Clinical and microbiological features of a cystic fibrosis patient chronically colonized with *Pandoraea sputorum* identified combining 16S rRNA sequencing and MALDI-TOF mass spectrometry

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Running title: *Pandoraea sputorum* chronic colonization in CF

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Abstract

Clonal isolates identified as various non-fermentative Gram-negative bacilli over a 5-year period from sputum cultures of a 30-year old cystic fibrosis patient were successfully re-identified as *Pandoraea sputorum* by combining 16S rRNA sequencing and MALDI-TOF MS. A decreased lung function improved after one-year of azithromycin and inhaled 7%-hypertonic saline treatment.

Key words: *Pandoraea sputorum*, MALDI-TOF MS, cystic fibrosis
CASE REPORT

A 30-year-old female cystic fibrosis (CF) patient (genotype F508del/D614G) with pancreatic sufficiency was transferred to our CF-Unit with a history of chronic bronchopulmonary colonization with *Pseudomonas aeruginosa* and *Candida albicans*. When first microbiological cultures were performed in our laboratory in October 2006, *Pandoraea sputorum* was detected but initially reported as non fermentative Gram-negative bacilli (NFGNB). At this time, her pulmonary function test revealed a forced vital capacity (FVC) of 2.87 L (85% predicted) and a forced expiratory volume in 1 second (FEV₁) of 2.22 L (75%). *P. sputorum* grew in twelve samples from October 2006 until May 2011. During these years she was treated with aerosolized colistin (2 MU twice daily with PARI Turbo-Boy compressor with a PARI LC-PLUS jet nebulizer, Pari Gräfelfing, Germany) and recombinant human DNase (2.5 mg daily). Until January 2008 she did not have any exacerbation and her pulmonary function remained stable. Along the next two years, her clinical condition worsened and she presented eleven exacerbations requiring 22 weeks of oral antibiotics, including trimethoprim-sulfamethoxazole (160/800 twice daily), and two weeks of intravenous antibiotics, including piperacillin-tazobactam (4/0.5 g every 8h). As a result of this, her lung function declined. In January 2010, FVC and FEV1 were 2.23 L (67% predicted) and 1.94 L (67% predicted), respectively, and oral azithromycin (500 mg every 48h) and inhaled 7% hypertonic saline with 0.1% hyaluronic acid (Hyaneb®, Praxis Pharmaceutical, Madrid, Spain) was initiated twice daily using an electronic vibrating mesh nebulizer (Pari eFlow rapid®, Pari). Ten months later, her clinical condition improved with a significant reduction in 24h sputum volume and in purulence and cough. During this time, the patient had only two pulmonary exacerbations and required 5 weeks of antibiotics (oral ciprofloxacin, 750 mg twice daily, and imipenem I.V., 500
mg every 8h). Her FVC and FEV1 increased up to 2.40 L (71% predicted) and 1.88 L (63% predicted), respectively.

Laboratory records indicated that sputum cultures during routine follow-up visits and/or patient’s exacerbations until 2009 yielded different NFGNB. Conventional identification of purple colonies recovered on Burkholderia cepacia complex selective agar plates (BCSA plates, BioMérieux, Marcy-L’Étoile, France) was performed using two phenotypic tests including the API 20NE gallery (BioMérieux) and WIDER semiautomatic system (Fco. Soria-Melguizo, Madrid, Spain). The API 20NE offered no identification (API code 0000443) and results from the WIDER system were inconsistent and isolates were reported as NFGNB (n=4), Achromobacter xylosoxidans (n=3) and P. aeruginosa (n=1). Afterwards, reassessment of bacterial identification was performed with a Matrix Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) method, using the Microflex LT mass spectrometer (Bruker Daltonics GmbH, Leipzig, Germany) with the FlexControl 3.0 and the MALDI BioTyper 2.0 and 3.0 software.

All of these isolates (n=8) and isolates subsequently obtained from sputum samples (n=4) (named, from this point, as CF-Pandoraea) recovered until May 2011 were initially identified by MALDI-TOF MS as P. pnomenusa, but with values indicating “secure genus identification, probable species identification” [i.e. a degree of similarity was found between the reference P. pnomenusa LMG 18817 HAM from the data base and the CF-Pandoraea isolates’ MALDI-TOF MS spectra]. A subsequently full characterization through PCR and sequencing of 16S rRNA genes were performed using the corresponding specific primers previously published (7, 14). Isolates were identified as Pandoraea sputorum in all of the cases. The length of the amplicon used for sequencing was ca. 1,500 bp, i.e. the complete gene, and corresponding percent
sequence homology with *P. sputorum* (LMG 18819 type strain) was 99% (accession no. AF139176). Consequently, the MALDI Biotyper 2.0 database (containing spectra of 3995 microorganism) was enlarged in our laboratory to include spectra for the initial *P. sputorum* isolate and that from *P. sputorum* LMG 18819 reference type strain (BCCM/ LMG bacteria collection, Gent, Belgium). All successive *Pandoraea* isolates recovered from our patient were identical and identified as *P. sputorum*. Hierarchical cluster analysis based on MALDI-TOF MS spectra comparison using updated MALDI Biotyper 3.0 software showed separate branches for *P. pnomenusa* and *P. sputorum*. Moreover our *P. sputorum* were clustered in the same branch as reference type strains (Fig. 1).

