



Original Article

Molecular epidemiology of *Aspergillus* species and other moulds in respiratory samples from Argentinean patients with cystic fibrosis

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Abstract

In cystic fibrosis (CF) patients, fungal colonization of the respiratory tract is frequently found. *Aspergillus fumigatus* is the most frequently recorded and is associated with loss of pulmonary function and allergic disease (ABPA). The knowledge on prevalence rates of filamentous fungi in CF patients in Latin America is scarce. One hundred and seventy-six fungal isolates recovered from the upper respiratory tract of CF patients from Argentina were identified to species by morphology and DNA sequencing. In total, 90% of CF patients were colonized by *Aspergillus* sp., followed by *Exophiala* sp. (14%) and *Scedosporium* sp. (10%). Among *Aspergillus*, six species complexes (*Fumigati*, *Flavi*, *Terrei*, *Nigri*, *Usti*, and *Nidulante*) and different cryptospecies were found. Among *Scedosporium*, three species were observed (*Scedosporium apiospermum*, *Scedosporium aurantiacum* and *Scedosporium boydii*). All *Exophiala* isolates were identified as *Exophiala dermatitidis*. Rare filamentous fungi were also found. All cases of ABPA were associated to the presence of *A. fumigatus*. Mixed colonization with other mould or rare fungi was observed in half of them. To our knowledge, this is the first prospective study of mould species in CF using molecular methods in Latin America. This study shows that *Aspergillus* sp., *E. dermatitidis* and *Scedosporium* sp. have a high frequency in CF patients from Argentina, and by far, *A. fumigatus* was the most commonly cultured species. Continuous clinical surveillance is required to detect the emergence of new fungal pathogens and to detect resistant or difficult-to-treat species capable of chronic colonizing the airways and of hematogenous dissemination in case of lung transplantation.

Key words: cystic fibrosis, molecular epidemiology, filamentous fungi, *Aspergillus*, *Scedosporium*, *Exophiala*.

Introduction

Recurrent lung infections that lead to chronic airway inflammation and respiratory failure remain the major prognostic problem in cystic fibrosis (CF) patients and accounts for over 90% of mortality.¹ Clinical research and therapy has almost been focused on the role of bacterial species with little attention paid to the role of fungi in the pathogenesis of lung function decline,

despite their frequent isolation in respiratory samples from CF patients.²

Aspergillus colonization is commonly observed in patients with this genetic disorder, with *Aspergillus fumigatus* most frequently encountered, particularly late in the course of these patients, and is associated with loss of pulmonary function and allergic disease.³

Clinically recognized CF-related lung diseases manifested by *A. fumigatus* include *Aspergillus* sensitization and allergic bronchopulmonary aspergillosis (ABPA), persistent *Aspergillus* colonization (infection) and bronchitis.⁴ ABPA is a serious complication that affects 10–12% of CF patients and is the result of a Th2-mediated hypersensitivity response to *Aspergillus* allergens that leads to respiratory exacerbations, and over time it can result in bronchiectasis, lung fibrosis (partly mediated by Th9 responses), and respiratory failure.⁵ Persistent *Aspergillus* colonization has a prevalence rate that ranges from 5.9 to 57% and is associated with lung function deterioration even without ABPA and more frequent hospitalizations compared with noncolonized CF patients.⁷ However, the impact of persistent *Aspergillus* -and other filamentous fungi- colonization (infection) on the progression and severity of pulmonary disease in CF patients in terms of clinical exacerbations and lung function has not been studied sufficiently. *Aspergillus* bronchitis is a clinical entity defined in CF patients characterized by the presence of high fungal burden without evidence of a hypersensitivity response.⁸ CF patients with *Aspergillus* bronchitis display a significant clinical improvement with the use of antifungal therapy.⁹

During the last few years, more reports about infections caused by *Scedosporium* complex species¹⁰ and other emerging fungi such as *Exophiala dermatitidis*,¹¹ *Rasamsonia argillacea*,¹² and *Trichosporon mycotoxinivorans*¹³ have been identified in respiratory specimens from CF patients. In particular, *Scedosporium* species have attracted much attention, partly due to the reduced possibilities for antifungal therapy.¹⁰

The knowledge on prevalence rates of filamentous fungi in CF patients in Latin America is scarce. The aim of the present study was to give an overview of the diversity and molecular epidemiology of fungal species in CF patients from Argentina.

