Prevalence, geographic risk factor, and development of a standardized protocol for fungal isolation in cystic fibrosis: Results from the international prospective study "MFIP"

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ABSTRACT

Background: Fungi are increasingly recognized for their potential role in contributing to pulmonary damage in Cystic Fibrosis (CF). We therefore designed a prospective international study aimed at (i) determining the prevalence of fungi isolated from sputum samples collected from a large CF population, (ii) comparing the performance of different media used for fungal culture, and (iii) proposing a standardized protocol suitable for CF routine microbiology.
Methods: An international, consensually designed prospective study was set up (https://www.ecfs.eu/special-projects/mucofong-international-project). All centers worked according to the same protocol approved by Lille Ethical Committee. CF sputa were inoculated onto eight semi-selective media incubated at 37 °C or 25 °C–30 °C for 15 days, and inspected twice weekly for fungal growth.
Results: A total of 469 sputa were collected from patients at 18 European and one Australian CF centers. Positive cultures for fungal growth were significantly associated with patient ages. *Aspergillus fumigatus* was the most frequently isolated mold. We identified a growing European North-to-South gradient of *Scedosporium* prevalence, while yeasts, *Aspergillus* section *Fumigati*, *Cladosporium* and *Penicillium* were significantly more prevalent in the Northern regions.
Conclusions: According to the CHi-squared Automatic Interaction Detector method, we propose a consensual

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protocol based on two media (YPDA or Sabouraud medium, and B(+) medium) to detect the main opportunistic molds in CF context; the use of an additional medium being recommended according to the patient's clinical status. This standardized protocol allows us to have an accurate overview of the respiratory mycobiome on the culturomic side in CF.

1. Introduction

Cystic Fibrosis (CF) represents the most common life-shortening autosomal recessive disorder in the Caucasian population, affecting about 70,000 people in the world for whom fungal respiratory colonization has emerged as a new concern [1].

As bacterial infections are responsible for the majority of pulmonary damage in CF, considerable attention has been given on their prevention and treatment by antibiotics, which has resulted in a rising life expectancy of CF patients [1–3]. Consequently, CF patients are more and more at risk of airway colonization or respiratory infection by fungi [1]. Currently, there is an increasing recognized role of fungi, as both key players in the natural course of the CF lung disease and/or real threats in case of lung transplantation [1,4]. Prevalence of fungi isolated from CF respiratory samples has significantly increased over recent decades; *Aspergillus fumigatus* complex being the most predominant species, responsible for colonization, *Aspergillus* sensitization (AS), and allergic bronchopulmonary aspergillosis (ABPA) [1,4]. In this context, more and more attention has been drawn toward filamentous fungi, as well as certain yeasts, reaching the status of emerging or re-emerging fungi [1].

However, the heterogeneity in study designs (mainly retrospective and observational) and the lack of standardization in the procedures for mycological analysis limit reliable epidemiological investigations and hold back from drawing a full picture of the current fungal situation in CF [1,4,5].

Therefore, we aimed at setting-up an international prospective study named MFIP for "MucoFong International Project" with the purpose of assessing fungus prevalence in CF airways, comparing performances of different media used for fungal culture, and developing an efficient standardized approach for the mycological examination of respiratory samples from CF patients. MFIP study was organized in the context of our international and multidisciplinary ECMM/ISHAM Working Group, and in collaboration with the European Cystic Fibrosis Society (https://www.ecfs.eu/special-projects/mucofong-international-project; https://www.ecfs.eu/sites/default/files/documents/MFIP-Synopsis-ESCF.pdf), with the aims of (i) determining the prevalence of fungi isolated from sputum samples collected from a large international CF population, (ii) evaluating and comparing the performance of media used for fungal cultures, and (iii) proposing a consensus standardized protocol suitable in routine clinical microbiological laboratories supporting CF units.

2. Methods

Taking into account our experience from the French multicentric study for mycological analysis of CF sputa, «MucoFong » program (PHRC-06/1902) [6], and advantage of the network based on our ECMM/ISHAM Working Group on "Filamentous fungi and chronic respiratory infections in CF", we collectively designed and prospectively realized the MFIP study as follows.

2.1. MFIP protocol design

As a first step, a general questionnaire focusing on each laboratory's current procedures was widely spread through our ECMM/ISHAM Working Group (Questionnaire is provided in the supplementary material pages 1–2, Appendix A); the corresponding data were then presented and discussed during the third meeting of our Working Group (Angers 2014). A consensus emerged for plating of CF respiratory secretions on a total of eight media incubated at two temperatures (37 °C and 25 °C–30 °C), sharing the same Excel database for reporting results, and producing robust data via the analysis and inclusion of results from approximately 500 sputa within the routine laboratory workflow in each center.

All the media (Table 1) were prepared at the Mycology laboratory of Lille Hospital according to published data [5–9], in two consecutive series during a six-week period, and were sent to each participating center (thirty-five plates of each mycological medium per center). To avoid seasonal bias, all the centers had the same period (six months, April to September) to include and plate consecutively about 30 sputa from CF patients.

All centers worked according to the same protocol approved by the Lille Ethical Committee (Observational study 2012–042). As the sputum samples analyzed in the framework of MFIP study were obtained from patients during routine check-up visits (secondary use of sample), this protocol was considered as "current care with secondary use of biological samples". According to French Bioethics rules, only the absence of patient's opposition for participating to MFIP-study was required (Notices are provided in the supplementary material pages 3–9, Appendix A).

