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Short communication

Susceptibility to five antifungals of *Aspergillus fumigatus* strains isolated from chronically colonised cystic fibrosis patients receiving azole therapy

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ABSTRACT

Exposure of *Aspergillus fumigatus* to stressful antifungal therapies may result in decreased susceptibility. The aim of the present work was to evaluate the susceptibility to azole and non-azole antifungals of 159 isolates of *A. fumigatus* collected from cystic fibrosis (CF) patients receiving azole antifungal therapy. The genetic diversity of the fungal isolates was assessed using microsatellite genotyping, and some strains were found in patient's sputum samples more than 4 years apart. No resistant isolates [minimal inhibitory concentration (MIC)/minimal effective concentration (MEC) ≥4 μg/mL] were identified to the antifungals amphotericin B, caspofungin, itraconazole and voriconazole. A single *A. fumigatus* isolate was identified outside of the epidemiological cut-off of 0.25 μg/mL for posaconazole. Susceptibility of the recurrent isolates was in agreement with the susceptibility of the first isolate identified (100% essential agreement). Even after azole exposure, several recurrent *A. fumigatus* strains were detected in the subsequent sputum samples. Development of resistance in *A. fumigatus* to antifungals appears to be rare amongst CF patients. However, it remains crucial to evaluate the importance of antifungal agents for allergic fungal diseases.

1. Introduction

Cystic fibrosis (CF) patients are continuously exposed to microorganisms that may colonise their lungs, reducing respiratory function and enhancing pulmonary problems. Allergic bronchopulmonary aspergillosis (ABPA) is an intense allergic reaction, mainly in response to the presence of *Aspergillus fumigatus*, which can affect up to 15% of CF patients [1]. This allergic disease is normally treated with prescription of oral corticosteroid therapy and in a few critical clinical cases azole antifungals may be used [1,2].

Aspergillus fumigatus is one of the most frequently isolated fungi from the lungs of CF patients [1]. It is commonly susceptible to the commercially available antifungals such as amphotericin B, caspofungin, itraconazole, voriconazole and posaconazole [3]. It was recently shown that CF patients may be chronically colonised with the same *A. fumigatus* strain over several years [4,5]. Exposure of *A. fumigatus* strains to distinct conditions and stressful therapies over long periods may sporadically lead to alterations in their susceptibility and to treatment failure [6,7]. The objective of the present work was to evaluate the susceptibility to azole and non-azole antifungals of 159 isolates of *A. fumigatus* collected from 11 patients, some of whom had been treated with azole antifungal therapy.

2. Materials and methods

2.1. Patients

A group of 11 patients admitted to Hospital S. João (Porto, Portugal) and diagnosed with CF were studied. The diagnosis of CF was confirmed by screening mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein and positive evaluation of the sweat test. Patient age ranged from 15 years to 51 years. Patients were selected for this study following confirmation of chronic colonisation with *A. fumigatus* (more than three sputum samples with *A. fumigatus* in a previous year). Sputum samples were then collected from the patients between January 2005 and May 2009 (with a minimum of two samples from each patient at different times). Seven patients never received antifungal treatment, whilst four of the patients (patients B, C, D and E shown in Table 1) received azole antifungal therapy in addition to the recommended antibacterial treatment and oral corticosteroid

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Table 1
Susceptibility to amphotericin B (AMB), caspofungin (CAS), itraconazole (ITC), voriconazole (VRC) and posaconazole (PSC) of 159 *Aspergillus fumigatus* isolates collected from the sputa of 11 cystic fibrosis patients.

Patient (*n*)[a]	AMB	CAS	ITC	VRC	PSC
A (39)	0.25–1	≤0.03–0.25	0.5–2	0.25–1	0.06–0.5
B (54)[b]	0.25–1	≤0.03–0.25	0.5–2	0.25–1	0.06–0.25
C (5)[b]	0.5–1	0.06–0.125	0.5–2	0.25–0.5	0.25
D (14)[b]	0.5–1	≤0.03–0.125	0.5–2	0.25–0.5	0.125
E (13)[b]	0.5–1	≤0.03–0.06	0.5–1	0.25–0.5	0.125–0.25
F (4)	0.25–0.5	0.125–0.25	0.5–1	0.5	0.125
G (1)	0.5	0.06	0.5	0.25	0.125
H (15)	0.5–2	≤0.03–0.125	0.5–1	0.25–0.5	0.06–0.25
I (10)	0.25–1	≤0.03–0.06	1–2	0.5	0.125–0.25
J (2)	0.5–1	0.06	0.5–1	0.5	0.125–0.25
K (2)	0.5	0.125–0.25	0.5	0.25–0.5	0.125

[a] Number of isolates recovered from each patient in parenthesis.
[b] Patients B, C, D and E received antifungal azole therapy (details in Section 2.2).

therapy. Four of the studied patients (patients A, D, E and K shown in Table 1) matched with the modified criteria for diagnosis of ABPA in CF patients [2].

2.2. Antifungal therapy

Patient B received itraconazole between December 2007 and April 2008 (5 months) at a daily dose of 200 mg due to a serious decrease of respiratory function and chronic colonisation with *A. fumigatus* and *Scedosporium apiospermum*. In April 2008, an infection by tuberculosis mycobacteria was diagnosed and treated and itraconazole therapy was suspended to avoid liver dysfunction. Because the decrease in respiratory function persisted, patient B started voriconazole therapy in June 2009 (200 mg/12 h). The patient is currently stabilised and remains on voriconazole therapy.

