Clinical Microbiology and Infection 29 (2023) 539.e1–539.e7

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

Fungal microbiota dynamics and its geographic, age and gender variability in patients with cystic fibrosis

Sara Martínez-Rodríguez [1, 11], Vicente Friaza [1, 2], Rosa M. Girón-Moreno [3], Esther Quintana Gallego [4, 5], Antonio Salcedo-Posadas [6], Joan Figuerola-Mulet [7], Amparo Solé-Jover [8], Elena Campano [1], Ruben Morilla [1, 2, 9], Enrique J. Calderón [1, 2, 10, 11], Francisco J. Medrano [1, 2, 10, 11, *], Carmen de la Horra [1, 2]

[1] *Instituto de Biomedicina de Sevilla (Hospital Universitario Virgen del Rocío/CSIC/ Universidad de Sevilla), Seville, Spain*
[2] *Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública, Instituto de Salud Carlos III, Madrid, Spain*
[3] *Unidad de Fibrosis Quística-Bronquiectasias, Instituto de Investigación Sanitaria de La Princesa, Madrid*
[4] *Unidad Médico-Quirúrgica de Enfermedades Respiratorias, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/Universidad de Sevilla, Seville, Spain*
[5] *Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain*
[6] *Servicio de Neumología Pediátrica, Hospital Niño Jesús, Madrid, Spain*
[7] *Servicio de Pediatría, Hospital Universitari Son Espases, Balearic Islands Health Research Institute (IdISBa), Palma de Mallorca, Spain*
[8] *Unidad de Trasplante Pulmonar y Fibrosis Quística. Universitat de Valencia, Hospital Universitario La Fe, Valencia, Spain*
[9] *Departamento de Enfermería, Universidad de Sevilla, Spain*
[10] *Servicio de Medicina Interna, Hospital Universitario Virgen del Rocío, Seville, Spain*
[11] *Departamento de Medicina, Facultad de Medicina, Universidad de Sevilla, Spain*

ARTICLE INFO

Article history:
Received 19 May 2022
Received in revised form
31 October 2022
Accepted 3 November 2022
Available online 10 November 2022

Editor: E. Roilides

Keywords:
Cohort studies
Cystis fibrosis
DNA
Mycobiota
Pneumocystis jirovecii
Sequence analysis

ABSTRACT

Objectives: In cystic fibrosis (CF), there is a predisposition to bronchial colonization by potentially pathogenic microorganisms, such as fungi. Our aims were to describe the dynamics of respiratory mycobiota in patients with CF and to evaluate the geographic, age and gender variability in its distribution.
Methods: Cohort study in which 45 patients with CF from four hospitals in three Spanish cities were followed up during a 1-year period, obtaining spontaneous sputum samples every 3 to 6 months. Fungal microbiota were characterized by Internal Transcribed Spacer sequencing and *Pneumocystis jirovecii* was identified by nested PCR in a total of 180 samples.
Results: The presence of fungi were detected in 119 (66.11%) of the 180 samples and in 44 (97.8%) of the 45 patients: 19 were positive and 1 negative throughout all follow-ups and the remaining 25 presented alternation between positive and negative results. A total of 16 different genera were identified, with *Candida spp.* (50/180, 27.78%) and *Pneumocystis spp.* (44/180, 24.44%) being the most prevalent ones. The distribution of fungal genera was different among the evaluated centres ($p < 0.05$), by age (non-adults aged 6–17 years vs. adults aged ≥18 years) ($p < 0.05$) and by gender ($p < 0.05$).
Discussion: A high prevalence of fungal respiratory microbiota in patients with CF was observed, whose dynamics are characterized by the existence of multiple cycles of clearance and colonization, reporting the existence of geographic, age and gender variability in the distribution of fungal genera in this disease.
Sara Martínez-Rodríguez, Clin Microbiol Infect 2023;29:539.e1–539.e7

Introduction

Cystic fibrosis (CF) is the most important severe inherited disease in the Caucasian population [1,2]. It is caused by mutations in the gene that codes for the CF transmembrane conductance regulator protein that leads to the accumulation of thick mucus in the

* Corresponding author. Francisco J. Medrano, Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío, Avda. Manuel Siurot s/n, 41013 Seville. Spain.
E-mail address: medrano@cica.es (F.J. Medrano).

https://doi.org/10.1016/j.cmi.2022.11.001
1198-743X/

affected organs, causing obstructions and systemic abnormalities [2,3]. In the case of the respiratory tract, thicker bronchociliary mucus causes a lack of mucus clearance, a failure in microbial clearance and predisposition to bronchial colonization by potentially pathogenic microorganisms [3,4].

The morbidity and mortality of this disease depend mainly on the state of the respiratory system [5]. In addition, it is a progressive disease in which the likelihood of suffering any complications increases with age. Because of the improved life expectancy of patients, it is expected that the incidence and severity of these complications will increase; the most important of which is the chronic colonization of the airways by pathogens [1].

The impact of fungal colonization in the respiratory tract has not yet been fully elucidated; however, a growing number of evidence suggests that molds and yeasts play an important role in this process [5,6]. Patients with CF are new pathogen reservoirs that will probably serve as an infection source for other susceptible individuals [7]. Thus, we faced the need for a descriptive study that identifies fungi in patients with CF from different geographic locations.