Patients were included if their CF diagnosis was documented, and if they underwent a mycological analysis as part of either the annual disease checkup or the clinical management of an acute pulmonary exacerbation in one of the participating centers. Patients were excluded from the study if they were unable to produce sputum. Briefly, after patient inclusion, MFIP mycological procedure included a digestion of sputum samples using mucolytic agent (Dithiothreitol), then 20 μL of digested or digested - 1:10 diluted sample were inoculated per plate, as previously described [5,6]. Samples were inoculated on eight culture media as follows: undiluted sputum sample on Chromogenic medium, Yeast Peptone Dextrose Agar (YPDA) or Sabouraud medium, Dichloran-rose-Bengal-Benomyl agar, *Scedosporium*-selective medium, YPDA or Sabouraud medium supplemented with chloramphenicol and cycloheximide, B(+) medium, and Erythritol medium, plus 1:10 diluted sputum sample on YPDA or Sabouraud medium. Media were incubated for fifteen days at 37 °C, except B(+) medium and Erythritol medium that were incubated 25 °C–30 °C (Table 1). All media were checked daily the first 4 days, and twice a week between the 5th and 15th day (Recommended check days were on Friday and Tuesday, plus an estimate of colony numbers at day 8 and 15 post inoculation) for fungal growth. For each plate, each fungal isolate detected was identified to species level. For each isolate, the estimated time to first detection was reported. Data were compiled and statistically analyzed at Lille, in collaboration with Lille Statistics Department.

2.2. Statistical analysis

For a given species and a given sputum sample, cultures were considered positive when the species growth was evidenced on at least one of the eight plated media. For each fungal species, the population reference was assessed by counting as positive each sample in which the given species was isolated at least from one of the 8 media. Using this medium combination, prevalence of each fungal species was then

Table 1
Composition, characteristics and incubation temperatures of the growth media used in MFIP.

Medium	Composition for 1 L of distilled water	Characteristics	Incubation Temperature
Chromogenic [5,6]	Peptone, 10 g; Glucose, 20 g; Agar-agar, 15 g; Chloramphenicol, 0.5 g; Chromogenic substrate 2 g	Medium based on the hydrolysis of chromogenic substrates by some *Candida* species, when incubated at 37 °C.	37 °C
YPDA or Sabouraud	YPDA: Yeast extract, 5 g; Peptone, 10 g; Glucose, 20 g; Agar, 20 g Sabouraud medium: Enzymatic digest of casein, 5 g; Peptone, 5 g; Glucose, 40 g; Agar, 20 g	These 2 media are widely used for mycology analysis.	37 °C plated with either undiluted or 1:10 diluted sputum sample in order to compare diluted and un-diluted samples for the recovery of minor fungal populations (1:10 dilution is used for detection of beginning colonization; Dr. Borman's personal communication)
Dichloran-rose-Bengal-Benomyl agar [6]	Dichloran-rose-Bengal agar base, 31.5 g; Chloramphenicol, 0.5 g; Glucose, 5 g; Benomyl, 0.008 g	As benomyl inhibits the microtubule network of fungal cell, this medium is well-adapted for the detection of slow-growing fungi.	37 °C
Scedosporium-s elective agar [7]	Mono-potassium-phosphate, 1.25 g; Magnesium sulfate 7 H2O, 0.625 g; Maltose, 6.25 g; Yeast extract, 1 g; Soy peptone, 0.625 g; Ciprofloxacin, 0.1 g; Streptomycin sulfate, 0.1 g; Chloramphenicol, 0.1 g; Dichloran, 2 mg; Benomyl,6 mg; Agar, 20 g	This semi-selective medium facilitates the growth and detection of *Scedosporium* species.	37 °C
YPDA or Sabouraud medium plus chloramphenicol and cycloheximide [6]	YPDA or Sabouraud composition plus Chloramphenicol, 0.5 g and Cycloheximide, 0.5 g	This semi-selective medium facilitates the growth and detection of slow-growing fungi that are resistant to cycloheximide like *Scedosporium* species.	37 °C
B(+) agar [8]	Yeast extract, 30 g; Glucose, 16.7 g; Agar, 20 g; Peptone, 6.8 g; Cotrimethoxazole, 0.128 g; Chloramphenicol, 0.05 g; Ceftazidime, 0.032 g; Colistin, 0.024 g	Given the supplementation with 4 broad spectrum antibiotics, this medium largely suppressed any bacterial growth.	<30 °C
Erythritol agar [6,9]	Yeast nitrogen base, 6.7 g; Meso-erythritol, 10 g; Chloramphenicol, 0.5 g; Agar, 25 g	This selective medium facilitates the isolation of *Exophiala dermatitidis* based on its ability to use erythritol as carbon substrate	<30 °C

estimated among the entire population, and among adult (15 years-old patients or older) and paediatric (patients under 15 years old) sub-populations. Performances of each growth medium were individually assessed, based on sensitivities (Se, referring to the probability that a given medium will be positive for a given species if this species is isolated at least from one of the eight plated media) and negative predictive values (NPV, referring to the probability that the species will be absent from the sample when the culture of this species is negative for a given medium) that were established for each medium, focusing on fungal species isolated at least in five samples over the whole sputum collection.

The Wilcoxon signed-rank test and the Kruskal-Wallis test by ranks were used for comparison of groups of continuous data. Fisher's exact test was used to compare groups of categorical data. To define the optimal combination of growth media able to detect up to 90% of the opportunistic fungal pathogens, we used decision trees obtained by the Chi-squared Automatic Interaction Detector (CHAID) method [6,10]. This statistical detection tree technique is based upon adjusted significance testing and is providing highly predictive algorithms of optimal fungal detection.