Patient C received itraconazole between October 2006 and September 2007 (12 months) at a daily dose of 400 mg due to a decrease in respiratory function and clinical and radiological deterioration (mycetoma). The patient was chronically colonised with *A. fumigatus* and *S. apiospermum*. The clinical picture persisted after 1 year of itraconazole and in September 2007 the patient started voriconazole at 200 mg/12 h. Sputum samples showed the absence of fungal colonisation following voriconazole treatment. Between January and March 2008 the patient developed hepatitis and voriconazole therapy was suspended. Patient C re-started voriconazole therapy in April 2008 without any further complication and remains on the antifungal treatment until now (August 2009).

Patient D received voriconazole therapy for 12 months (from September 2007 to September 2008) at a dose of 200 mg/12 h due to serious haemoptysis and diagnosis of ABPA. The patient recovered and is currently stabilised.

Patient E received voriconazole therapy for 8 months (between June 2006 and February 2007; dose of 200 mg/12 h) due to ABPA, with a favourable outcome. Presently (August 2009), the patient is receiving itraconazole therapy (daily dose of 200 mg initiated in September 2008) due to a serious haemoptysis and no criteria for ABPA. The patient is presently stabilised.

2.3. Fungal organisms

Sputum samples were cultured in Sabouraud agar for 10 days at 30 °C and 37 °C. A total of 159 isolates of *A. fumigatus* were recovered from the clinical samples. The isolates were identified based upon macroscopic and microscopic morphological characteristics following standard mycological procedures. Two *A. fumigatus* reference strains (ATCC 46645 and MYA 772) were included in the study. Prior to DNA isolation, moulds were grown for 5 days on Sabouraud dextrose agar slants (Difco, Detroit, MI) at 30 °C. A sodium hydroxide-based method was used to extract DNA from conidia, as previously described [8]. DNA was suspended in 50 μL of sterile water and stored at −20 °C.

2.4. Antifungal susceptibility testing

Determination of minimal inhibitory concentrations (MICs) of amphotericin B (Bristol-Myers SP, Dublin, Ireland), caspofungin (Merck Sharp & Dohme, Whitehouse Station, NJ), itraconazole (Janssen-Cilag, High Wycombe, UK), voriconazole (Pfizer Inc., New York, NY) and posaconazole (Schering-Plough Farma, Cacém, Portugal) was performed according to the Clinical and Laboratory Standards Institute broth microdilution method [9]. MICs were defined as the absence of visible growth after 48 h in the presence of the antifungals amphotericin B, itraconazole, voriconazole and posaconazole. For caspofungin, the minimal effective concentration (MEC) was determined as previously recommended [3]. Quality control strains *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included each time testing was performed and antifungal MICs were within the expected limits [10,11]. Fungal strains were classified according to the recently proposed in vitro breakpoints for amphotericin B, caspofungin, itraconazole and voriconazole (susceptible, MIC/MEC ≤ 1 μg/mL; intermediate, MIC/MEC = 2 μg/mL; and resistant, MIC/MEC ≥ 4 μg/mL) [12]. For posaconazole, the fungal strains were classified according to recently proposed epidemiological cut-offs (wild-type, MIC ≤ 0.25 μg/mL; non-wild-type, MIC ≥ 0.5 μg/mL) [13]. Next, MICs of isolates with the same genotype were compared and were considered in essential agreement when the differences were no more than three dilutions (e.g. 0.5, 1.0 and 2.0 μg/mL).

2.5. Aspergillus fumigatus genotyping

Microsatellite multiplex polymerase chain reaction (PCR) was performed using previously selected primers allowing the identification of microsatellites based on trinucleotide, tetranucleotide and pentanucleotide motifs located on different chromosomes [8]. Multiplex PCR was performed using 1 μL of genomic DNA (1–5 ng/μL), 2.5 μL of Multiplex PCR Master Mix (Qiagen, Hilden, Germany) and 0.5 μL of a mix with eight primer pairs (final concentration of each primer 2 μM) in a final volume of 5 μL. The conditions for PCR amplification and sequencing reactions were as previously described [8].

2.6. Statistical analysis

Data were compared at a significance level of 0.05 by analysis of variance (ANOVA) using the Bonferroni correction, and Student's *t*-test for paired samples. Genotype diversity was calculated by Simpson's index of diversity. Statistical analysis for pair-wise linkage disequilibrium was performed by running the statistical software package Arlequin 3.1 (http://cmpg.unibe.ch/software/arlequin3/).

3. Results

Amongst the entire *A. fumigatus* collection, no resistant isolates (MIC/MEC ≥ 4 μg/mL) were identified to the antifungals amphotericin B, caspofungin, itraconazole and voriconazole (Table 1). The susceptibility of a few isolates was classified as intermediate to amphotericin B (3 isolates) and itraconazole (18 isolates), and a single *A. fumigatus* isolate was identified outside of the epidemiological cut-off of 0.25 μg/mL for posaconazole (this last isolate showed a susceptibility of 0.5 μg/mL for amphotericin B, 0.06 μg/mL for caspofungin, 2 μg/mL for itraconazole and