Methods

Samples and culture

Sputum samples of 50 Argentinean CF patients were processed using %5 N-acetyl-L-cysteine at a 1:1 ratio for liquefying and then subsequently plated undiluted on culture media. Culture conditions were as follows: beside standard media for bacterial culture, nonselective fungal medium (Sabouraud dextrose agar (SDA) supplemented with 500 mg/l chloramphenicol) and selective media were used. Among them, *Scedosporium*-agar (SceSel⁺-agar)¹⁴ and erythritol-chloramphenicol agar (ECA)¹⁵ to facilitate the detection of *Scedosporium* and *Exophiala* species, respectively, and the Remel inhibitory mould agar (IMA). All plates were incubated at 37°C for at least 10 days at 37°C. It should be pointed out that the frequency of sampling was a minimum of 4 samples per year. In the case of clinical

deterioration or during hospital stays, the frequency of sampling is higher.

All fungal isolates found in all microbiological specimen were then subcultured and maintained on SDA and/or potato dextrose agar at 37°C. Isolates were then identified by morphological criteria based on standard macroscopic and microscopic characteristics, including colonial form, growth rate, surface appearance and color, and microscopic assessment of hyphae (size, branching, and presence of septa), conidia, and conidia-bearing structures.

PCR and identification by β -tubulin, ITS, and calmodulin sequences

For this purpose, conidia from each strain were inoculated into 6 ml of YPD (yeast extract, peptone, dextrose) medium and grown for 24–72 hours at 37°C. Mycelial mats were recovered and DNA extraction was performed as previously described.¹⁶ The quality and quantity of genomic DNA was estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, San Francisco, CA, USA) and stored at –20°C until used. The BT2a/BT2b primers were used to amplify the partial β -tubulin gene whereas the ITS region was amplified using the universal fungal primers ITS1/ITS4 and the calmodulin (CaM) gene fragment was amplified using the set of primers CMD5 and CMD6.¹⁷ Amplifications were performed in a 25- μ l volume reaction containing 0.2 μ M of each primer and 20–50 ng of fungal genomic DNA.

Polymerase chain reaction (PCR) amplification was carried out in a thermal cycler (Perkin-Elmer, San Jose, CA, USA) using for the β -tubulin and ITS ribosomal regions one initial step of denaturation at 94°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 52°C (Bt2) or 54°C (ITS) for 30 seconds and 72°C for 2 minutes, and a last final extension step of 72°C for 10 minutes. For the CaM region, amplification included an initial step of denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 2 minutes, with a last final extension step of 72°C for 10 minutes.

Then, PCR products were purified and bidirectionally sequenced using ABI BigDye Terminator v.3.1 (Macrogen Inc., South Korea). The resulting nucleotide sequences were edited using the program Codon Code Aligner v7.1.2. DEMO (Codon-Code Corporation, www.codoncode.com) and aligned using the Muscle algorithm contained in the Unipro UGENE v1.29.0 software. The sequences identified for all fungal isolates were verified by BLAST search (<http://blast.ncbi.nlm.nih.gov>) and compared with sequences published in the NCBI GenBank database and in the CBS-KNAW Fungal Biodiversity Institute database. The ITS sequences were used to identify isolates to the species complex level, whereas the partial β -tubulin and CaM sequences were used to identify them to the species level.

