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Pseudomonas aeruginosa chronic infections in patients with bronchiectasis: a silent reservoir of carbapenemase-producing epidemic high-risk clones

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Objectives: *Pseudomonas aeruginosa* is one of the major drivers of morbidity and mortality in patients with chronic underlying diseases. Whereas cystic fibrosis (CF) *P. aeruginosa* strains have been well studied, non-CF bronchiectasis isolates have received less scientific attention.

Methods: We determined the antibiotic susceptibility profiles of a collection of 100 *P. aeruginosa* isolates recovered from a total of 100 non-CF bronchiectasis patients attending a Catalonian hospital. All carbapenemase-producing isolates were characterized by WGS.

Results: Twelve isolates were classified as MDR (12%) and six were found to be carbapenemase (VIM-2) producers (6%). Of note, two of the VIM-2-producing isolates were carbapenem susceptible due to the presence of inactivating mutations in MexAB-OprM efflux pump components. These isolates exhibited properties of chronic *P. aeruginosa* isolates, such as mutator or mucoid phenotypes that are associated with persistent infections despite intensive antibiotic therapies. The phylogenetic analysis evidenced that all VIM-2 isolates belonged to the high-risk clone ST235. Core-genome MLST analysis revealed 7–260 allelic differences, arguing against recent transmission but a common source of infection or an ancient interpatient transmission event could not be ruled out.

Conclusions: Altogether, these findings suggest that *P. aeruginosa* chronic respiratory infections can be an important and silent reservoir of transferable resistance determinants and *P. aeruginosa* high-risk clones, thus contributing to their increased resistance and worldwide dissemination.

Introduction

Pseudomonas aeruginosa is a ubiquitous and opportunistic pathogen responsible for many acute hospital-acquired infections, particularly affecting immunocompromised and ICU patients.[1] Also, *P. aeruginosa* is a major cause of chronic respiratory infections (CRIs), leading to significant morbidity and mortality in individuals with chronic inflammatory airway diseases such as cystic fibrosis (CF), COPD, asthma and non-CF bronchiectasis.[2]

A defining characteristic of *P. aeruginosa* CRI is its long-term persistence despite aggressive antimicrobial therapy, with resistance to antibiotics arising from multiple factors. Key contributors to antibiotic resistance include the transition from the planktonic to the biofilm mode of growth and the acquisition of chromosomal antibiotic resistance mutations, triggered by the presence of mutator strains. Moreover, the acquisition of transmissible epidemic strains or transferable resistance genetic elements can also significantly contribute to antibiotic resistance.[3]

Compared with CF, *P. aeruginosa* CRI in patients with non-CF bronchiectasis has received much less scientific attention. Therefore, the aim of this study was to determine the antibiotic susceptibility profiles of a collection of 100 *P. aeruginosa* isolates

recovered from patients with non-CF bronchiectasis, assess the prevalence of carbapenemase-encoding genes and conduct an in-depth characterization of carbapenemase-producing strains.

Materials and methods

P. aeruginosa collection, antibiotic susceptibility testing and carbapenemase detection

The collection included 100 *P. aeruginosa* isolates recovered from respiratory samples of 100 different patients with non-CF bronchiectasis who attended consecutively the Hospital Universitari Arnau de Vilanova de Lleida (Catalonia, Spain) between September 2019 and May 2021. Different colony morphotypes were observed in some of the patients; however, just one colony morphotype per patient was randomly selected for further characterization. Twenty patients were hospitalized whereas 80 were outpatients.

MICs of piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin and colistin were determined by broth microdilution using the MicroScan WalkAway® system (NEG MIC 44 panels). EUCAST v14.0 clinical breakpoints were applied for interpretation of S/I/R categories and ECDC-established recommendations were used to define MDR profiles.[4]

In all MDR and/or meropenem-resistant isolates, a commercially available multiplex real-time PCR assay (Xpert Carba-R assay, Cepheid), which detects the bla_{VIM}, bla_{NDM}, *bla*KPC, bla_{IMP-1} and bla_{OXA-48} genes, as well as an in-house multiplex PCR that additionally detects *bla*GES genes,[5] was performed.