Statistical analysis was done using software SAS© version 9.3, considering $p < .05$ as significant.

3. Results

3.1. General epidemiology

A total of 469 sputum samples were collected from 469 patients followed-up in eighteen European and one Australian CF centers enrolled in MFIP study (Fig. 1). Patients were 5- to 67-years-old, with a sex ratio at 1.16 (252 males vs. 217 females). About 78% of sputum samples cultured were positive for at least one fungus. CF patients with positive mycological cultures were significantly older than patients with negative cultures (mean age: 25.8 ± 11.3 y.o. vs. 17.5 ± 11.8 y.o., and median age: 24.0, Q1: 19.0 - Q3: 32.0 y.o. vs. 14.5 Q1: 8.0 - Q3: 23.0 y.o.) (Fig. 2). However, the number of fungal species isolated per patient sample was not significantly correlated with patient ages. As we didn't collect clinical data from MFIP cohort, we couldn't go further to document the relation between patient age and fungal colonization.

3.2. Species prevalence and geographical distribution

Among the whole MFIP population, *A. fumigatus* was the most frequently isolated filamentous fungus (34.5%), followed by *Scedosporium* species (5.1%). *Candida albicans* was the most frequently isolated yeast (47.7%). Moreover, prevalence of *C. albicans*, *Aspergillus* section *Fumigati*, and species belonging to *Scedosporium apiospermum* complex were significantly higher in the adult population compared to the paediatric population (Table 2).

Regarding geographical distribution, the analysis focused exclusively on European countries (Fig. 1). Species distributions were studied according to the latitude coordinates of MFIP centers, grouped into centers with either a latitude coordinate above 50°, between 49° and 45°, or under 45°. *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *Geotrichum* sp., *Aspergillus* section Fumigati, *Cladosporium* and *Penicillium* species were significantly more prevalent in the Northern European centers (Table 3). Conversely, a growing north-south gradient of the prevalence of *Scedosporium* and *Lomentospora* species was identified (Table 3). Of note, Australian fungal prevalence was estimated at: *A. fumigatus* 56.3%, *Candida* sp. 12.5%, *A. flavus* 12.5%, *A. niger* 6.3% and *S. apiospermum* species 6.3%, in our cohort of CF patients from Sydney.

3.3. Individual performances of each growth media

Values of sensitivity and NPV of each culture medium regarding detection of each fungal species are summarized in Table 4. Plating on

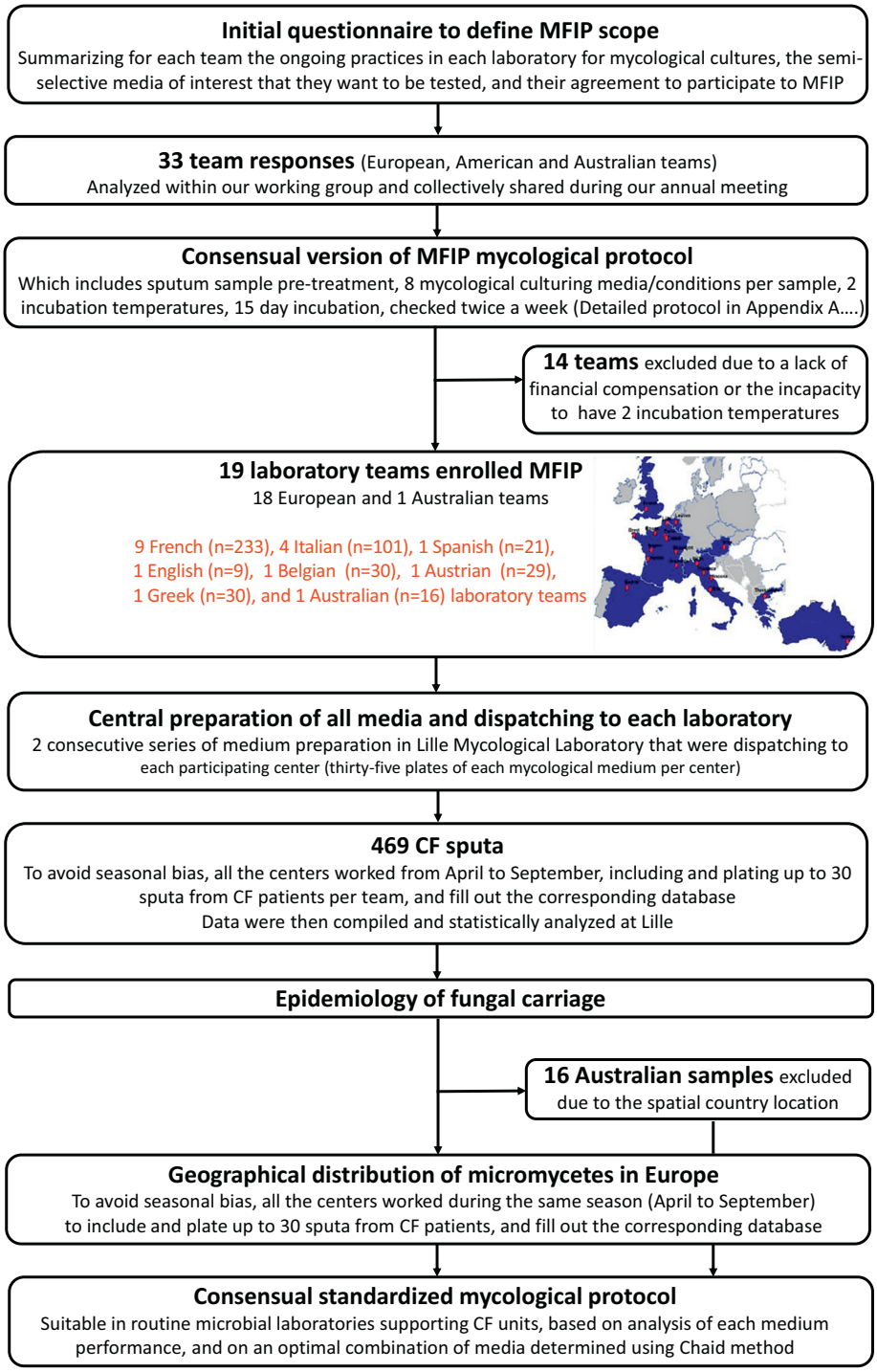

Fig. 1. Flow diagram of MFIP study.

