Scedosporium species and Lomentospora prolificans in Italian cystic fibrosis patients: prevalence and distribution in seven centers using a selective medium

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Summary

Background and aims. *Scedosporium* species and *Lomentospora prolificans* are the filamentous fungi isolated more frequently from the cystic fibrosis (CF) lower airways, after *Aspergillus fumigatus*. Previous studies showed that, in CF patients, *Scedosporium* species and *Lomentospora prolificans* are responsible for airways colonization/infection, enhancing pulmonary deterioration, and for severe invasive infections in CF patients undergoing lung transplantation. Aims of our project were to evaluate the prevalence of *Scedosporium* and *Lomentospora* species in Italian patients with CF, and to evaluate the interest of the *Scedosporium*-selective colture medium SceSel compared to routinely used agar-based culture media, in order to improve laboratory diagnostic performances.

Materials and methods. A total of 1977 sputum samples from 1154 CF patients were collected and processed according to CF Italian recommendations in the year 2017 (January 1st to December 31st). The SceSel agar was used in addition to the routine culture procedures to burst growth and isolation of *Scedosporium*/*Lomentospora* species. The fungal isolates were identified by classical mycological methods and polymerase chain reaction-based DNA sequencing of ITS1 and ITS4 regions. Susceptibility of the isolates to antifungal drugs was investigated by E-test method.

Results. Among the 1154 enrolled patients, 62 (5.3%) were positive for *Scedosporium*/*Lomentospora* species (median age: 27y; range 8-64y; 33 male). Out of 1977 samples, 93 were positive for *Scedosporium*/*Lomentospora* species, and 13 (13.9%) were recovered only on SceSel agar.

According to molecular analyses, isolation rates of each species were: *S. apiospermum* 53.1%, *S. boydii* 37.5%, *L. prolificans* 6.3% and *S. aurantiacum* 3.1%.

Amphotericin B minimum inhibitory concentration (MIC) values were above or equal to 2 mg/L in all strains. Voriconazole MIC values were below or equal to 0.38 mg/L in almost all strains, proving this drug to be the most effective antimycotic for members of *Scedosporium* species. Otherwise, *Lomentospora prolificans* showed to be resistant to all considered antifungal drugs, only Voriconazole seems to be active on some *Lomentospora prolificans* isolates.

Conclusions. Our results suggest that SceSel agar should be used in combination with routine media as standard microbiological protocol and procedures are not always adequate to isolate *Scedosporium* and *Lomentospora* species in CF respiratory samples. Molecular identification and susceptibility tests are needed, especially for strains isolated from critical patients and those considered for transplantation, as they might develop invasive scedosporiosis.

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Introduction

Cystic fibrosis (CF) is a genetic disorder that affects mostly the lungs, but also the liver, pancreas, kidneys, and intestine. Particularly, in the CF lung, an impaired mucociliary clearance and thick mucus create an ideal environment for microbial colonization. Bacteria typically associated with CF bronchopulmonary disease are *Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepacia* complex and other emerging CF pathogens (4).

Besides bacteria, moulds and yeasts are isolated with increasing frequency in CF patient's respiratory secretions. The thick mucus in CF airways represents a source of nutrients supporting the germination of inhaled spores, ubiquitous in the environment, and the growth of fungi. Among yeast, *Candida albicans* can be isolated in up to 75% of CF clinical specimens; occasionally other *Candida* species, such as *C. parapsilosis* and *C. glabrata,* may be found (2). Members of *Scedosporium* species and *Lomentospora prolificans* are the second most frequently isolated (6-15%) filamentous fungi from the CF lower airways next to *Aspergillus fumigatus* (8,13). Previous studies showed that, as *A. fumigatus*, *Scedosporium/Lomentospora* species contribute to the progression of pulmonary deterioration of CF patients and are responsible for opportunistic infections in immunocompromised patients as well as in healthy subjects. Moreover, as lung transplantation remains the ultimate treatment for end-stage lung disease in CF patients, susceptibility to fungal infection secondary to immunosuppression has become one of the major challenges to deal with (1).