Characterization of carbapenemase-producing *P. aeruginosa* isolates

MICs of ceftolozane/tazobactam, ceftazidime/avibactam, imipenem/relebactam, meropenem/vaborbactam and cefiderocol were additionally determined for the carbapenemase-producing strains. Rifampicin (300 mg/L) resistance mutant frequencies were also determined following previously established procedures,[6] and mucoidity was visually investigated.

In addition, whole-genome sequences were obtained. Total genomic DNA was extracted using a commercially available kit (High Pure PCR template preparation kit; Roche Diagnostics) and indexed paired-end libraries were prepared (Illumina DNA Prep®, Illumina) and then sequenced on an Illumina MiSeq® using the MiSeq reagent kit v3 and 600 cycles. For the genomic characterization, paired-end reads were *de novo* assembled with SPAdes using default options in order to infer the ST, and for the detection of acquired resistance determinants (MLST v2.0.4, ResFinder v4.1.0; http://www.genomicepidemiology.org/services). A variant calling analysis using the PAO1 reference genome (NC_002516.2) was also performed to explore the mutational resistome and the genetic basis of mutator and mucoid phenotypes, as well as to explore the integrity of the quorum-sensing (QS) system regulator LasR.[7,8,9,10] For this purpose, reads were first aligned to the reference genome using Bowtie2 v2.2.4, and pileup and raw files were then obtained using SAMtools v0.1.16 and the Genome Analysis Toolkit GATK v3.4-46. SNPs were extracted from the raw files if they met the following criteria: a quality score of at least 50; a root-mean-square mapping quality of at least 25; and a coverage depth of at least 10 reads, excluding all ambiguous variants. SNPs and indels in a set of genes related to *P. aeruginosa* mutational resistance ($n=40$), hypermutation ($n=15$) and mucoidity (*mucA*/*mucB*) were eventually extracted and annotated using the SnpEff v4.2 software.

Finally, to infer the genetic relatedness and possible transmission events among patients, a core SNP-based maximum-likelihood tree was constructed using Parsnp v1.2 from the Harvest Suite package, forcing the inclusion (-c) of all genomes. Core-genome MLST (cgMLST) was also performed using the open-source algorithm chewBBACA, and allele distances were calculated.[11] To perform the cgMLST, six additional genomes from isolates belonging to the same ST and recovered from other Spanish hospitals were included.[5]

Results

Antibiotic resistance rates are shown in Table 1. Higher resistance rates were documented for ciprofloxacin and levofloxacin, whereas colistin, amikacin and the carbapenems imipenem and meropenem were the most active compounds.

A total of 12 isolates were classified as MDR, 6 of them being susceptible to meropenem. Six of the MDR isolates were positive for VIM carbapenemase production in both PCR assays used, but only four of them were resistant to meropenem.

All VIM-producing isolates were determined to belong to the high-risk clone ST235. By WGS, a bla_{VIM-2} gene within the same class 1 integron was detected: *intI1-*bla_{VIM-2}*-aac(6′)-IIa-catB3-aadA1-qacE-sul1*. Furthermore, all VIM-2-producing isolates presented a common set of chromosomic mutations: an amino acid substitution in the major regulator of the MexXY efflux pump system (*mexZ*-L109F), mutations in the QRDRs of DNA gyrase subunit A (*gyrA*-T83I) and in the DNA topoisomerase *parC* (S87L), and a mutation likely involved in the overexpression of the chromosomally encoded cephalosporinase AmpC (*ampD*-Y127H) (Figure 1).

All isolates were phenotypically resistant to ciprofloxacin, tobramycin, piperacillin/tazobactam, ceftolozane/tazobactam and imipenem/relebactam, remaining susceptible to cefiderocol,

Table 1. Antimicrobial susceptibility of the *P. aeruginosa* bronchiectasis collection

Antibiotic	S (%)	I (%)	R (%)
TZP	—	87	13
CAZ	—	90	10
FEP	—	87	13
IPM	—	94	6
MEM	93	1	6
TOB	86	—	14
AMK	95	—	5
CIP	—	70	30
LVX	—	68	32
CST	98	—	1

S, susceptible; I, susceptible, increased exposure; R, resistant; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; CST, colistin.