Sabouraud or YPDA medium a 1:10 diluted sputum sample to increase chances of isolating minority fungal population and therefore of detecting a beginning colonization did not appear to be as efficient as expected in our CF population. Conversely, plating on Sabouraud or YPDA medium with undiluted samples exhibited the highest sensitivity (69.4%) and negative predictive value (86.3%) for isolating *Aspergillus* section *Fumigati*. For *Scedosporium* species, the *Scedosporium*-selective agar confirmed the high performance previously reported [7]. To isolate other molds than *Aspergillus* section *Fumigati*, media that were incubated between 25 °C and 30 °C such as B(+) or Erythritol agar appeared to be more efficient, in agreement with the mesophilic characteristics of these environmental molds.

Additionally, chromogenic medium exhibited the best performances for detection of some non-albicans*Candida* species, such as *C. glabrata* and *C. parapsilosis*, as previously reported [5,6]. Surprisingly, the best medium to isolate *C. albicans* was the Dichloran-rose-Bengal-Benomyl agar (Se of 88.4%, NPV of 90.4%). Finally, (B+) media showed high sensitivities and negative predictive values for detecting numerous yeast and mold species, described earlier [8].

We documented the incubation time (expressed in days) required for culture positivity (Fig. 3). While *Candida* species required similar short delays and median times compared to *Aspergillus* species (about 3 days), *Scedosporium* and *Exophiala* species required a longer incubation time with a median of 5 days; 41.7% of *S. apiospermum* complex

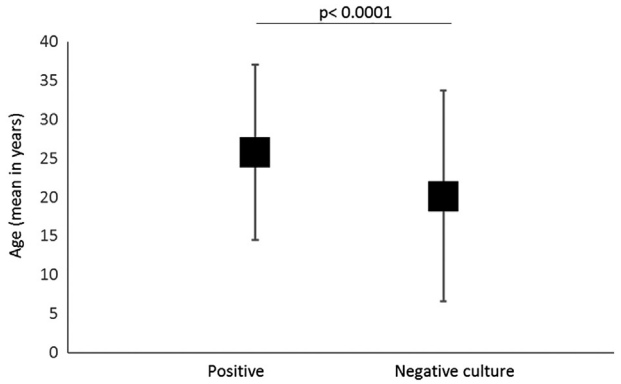

Fig. 2. Mean age in years of patients with positive and negative mycological cultures.

isolates and 33.3% of *Exophiala* isolates requiring >6 days of incubation to achieve detectable growth. In fact, a notable proportion (between 11% to 42%) of the molds recovered in MFIP study need more than one week of incubation (Fig. 3), which may let us propose a two-weeks-incubation time, especially when *Scedosporium* or *Exophiala* species are suspected.

3.4. Determining the optimal combination of growth media for mycological analysis of sputum sample in the context of CF

We used decisional trees to provide optimal combinations of media that allows us to detect fungi selected on the basis of their pathogenicity in CF as follows: all *Aspergillus* isolates, *Scedosporium* species plus *L. prolificans* isolates, *Exophiala* species, isolates belonging to *Rasamsonia argillacea* complex and *Cladosporium* isolates. Algorithms were built according to the CHAID method, based on our categorical variable (i.e. presence of a given fungus in a given medium), and on the individual performances of each medium previously determined.

To categorize each medium as the best choice (or not) for isolating the selected opportunist fungi, we applied CHAID method using two complementary approaches. The first one considered each pair of patient and sputum as an event and the corresponding sputum analysis as positive or negative regarding the growth of one of our fungus selection, for each of the eight media (216 sputa with positive cultures for at least one of our fungus selection among the 366 sputa with positive cultures). The second approach took into account each of the selected opportunistic fungal pathogens isolated in one of the sputa as events, the corresponding analysis being based on the presence or absence of fungi for each medium (278 species identified as either *Aspergillus*, or *Scedosporium* or *Lomentospora*, or *Exophiala*, or *Rasamsonia argillacea* complex, or *Cladosporium* species). Both approaches gave the same optimal combination of growth media (Fig. 4). The best medium to isolate one of the selected opportunistic fungi was Sabouraud or YPDA medium, which established the positive culture of 152 sputum samples among the 216 sputa with positive culture. The next step to maximize the chance of isolating fungi resided into plating B(+) medium, which established the positive culture for an additional set of 34 sputa. Then, plating erythritol medium allowed to identify 16 more sputa with positive cultures, leading to a combination of 3 media able to recover 93.2% of the sputum samples with positive cultures in our CF population.

Table 2
Prevalence of fungi among paediatric and adult sub-populations.