The aims of this study were to describe the respiratory mycobiota of patients with CF; to know the dynamics of fungal colonization in time; and to evaluate the geographic, age and gender variability in the distribution of the different fungal species.

Methods

Study population

In this Spanish multicentre cohort study, all patients with CF who met the inclusion criteria and who were followed up between January and February 2011 at the Virgen del Rocío University Hospital (Seville), and between March and June 2014 at the following hospitals: La Princesa University Hospital (Madrid 1), Niño Jesús University Children's Hospital (Madrid 2) and Son Espases University Hospital (Mallorca) were included.

The inclusion criteria established were: (a) the previous diagnosis of CF (two confirmed CF transmembrane conductance regulator gene mutations or two consecutive positive sweat chloride tests (>60 mmol/l)); (b) patient aged >6 years; (c) informed consent signed by the patient or by their legal guardian/s; (d) clinical stability and possibility of obtaining spontaneous sputum samples; (e) absence of a concomitant diagnosis of a lung disease; and (f) absence of taking antifungal drugs in the 6 months before the initiation of the study.

Patients were followed up for 1-year period and underwent a clinical and biological examination every 3 to 6 months, obtaining spontaneous sputum following a standardized protocol in each visit, which was subsequently stored in aliquots at −20 °C. Another aliquot was sent to the Microbiology Department from each Hospital where a cellular count was performed, and only the samples with squamous epithelial cells count <15% were included in the study. Every 6 months during the study, frozen samples were sent to the laboratory of the Biomedicine Institute of Seville, where DNA extractions and PCRs were performed at the same time. Every 6 months 10% of randomly selected samples were retested, a quality control discordance >5% led to the revision of the samples evaluated during this period.

The study was reviewed and approved by the Ethics Committee of Virgen del Rocío University Hospital (Seville, Spain).

DNA extraction

Sputum samples were mechanically disaggregated, and DNA extraction was performed using the commercial NucleoSpin Tissue kit (Machenary Nagel).

Pneumocystis. jirovecii detection

P. jirovecii identification was performed using nested PCR with Biotaq DNA polymerase (Bioline) by amplification of the mitochondrial large subunit rRNA gene [8].

Fungal identification

For fungal identification, the nuclear ribosomal Internal Transcribed Spacer (ITS) sequence was amplified using semi-nested PCR with ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCG CTTATTGATATGC-3′) pair of primers in the first PCR round, and ITS-3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS-4 (5′-TCCTCCGCTTAT TGATATGC-3′) in the second one. The first round was conducted in 35 cycles, whereas the second round was executed in similar conditions and throughout 40 cycles.

The PCR products were resolved by 2% agarose gel electrophoresis. The bands were purified following the protocol of MEGA-quick-spin™ plus Fragment DNA Purification Kit (iNtRON Biotechnology). The isolated DNA was sequenced using a BigDye Terminator v3.1-kit from Applied Biosystems and the AB3500 Genetic Analyzer (Applied Biosystems).

Sequences were processed with the Finch TV 1.4 software and were subsequently compared with the sequences included in GenBank and European Molecular Bilogy Laboratory (EMBL) databases using the Basic Local Alignment Search Tool (BLAST) software.

To avoid false positives due to contamination, filter pipette tips were used at all stages. DNA extraction, preparation of the reaction mixture, PCR amplification and detection were performed in different areas. Autoclaved water was employed in the PCR mixture as a PCR-negative control.

Data processing and statistical analysis

Specific computer support was designed in ACCESS for data archiving in compliance with the confidentiality and privacy standards required by the current Spanish regulations (Organic Law 3/2018 on the Protection of Personal Data).

The Fisher exact test was performed by IBM SPSS Statistic 25 to detect the differences between the participating hospitals and age and gender variability. A p value of <0.05 was considered statistically significant. Those genera for which there was only one positive pathogen sample were grouped in the category ‘minority fungi’.

Mycobiota/chronic or persistent colonization

Chronic or persistent colonization for bacteria, such as *Pseudomonas aeruginosa* is defined by the European consensus as “Presence of *P. aeruginosa* in the bronchial tree for at least 6 months, based on at least three positive cultures with at least 1-month intervals between them” [9,10]. There are no similar consensuses to define persistent colonization of fungi; therefore, in our case, it has been defined as: ‘Presence of a specific fungus in at least two consecutive samples in a period of 6 months’. To refer to the rest of the identified fungi we will use the term ‘mycobiota’.

Results

A total of 180 samples from 45 patients were included in the present study, of which 119 (66.11%) were positive for fungi. On the other hand, 44 patients (97.8%) were positive in at least one clinical visit during the follow-up. None of the patients had a previous history of *P. jirovecii* pneumonia (PCP), nor invasive fungal disease or developed it during the follow-up.

The highest rate of fungi positive samples was observed in Seville in which 60 samples were analysed and 49 (81.67%) were positive and the lowest was in Madrid 2 with a prevalence of 50% (15 fungi positive samples out of 30 were analysed). The results obtained in the different centres are shown in Table 1.