Detection of *Scedosporium/Lomentospora* species infection/colonization in CF patients depends on adequate cultural and molecular diagnostic protocols. Routinely, sputum cultural analysis is carried out on media not specifically selective for *Scedosporium/Lomentospora* species as Sabouraud plus chloramphenicol (Sab). On these media, the rapid growth of gram-negative bacteria, especially *Pseudomonas aeruginosa* mucoid phenotype, or *A. fumigatus* and *Candida spp* at high load may interfere with the slow development of *Scedosporium/Lomentospora* species colonies (14). In a previous multicenter study, conducted by CF Italian centers during 2009 and 2010 (data not published), the prevalence of *Scedosporium/Lomentospora* species accounted for around 4%, consistent with the values (3-18.6%) reported in other European countries (2,13). Presumably, the prevalence rate may have been underestimated as none of the involved centers, at that time, made use of any *Scedosporium/Lomentospora* selective agar. Therefore, an Italian multicenter study, that includes a culture medium selective for *Scedosporium/Lomentospora* species, has been planned. The aim of this study is to evaluate the growth of *Scedosporium/Lomentospora* species on a selective agar versus the growth on routinely used agar, in order to determine the prevalence value and compare it with the one obtained in the previous study. Molecular analysis was also carried out to define isolation frequencies of each species.

In addition, we carried out susceptibility tests for the top five antifungal drugs commonly used in clinical practice.

Materials and Methods

Cultural analysis

Over a one-year period, starting from January 2017 to December 2017, at the microbiology laboratories of seven Italian CF centers adhering to this study (Ancona, Atri, Firenze, Genova, Milano, Roma, Napoli), sputum samples were collected and processed according to CF Italian recommendations (15).

Briefly, samples were diluted 1:5 with a mucolytic agent (Dithiothreitol, Copan,USA) and 20 µl of diluted sample were plated on routine agar media, including Sabouraud agar with chloramphenicol (Sab) (Biomerieux, France) and *Burkholderia cepacia* selective agar (BCSA) (Becton Dickinson, USA). A SceSel agar (Liofilchem, Italy) was added to burst growth and isolation of *Scedosporium/Lomentospora* species isolates. We decided to consider BCSA plates also, as this medium allows the growth of almost all species of fungi. After an early 2-days incubation at 35°C and a first examination of Sab, BCSA and SceSel, follow-up inspections after 10 and 15 days since inoculation were carried out. All species of moulds, number of colonies of each species and Gram-negative bacteria colonies were recorded at each time point.

All *Scedosporium/Lomentospora* species isolates were identified, on genus level, by macroscopical morphology of colonies and microscopical features on lactophenol cotton blue mount. Species molecular identification by means of Sanger sequencing method, as well as susceptibility tests, were performed at the microbiology laboratory of Milano.

DNA extraction

Total genomic DNA from the fungal mycelium after a 48h incubation in Brain-heart infusion Broth (BHI) was extracted, using the UltraClean® Microbial DNA Isolation kit (Mo Bio). Extracted DNA was quantified with Nanodrop Spectrophotometer (Thermo Scientific) to obtain a final concentration of 30 ng/µL, suitable for performing the next step of amplification.

PCR and sequences analysis

Three primers, previously selected for their efficiency in discriminating species within *Scedosporium/Lomentospora* species , were used to amplify total DNA samples. The PCR involved the amplification of the ITS region, using forward and reverse primer, ITS1 and ITS4. Amplification was carried out in a thermocycler (mastercycler Nexus gradient, Eppendorf) programmed for 40 cycles, each consisting of 30 seconds denaturation step at 94°C, 1 min annealing step of at 55°C and 1 min elongation step of at 72°C. Amplicons were separated by electrophoresis in 2% agarose. When the PCR provided insufficient amount of amplicon, a seminested PCR reaction was performed using the forward ITS86 and the reverse primer ITS4. After a purification step, two sequence reactions for each purified amplicon have been carried out, using separately the reverse primer ITS4 and the forward primer ITS1, or ITS86. The sequence reactions were performed for 25 cycles, each consisting of denaturation step of 10 seconds at 96°C, annealing step of 5 seconds at 50°C and elongation step of 4 min at 60°C.