Species[a]	Paediatric population (<15 y.o.)		Adult population (≥15 y.o.)		*p* value[c]
	Isolate number	Prevalence[b]	Isolate number	Prevalence	
Candida albicans	28	28%	195	55.4%	<0.001
Candida dubliniensis	2	2%	19	5.4%	NS[d]
Candida glabrata	3	3%	19	5.4%	NS
Candida parapsilosis	4	4%	17	4.8%	NS
Other *Candida* species	6	6%	31	8.8%	NS
Geotrichum sp.	1	1%	6	1.7%	NS
Exophiala sp.	2	2%	14	4%	NS
Aspergillus section *Fumigati*	16	16%	134	38%	<0.001
Aspergillus section *Circumdati*	1	1%	13	3.7%	NS
Aspergillus section *Nidulantes*	1	1%	12	3.4%	NS
Aspergillus sp.	3	3%	9	2.5%	NS
Species belonging to *S. apiospermum* complex	1	1%	22	6.2%	0.037
Other *Scedosporium* species plus *Lomentospora prolificans*	3	3%	16	4.5%	NS
Rasamsonia argillacea species complex	0	0%	5	1.4%	NS
Penicillium sp.	4	4%	39	11.1%	NS
Cladosporium sp.	1	1%	8	2.3%	NS
Other filamentous fungi	1	1%	18	5.1%	NS

[a] For statistical purposes, *Aspergillus* species belonging to section *Fumigati*, to section *Circumdati*, to section *Nidulantes* were grouped, as well as *Candida* species that differed from *Candida albicans*, *Candida dubliniensis*, *Candida glabrata* and *Candida parapsilosis*. Other *Scedosporium* species plus *Lomentospora prolificans* refered to *Scedosporium* species not formally identified as species belonging to the *S. apiospermum complex* plus *Lomentospora prolificans* species. Other filamentous fungi grouped species identified as *Arthrographis kalrae*, *Chrysosporium* sp., *Fusarium oxysporum*, *Fusarium* sp., *Byssochlamys spectabilis* (formerly *Paecylomyces variotii*), *Acremonium* sp., and *Rhizomucor* sp.
[b] Prevalence was estimated among the whole population ($n = 469$).
[c] Fisher's Exact Test.
[d] NS: Not significant *p* value.

Table 3
Prevalence of fungi according to the latitude coordinates of MFIP centers.

Species[a]	Latitude coordinate of MFIP centers			p value[b]
	>50°	49–45°	44–40°	
Candida albicans	55.6%	54.0%	37.4%	0.0024
Candida dubliniensis	11.1%	4.4%	1.5%	0.0107
Candida glabrata	8.3%	6.0%	07%	0.0101
Candida parapsilosis	1.4%	4.8%	6.1%	NS[c]
Other *Candida* species	11.1%	3.6%	16.0%	<0.0001
Geotrichum sp.	5.6%	0.8%	0.8%	0.0194
Exophiala sp.	5.6%	4.4%	0.8%	NS
Aspergillus section *Fumigati*	43.1%	34.0%	26.7%	0.0479
Aspergillus section *Circumdati*	2.8%	1.6%	6.1%	NS
Aspergillus section *Nidulantes*	0%	3.6%	3.1%	NS
Aspergillus sp.	1.4%	2.0%	4.6%	NS
Species belonging to *S. apiospermum* complex	1.4%	2.0%	14.0%	<0.0001
Other *Scedosporium* species plus *Lomentospora prolificans*	2.8%	7.2%	16.8%	0.0019
Rasamsonia argillacea species complex	0%	0.8%	2.3%	NS
Penicillium sp.	19.4%	10.4%	2.3%	<0.0001
Cladosporium sp.	5.6%	2.0%	0%	0.0202
Other filamentous fungi	11.1%	2.8%	3.1%	0.0126

[a] For statistical purposes, *Aspergillus* species belonging to section *Fumigati*, to section *Circumdati*, to section *Nidulantes* were grouped, as well as *Candida* species that differed from *Candida albicans*, *Candida dubliniensis*, *Candida glabrata* and *Candida parapsilosis*. Other *Scedosporium* species plus *Lomentospora prolificans* refered to *Scedosporium* species not formally identified as species belonging to the *S. apiospermum complex* plus *Lomentospora prolificans* species. Other filamentous fungi grouped species identified as *Arthrographis kalrae*, *Chrysosporium* sp., *Fusarium oxysporum*, *Fusarium* sp., *Byssochlamys spectabilis* (formerly *Paecylomyces variotii*), *Acremonium* sp., and *Rhizomucor* sp.
[b] Fisher's Exact Test adjusted for age.
[c] NS: Not significant p value.

4. Discussion

The MFIP study was designed to assess fungus prevalence in CF airways, to compare performances of different media used for fungal culture, and therefore to propose an optimal culture protocol efficient for the detection of all fungal opportunistic pathogens present in CF sputa at routine clinical laboratory level. It emerged from a collaborative

Table 4
Sensitivities (Se) and negative predictive values (NPV) of each medium for each fungal species.