Regarding the dynamics of the fungal microbiota genera, the results obtained for each patient during the follow-up are summarized in Table 2. Overall, 19 (42.22%) patients were fungi positive throughout the follow-up study, being most of them (12) were from Seville. Nevertheless, only 1 (2.22%) patient remained negative for fungi throughout the entire study. During the follow-up, the rest of the patients showed an alternation of positive and negative results in different visits (Table 3).

During the follow-up, 11 patients presented with chronic colonization by fungi: 4 by *Candida albicans* (Seville), 3 by *P. jirovecii* (Seville), 1 by *Cladosporium cladosporoides* (Seville), 1 by C. *parapsilosis* (Seville), 1 by C. *tropicalis* (Madrid 1) and 1 presented concomitant chronic colonization by *P. jirovecii* and *Exophiala dermatitidis* (Madrid 1).

The overall distribution of fungal genera identified in the samples evaluated is shown in Fig. 1. A total of 30 different 'operational taxonomic units' were identified, of which only their genus could be identified in 8, whereas their species were identified in the remaining 22. Sixteen fungal genera were present in the tested samples; being the most prevalent were *Candida spp.* (50/180, 27.78%) and *Pneumocystis spp.* (44/180, 24.44%).

The geographic variability of fungal genera is shown in Fig. 2. The most prevalent fungal genera in all centres are *Candida spp.* and *Pneumocystis spp.* There were significant differences in the distribution of fungi genera by centres ($p < 0.05$, Fisher exact test). The genus *Scedosporium spp.* is present in Madrid but has a low prevalence in Mallorca, and it is not detected in Seville. The genus *Cladosporium spp.* has high prevalence in Seville but low frequency in Mallorca and does not appear in Madrid. For its part, the genus *Exophiala spp.* is present in Madrid and Mallorca but not in Seville.

There were significant differences in the distribution of fungi genera by age (non-adults aged 6–17 years vs. adults aged ≥18 years; $p < 0.05$, Fisher exact test) being *Candida spp.* and *Pneumocystis spp.* the most frequent in both groups (please see supplementary material) (Fig. S1), *Exophiala spp.* is only found in adults, whereas the *Saccharomyces spp.* genus only appears in a low proportion in patients between 6 and 17 years old but it is not found in adults. Besides, there were also significant differences in the distribution of fungi genera by gender ($p < 0.05$, Fisher exact test). *Aspergillus spp.* and *Exophiala spp.* only appear in a low proportion among women whereas *Candida spp.* is more common among men.

Discussion

In the present study, the respiratory mycobiota dynamics and its age, geographic and gender variability in patients with CF were described. Sixteen different genera of fungi were identified, observing a high prevalence of fungal microbiota (66.11%) in the sputum of these patients. This is not entirely consistent with the findings of previous studies. As an example, Delhaes et al. [11] found 100% fungal colonization when analysing eight samples from four patients with CF, whereas Kramer et al. [12] found that 89% of 72 samples were positive for fungus. The non-coincidence of the colonization rates between our study and others performed earlier may be due to the low number of samples used in previous studies, which could have overestimated these rates.

Although ITS-PCR is the most used test for fungal identification, *P. jirovecii* only presents one copy of this gene, that is why the sensitivity of this technique is reduced for this fungus [11]. Because of this, for the identification of *Pneumocystis*, a mitochondrial large subunit rRNA gene PCR is assessed. Otherwise, the knowledge of the presence of *Pneumocystis* in samples from patients with CF is especially important owing to the impact it has on the lung pathophysiology [4]. It is well known that *P. jirovecii* causes severe pneumonia in immunocompromised patients called *Pneumocystis* pneumonia (PCP). Nonetheless, *Pneumocystis* infections can manifest in different ways, with PCP being a small part of them. Moreover, *Pneumocystis* can colonize both immunocompromised and immunocompetent patients with underlying lung diseases without signs or symptoms of acute pneumonia; nevertheless, it has been suggested that *P. jirovecii* could be a morbidity cofactor [4]. In addition, it may be involved in the development or transmission of disease acting like a reservoir [4,13].

It is worth mentioning that in the present study the overall rate of colonization by *P. jirovecii* (44/180, 24.44%) was similar to that obtained in previous studies conducted in Spain (21.5%) [14] and higher than that obtained in other European countries, such as Germany (7.4%) [15] or France (1.3%) in Brest [16], 3.5% in Rennes [17] and 12.5% in a multicentre study [18] or the United Kingdom (8.1%) [19], but lower than that obtained in Brazil (38.2%) [20]. Given that the highest prevalence is found in warm countries, these findings suggest that the geographic variability of this pathogen could be related to climatic factors, as it has been described in some studies for PCP [21,22], although others show discrepant results [23].

Additionally, the distribution of the different genera of fungi was different in the three locations studied, reporting the existence of geographic variability in adults with CF. The hospitals with the highest number of genera identified were located in Seville and Mallorca.

The predominant genus along with *Pneumocystis spp.* was *Candida spp.* Although, Madrid has a high prevalence of *Scedosporium spp.*, in the Sevilla Hospital this genus does not appear. In contrast, *Cladosporium spp.* genus is much more present in the hospital of Seville, whereas in Madrid it hardly ever appears, and it is relegated to non-adult patients. Additionally, the genus *Exophiala spp.* seems to be relevant in hospitals located in Madrid and Mallorca and is not observed in Seville.