MIC (mg/L)															WGS resistome
Isolate ID	FEP	TZP	CAZ	ATM	IPM	MEM	C/T	CZA	IMR	MEV	FDC	CIP	TOB	AMK	CST	
BQ01	>16	>32	>16	>32	>8	>16	>8	>16	>8	>16	≤0.06	>1	>4	≤2	≤0.5	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H)
BQ05	16	>32	>16	2	4	2	>8	2	4	4	≤0.06	1	>4	4	≤0.5	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H), ***mexA* (R3X)**
BQ06	8	>32	4	2	4	1	>8	1	4	2	≤0.06	1	>4	4	1	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H), ***mexB* (Q575X)**
BQ03	>16	>32	>16	>32	>8	>16	>8	>16	>8	>16	0.5	>1	>4	16	1	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H)
BQ02	>16	>32	>16	>32	>8	16	>8	>16	>8	16	0.5	>1	>4	8	≤0.5	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H)
BQ04	16	>32	>16	1	>8	>16	>8	16	>8	>16	0.12	>1	>4	16	2	VIM-2, *aac(6')-IIa*, *aadA1*, *mexZ* (L109F), *gyrA* (T83I), *parC* (S87L), *ampD* (Y127H), ***mexB* (G217S)**, ***mexX* (nt1159InsC)**, ***mexY* (G270S)**, ***parS* (A252T)**

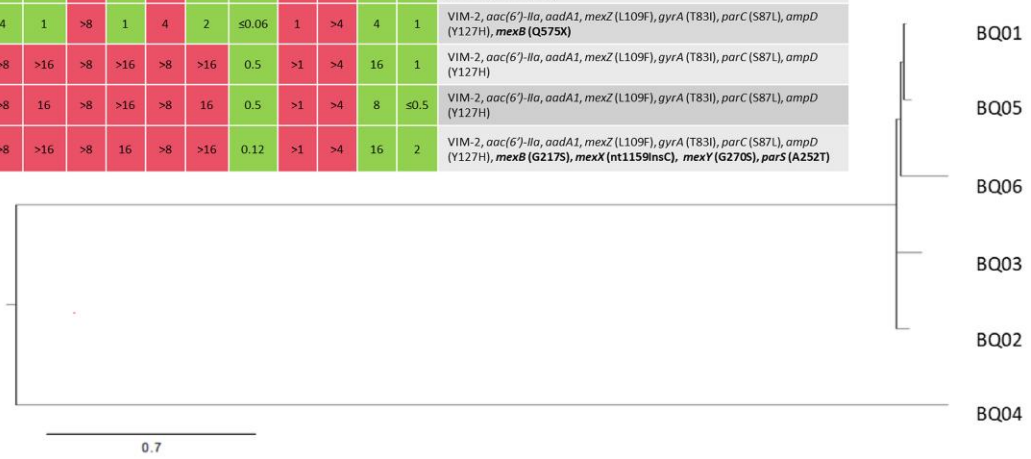

Figure 1. Antibiotic susceptibility profiles, isolate-specific resistome mutations and genetic relatedness of the VIM-2-producing isolates. FEP, cefepime (S ≤ 0.001; R > 8); TZP, piperacillin/tazobactam (S ≤ 0.001; R > 16); CAZ, ceftazidime (S ≤ 0.001; R > 8); ATM, aztreonam (S ≤ 0.001; R > 16); IPM, imipenem (S ≤ 0.001; R > 4); MEM, meropenem (S ≤ 2; R > 8); C/T, ceftolozane/tazobactam (S ≤ 4; R > 4); CZA, ceftazidime/avibactam (S ≤ 8; R > 8); IMR, imipenem/relebactam (S ≤ 2; R > 2); MEV, meropenem/vaborbactam (S ≤ 8; R > 8); FDC, cefiderocol (S ≤ 2; R > 2); CIP, ciprofloxacin (S ≤ 0.001; R > 0.5); TOB, tobramycin (S ≤ 2; R > 2); AMK, amikacin (S ≤ 16; R > 16); CST, colistin (S ≤ 4; R > 4). Green colour indicates clinical susceptibility at standard and increased dosing, while red indicates resistance.