Species[a]	Se – NPV in % of							
	Chromogenic	YPDA or Sabouraud plated with 1:10 diluted sputum sample	YPDA or Sabouraud medium	Dichloran-rose-Bengal-Benomyl	Scedosporium selective agar	YPDA or Sabouraudmedium pluschloramphenicoland cycloheximide	B(+) medium	Erythritol medium
Candida albicans	81.7–85.0	40.5–65.2	79.0–83.9	88.4–90.4	79.5–84.2	77.2–72.8	75.5–81.7	63.8–75.1
Candida dubliniensis	66.7–98.4	38.1–97.2	71.4–98.7	61.9–98.2	61. –98.2	61.9–98.2	80.9–99.1	71.4–98.7
Candida glabrata	90.1–99.5[b]	50.0–97.6	81.8 –99.1	72.7–98.7	27.3–98.9	40.9–97.2	72.7–98.7	36.4–97.0
Candida parapsilosis	85.7–99.3	52.4–97.8	66.7–98.5	71.4–98.7	71.4–98.7	42.9–97.4	71.4–98.7	52.4–97.2
Other *Candida* species	60.7–97.5	52.5–95.8	72.5–97.5	75.0–97.7	70.0–97.3	62.5–96.6	77.5–97.9	65.0–96.8
Geotrichum sp.	14.3–98.7	28.6–98.9	28.6–98.9	28.6–98.9	28.6–98.9	28.6–98.9	85.7–99.8	57.1–99.3
Exophiala sp.	43.7–98.0	37.5–97.8	43.7–98.0	0–0	12.5–97.0	31.2–97.6	87.5–99.6	68.7–98.9
Aspergillus section *Fumigati*	65.6–84.3	43.7–77.4	69.4–86.3	6.2–67.3	5.0–67.0	49.4–79.2	59.4–82.6	61.9–83.5
Aspergillus section *Circumdati*	53.3–98.4	26.7–97.6	53.3–98.5	0–0	0–0	33.3–97.8	26.7–97.6	26.7–97.6
Aspergillus section *Nidulantes*	61.5–98.9	46.1–98.5	61.5–98.9	0–0	0–0	28.1–97.8	69.2–99.1	69.2–99.1
Aspergillus sp.	44.4–98.9	58.3–98.9	58.3–98.9	16.7–97.8	16.7–97.9	50.0–98.7	75.0–99.3	58.3–98.9
Species belonging to *S. apiospermum* complex	56.5–97.7	52.2–97.6	73.9–98.7	82.6–99.1	87.0–99.3	65.2–98.2	82.6–99.1	78.3–98.9
Other *Scedosporium* species plus *Lomentospora prolificans*	45.0–97.5	45.0–97.1	55.0–98.0	80.0–99.1	95.0–99.8	40.0–97.4	70.0–98.7	50.0–97.8
Rasamsonia argillacea species complex	60.0–99.6	40.0–99.4	80.0–99.8	40.0–99.4	40.0–99.4	80.0–99.8	60.0–99.6	80.0–99.8
Penicillium sp.	4.6–91.0	4.6–91.2	7.0–91.4	4.6–91.0	4.6–91.2	4.6–91.2	55.8–95.4	72.1–97.3
Cladosporium sp.	0–0	0–0	0–0	0–0	0–0	0–0	44.4–98.9	67.7–99.3
Other filamentous fungi	12.5–96.9	15.8–96.5	31.9–97.2	10.5–96.4	21.0–96.8	31.6–97.2	36.9–97.4	52.6–98.0

[a] For statistical purposes, *Aspergillus* species belonging to section *Fumigati*, to section *Circumdati*, to section *Nidulantes* were grouped, as well as *Candida* species that differed from *Candida albicans*, *Candida dubliniensis*, *Candida glabrata* and *Candida parapsilosis*. Other *Scedosporium* species plus *Lomentospora prolificans* refered to *Scedosporium* species not formally identified as species belonging to the *S. apiospermum complex* plus *Lomentospora prolificans* species. Other filamentous fungi grouped species identified as *Arthrographis kalrae*, *Chrysosporium* sp., *Fusarium oxysporum*, *Fusarium* sp., *Byssochlamys spectabilis* (formerly *Paecylomyces variotii*), *Acremonium* sp., and *Rhizomucor* sp.
[b] Underlined values referred to the best values of Se and NPV for a given species among the eight growth conditions tested.

work from our international and multidisciplinary ECMM/ISHAM Working Group, in collaboration with the European Cystic Fibrosis Society (Fig. 1). For the 469 patients able to expectorate that were included in the MFIP observational study, 78% of sputum samples were positive for at least one fungus, with a significant increased age observed in CF patients with positive mycological cultures compared to patients with negative cultures, in agreement with previous studies (Fig. 2) [5,6,8,11,12]. However, collecting exclusively sputa may lead to bias, as sputum samples represent a noninvasive sampling method to assess microbial diversity of upper airways, but with some limitations such a slightly over-representation of mouth flora [13,14]. Although it is difficult to compare our results with previous published data given the heterogeneity of study designs [1,5,6,8,11,12,15–20], this international prospective study on sputum analysis provided robust data among different participant centers.

Among MFIP findings, culture positivity in older CF patients compared to patients with negative cultures confirmed the crucial role of fungal pathogens within CF disease outcome, that may be associated with the use of antibiotics, and/or corticosteroids [6,8,12,21]. However, we couldn't confirm that age or some treatments were associated with fungal isolation independently, as MFIP study didn't collect clinical data (excepted age and sex), due to the Ethics discrepancy between the countries. In addition, CF patients were included in MFIP study only if they were expectorators, which may represent major limitations since it is more common for older children and adults to be expectorators. Moreover, the period chosen to conduce MFIP study (April to September) was adequate to minimize seasonal bias; however, these months also encompass seasons in which respiratory illnesses are less common (versus the winter months/colder temperatures).

Despite discrepancies in study designs, the relative prevalence of the principal opportunistic fungal pathogens identified in MFIP survey were comparable to those established in recent published data [5,6,17,23,24]. While clinical relevance of *C. albicans* colonization is still matter of debate [1], *C. albicans* was the most prevalent fungus isolated in our population (47.7%). We confirmed the high prevalence of *A. fumigatus* (34.5%), and of *Aspergillus* section *Fumigati* (34.8%), the notable prevalence of *Exophiala* (3.4%) and *R. argillacea* complex (1.1%) species, as well as the high performances of both B(+) and erythritol media as previously reported [1,5–9,11].