Table 1
Prevalence of fungal microbiota in the sputum samples, gender and age groups of patients with cystic fibrosis by centre

	No. of patients	Gender (man/woman)	Age (adults/paediatrics)	No. of samples	*Positive samples,* n *(%)*	*Pneumocystis jirovecii,* n *(%)*	*Other fungi,* n *(%)*
Seville	20	8/12	12/8	60	49 (81.67)	19 (31.67)	44 (73.33)
Madrid 1	10	2/8	10/0	45	29 (64.44)	12 (26.67)	24 (53.33)
Madrid 2	6	5/1	0/6	30	15 (50)	5 (16.67)	11 (36.67)
Mallorca	9	6/3	3/6	45	26 (57.78)	8 (17.78)	23 (51.11)
Total	45	21/24	25/20	180	119 (66.11)	44 (24.44)	102 (56.67)

Adults aged ≥18 years; paediatrics aged 6 to 17 years old. Seville, Virgen del Rocío University Hospital; Madrid 1, La Princesa University Hospital; Madrid 2, Niño Jesús University Children's Hospital; Mallorca, Son Espases University Hospital; Positive samples, positive samples for *P. jirovecii* and/or other fungi.

Table 2
Dynamics of pathogen identified in sputum samples during the follow-up of patients with cystic fibrosis

Hospital	Patient code	Follow-up					Age (y)	FEV1 (%)	Pseudomonas. aeruginosa colonization
		0 moths	3 months	6 months	9 months	12 moths			
Virgen del Rocío University Hospital (Seville)	HVR1	*Aspergillus spp.*		*A. restrictus*		Uncultured fungus	12	42	0
	HVR2	*Candida albicans/P. jirovecii*		*C. albicans*		C. albicans	18	61	0
	HVR3	*Cladosporium spp.*		-		-	21	42	1
	HVR4	*C. parapsilosis/P. jirovecii*		-		-	27	68	1
	HVR5	*C. albicans/P. jirovecii*		*Alternaria spp./W. sebi*		C. albicans/P. jirovecii	33	62	1
	HVR6	*C. flavescens/P. jirovecii*		*C. herbarum*		Uncultured fungus	32	78	1
	HVR7	Uncultured fungus		*P. jirovecii*		C. parapsilosis/P. jirovecii	28	25	1
	HVR8	*C. albicans/P. jirovecii*		*C. glabrata/P. jirovecii*		Candida spp.	18	51	1
	HVR9	*C. parapsilosis*		*C. cladosporioides*		C. cladosporioides	25	39	1
	HVR10	*C. glabrata*		*C. albicans*		C. albicans	21	33	0
	HVR11	*C. albicans*		*C. albicans*		-	28	31	1
	HVR12	Uncultured fungus		*C. albicans*		P. jirovecii	22	23	1
	HVR13	-		*P. jirovecii/ Zygosaccharomyces spp.*		-	9	78	1
	HVR14	*Aspergillus spp.*		*P. jirovecii*		Uncultured fungus	9	90	0
	HVR15	*A. alternae/P. jirovecii*		*C. albicans*		C. albicans	12	63	0
	HVR16	*C. albicans/C. parapsilosis*		*C. parapsilosis/P. jirovecii*		-	10	86	0
	HVR17	*C. cladosporioides*		*C. ramotenellum*		-	21	47	0
	HVR18	*C. cladosporioides*		-		P. jirovecii	13	67	0
	HVR19	*C. albicans/P. jirovecii*		-		C. parapsilosis	7	41	0
	HVR20	*Cladosporium spp./P. jirovecii*		*C. glabrata/P. jirovecii*		P. jirovecii	14	85	0
La Princesa University Hospital (Madrid 1)	HPP1	*C. albicans*	-	*C. albicans*	C. albicans		28	83	1
	HPP2	*P. jirovecii*	-	*P. jirovecii/ Scedosporium prolificans*	S. prolificans	-	20	59	0
	HPP3	*C. tropicalis*	*C.tropicalis*	*C. tropicalis*	C. tropicalis/P. jirovecii	-	32	69	1
	HPP4	-	-				19	82	1
	HPP5	*S. prolificans*	-	*P. jirovecii*	-	P. jirovecii/L. elongisporus	25	66	0
	HPP6	*Exophiala dermatitidis*	*E. dermatitidis/P. jirovecii*	*E. dermatitidis/P. jirovecii*	E. dermatitidis/P. jirovecii	E. dermatitidis/P. jirovecii	18	48	1
	HPP7	-	*C. albicans*	*S. apiospermum*	C. albicans/P. jirovecii	S. apiospermum	22	72	1
	HPP8	-	*S. apiospermum*	*P. jirovecii*	P. jirovecii	S. boydii	22	60	0
	HPP9	-	-	-	C. albicans	S. boydii	31	63	1
	HPP10	*P. jirovecii*	-	-	-		22	85	0
Niño Jesús University Children's Hospital (Madrid 2)	HNJ1	*C. parapsilosis*	-	*C. albicans*	S. prolificans	S. apiospermum	17	80	0
	HNJ2	-	-	*C. herbarum*	-	Uncultured fungus	13	90	1
	HNJ3	*C. parapsilosis/P. jirovecii*	*C.albicans/C. glabrata*	*S. cereviseae*	P. jirovecii	S. cereviseae	15	103	0
	HNJ4	*P. jirovecii*	-	-	-	-	15	81	1
	HNJ5	*Trichosporon spp*	-	-	-	P. jirovecii	15	78	0
	HNJ6	-	-	-	-	P. jirovecii	12	96	0
Son Espases University Hospital	HSE1	-	*C. ramotenellum*	*C. albicans*	Sporobolomyces spp.	Uncultured fungus	13	90	0
	HSE2	*C. albicans/C. flagrans/M. restricta/P. jirovecii*	*C. albicans*	Uncultured fungus	-	-	10	9	0
	HSE3	-	-	*C. albicans/P. jirovecii*	-	C. lusitaniae	12	90	0