In addition, genetic relatedness was performed by *XbaI*-pulsed-field gel electrophoresis (PFGE) analysis (13) revealing indistinguishable patterns among all isolates (Fig. 2). Susceptibility patterns (standard microdilution, CLSI) were also almost identical for all isolates. In the absence of interpretive susceptibility criteria for *Pandoraea*, results were interpreted using CLSI criteria for “other non-Enterobacteriaceae” isolates (5). They were resistant to amoxicillin, amoxicillin/clavulanate, cefotaxime, ceftazidime, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, colistin and azithromycin and were susceptible to piperacillin/tazobactam (MIC ≤16/4 mg/L), imipenem (MIC 4 mg/L) and trimethoprim-sulfamethoxazole (MIC 2/18 mg/L).

*Pandoraea* species are considered as emerging pathogen in CF (2, 9). Nevertheless, partially due to the difficulties in their recognition in routine bacteriological cultures,
data from the clinical course and outcome in patients colonized/infected with this organism are still scarce. The genus *Pandoraea* was created in 2000 to accommodate organisms from *Pseudomonas* rRNA-homology group II. Five species were described, including *Pandoraea apista*, *Pandoraea norimbergensis*, *Pandoraea pulmonicola*, *P. sputorum* and *P. pnomenusa*. They are motile, aerobic, non-fermentative Gram-negative rods able to grow on BCSA plates. When applying conventional identification phenotypic methods, the microbiology laboratory commonly misidentifies this pathogen as *Ralstonia* or *Burkholderia* species, the two genera that are phylogenetically most closely related to *Pandoraea* spp. (6, 11, 14). In CF, correct identification of the different pathogens is extremely important for the clinical management of the patient colonized by these organisms which might exhibit distinct degrees of pathogenicity (4, 8).

*Pandoraea* has been isolated from environmental samples and occasionally recovered, but at an increasing rate, from the respiratory tract of CF-patients and is considered an emerging opportunistic pathogen. Also, it has recovered from blood cultures from non-cystic fibrosis patients (8, 9, 13). Within this genus, *P. apista* has been isolated from the lungs and blood cultures of CF-patients, supporting, unlike *P. aeruginosa*, an invasive role for this pathogen in this disease (12). In addition, chronic bronchopulmonary infection with a single clone of *P. apista* has been associated with decreased lung function (13) and different *P. apista* strains have been implicated in the deterioration of lung function, although it is unclear whether this deterioration could be directly attributed to this organism (1). Interestingly, *P. sputorum* was persistently recovered from respiratory secretions of a CF-patient under consideration for lung transplantation (14). Furthermore, cross-infection with *Pandoraea* spp. has been reported in winter-camps and/or hospitals (13). More recently, among the Irish CF-

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population, *P. pulmonicola* was the most predominantly species identified but, as all
Pandoraea-colonized patients were co-colonized with other CF-pathogens it was
difficult to establish clinical correlations between microorganisms and severity of
pathogenic colonization (8).

Here we report the chronic bronchopulmonary colonization with a *P. sputorum*
iso late that was successfully identified by combining a molecular technique based on
16S rRNA PCR and sequencing and a mass-spectrometry method. This organism,
previously identified in respiratory secretions of CF-patients (6, 14), has demonstrated
pathogenicity in an *in vitro* model with lung cells (4). *P. sputorum* isolates triggered a
pronounced pro-inflammatory response, with elevation of interleukin (IL)-8 similar to
other Pandoraea species and well-established CF-pathogens such as *P. aeruginosa* and
Bcc (4). A lung function decline with frequent exacerbations was observed in our
patient. Azithromycin and inhaled 7%-hypertonic saline were used and patient lung
function improved with no further exacerbations. As in chronically colonized patients
with *P. aeruginosa* the potential role of azithromycin requires further investigation that
might interfere with biofilm formation and exert an immunomodulatory effect.

As it is known that different Pandoraea species are capable of causing infections
in CF-subjects, the correct identification of these organisms is important. Pandoraea
species, including *P. sputorum* have never been identified by conventional methods in
our laboratory, even when using a widely accepted phenotypic method such as the API
20NE gallery (3, 10). This fact has been reinforced in a recent quality assurance trial of
CF-microbiology in which a *P. pnomenusa* strain was not even detected or was
misclassified by many laboratories (11). In addition, not all the 16S rRNA PCR and
sequencing schemes are able to correctly identify all the Pandoraea species (14). One
factor limiting the use of MALDI-TOF MS is the scant reference data sets for
microorganisms that are infrequently isolated from clinical specimens. Nevertheless, the possibility of updating the database by enlarging it with new bacterial species is valuable (2). In our CF-patient, all isolates were accurately identified as *P. sputorum* combining 16S rRNA PCR and sequencing and the MALDI-TOF MS proteomic platform after the extension of its reference database.

Finally, to determine persistence over time of the same *P. sputorum* isolate as generally occurs with *P. aeruginosa* in CF-patients, a molecular epidemiological analysis of *P. sputorum* isolates was performed. The PFGE showed indistinguishable patterns among all isolates indicating chronic colonization with a single clone rather than repeated infections (Fig. 3).

In conclusion, the presence of *Pandoraea* spp. can be underestimated in CF-patients when using conventional identification methods. MALDI-TOF MS appears to be a promising mean for the accurate identification of organisms of this genus which may have a potential role in pulmonary function deterioration when chronically colonizing CF-patients.

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REFERENCES


FIG. 1. Cluster analysis from MALDI-TOF MS spectra of Pandoraea isolates including seven reference strains (MALDI Biotyper 3.0 database), one *P. sputorum* LMG 18819 reference type strain and one clinical isolate (*Pandoraea sputorum* CF). Distance is displayed in relative units.
FIG. 2. PFGE of XbaI-digested genomic DNA of 8 selected clinical P. sputorum isolates recovered from 2006 to 2011. Lane M, bacteriophage lamda ladder PFGE marker (New England Biolabs)