However, a novel finding from the current study was the existence of geographical "prevalence gradients" for several of the key pathogenic fungi isolated from CF respiratory secretions, with *Aspergillus* section Fumigati, *Cladosporium* and *Penicillium* species significantly more prevalent in the Northern European centers, whereas the reverse was identified for *Scedosporium* and *Lomentospora* species (Table 3). It is interesting to speculate that the increased prevalence of *Aspergillus* section Fumigati, *Cladosporium* and *Penicillium* species in CF respiratory secretions from patients from Northern latitudes might result from higher exposures to spores of these fungi in the colder and damper indoor environments that predominate in Northern Europe [21–23], or from differences in CF patient management such as the use of inhaled antibiotics that may impact fungal colonization [2,24–27]. Conversely, the north-to-south increased prevalence of *S. apiospermum* complex and *Lomentospora* species may be explained by the climatic characteristics of these genera which are composed of thermophiles species having a geographic distribution directly related to organic pollution [28]. The observed distribution of

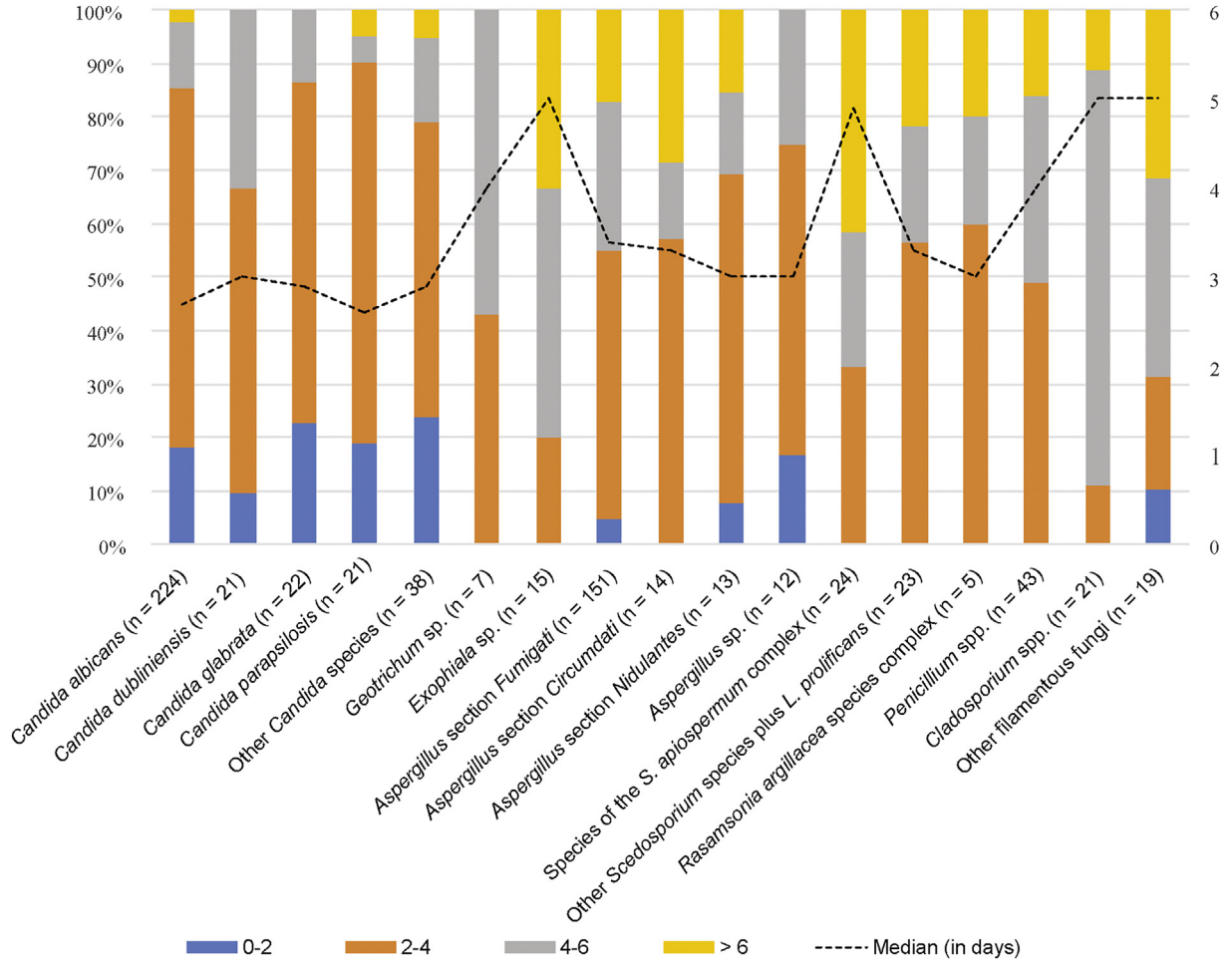

Fig. 3. Estimated delay for the first detection reported in any of the plated media and proportion of corresponding isolates.