HSE4	*Exophiala spp.*	Uncultured fungus	Uncultured fungus	—	E. dermatitidis	21	NA	NA
HSE5	*C. albicans/C. deformans*	-	-	P. jirovecii	P. jirovecii	23	NA	NA
HSE6	*C. albicans/C. deformans*	*C. tropicalis*	*C. albicans*	C albicans/C. glabrata	P. jirovecii/S. apiospermum	11	47	0
HSE7	-	*S. apiospermum*	-	-	-	17	92	1
HSE8	*P. jirovecii*	Uncultured fungus	-	-	-	9	84	0
HSE9	-	*C. albicans/P. jirovecii*	-	C. albicans	-	22	NA	NA

FEV1 (%), forced expiratory volume in one 1 second (%); HVR, Hospital Virgen del Rocío; HPP, Hospital La Princesa; HNJ, Hospital Niño Jesús; HSE, Hospital Son Espases; NA, not available; 0, no; 1, yes.

Table 3
Patterns of evolution over time of fungal microbiota in the study patients with cystic fibrosis

Pattern	No. of patients (%)	No. of patients by hospital (%)			
		Seville	Madrid 1	Madrid 2	Mallorca
+++	19 (42.22)	12 (60)	3 (30)	2 (33.33)	2 (22.22)
++−	7 (15.56)	3 (15)	2 (20)	0	2 (22.22)
+−+	4 (8.9)	2 (10)	0	1 (16.67)	1 (11.11)
+−−	4 (8.9)	2 (10)	1 (10)	1 (16.67)	0
−++	6 (13.33)	0	3 (30)	1 (16.67)	2 (22.22)
−+−	3 (6.67)	1 (5)	0	0	2 (22.22)
−−+	1 (2.22)	0	0	1 (16.67)	0
−−−	1 (2.22)	0	1 (10)	0	0
Total	45 (100)	20 (100)	10 (100)	6 (100)	9 (100)

+, positive; −, negative; the first symbol represents the situation at the beginning. The second the situation during the follow-up (those with at least one sample + are considered positive) and the last symbol the final situation; Seville, Virgen del Rocío University Hospital; Madrid 1, La Princesa University Hospital; Madrid 2, Niño Jesús University Children's Hospital; Mallorca, Son Espases University Hospital. No, Number.

Furthermore, in agreement with what was previously described [24], there are clearance and reinfection cycles for *P. jirovecii*. In our study, this condition is extended to the rest of fungi that are only present in some samples of each patient, alternating negative and positive samples over time in most of them.

Eleven of the patients presented chronic colonization by fungi: 4 by C. *albicans*, 3 by *P. jirovecii*, 1 by *Cladosporium cladosporioides*, 1 by C. *parapsilosis*, 1 by C. *tropicalis* and 1 presented concomitant chronic colonization by *P. jirovecii* and *Exophiala dermatitidis*.

Candida spp. colonization is very common in patients with CF. Usually, *Candida spp.* does not seem to cause pulmonary exacerbations; however, a recent retrospective analysis revealed that colonization by *C. albicans* was associated with a decline in lung function [25,26]. The roles of *C. parapsilosis* and *C. tropicalis* in this disease are not yet clarified. The low prevalence of *Aspergillus spp.* observed in our study is striking, a finding that could be related to the low sensitivity of using only the ITS gene to detect this fungus.

Meanwhile, only 1 (2.22%) patient remained negative for fungi throughout the entire study. It is important to highlight that only 2 of 5 expected samples could be obtained from this patient, which could be indicating an improvement in the progression of the disease and the clearance of pulmonary microorganisms.

Interestingly, in our study fungi are geographically distributed according to a criterion that is still unknown, but could be related to climate or a preferred habitat that favours the local spread of some fungal genera.

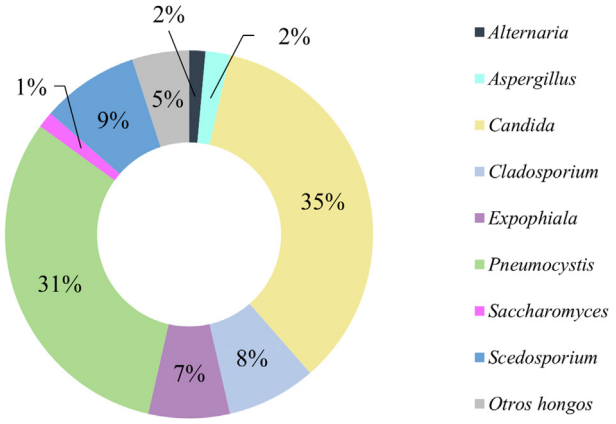

Fig. 1. Global distribution of fungal genera in sputum samples of patients with cystic fibrosis from Spain.