Results

In total, 176 fungal isolates were recovered from the upper respiratory tract of 50 CF patients from Argentina. All samples were obtained from spontaneous sputum. A median of four samples was provided by each patient. The sample cohort was widely distributed in age (mean 15.6, range 6–52 years) with 21 (42%) adults (≥ 18 years old at time of sample acquisition) and 29 (58%) pediatric patients (15 of them [51.7%] were ≥ 12 years old); there was a female preponderance (23 males, 27 females). A wide range of pulmonary disease severity was observed. Most of the samples were obtained when patients were in clinically stable status ($n = 37$) whereas 12 patients had samples obtained during an exacerbation or ABPA, and 1 patient was at end of life in respiratory failure. The most common cocolonizing microbes were *Staphylococcus aureus* (87.2% of patients), *Candida albicans* (72.3% of patients), *Pseudomonas aeruginosa* (40.4% of patients), *Stenotrophomonas maltophilia* (25.5% of patients), *Moraxella catarrhalis* (10.6% of patients), *Klebsiella pneumoniae* (8.5% of patients), *Burkholderia cepacia* (2.1% of patients), and *Mycobacterium tuberculosis* (2.1% of patients). In summary, this sample cohort represents a broad spectrum of CF patients with respect to age, clinical status and microbiology, suggesting generalizability of the molecular epidemiology of *Aspergillus* species and other molds in respiratory samples from Argentinean patients with CF.

Overall, about 90% of CF patients (45/50) were colonized by *Aspergillus* sp., followed by *E. dermatitidis* (14%, 7/50), and *Scedosporium apiospermum* complex species (10%, 5/50). Among *Aspergillus*, six species complexes were found: 63.2% belonged to section *Fumigati* (76 *Aspergillus fumigatus* and 3 *Aspergillus lentulus*), 16% to section *Flavi* (18 *Aspergillus flavus*, 1 *Aspergillus parasiticus*, and 1 *Aspergillus pseudonomius*), 12.8% to section *Terrei* (14 *Aspergillus terreus*, 1 *Aspergillus pseudoterreus*, and 1 *Aspergillus alabamensis*), 6.4% to section *Nigri* (eight *Aspergillus niger*) and 0.8% to section *Usti* (one *Aspergillus calidoustus*) and section *Nidulante* (one *Aspergillus nidulans*). Among *S. apiospermum* complex species, 43.75% were *Scedosporium apiospermum*, 31.25% were *Scedosporium aurantiacum*, and 25% were *Scedosporium boydii*.

Rare fungi in CF patients like *Trichosporon asahii*, *Trichosporon mycotoxinivorans*, *Rasamsonia aegroticola* (which is a species of the *R. argillacea* complex) *Curvularia coateciae*, *Fusarium oxysporum*, *Alternaria nobilis*, and *Paecilomyces variotti* were isolated (Table 1).

Mixed colonization was also quite common (40% of CF patients). Of them, 85% ($n = 17$) were colonized with at least two different fungal species, whereas in the remaining 15% ($n = 3$) five fungal species were recovered from the same sputum sample. *A. fumigatus/A. flavus* was the most frequent mixed colonization observed (29.4%), followed by *A. fumigatus/A. terreus* (23.5%) and *A. fumigatus/A. niger* (11.8%). Mixed colonization by *S. apiospermum* complex and *A. fumigatus*, which is

quite common, was not observed. Noteworthy, all cases of ABPA were associated with the presence of *A. fumigatus*; of them four cases were cocolonized with *A. flavus*, one case was cocolonized with *A. alabamensis*, and one case was cocolonized with *T. mycotoxinivorans*.

Discussion

The isolation of *Aspergillus* species, in particular, *A. fumigatus*, in respiratory secretions of patients with CF is a widespread occurrence and it is associated to their ubiquity in the environment and their conidia dispersion and inhalation in the breathed air.¹⁸ *Aspergillus* infection and disease in CF is considered to become a problem during adolescence and adulthood. Moreover, chronic lung infections in CF patients drastically influence morbidity and mortality.

In CF patients, *Aspergillus* and other fungal conidia become easily trapped, partly because the mucociliary clearance activity is impaired and partly because the airways are filled with thick mucus. Several reports have shown a high variety in prevalence rates of filamentous fungi colonizing the upper respiratory tract of CF patients, and this most likely reflects the lack of standardization in culture techniques, the periodicity of sampling, the interpretation of the culture results, the geographical area, among others.^{10,19} There is a limited knowledge of the fungal epidemiology and diversity in CF patients in Latin America.