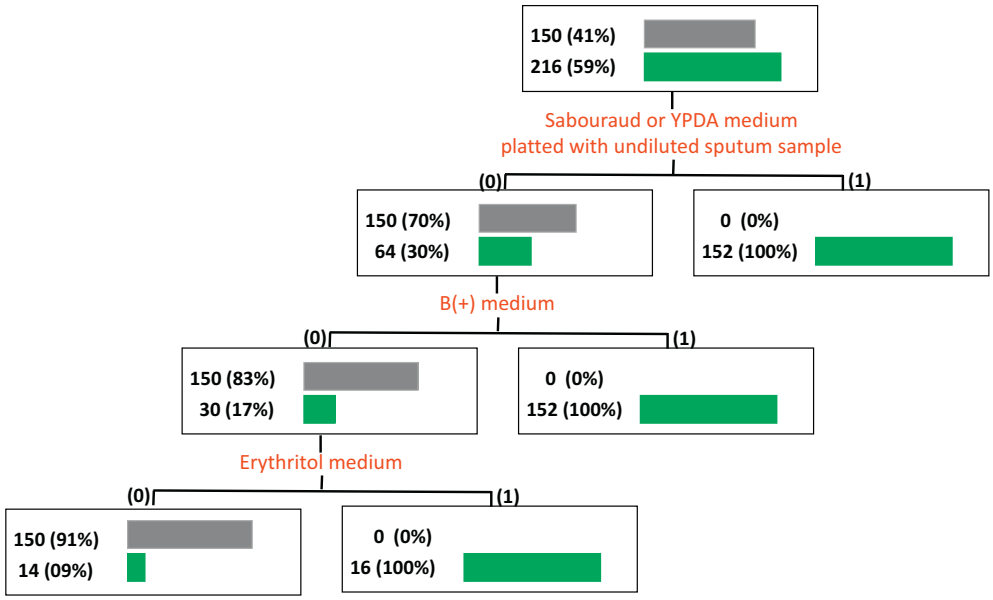

Fig. 4. CHAID deduced combination of three media for routine mycological analysis of sputum samples in the context of CF. (Among sputum samples with positive cultures, 216 sputa (▮) corresponded to those with isolates identified as either *Aspergillus*, *Scedosporium*, *Lomentospora*, *Exophiala*, *Rasamsonia argillacea* complex, *Cladosporium* species and 150 (▮) to the sputum sample with negative culture regarding our selection. For each node, dichotomic branches referred to a successful identification of a positive sample (1) versus a failure (0) to identify a sputum with positive culturing for any of the selected fungal species. The CHAID statistical detection tree technique is based upon adjusted significance testing, providing highly predictive algorithms of optimal detection of selected fungal species).

CFTR mutations that show a decreasing gradient of Delta F508 mutation in Europe with an increasing gradient along the North-South axis of G542X mutation plus mutation heterogeneities in Mediterranean countries may also be considered as a factor promoting fungal colonization [29–31]. However due to the ethical complexity of setting up an international study, exclusively age and sex of CF patients were collected, which limit the clinical interpretation of our findings.

As recently proposed [5,6,11], optimizing and standardizing routine laboratory culture methods to ensure an efficient recovery of fungi from CF sputum sample is nowadays warranted. Based on MFIP results and CHAID method analysis, after a mucolytic chemical pre-treatment of sputum samples, a protocol combining at least YPDA or Sabouraud, and B(+) media incubated at 37 °C and 25 °C–30 °C for 15 days appeared as the best protocol to accurately detect the most frequent and clinically relevant molds in CF context. The use of an additional medium is highly recommended according to the patient's clinical status, as follows: (i) in the absence of bacterial etiology of respiratory alterations, YPDA or Sabouraud medium supplemented with chloramphenicol and cycloheximide should be added, (ii) when patient is colonized with *Exophiala* species, plating an erythritol medium is recommended, and (iii) when patient is chronically colonized *S. apiospermum* complex or *Lomentospora* species, the use of *Scedosporium*-selective medium is recommended.

On the whole, MFIP study has provided an evidence-base for national and international laboratory guidelines, to adopt optimal culture-based isolation protocols. To date, there has been a paucity of information regarding those methods that clinical microbiology laboratories, serving CF centers, have been performing. Two very recent reports examining current laboratory practice from the UK and the Spain showed respectively that 91% and 100% of laboratories currently employ Sabouraud medium and none of the other additional media employed in the current study [32,33]. MFIP survey (provided in the supplementary material pages 1–2) also revealed wide variation in laboratory practice: (i) employment of different media and additional antibiotics, (ii) incubation temperature, and (iii) incubation duration. Equally, MFIP study also indicated that there was a willingness for such laboratories to modify their practice in the presence of robust new evidence, as reported here.

In fact, given the fundamental role of fungi into the lung microbiome [34,36], having an exhaustive picture of the lung mycobiome is of major interest to decipher interactions between the mycobiome and other lung microbes especially bacteria flora, with host physiology, and in pathogenic or mutualistic phenotypes. Beside molecular evidences that the lung mycobiome has a significant impact on clinical outcome of chronic respiratory diseases such as CF, developing mass spectrometry and new culture methods may allow rapid progress to identify, to characterize, and to phenotype new fungal species emerging as clinically relevant for CF patients, culturomics being complementary to metagenomics.

To conclude, this is the first study organized at an international level, aiming at sharing and coalescing our experience to investigate fungal risk in CF. In fine, the use of such standardized protocol may allow us to overcome the major bias associated to the wide heterogeneity of current mycological procedures, to have an accurate overview of the fungal risk and therefore to have a better analysis of the lung mycobiome on the culturomics side. Implementing such standardized protocol is of major interest for understanding the fungal airway colonization determinants and for drawing an exhaustive map of the CF mycobiome at the international scale.

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Contributors

LD was the main author of the article. LD and J-PB contributed to the study design. LD, and KT coordinated this observational study analysis, from collecting to analyzing the data. All the authors participated to the study and worked on subsequent drafts of the manuscript. The authors would like to thank Professor John E. Moore for his helpful discussion.

Declaration of interests

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2018.10.001.

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