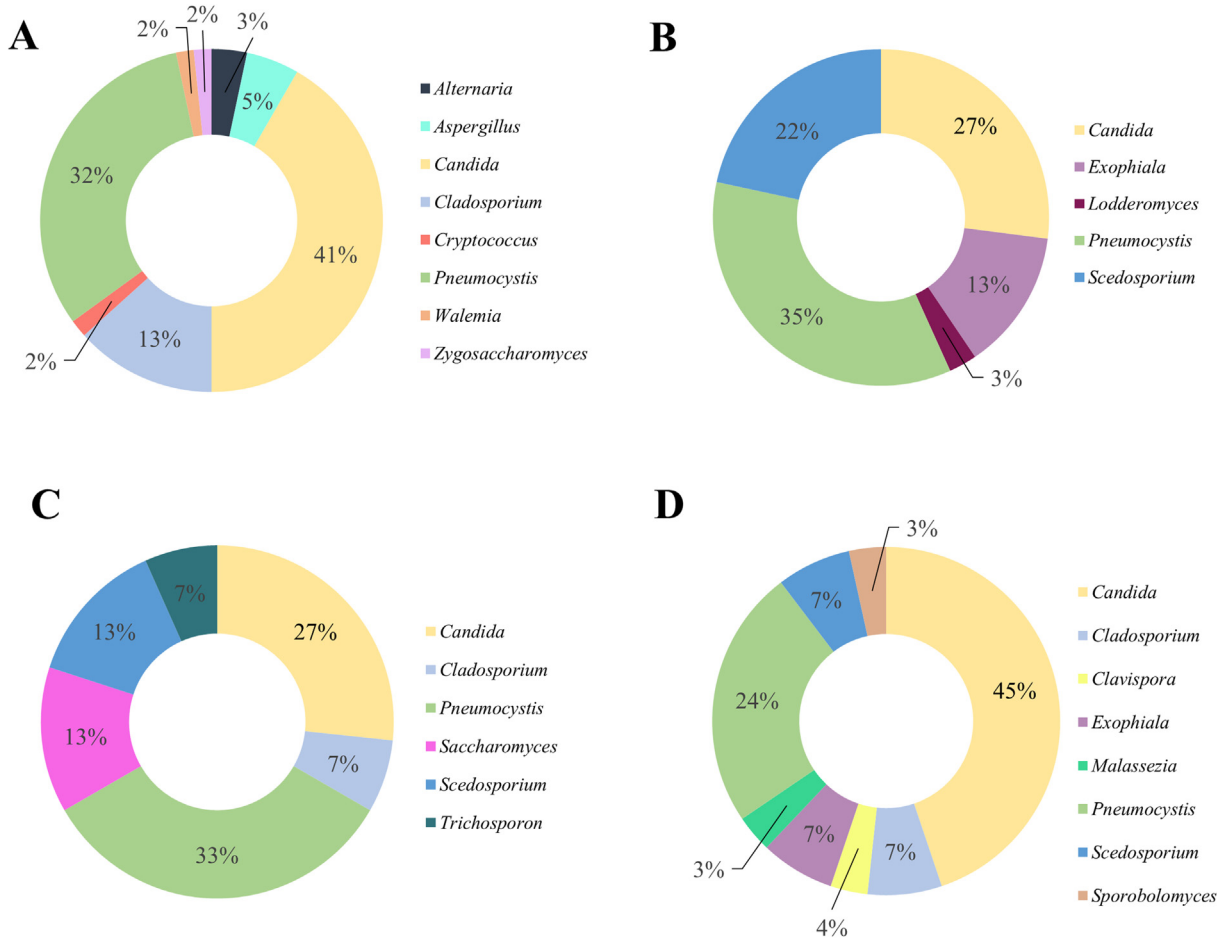

Fig. 2. Distribution of fungal genera in sputum samples of patients with cystic fibrosis from Spain by Center. A. Virgen del Rocío University Hospital (Seville); B. La Princesa University Hospital (Madrid 1); C. Niño Jesús University Children's Hospital (Madrid 2); D. Son Espases University Hospital (Mallorca).

Another novel aspect of our study is the finding that what is observed for the bacterial microbiota [27] is also true for the distribution of fungi: the fungal microbiota changes significantly with age in patients with CF. Although the main fungi, such as *Candida spp.* and *Pneumocystis spp.*, predominate in both adults and non-adult patients, others like *Exophiala spp.* are specific to adults like previously reported [28], whereas *Saccharomyces spp.* is only found in the non-adult population.

On the other hand, we have also found a different distribution of fungi by gender, not previously described, especially of uncertain origin and significance. However, we hypothesize that it could be because of a different susceptibility because of certain characteristics of the host linked to sex.

The present study had some limitations. Regarding the type of sample, sputum is not considered the most representative sample of the lung microbiota, whose reference standard, is bronchoalveolar lavage. Sputum is frequently used in epidemiological studies of the lung microbiota in subjects with pulmonary affections because of its null invasiveness. Another limitation is that those samples from Virgen del Rocío University Hospital, previously analysed, are asynchronous regarding the samples from other participating hospitals.