In the present study, carried out in Argentina, *Aspergillus*, *Scedosporium*, and *Exophiala* species ranked the first, second, and third among the filamentous fungi colonizing the airways of patients with CF, respectively. Standardized culture procedures were used to improve species identification, as recently recommended by the European Confederation of Medical Mycology/International Society for Human and Animal Mycology (ECMMISHAM) working group *Fungal respiratory infections in cystic fibrosis*.²⁰

This work also demonstrated that *Scedosporium* and *Exophiala* can be recovered from colonized CF patients with a similar frequency as *Aspergillus* section *Flavi* and *Aspergillus* section *Terrei*, respectively. A total of 12 different fungal genera were found in respiratory samples from 50 CF patients (in order of frequency, *Aspergillus*, *Scedosporium*, *Exophiala*, *Penicillium*, *Talaromyces*, *Trichosporon*, *Alternaria*, *Rodothorula*, *Rasamsonia*, *Fusarium*, *Curvularia*, and *Paecilomyces*).

Approximately, 78% of CF patients were colonized with *A. fumigatus* and all cases of ABPA or disease exacerbation were associated to its presence. Mixed colonization with other mold or rare fungi was observed in almost half of them. The most frequent cocolonizing mould was *Aspergillus* from section *Flavi* (4 patients were cocolonized with *A. flavus*), 1 with *A. alabamensis* (section *Terrei*), and the remaining one was cocolonized with *T. mycotoxinivorans*. Therefore, the role of mixed colonization on lung function or ABPA should be further studied. Many studies

Table 1. Fungal isolates found in sputa from CF patients in Argentina and clinical characteristics associated to their presence.

CF patients													
Species	Total number of isolates	Total number of patients	Median age at time of sampling	Sex	Concomitant colonization							Clinical status	
					SA	CA	PA	SM	MC	KP	BC		
<i>Alternaria nobilis</i>	1	1	17	M	X	X		X					Stable
<i>Aspergillus alabamensis</i>	1	1	17	M	X	X							ABPA
<i>Aspergillus calidoustus</i>	1	1	23	F	X								Stable
<i>Aspergillus flavus sensu stricto</i>	18	10	10.1	44.4% F, 48.7% M	X	X	X	X					55.6% stable, 44.4% ABPA
<i>Aspergillus fumigatus sensu stricto</i>	76	40	15.8	51.3% F, 48.7% M	X	X	X	X	X	X	X	X	69.2% stable, 30.8% ABPA
<i>Aspergillus lentulus</i>	3	3	8.3	0% F, 100% M	X	X	X	X					100% stable
<i>Aspergillus nidulans</i>	1	1	32	F	X		X						Stable
<i>Aspergillus niger sensu stricto</i>	8	6	13.9	50% F, 50% M	X	X	X	X					100% stable
<i>Aspergillus parasiticus</i>	1	1	24	F	X	X	X						ABPA
<i>Aspergillus pseudonovius</i>	1	1	12	F	X	X			X	X			Stable
<i>Aspergillus pseudoterreus</i>	1	1	16	M	X	X							Stable
<i>Aspergillus terreus sensu stricto</i>	14	6	22.2	83.3% F, 16.7% M	X	X	X	X					83.3% stable, 16.7% ABPA
<i>Curvularia coactiae</i>	2	1	6	M		X							Stable
<i>Exophiala dermatitidis</i>	14	7	21.1	80% F, 20% M	X	X	X	X	X	X			100% stable
<i>Fusarium oxysporium</i>	1	1	24	F	X	X	X						Stable
<i>Paecilomyces variotti sensu stricto</i>	1	1	16	M	X	X							Stable
<i>Penicillium canescens</i>	1	1	23	F	X								Stable
<i>Penicillium chrysogenum</i>	1	1	7	F	X	X	X		X				Stable
<i>Penicillium georgiense</i>	1	1	17	M	X	X							Stable
<i>Penicillium rubens</i>	1	1	7	F	X	X	X		X				Stable
<i>Rasamsonia aegroticola</i>	1	1	24	F	X	X	X						Stable
<i>Rhodotorula rubra</i>	2	2	16.6	100% F	X	X		X	X		X		100% stable
<i>Scedosporium apiospermum</i>	7	1	24	F	X								Stable
<i>Scedosporium aurantiacum</i>	5	1	28	F	X	X	X						Stable
<i>Scedosporium boydii</i>	4	4	21.6	50% F, 50% M	X	X	X	X		X			Stable
<i>Talaromyces columbinus</i>	1	1	32	M	X	X		X	X		X		Stable
<i>Talaromyces stollii</i>	4	3	33.7	66.7% F, 33.3% M	X	X	X	X		X			Stable
<i>Trichosporon asahii</i>	2	1	35	M	X	X							Stable
<i>Trichosporon mycotoxinivorans</i>	2	1	9	F	X	X	X						ABPA