After a further purification stage, sequences were obtained by the ABI Prism 3100 Genetic Analyzer capillary electrophoresis system (Applied Biosystem), analysed with the specific program BioEdit and compared with those recorded in GenBank database.

Antifungal susceptibility testing

In vitro susceptibility to antifungals was determined on RPMI agar supplemented with 2% glucose (Biomerieux, France) using E-test strips of Voriconazole (VCZ), Posaconazole (PCZ), Itraconazole (ITC), Amphotericin B (AMB) and Caspofugin (CAS) containing concentration gradients from 0.002 to 32 mg/L for each drug. The conidial inocula were set at 0.5 McFarland and then diluted 1:5 in saline water. The obtained suspensions were plated with a cotton sterile swab on RPMI agar plates and antifungal strips were then deposited on the plates; at the same time, a sab

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agar plate was inoculated with the same conidia suspension to monitor growth and density of the inoculum.

MIC values were evaluated after a 24/48 hours of incubation at 35°C, as the point of intersection of the border of the elliptical inhibition zone and the E-test strip. For CAS, MEC values were evaluated as the point of intersection of the border between the normal and the anomalous growing colonies and the strip.

As reference strain, *A. fumigatus* ATCC 204305 was tested: results were within the recommended CLSI range.

Results

A total of 1977 sputum samples from 1154 CF patients (median age: 25y; range 8-64y; 593 male), out of the 2281 attending the involved centers, were evaluated in this study.

The number of the positive samples per each medium and center is shown in Table 1.

Sixty-two patients were positive for *Scedosporium/ Lomentospora* species (median age: 27y; range 8-64y; 33 male). No statistically significant difference was observed between male and female patients irrespective of age, as calculated using the t student test (t=0.03; p>0.1). The overall prevalence of *Scedosporium/ Lomentospora* species was 5.3%, ranging within the seven centers from 1.9% to 9.8% (Table 2).

Ninety-three samples were positive for *Scedosporium/ Lomentospora* species: 13 (13.9%) were recovered only on SceSel agar. Four samples (4.3%) showed *Scedosporium/ Lomentospora* species growth only on Sab: for three samples, *Scedosporium/ Lomentospora* species failed to grow on SceSel, while in one case the massive growth of other microorganisms covered *Scedosporium/ Lomentospora* species colonies (Table 1).

According to molecular analysis, rates of isolation of each species were: *S. apiospermum* 53.1%, *S. boydii* 37.5%, *L. prolificans* 6.3% and *S. aurantiacum* 3.1%. (Table 3). No other *Scedosporium* species were detected.

In six patients we could recognise two strains phenotypically different but belonging to the same species: *S. apiospermum* in 3, *S. boydii* in 2 and *S. aurantiacum* in 1 patient, respectively.

Resistance pattern of *Scedosporium/Lomentospora* species isolates is reported in Table 4 and Figure 1. Only VCZ reveals low MIC values for all species; all isolates display high MIC values to AMB. *Lomentospora prolificans* proves to be resistant to all considered antifungal drugs.

Discussion and Conclusions

The focus of the microbiological follow-up of CF patients is almost exclusively on bacteria. Besides the bacteria, many different fungal species are also isolated from the CF airways, and an increasing body of evidence support the role of fungi in the pathogenesis of CF disease. The prevalence rates vary considerably between CF centers, above all because of differences in the methodology used in fungi recovery and identification. Previous studies showed that nonselective culture media are inadequate for an accurate diagnosis of fungal infection and that selective fungal culture media and prolonged incubation periods can provide significantly higher rates of fungi detection (6). In particular, some selective agar have been conceived to enhance growth and isolation of *Scedosporium/Lomentospora* species isolates (7,3). In this study, we tested SceSel agar with the composition published by Rainer et al. in 2008. SceSel is a variation of modified Leonian's agar containing dichloran and benomyl, wich, inhibiting Hyphomycetes (mainly *Aspergillus fumigatus*) and Zygomycetes, showed a superior performance for isolating *Scedosporium* (7).

