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Antibiotic resistance and population structure of cystic fibrosis *Pseudomonas aeruginosa* isolates from a Spanish multi-centre study

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ABSTRACT

The first Spanish multi-centre study on the microbiology of cystic fibrosis (CF) was conducted from 2013 to 2014. The study involved 24 CF units from 17 hospitals, and recruited 341 patients. The aim of this study was to characterise *Pseudomonas aeruginosa* isolates, 79 of which were recovered from 75 (22%) patients. The study determined the population structure, antibiotic susceptibility profile and genetic background of the strains. Fifty-five percent of the isolates were multi-drug-resistant, and 16% were extensively-drug-resistant. Defective *mut*S and *mut*L genes were observed in mutator isolates (15.2%). Considerable genetic diversity was observed by pulsed-field gel electrophoresis (70 patterns) and multi-locus sequence typing (72 sequence types). International epidemic clones were not detected. Fifty-one new and 14 previously described array tube (AT) genotypes were detected by AT technology. This study found a genetically unrelated and highly diverse CF *P. aeruginosa* population in Spain, not represented by the epidemic clones widely distributed across Europe, with multiple combinations of virulence factors and high antimicrobial resistance rates (except for colistin).

1. Introduction

The lower respiratory tract of patients with cystic fibrosis (CF) is usually chronically colonised by a complex microbial ecosystem. This colonisation triggers an inflammatory response that produces respiratory symptoms and acute exacerbations, and influences the patients' clinical course and outcome. *Pseudomonas aeruginosa* is the most relevant micro-organism in this process. During the first stages of the disease, CF *P. aeruginosa* isolates are almost identical to environmental isolates. The evolved disease is characterised by mucoid colonies and/or multi-drug-resistant isolates [1] that result from the particular CF lung environment, a compartmentalised hostile niche for *P. aeruginosa* that forces the bacteria to an ecological adaptation [2], and frequent mutator phenotypes [3].

Previous epidemiological studies on CF *P. aeruginosa* isolates have been performed using different molecular typing tools. For instance, the use of multi-locus sequence typing (MLST) has allowed the identification of international epidemic CF clones, such as the well-known Liverpool epidemic strain or Clone C. Moreover, several hypertransmissible CF *P. aeruginosa* strains have been described [4], the detection of which should alert clinicians to prevent transmission between patients, including siblings [5] and patients from the same or different centres [6]. By using the array tube (AT) multi-marker array, some genotypes have been found to be most abundant in the global *P. aeruginosa* population, particularly AT genotypes 0C2E, 2C22, C40A, D421 and F429 that have been detected in both clinical and environmental isolates [7–13].

In Spain, the genetic background of *P. aeruginosa* isolates obtained from two different CF units has been reported previously [14,15], with ST274 and ST395 identified as endemic clones at each centre. This study, the first Spanish multi-centre study on the microbiology of CF, was conducted from 2013 to 2014, and included a representative patient population from across Spain [16]. The aim of this study was to characterise *P. aeruginosa* isolates to complete the microbiological description of this micro-organism in patients with CF in Spain.

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2. Materials and methods

2.1. Study design

This study involved 24 CF units (12 paediatric and 12 adult) from 17 hospitals [16]. Fifteen consecutive unselected patients per CF unit were recruited, and a single sputum sample from each patient was frozen immediately after collection at –80°C. The frozen samples were sent to Ramón y Cajal University Hospital and, after slow defrosting, were seeded in plates in the appropriate culture medium (see details in [17]). The plates were examined at 24 and 48 h, and the incubation time was extended to 5 days in order to identify potentially slow-growing bacteria. Colonies with compatible *P. aeruginosa* morphology were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany) and stored for further studies.

2.2. Antibiotic susceptibility

Antibiotic susceptibility was determined by disk diffusion, except for fosfomycin, for which the agar dilution method was performed [17]. The tested compounds included piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, colistin, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin and fosfomycin. The European Committee on Antimicrobial Susceptibility Testing clinical breakpoints for systemic infections were applied (www.eucast.org), except for fosfomycin. PAO1 and ATCC 27853 *P. aeruginosa* reference strains were used as controls. Consensus recommendations [18] were used to evaluate the proportion of multi-drug-resistant (MDR, not susceptible to at least three antimicrobial classes), extensively-drug-resistant (XDR, only susceptible to one or two antimicrobial classes) and pandrug-resistant (PDR; not susceptible to any antibiotics) strains, considering the following seven antimicrobial classes: cephalosporins (ceftazidime and/or cefepime), penicillin-β-lactamase inhibitor combinations (piperacillin-tazobactam), monobactams (aztreonam), carbapenems (imipenem and/or meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, tobramycin, and/or amikacin) and colistin. For percentages of pseudomonas colonisation and antibiotic resistance, 95% confidence intervals (CI) were calculated using the Exact formula.