ABPA, allergic bronchopulmonary aspergillosis; CF, cystic fibrosis; F, female; M, male; S, stable.

SA, *Staphylococcus aureus*; CA, *Candida albicans*; PA, *Pseudomonas aeruginosa*; SM, *Stenotrophomonas maltophilia*; MC, *Moraxella catarrhalis*; KP, *Klebsiella pneumoniae*; BC, *Burkholderia cepacia*.

showed that *Aspergillus* colonized patients displayed more pulmonary exacerbations requiring hospitalizations and/or significant lower lung function parameters and/or steeper lung function decline in contraposition to those noncolonized CF patients.⁷ Indeed, a survey carried out by Boyle et al. showed that *Aspergillus* colonization is considered by most CF professionals to be potentially harmful in CF patients recommending its antifungal treatment.²¹ In order to accurately guide the management of fungal infection in CF patients, fungal identification at species level is important because different species may significantly vary in their susceptibility profile to amphotericin B or azole drugs. For instance, some *Aspergillus* species may be intrinsically resistant to specific classes of antifungal agent such as *A. lentulus* which shows reduced susceptibility to amphotericin B, azoles and variable susceptibility to caspofungin.²² Moreover, it has been recently shown that *A. fumigatus* can be recovered from cough aerosols of colonized CF patients, suggesting droplet or airborne transmission as potential routes of patient to patient transmission.²³ There is a greater likelihood that this observation might be extended to other mould species. However, the most concerning point is that threats, such as azole resistance or species intrinsically resistant to antifungal drugs, might spread through cough aerosols from patients to patient or from patient to environment.

Reports describing the relative number of *A. fumigatus* versus non-*fumigatus* *Aspergillus* species have shown that between 36% and 58% of *Aspergillus* colonization in CF patients is caused by *A. fumigatus*. In the present study, 90% of patients were colonized by *Aspergillus* sp, of them, 78% were from section *Fumigati*. A high percentage of non-*Fumigati* were also observed, since 74% of CF patients were colonized by non-*Fumigati* species (24% were colonized by section *Flavi*, 14% by section *Terrei*, 12% by section *Nigri*, and 2% by section *Usti* and *Nidulante*). Noteworthy, mixed *Aspergillus* species occurred in 36% of patients. A similar percentage was reported by Jubin et al., who showed that 33% of patients were colonized by mixed *Aspergillus* species.²⁴ Noteworthy, *S. aureus*, *C. albicans*, *P. aeruginosa*, and *S. maltophilia* were frequently isolated alongside the fungus in *Aspergillus*-positive samples, as well as in *Scedosporium*- and *Exophiala*-positive samples. Therefore, no association was observed between a particular fungal genus and a particular bacterial colonization in CF patients.

So far, little attention has been paid to the role in lung disease in CF of the other filamentous fungi frequently isolated from the upper tract of this group of patients, such as *Scedosporium* and *Exophiala*.