The cohort design that allows the sequential evaluation of patients and the number of subjects and samples analysed, being much higher than those previously described in the literature [4,14–20], is a remarkable strength that increases the validity of the results obtained.

It is now evident that lung bacterial colonization has an important impact on the evolution of CF. In the present study, we have been able to demonstrate the existence of a high prevalence of fungal respiratory microbiota in patients with CF, whose dynamics are characterized by the existence of multiple cycles of clearance and recolonization, reporting the existence of geographic, age and gender variability in the distribution of fungal genera in this disease. Further larger prospective studies are required to confirm these findings and define their clinical relevance.

Author contributions

S.M.R., V.F., F.J.M, A.S.J., E.J.C. and C.H. conceived and designed the research. R.M.G.M., E.Q., A.S.P. and N.B. collected the samples and followed up the patients. S.M.R., V.F., E.C., R.M. and C.H. analysed the samples. S.M.R. wrote the initial draft of the manuscript. V.F., F.J.M., E.J.C. and C.H. contributed to the development of the study and data interpretation. All authors reviewed and approved the final version of the manuscript. S.M.R. and V.F. are joint first authors.

Transparency declaration

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding

The study was supported by the Spanish Ministry of Science and Innovation (grant number FIS-PS09/00957), by Plan Andaluz de Investigación, Desarrollo e Innovación, Consejería de Economía Conocimiento, Empresas y Universidad de la Junta de Andalucía (grant number PS20_00894), and by Consejería de Salud y Familia, Junta de Andalucía (grant number CSyF Exp. RH-0061/2021).

Acknowledgements

Our deep gratitude to Francisco Javier Dapena, founder of the Cystic Fibrosis Unit of the Virgen del Rocío University Hospital, who died in 2017, for making the development of this study possible and for his contribution to the fight against cystic fibrosis in Spain. The authors are grateful to Reyes Morales for English revision of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2022.11.001.

