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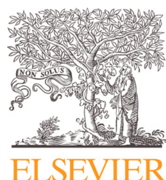

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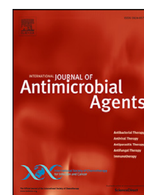

Phenotypic and genetic resistance traits of *Pseudomonas aeruginosa* strains infecting cystic fibrosis patients: A French cohort study

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ABSTRACT

Pseudomonas aeruginosa is responsible for chronic respiratory tract colonisation and acute exacerbations in cystic fibrosis (CF) patients. This Gram-negative bacterium often develops multidrug resistance, which represents a therapeutic challenge. The objective of this study was to characterise the phenotypic and genetic β-lactam resistance traits of *P. aeruginosa* strains isolated from CF patients at Grenoble Alpes University Hospital (Grenoble, France). The susceptibility to β-lactam compounds of 123 *P. aeruginosa* strains collected from the lower respiratory tract of 45 CF patients between 2010–2014 was evaluated. Genetic analyses focused on characterisation of the presence of carbapenemase- and extended-spectrum β-lactamases (ESBL)-encoding genes as well as alterations in the *oprD* gene encoding the OprD porin. Among the 123 *P. aeruginosa* strains evaluated, 25 were susceptible to both ceftazidime (CAZ) and imipenem (IPM), 9 only to IPM and 36 only to CAZ; 53 strains were resistant to both drugs. CAZ resistance could be reverted by cloxacillin in 29 strains, indicating overproduction of cephalosporinase. Genetic analyses performed for 79 *P. aeruginosa* strains revealed no ESBL- or carbapenemases-encoding genes. Among the 74 IPM-resistant strains, 42 (56.8%) displayed major alterations in the OprD protein sequence. This study shows that in this CF patient cohort, cephalosporinase overproduction and OprD alterations were the main resistance mechanisms of *P. aeruginosa* to CAZ and IPM, respectively. No genes coding for ESBLs or carbapenemases were detected, but monitoring of the emergence of such resistance genes in CF patients is warranted owing to their ability to rapidly spread by horizontal gene transfer.

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1. Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium of the aquatic environment and a major opportunistic human pathogen [1]. This organism is responsible for nosocomial infections, which may be life-threatening especially in immunosuppressed patients [1]. It is also the leading cause of morbidity and mortality in cystic fibrosis (CF) patients, causing chronic respiratory tract infections with acute exacerbations, which progressively impair respiratory function [1]. *Pseudomonas aeruginosa* is naturally resistant to many antibiotics [2] and can develop multidrug resistance, which represents a therapeutic challenge [3]. β-Lactam antibiotics active against wild-type strains of *P. aeruginosa* include carboxypenicillins (such as ticarcillin), ureidopenicillins (such as piperacillin), some broad-spectrum cephalosporins (including ceftazidime and cefepime), monobactams (aztreonam) and carbapenems (imipenem, meropenem and doripenem) [2]. Natural resistance to other β-lactam compounds is mainly caused by membrane impermeability, efflux systems and chromosomally-encoded β-lactamases such as the AmpC cephalosporinase encoded by *ampC* [2].

A large number of acquired resistance mechanisms to β-lactams have also been characterised in *P. aeruginosa*, including mutations leading to AmpC overproduction and extended-spectrum AmpC (inactivating broad-spectrum cephalosporins but also carbapenems), acquisition of genes encoding extended-spectrum β-lactamases (ESBLs) or carbapenemases, and overexpression of efflux systems [2,4–7]. Alteration in expression of the porin OprD, mainly caused by mutations or insertions in the encoding gene *oprD*, leads to selective resistance to carbapenems [8–11]. However, the combination of these different resistance mechanisms may lead to resistance to almost all β-lactam compounds [12].

The worldwide epidemiology of these resistance mechanisms is only partially known, especially the prevalence of *P. aeruginosa* strains producing carbapenemases, which may vary greatly

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Table 1
β-Lactam resistance phenotype for 123 *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients, and genetic alterations found for each category.

Phenotype	Susceptibility to:		No. of strains	CLO[a]	Genetic alterations[b]		
	CAZ	IPM			ESBL	CARB	*oprD*
CAZ^SIPM^S	S	S	25	NA	NA	NA	NA
CAZ^RIPM^S	R	S	9	4	0/5	NA	0/5
CAZ^SIPM^R	S	R	36	NA	0/4	0/5	20/31
CAZ^RIPM^R	R	R	53	25	0/12	0/35	22/43

CAZ, ceftazidime; IPM, imipenem; S, susceptible; R, resistant or intermediate-susceptible; NA, not applicable.
[a] Number of strains with reversion of CAZ resistance to susceptibility by cloxacillin.
[b] Extended-spectrum β-lactamase-encoding genes (ESBL), carbapenemase-encoding genes (CARB) and major alterations in the outer membrane porin D2-encoding gene *oprD* (no. of strains/no. of strains tested).

depending on the country and regions considered [13,14]. Moreover, *P. aeruginosa* strains isolated from the respiratory tract of CF patients are phenotypically different from those responsible for acute nosocomial infections and environmental strains [15,16]. Only a limited number of studies have focused on the epidemiology of β-lactam resistance mechanisms in *P. aeruginosa* strains infecting CF patients [4,13,17,18]. In the present study, the phenotypic and genetic β-lactam resistance traits of *P. aeruginosa* strains isolated from the respiratory tract of CF patients at Grenoble Alpes University Hospital (Grenoble, France) were characterised.