Out of the 1.977 sputum samples we could evaluate, 93 were found positive and the 13.9 % of positive samples were recovered

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Table 1. Number of positive samples on each medium and for each center.

	Milano	Teramo	Genova	Roma	Firenze	Napoli	Ancona	Total
Studied samples	567	77	136	303	259	550	85	1977
Positive samples	27	6	3	28	13	3	13	93
Positive samples on Scesel	26	6	3	26	13	3	12	89
Positive samples on Sab	24	6	3	24	8	3	12	80
Positive samples on BCSA	18	0	0	1	2	3	8	32
Positive samples only on Scesel	3	0	0	4	5	0	1	13
Positive samples only on Sab	1	0	0	2	0	0	1	4

Table 2. Characteristics of enrolled and positive patients in each CF Center.

	Milano	Teramo	Genova	Roma	Firenze	Napoli	Ancona	Total
Total enrolled pz								
N.	345	55	127	173	233	160	61	1154
Male-Female	189-156	25-30	65-62	76-97	126-107	86-74	26-35	593-561
Median age (range)	27 (1-63)	20 (8-52)	27 (1-67)	20 (4-48)	28 (6-57)	22 (4-73)	19 (3-64)	25 (1-73)
Scedosporium/Lomentospora culture positive samples								
N.	17	3	3	16	14	3	6	62
Male-Female	10-7	1-2	1-2	9-7	9-5	1-2	2-4	33-29
Median age (range)	27 (12-47)	13 (11-14)	26 (19-49)	21 (8-41)	24 (16-50)	32 (29-33)	39 (15-64)	27 (8-64)
Prevalence, %	4.9	5.4	2.4	9.2	6	1.9	9.8	5.3

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Table 3. Isolation rates of each *Scedosporium/Lomentospora* species in each center: numbers and percentages.

	Milano	Teramo	Genova	Roma	Firenze	Napoli	Ancona	Total
S. boydii (%)	9 (47.3)	1 (25)	1 (33.3)	5 (2.09)	4 (36.3)	2 (66.6)	2 (28.5)	25 (37.5)
S. apiospermum (%)	7 (36.8)	3 (75)	2 (66.6)	11 (56.5)	7 (63.6)	1 (33.3)	4 (57.1)	36 (53.1)
S. aurantiacum (%)	1 (5.2)			2 (1,7)				3 (3.1)
L. prolificans (%)	2 (10.5)			1 (4.3)			1 (14.2)	4 (6.3)
Total	19	4	3	18	11	3	7	68 (100)

Table 4. *In vitro* susceptibility of *Scedosporium/Lomentospora* isolates to triazole MIC (Minimal inhibitory concentration), caspofungin MEC (Minimal effective concentration) and mean values (mg/l).

Species	n	VCZ			PCZ			ITC			CAS			AMB		
		range	MIC 90	mean	range	MIC 90	mean	range	MIC 90	mean	range	MEC 90	mean	range	MIC 90	mean
S. apiospermum	28	0.008-0.38	0.38	0.149	0.008- >32	>32	4.57	0.25- >32	>32	17.8	0.38- >32	>32	10.83	2- >32	>32	27.17
S. boydii	22	0.012-0.125	0.94	0.048	0.047-2	1,5	0.67	0.032- >32	>32	9.06	0.25- >32	>32	11.85	3- >32	>32	28.59
S. aurianticum	3	0.032-0.094	0.094	0.073	1-1.5	1,5	1.33	>32	>32	32	0.5- >32	>32	11.08	>32	>32	32
L. prolificans	2	0.75- >32	>32	16.375	>32	>32	32	>32	>32	32	12- >32	>32	22	>32	>32	32

VCZ, Voriconazole; PCZ, Posaconazole; ITC, Itraconazole; CAS, Caspofungin; AMB, Amphotericin B.