amikacin and colistin. The presence of *gyrA* and *parC* gain-of-function mutations correlated with the documented resistance to ciprofloxacin,[12] while tobramycin resistance was explained by the acquired aminoglycoside acetyltransferase *aac(6′)-IIa*,[13] along with the up-regulation of the MexXY efflux pump system.[12] By contrast, the presence of the MBL VIM-2, along with the overexpression of the intrinsic cephalosporinase AmpC, should confer resistance to all β-lactams including the novel β-lactam/β-lactamase inhibitors (BL/BLIs).[12,14] However, BQ05 and BQ06 isolates were determined to be susceptible to both carbapenems as well as to ceftazidime/avibactam, meropenem/vaborbactam and aztreonam (Figure 1). These isolates harboured truncating mutations in either the MexA or MexB components of the MexAB-OprM efflux pump system, which plays a major role in the basal resistance level to most β-lactams, including the novel BL-BLI combinations and, thus explaining their susceptibility profiles. Indeed MexAB-OprM overexpression plays a significant role in resistance to ceftazidime/avibactam, imipenem/relebactam and meropenem/vaborbactam, because of the ability of this system to extrude both β-lactams and β-lactamase inhibitors.[12,15,16,17]

One of the most common characteristics of *P. aeruginosa* CRIs is the high frequency of hypermutable strains, which play a crucial role as drivers of antibiotic resistance development.[6] Of note, isolate BQ04 showed a higher number of resistome mutations compared with all other VIM-2-producing isolates (Figure 1) and was the only isolate that exhibited a mutator phenotype, being an inactivating mutation in *mutS* (nt637Δ23) the genetic cause of hypermutation.

Along with the mutator phenotype, the conversion to a mucoid phenotype, which leads to an increased tolerance and resistance against antibiotics, is another hallmark of *P. aeruginosa* CRIs. MucA and MucB are critical negative modulators of the sigma factor AlgU and regulate the mucoid conversion of *P. aeruginosa*.[10] Isolate BQ05 exhibited inactivating mutations in *mucA* (V147X), and BQ06 in *mucB* (W118X); however, just isolate BQ06 presented a mucoid phenotype.

Finally, another common trait of *P. aeruginosa* CRI is the loss of the QS system regulation, which promotes biofilm formation. Notably, inactivating mutations in the QS regulator LasR were detected in four of these carbapenemase-producing isolates: BQ05 (W187X), BQ06 (Q45X) and BQ02/BQ04 (nt93Δ8).

Finally, since all patients except one had been hospitalized during the VIM-2-producing *P. aeruginosa* episode, the genetic relatedness among these isolates was studied. The core SNP-based maximum-likelihood tree revealed that isolates were closely related, displaying that the mutator BQ04 the highest genetic divergence among them (Figure 1). For the cgMLST analysis, six additional ST235 *P. aeruginosa* isolates recovered from six different Spanish hospitals were included (BAL03-041, CAT09-001, CLE02-004, GAL04-002, GAL05-002 and PVA01-035).[5] Across all isolates, the median allele difference was 128.5 (range 7–326) whereas within the BQ isolates this median was 46 (range 7–260), and even lower when excluding the mutator isolate BQ04 (26, range 7–46). Thus, while all these results argue against a recent event of direct patient transmission, a common source of infection or an ancient transmission event could not be excluded.

Discussion

In recent years, MDR and carbapenem-resistant *P. aeruginosa* infections have significantly increased worldwide, limiting

therapeutic options. The dissemination of epidemic high-risk clones, such as ST235, further exacerbates this threat, particularly in nosocomial settings where the antibiotic selection pressure can contribute to their persistence.[18,19,20]

A VIM-2-producing ST235 clone was found to be the cause of CRIs in six of the patients with non-CF bronchiectasis, a finding that is an alert that these long-term respiratory infections could act as a reservoir for the dissemination of transferable resistance determinants and high-risk clones. In fact, previous work has already pointed out that COPD and bronchiectasis patients with CRI by mucoid MBL-producing *P. aeruginosa* strains could act as a reservoir as these strains are extremely difficult to eradicate despite intensive antimicrobial therapies.[21,22] More worrisome, in this work, we found that two of the MBL isolates did not exhibit resistance to carbapenems due to the presence of truncating mutations in components of the MexAB-OprM efflux pump, a system that is frequently impaired in chronic *P. aeruginosa* isolates, a circumstance that makes their suspicion and detection difficult.[23,24]

Altogether, this work is an alert that chronic *P. aeruginosa* isolates can be an important and silent reservoir of undetected transferable resistance determinants and *P. aeruginosa* high-risk clones, contributing to their worldwide dissemination.

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Transparency declarations

The authors declare no conflicts of interest.

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