of oprD gene of previously *P. aeruginosa* strains isolated from the same patient:

❑ Retrospectively, investigation of five *P. aeruginosa* strains isolated from the same patient prior to the isolation of the **Strain_02**, reveals that one of these five strains (**PA2242 strain**) was imipenem-susceptible with MIC=0.75µg/ml.

❑ PCR targeting the *oprD* gene was positive for this isolate (**PA2242 strain**) with an expected size of 1,468-bp (**Table 2 and Fig. 4**) whereas PCR products for the remaining four imipenem-resistant strains were of about 2.8-Kb like **Strain_02** (**Table 2 and Fig. 4**).

❑ The specific PCR targeting *IS*Pa46 was positive for all strains except **PAO1 strain**, suggesting therefore the presence of this insertion sequence *IS*Pa46 specifically in all strains isolated from this patient (**Fig. 4**).



γ: DNA ladder; **1:** PA414; **2:** PA684; **3:** PA1381; **4:** PA2242; **5:** PA2250; **6:** Strain_02; **7:** PAO1.
oprD-F/R: 5'-GGAACCTCAACTATCGCCAAG-3'/3'-GTTGCCTGTCGGTCGATTAC-5' (Wolter *et al.* 2004);
*IS*Pa46-F/R: 5'-TTTTGCTGATGTTGCGTAGC-3'/3'-CAGATCGTCGAGAAGCACCA-5'; and
*IS*Pa46-*oprD*-F/R: 5'-CAGGCCTTCACTTCATCG-3'/3'-GTCGTTCATCACTGGCAGGT-5' (this study).

Figure 4: Investigation of the insertion of ISPa46 in oprD gene in the five strains isolated in 2008-2009 from the same patient

❑ **Interestingly**, PCR checking the insertion of *IS*Pa46 in *oprD* was negative only for imipenem-susceptible **PA2242 strain** (**Fig. 4**). This result that *IS*Pa46 was present in the genome of these clinical isolates but transposed in *oprD* gene only for the imipenem-resistant isolates.

❑ Moreover, complete MLST analysis reveals that all strains from this patient are the same clone with sequence type ST540/551 (**Table 2**).

Table 2: Imipenem susceptibility, oprD PCR investigation and MLTS analysis of strains isolated from the same patient in 2008-2009

	Isolated in 2008-2009					Isolated in 2011	
Strains	PA414	PA684	PA1381	PA2242	PA2250	Strain_02	PAO1
Etest Imipenem	32 µg/ml	16 µg/ml	32 µg/ml	0.75 µg/ml	16µg/ml	32 µg/ml	0.125 µg/ml
OprD PCR	+ (2850-bp)	+ (2850-bp)	+ (2850-bp)	+ (1450-bp, normal size)	+ (2850-bp)	+ (2850-bp)	+ (1450-bp, normal size)
ISPa46 PCR	+	+	+	+	+	+	-
ISPa46/OprD PCR	+	+	+	-	+	+	-
Sequence type (ST)	540	540	540/692	540/551	540/551	540/692	PAO1

CONCLUSIONS

❖ To date, the presence of IS elements disrupting the *oprD* gene as a cause of carbapenem resistance in *P. aeruginosa* has been reported only in United States **[3]**; in South Africa **[4]**; in Spain **[5]** and in China **[6]**.

❖ Selection of carbapenem-resistant *P. aeruginosa* in the absence of carbapenem therapy in the present study is worrying and is an example of a collateral effect of the use of unrelated antibiotics that may select for multidrug resistance without direct and specific antibiotic selective pressure.

❖ Random transposition of IS elements is known to be a driver of adaptation of bacteria to environmental changes **[7]** especially it has been demonstrated that IS elements are a key factor for bacterial genome evolution and adaptation of *P. aeruginosa* isolates in patients with CF **[8].**

❖ In conclusion, we report a novel IS element, *IS*Pa46, in a *P. aeruginosa* clinical isolate of a CF patient in Marseille, France, that was associated with carbapenem resistance and selected in the absence of carbapenem treatment.

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