2. Materials and methods

2.1. Bacterial strains

A total 123 *P. aeruginosa* strains (Pae1 to Pae123) isolated between 2010–2014 from lower respiratory tract samples collected for routine diagnostic purposes in 45 CF patients followed at the 'Centre de Ressource et de Compétences de la Mucoviscidose' (CRCM) of Grenoble Alpes University Hospital were selected. Three *P. aeruginosa* strains previously characterised in our laboratory as carrying either the bla_{VIM-1} and bla_{OXA-2} genes, the bla_{VIM-2} and bla_{OXA-10} genes, or the bla_{IMP} gene were used as controls for PCR experiments. All of these *P. aeruginosa* strains were stored at −80 °C. Isolates were grown on tryptic soy agar plates (bioMérieux, Marcy-l'Étoile, France). Bacterial identification was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) using a microflex® system (Biotyper®; Brucker Daltonics, Wissembourg, France). A likelihood score of >2 for *P. aeruginosa* species was considered as a confirmed identification.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion assay (Kirby–Bauer technique) according to the 2015 European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [19,20]. A bacterial inoculum was prepared in sterile distilled water to obtain a turbidity of 0.5 MacFarland standard using a DensiCHEK® system (bioMérieux). For each strain, two antibiograms were performed: one using a standard Mueller–Hinton agar plate (bioMérieux); and another using a Mueller–Hinton agar plate containing 250 mg/L cloxacillin (bioMérieux) to inhibit the chromosomally-encoded cephalosporinase of *P. aeruginosa*. Twelve antibiotics were tested, including nine β-lactams [ticarcillin, piperacillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime (CAZ), cefepime, aztreonam, imipenem (IPM) and meropenem], an aminoglycoside (amikacin), a fluoroquinolone (ciprofloxacin) and fosfomycin. Diameters of the zone of inhibition were read after 24 h of incubation of the plates at 37 °C in an ambient atmosphere and were interpreted using the 2015 EUCAST susceptibility and resistance breakpoints.

Antimicrobial susceptibility testing results were used to sort the strains into six different phenotypic categories according to their susceptibility to the third-generation cephalosporin CAZ and the carbapenem IPM and the effect of cloxacillin on the CAZ resistance phenotype (Table 1). These categories were meant to guide the choice of the more appropriate genetic analyses to perform in order to identify the involved β-lactam resistance genetic determinants (see below).

2.3. Characterisation of genetic determinants of β-lactams resistance

DNA extraction from bacterial suspensions was performed using a QIAamp® DNA Mini Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. The concentration and quality (ratio of the absorbance at 260 nm and 280 nm) of DNA extracts were evaluated using a NanoDrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA). For PCR runs, a 15 ± 5 ng/µL DNA concentration was used by diluting DNA extracts in DNA-free water. PCR tests were designed to detect specifically ESBL- and carbapenemase-encoding genes as well as alterations in the *oprD* gene.

For strains resistant to CAZ but susceptible to IPM (i.e. CAZ^RIPM^S phenotype), but with reversion of CAZ resistance to susceptibility in the presence of cloxacillin, overexpression of the chromosomally-encoded cephalosporinase was considered the most likely resistance mechanism to β-lactams and no further genetic analysis was performed. For strains with the CAZ^RIPM^S phenotype with no reversion of CAZ resistance in the presence of cloxacillin, the presence primarily of the following ESBL-encoding genes was checked: bla_{VEB}; bla_{PER}; bla_{GES}; bla_{TEM}; bla_{SHV}; bla_{BEL}; bla_{KPC}; bla_{OXA-2}; bla_{OXA1-0}; and bla_{OXA-18}. Primers used for amplification and sequencing of the genes encoding ESBLs were as previously described [6,21]. For strains with the CAZ^SIPM^R phenotype, the entire DNA sequence of the o*prD* gene was determined using four PCR sequencing assays and previously described primers [22]. For strains with the CAZ^RIPM^R phenotype, the presence of carbapenemase-encoding genes (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{GIM}) was checked as well as the presence of ESBL-encoding genes and *oprD* gene alterations. Primers used for amplification and sequencing of the genes encoding carbapenemases were as previously described [21]. For bla_{IMP}, bla_{SPM} and bla_{GIM} genes, because of a lack of specificity of these primers, a second set of primers was also used [22]. The different primers used in this study are presented in Supplementary Table S1.

PCR was performed using standard procedures. The PCR mix included 12.5 µL of Premium Ex *Taq* HotStart (TAKARA Bio Inc., Shiga, Japan), 1.25 µL of each primer (10 µmol final concentration), 8 µL of DNA-free water and 2 µL of the bacterial DNA extract at 15 ± 5 ng/µL. The amplification protocol was as follows: DNA denaturation at 98 °C for 30 s; followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 60 °C for 30 s and DNA extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min.

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