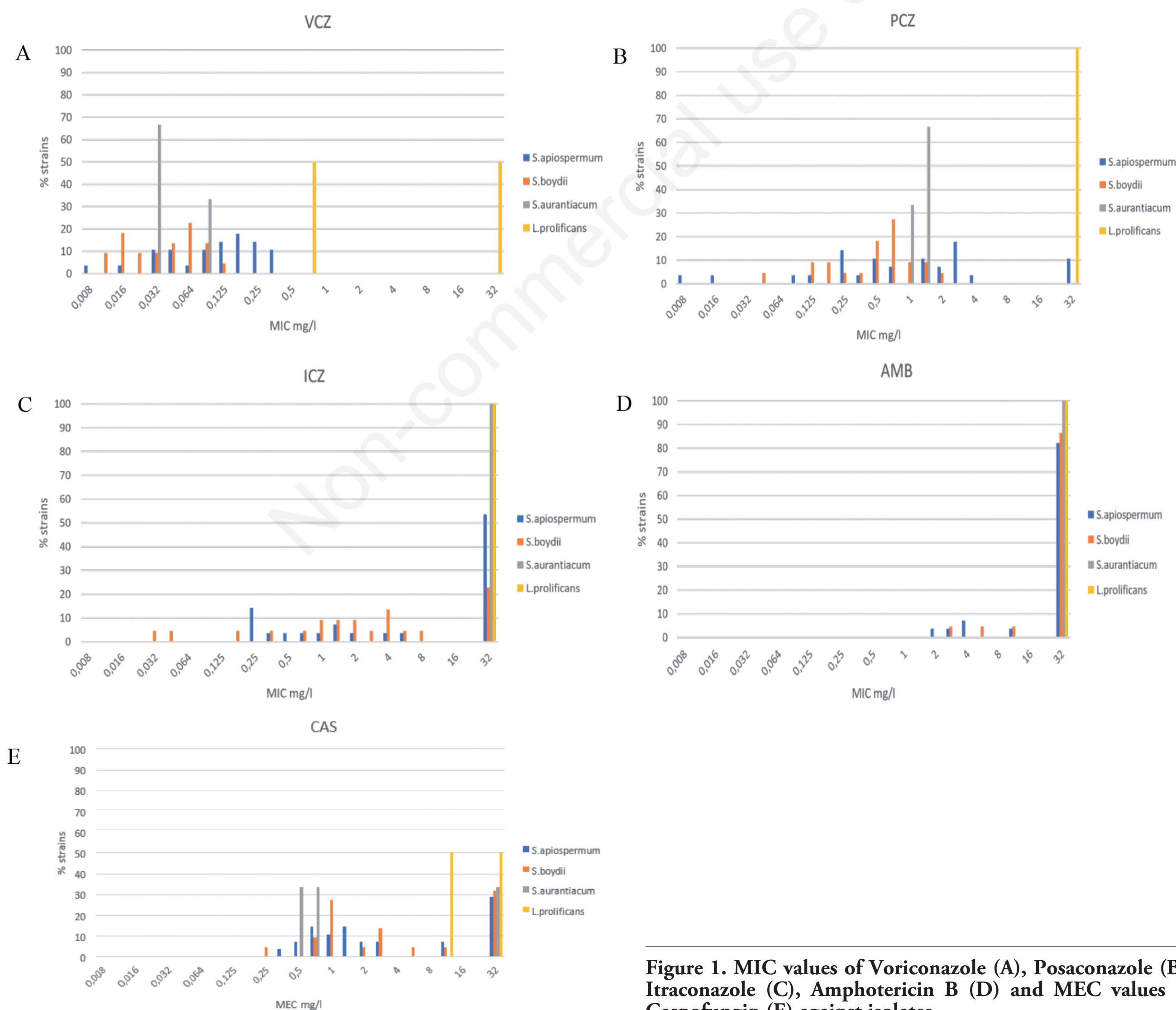

Figure 1. MIC values of Voriconazole (A), Posaconazole (B), Itraconazole (C), Amphotericin B (D) and MEC values of Caspofungin (E) against isolates.