2.3. Mutant frequencies and genetic basis for hypermutation

Mutant frequencies for rifampicin (300 mg/L) resistance were determined in triplicate for all strains following previously established procedures [3]. To explore the genetic basis of mutator isolates, previously described primers and protocols were employed to amplify and sequence the *mut*S and *mut*L genes [19]. Briefly, plasmid pUCPMS harbouring PAO1 wild-type *mut*S, plasmid pUCPML harbouring PAO1 wild-type *mut*L, and plasmid pUCP24, a control-cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased mutant frequencies for rifampicin resistance in two independent transformant colonies for each mutator isolate. Previously described primers and protocols [19] were used for the amplification and sequencing of *mut*S or *mut*L genes according to the results of complementation experiments.

2.4. Population structure

The genetic diversity of the isolates was explored initially by pulsed-field gel electrophoresis (PFGE) with the macrorestriction enzyme *Spe*I [20]. DNA separation was performed using a contour-clamped homogeneous electric field DRIII apparatus (Bio-Rad, La Jolla, CA, USA) under the following conditions: 6 V/cm^2 for 22 h with pulse times of 5–40 s. Finally, DNA macrorestriction patterns were interpreted according to visual criteria, and after constructing a dendrogram using the Dice coefficient and Phoretix 5.0 software.

All isolates were further genotyped by MLST (http://pubmlst.org/paeruginosa/) using available protocols and databases. MEGA6 software enabled phylogenetic analysis of the MLST alleles and their concatenate sequence. A minimum spanning tree was constructed using the goeBURST algorithm (www.phyloviz.net).

2.5. P. aeruginosa *AT genotyping*

The Clondiag (Alere Technologies GmbH, Jena, Germany) AT species-specific genotyping system was employed according to the manufacturer's protocol [13]. This species-specific micro-array enables the genotyping of *P. aeruginosa* strains using 13 informative single nucleotide polymorphisms at conserved loci, the *fli*Ca/*fli*Cb multi-allelic locus, and the presence or absence of the *exoS*/*exoU* marker gene. The AT system also includes 38 genetic markers from the accessory genome for defining intraclonal diversity.

2.6. exoS and exoU amplification assays

Polymerase chain reaction (PCR) assays for detecting *exoS* and *exoU* genes were performed using previously described primers and protocols [21], with slight modifications. PCR reactions were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a DNA thermal cycler (Arktic Thermal Cycler; Thermo Fisher, Waltham, MA, USA) under the following conditions: denaturation for 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s, and a final extension step of 10 min at 72 °C.

3. Results

3.1. Patients, samples and isolates

From a total of 341 respiratory samples (one per patient), 79 *P. aeruginosa* isolates were recovered from 75 patients (four patients presented colonies with two different morphologies). The global colonisation rate was 22% (95% CI 17–26), and was higher in the adult population (32.7%, 95% CI 25–40) than in the paediatric population (9.9%, 95% CI 5–15) ($P < 0.001$). *P. aeruginosa* colonisation status was defined as intermittent (one patient, 6%) or chronic (15 patients, 94%) for the paediatric population; the corresponding numbers were 10 patients (17%) and 49 patients (83%) for the adult population. The primary characteristics of the patients with CF are shown in Table 1. Clinical data from the entire CF population can be found elsewhere [16].

The classical CF mucoid morphotype was observed in 17 (21.5%, 95% CI 13–32) isolates, whereas the others were classified as metallic (23 isolates, 29.2%, 95% CI 19–40) or Enterobacteriaceae-like (23 isolates, 29.2%, 95% CI 19–40). Sixteen isolates (20%, 95% CI 12–30) presented small colony variant (SCV) morphology. Half of the isolates exhibited brown (16 isolates, 20.2%) or green (25 isolates, 31.6%) pigmentation after 48 h of incubation at 37 °C. In 36 (48%, 95% CI 34–57) of the 75 patients, co-existence of *P. aeruginosa* and *Staphylococcus aureus* was detected by classical culture in agar plates, with seven (9.3%) of the isolates resistant to methicillin.

3.2. Antibiotic susceptibility profiles

Overall (intermediate plus resistant) antibiotic resistance rates are shown in Table 2. Colistin was the most active compound, and only three isolates (4%) were classified as resistant to colistin. Considering co-resistances and excluding aztreonam, 15 isolates