References

[1] Regard L, Martin C, Chassagnon G, Burgel PR. Acute and chronic non-pulmonary complications in adults with cystic fibrosis. Expert Rev Respir Med 2019;13:23–38. https://doi.org/10.1080/17476348.2019.1552832.
[2] Ratjen F, Bell SC, Rowe SM, Goss CH, Quitnner AL, Bush A. Cystic fibrosis. Nat Rev Dis Primers 2015;1:15010. https://doi.org/10.1016/S0140-6736(16)00576-6.
[3] Brown SD, White R, Tobin P. Keep them breathing: cystic fibrosis pathophysiology, diagnosis, and treatment. JAAPA 2017;30:23–7. https://doi.org/10.1097/01.JAA.0000515540.36581.92.
[4] Bonnet P, Le Gal S, Calderon E, Delhaes L, Quinio D, Robert-Gangneux F, et al. *Pneumocystis jirovecii* in patients with cystic fibrosis: a review. Front Cell Infect Microbiol 2020;10:571253. https://doi.org/10.3389/fcimb.2020.571253.
[5] de Dios-Caballero J, Cantón R, Ponce Alonso M, García Clemente MM, Gómez G-de la Pedrosa E, López-Campos JL, et al. The human mycobiome in chronic respiratory diseases: current situation and future perspectives. Microorganisms 2022;10:810. https://doi.org/10.3390/microorganisms10040810.
[6] Cuthbertson L, Felton I, James P, Cox MJ, Bilton D, Schelenz S, et al. The fungal airway microbiome in cystic fibrosis and non-cystic fibrosis bronchiectasis. J Cyst Fibros 2021;20:295–302. https://doi.org/10.1016/j.jcf.2020.05.013.
[7] Gutiérrez S, Respaldiza N, Campano E, Martínez-Risquez MT, Calderón EJ, de La Horra C. *Pneumocystis jirovecii* colonization in chronic pulmonary disease. Parasite 2011;18:121–6. https://doi.org/10.1051/parasite/2011182121.
[8] Wakefield AE, Pixley FJ, Banerji S, Sinclair K, Miller RF, Moxon ER, et al. Amplification of mitochondrial ribosomal RNA sequences from *Pneumocystis carinii* DNA of rat and human origin. Mol Biochem Parasitol 1990;43:69–76. https://doi.org/10.1016/0166-6851(90)90131-5.
[9] Döring G, Conway SP, Heijerman HG, Hodson ME, Høiby N, Smyth A, et al. Antibiotic therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a European consensus. Eur Respir J 2000;16:749–67. https://doi.org/10.1034/j.1399-3003.2000.16d30.x.
[10] Lee TW, Brownlee KG, Conway SP, Denton M, Littlewood JM. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J Cyst Fibros 2003;2:29–34. https://doi.org/10.1016/S1569-1993(02)00141-8.
[11] Delhaes L, Monchy S, Fréalle E, Hubans C, Salleron J, Leroy S, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community–Implications for therapeutic management. PLoS One 2012;7:36313. https://doi.org/10.1371/journal.pone.0036313.
[12] Kramer R, Sauer-Heilborn A, Welte T, Guzman CA, Abraham WR, Höfle MG. Cohort study of airway mycobiome in adult cystic fibrosis patients: differences in community structure between fungi and bacteria reveal predominance of transient fungal elements. J Clin Microbiol 2015;53:2900–7. https://doi.org/10.1128/JCM.01094-15.
[13] Morilla R, Medrano FJ, Calzada A, Quintana E, Campano E, Friaza V, et al. *Pneumocystis jirovecii* among patients with cystic fibrosis and their household members. Med Mycol 2021;59:849–54. https://doi.org/10.1093/mmy/myab010.
[14] Respaldiza N, Montes-Cano MA, Dapena FJ, De La Horra C, Mateos I, Medrano FJ, et al. Prevalence of colonisation and genotypic characterisation of *Pneumocystis jirovecii* among cystic fibrosis patients in Spain. Clin Microbiol Infect 2005;11:1012–5. https://doi.org/10.1111/j.1469-0691.2005.01276.x.
[15] Sing A, Geiger AM, Hogardt M, Heesemann J. *Pneumocystis carinii* carriage among cystic fibrosis patients, as detected by nested PCR. J Clin Microbiol 2001;39:2717–8. https://doi.org/10.1128/JCM.39.7.2717-2718.2001.
[16] Le Gal S, Hery-Arnaud G, Ramel S, Virmaux M, Damiani C, Totet A, et al. *Pneumocystis jirovecii* and cystic fibrosis in France. Scand J Infect Dis 2010;42:225–7. https://doi.org/10.3109/00365540903447000.
[17] Nevez G, Robert-Gangneux F, Pougnet L, Virmaux M, Belleguic C, Deneuville E, et al. *Pneumocystis jirovecii* and cystic fibrosis in Brittany, France. Mycopathologia 2018;183:81–7. https://doi.org/10.1007/s11046-017-0172-2.
[18] Hernández-Hernández F, Fréalle E, Caneiro P, Salleron J, Durand-Joly I, Accoceberry I, et al. Prospective multicenter study of *Pneumocystis jirovecii* colonization among cystic fibrosis patients in France. J Clin Microbiol 2012;50:4107–10. https://doi.org/10.1128/JCM.01974-12.
[19] Green HD, Bright-Thomas RJ, Mutton KJ, Guiver M, Jones AM. Increased prevalence of *Pneumocystis jirovecii* colonisation in acute pulmonary exacerbations of cystic fibrosis. J Infect 2016;73:1–7. https://doi.org/10.1016/j.jinf.2016.05.001.
[20] Pederiva MA, Wissmann G, Friaza V, Morilla R, de La Horra C, Montes-Cano MA, et al. High prevalence of *Pneumocystis jirovecii* colonization in Brazilian cystic fibrosis patients. Med Mycol 2012;50:556–60. https://doi.org/10.3109/13693786.2011.645892.
[21] Varela JM, Regordán C, Medrano FJ, Respaldiza N, de La Horra C, Montes-Cano MA, et al. Climatic factors and *Pneumocystis jiroveci* infection in southern Spain. Clin Microbiol Infect 2004;10:770–2. https://doi.org/10.1111/j.1469-0691.2004.00937.x.
[22] Miller RF, Grant AD, Foley NM. Seasonal variation in presentation of *Pneumocystis carinii* pneumonia. Lancet 1992;339:747–8. https://doi.org/10.1016/0140-6736(92)90650-r.
[23] Pereira-Díaz E, Moreno-Verdejo F, de la Horra C, Guerrero JA, Calderón EJ, Medrano FJ. Changing trends in the epidemiology and risk factors of *Pneumocystis* pneumonia in Spain. Front Public Health 2019;7:275. https://doi.org/10.3389/fpubh.2019.00275.
[24] Montes-Cano MA, de La Horra C, Dapena FJ, Mateos I, Friaza V, Respaldiza N, et al. Dynamic colonisation by different *Pneumocystis jirovecii* genotypes in cystic fibrosis patients. Clin Microbiol Infect 2007;13:1008–11. https://doi.org/10.1111/j.1469-0691.2007.01789.x.
[25] Al Shakirchi M, Klingspor L, Bergman P, Hjelte L, De Monestrol I. A 16-year retrospective study on fungal prevalence and diversity in patients with cystic fibrosis: *Candida dubliniensis* was associated with a decline in lung function. Int J Infect Dis 2020;96:663–70. https://doi.org/10.1016/j.ijid.2020.05.063.
[26] Renner S, Nachbaur E, Jaksch P, Dehlink E. Update on respiratory fungal infections in cystic fibrosis lung disease and after lung transplantation. J Fungi (Basel) 2020;6:381. https://doi.org/10.3390/jof6040381.
[27] Zemanick ET, Wagner BD, Robertson CE, Ahrens RC, Chmiel JF, Clancy JP. Airway microbiota across age and disease spectrum in cystic fibrosis. Eur Respir J 2017;50:1700832. https://doi.org/doi:10.1183/13993003.00832-2017.
[28] Oliver A, Alarcón T, Caballero E, Cantón R. Diagnóstico microbiológico de la colonización-infección broncopulmonar en el paciente con fibrosis quística [Microbiological diagnosis of bronchopulmonary colonization-infection in cystic fibrosis]. Enferm Infecc Microbiol Clin 2009;27:89–104. https://doi.org/10.1016/j.eimc.2008.05.004.