The genus *Scedosporium*, is considered the second among the filamentous fungi colonizing the airways of CF patients in Europe. In our country, ranked third in frequency after *Aspergillus* and *Exophiala*. The colonization of the airways by *Scedosporium* sp. is thought to contribute to the inflammatory reaction leading to a progressive deterioration of the lung function,

similarly as it was described by *Aspergillus* sp. Moreover, chronic colonization by *Scedosporium* sp might lead to severe disseminated infections in case of lung transplantation.²⁵ These findings, taken together with the low susceptibility profile of these colonizing fungi to antifungal drugs, makes them a critical hazard in CF patients that should need lung transplantation.

Chronic colonization of the CF airways by the melanized dimorphic fungus *E. dermatitidis* was associated with recurrent pneumonia and hemoptysis in one report, and in another one it was associated to one case of fever and reduced clinical condition.⁶ However, its clinical role in progression of pulmonary disease in CF remains unclear.⁹ In this study, isolation of *E. dermatitidis* did not appear to be associated with clinical deterioration.

In two patients (4%), the basidiomycetous yeasts *T. asahii* and *T. mycotoxivorans*, were detected in at least 1 yearly sample. The presence of *T. asahii* was not associated with lung exacerbations. However, the presence of *T. mycotoxivorans* was associated to episodes of severe pulmonary exacerbation and ABPA requiring prolonged hospital stays in one patient that was cocolonized with *A. fumigatus*. The significance of *Trichosporon* species for CF patients has poorly been investigated. A study cohort performed in Munich demonstrated a potential association of *Trichosporon* spp. with severe exacerbations in CF patients.²⁶ Other reports in CF patients described non-*aspergillus* allergic bronchopulmonary mycosis due to *Trichosporon beigelii*,²⁷ fulminant pneumonia due to *T. mycotoxivorans*,¹³ fulminant disseminated infection with *T. mycotoxivorans* after lung transplantation in a CF patient coinfecting with *A. fumigatus* and *S. apiospermum*,²⁸ as well as chronic colonization without clinical deterioration.²⁹

Other filamentous fungi not associated with clinic worsening were sporadically isolated from the expectorations of CF patients (*R. aegroticola*, *C. coateciae*, *F. oxysporum*, *A. nobilis*, *P. variotti sensu stricto*, and those species ascribed to the genera *Talaromyces* and *Penicillium* which are generally considered as putative contaminant fungi).

However, it should be noticed that some of the detected species of the genus *Penicillium*, *Talaromyces*, and *Rasamsonia* are associated with human infections, and thus it should be figured out if the isolation of these species represents incidentally inhaled spores that got trapped in the thick mucus or moulds that are actively proliferating in the respiratory tract. For instance, *T. columbinus* has previously been reported as causal organism in certain human lung infections under the name of *Penicillium piceum*, which is a relatively uncommon but widely distributed species.³⁰ *T. stollii* was also reported to be recovered from pulmonary and invasive infections in humans.³¹ *P. chrysogenum*, the most common species of *Penicillium*, was reported to be an agent of onychomycosis, keratomycosis, allergic bronchopulmonary mycosis and asthma.³² Also, a single case was reported of clinical deterioration and lung function decline in CF patients colonized with species of the *R. argillacea* complex.³³

Further studies, such as long-time surveillance of sputa by metagenomic and transcriptomic studies, are needed to give further insight into the complex microbial dynamics and microbial-host interactions, so as to unravel the mechanisms developed by the different pathogens to establish within the respiratory tract of CF patients and in turn to specifically adapt to the physico-chemical conditions encountered in this clinical setting (acid pH, hypoxia, hypercapnia, thick bronchial mucus) as well as to define novel antifungal targets.

To the best of our knowledge, this epidemiological study is the first in Latin America in describing the mould species frequently recovered from the respiratory secretions of CF patients using molecular methods. Correct identification at species level should be of medical importance because of its implications for antifungal therapy and the detection of resistant or difficult to treat species capable of chronic colonization of the airways and of hematogenous dissemination in case of lung transplantation. Continuous surveillance is also required in order to detect the emergence of new fungal pathogens.

Finally, a systematic approach, in which standardized fungal diagnostic measurements are applied in a longitudinal way to a large cohort of patients with CF, is urgently needed to assess the impact of persistent mould colonization and the need of therapeutic interventions.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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