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only by means of SceSel agar, because the faster growth of other microorganisms, as *A. fumigatus* and *P. aeruginosa* , often prevented growth of *Scedosporium/Lomentospora* species on sab agar plates. In contrast, four samples revealed to be positive for *Scedosporium/Lomentospora* species only on Sab medium, and were missed on SceSel, probably due to low load and of the profuse growth of *Aspergillus* spp on SceSel. These results support a multimodal approach for fungi detection from CF bronchial secretions. Using a combination of SceSel and Sab, we may improve our possibility to recognize *Scedosporium/Lomentospora* species colonization/infection, a critical issue considering the fungi impact on the lung disease progression and transplantation in CF patients (12,16).

In this study we could enrol 50.5% of patients in regular follow-up at the CF centers involved and we documented an overall prevalence value of 5.3%, higher than the one we reported in 2010 when only Sab was used as culture medium. Prevalence varied among CF centers: the lower values were found in Genova and Napoli, the higher in Roma and Ancona. Further information is needed to understand if these differences in prevalence are linked to variation in environmental spread of species and/or different therapeutic regimens among centers.

The *Scedosporium* taxonomy is continuously under review. Several species are now recognized, including *S. angustum, S. minutisporum*, *S. dehoogii*, *S. aurantiacum*, *S. desertorum*, *S. cereisporum*, *S. ellipsoideum*, and *S. fusoideum*. *Scedosporium* also comprises *S. apiospermum* and *S. boydii* that are the main clinically relevant species. *S. prolificans* is now identified as a different species and renamed *Lomentospora prolificans,* on account of its distinctive characteristics (10).

The most frequently isolated specie in our study was *S. apiospermum* but, in contrast with the national trend, *S. boydii* was more represented in Milano and in Napoli centers, similarly to epidemiological data reported in 2013 by Zouhair et al. in France (17).

In Italian centers, *L. prolificans* was isolated with a lower rate than in France and Germany (9,11). Moreover, no other species, e.g. *S. minutisporum,* have been identified, except the four mentioned above. Furthermore, contrarily to data reported by Schwarz C. et al. none of our patients showed to be colonized by two different species of *Scedosporium/Lomentospora* species (11).

As described before, resistance pattern was evaluated with the E-test method.

Several studies have compared the E-test with reference methods and have found it suitable for routine work in the clinical mycology laboratory as it is simpler, less time consuming and reproducible (5).

Lomentospora prolificans shows to be resistant to all drugs; in the present study we could test only two of the recovered *Lomentospora prolificans* strains, one of them display low MIC value to VCZ. For the *Scedosporium* isolates, VCZ MICs were below 0.5 mg/L and MIC90 values below 1 mg/L so VCZ proved to be the most effective antimycotic drug against members of the complex. However, comparing our MIC data with those obtained by broth microdilution and published in literature (9), we can see that they are substantial lower. This observation needs to be clarified as VCZ is widely used in antifungal therapy.

Regarding MIC90, *S. apiospermum* shows values of 32 mg/L for both PCZ and ITC, while *S. boydii* only for ITC; however, there were a certain number of strains of *S. apiospermum* and *S. boydii* with low MIC values. S. *aurantiacum* strains seem to be resistant to ITC, showing MIC values higher than 32 mg/L, but PCZ MIC90 value reach 1.5 mg/L.

High MIC values for AMB were confirmed for all strains as well known.

As *Lomentospora* members clearly show to differ from *Scedosporium* members in resistance pattern, and a certain variability is recognized also among strains within the same group, molecular identification and susceptibility tests are recommended, especially for strains isolated from patients with progressive lung deterioration and those considered for transplantation, as they might develop invasive scedosporiosis.

This study clearly demonstrates that an approach based on the use of multiple media enhances the identification of *Scedosporium/Lomentospora* species, emerging pathogens in CF.

Moreover, our expectation is that all the Italian CF centers may apply the same mycological approach in order to better define the *Scedosporium/Lomentospora* species prevalence in all the geographical areas of Italy, first step to settle basis for further insights regarding differences in *Scedosporium/Lomentospora* species prevalence due to environmental conditions.

Since *Scedosporium/Lomentospora* species may strongly affect CF patient care, we may need a better consensus about susceptibility tests especially regarding cheaper and faster methods and the improvement of routinely used susceptibility tests for *Scedosporium/Lomentospora* species in CF microbiological labs.

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