Fungal respiratory infections in cystic fibrosis: diagnostic challenges in the clinical laboratory

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Host-Pathogen Interaction Study Group, EA 3142
Angers University, Angers, France

Anvers, Belgium, 26 October 2014

Fungal respiratory infections in CF

Morbidity and mortality in cystic fibrosis essentially due to chronic respiratory infections

Bacteria, especially *Pseudomonas aeruginosa*: the major cause of these infections

Over recent decades, considerable attention has been paid to :

- **prevention and treatment of bacterial respiratory infections**
- **development of an early diagnosis of CF**
- **improvement in the nutritional status of the patients**

Marked increase in life expectancy

Fungal respiratory infections in CF

Later in age, the respiratory tract of CF patients may also be colonized by various fungal species

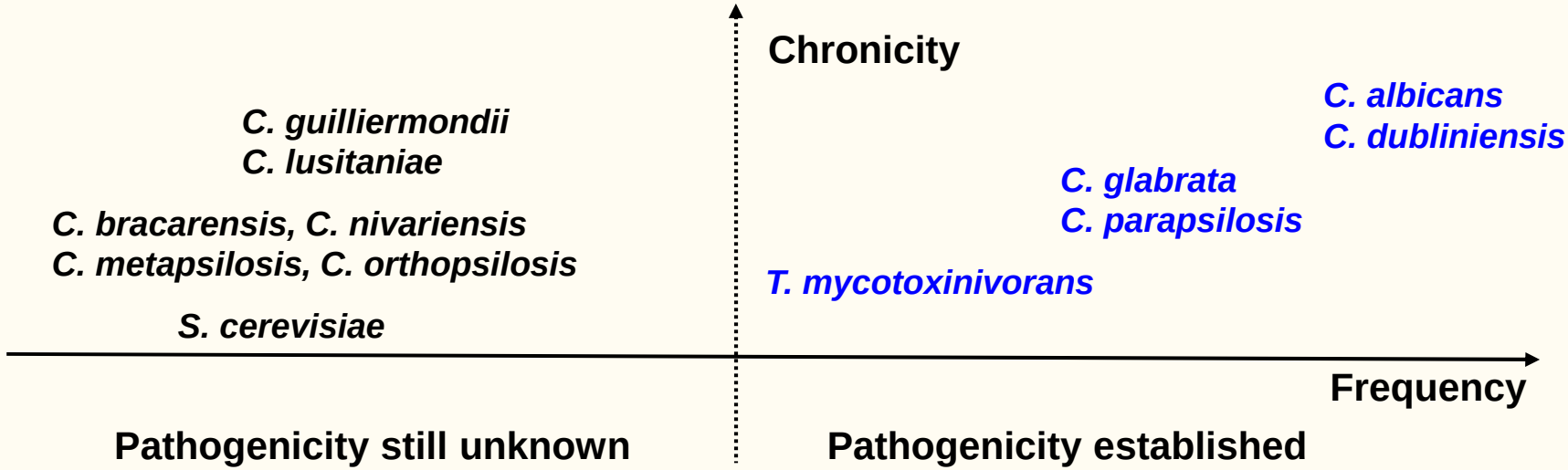
Fungal colonization of the airways facilitated by repeated cures of antibiotics and use of corticosteroids

True respiratory infections the frequency of which regularly increased

However, relatively little progress with regards to fungal respiratory infections.

Yeasts and moulds in CF

Fungal biota in CF: a great diversity



DNA detection from sputum samples : *Malassezia* species

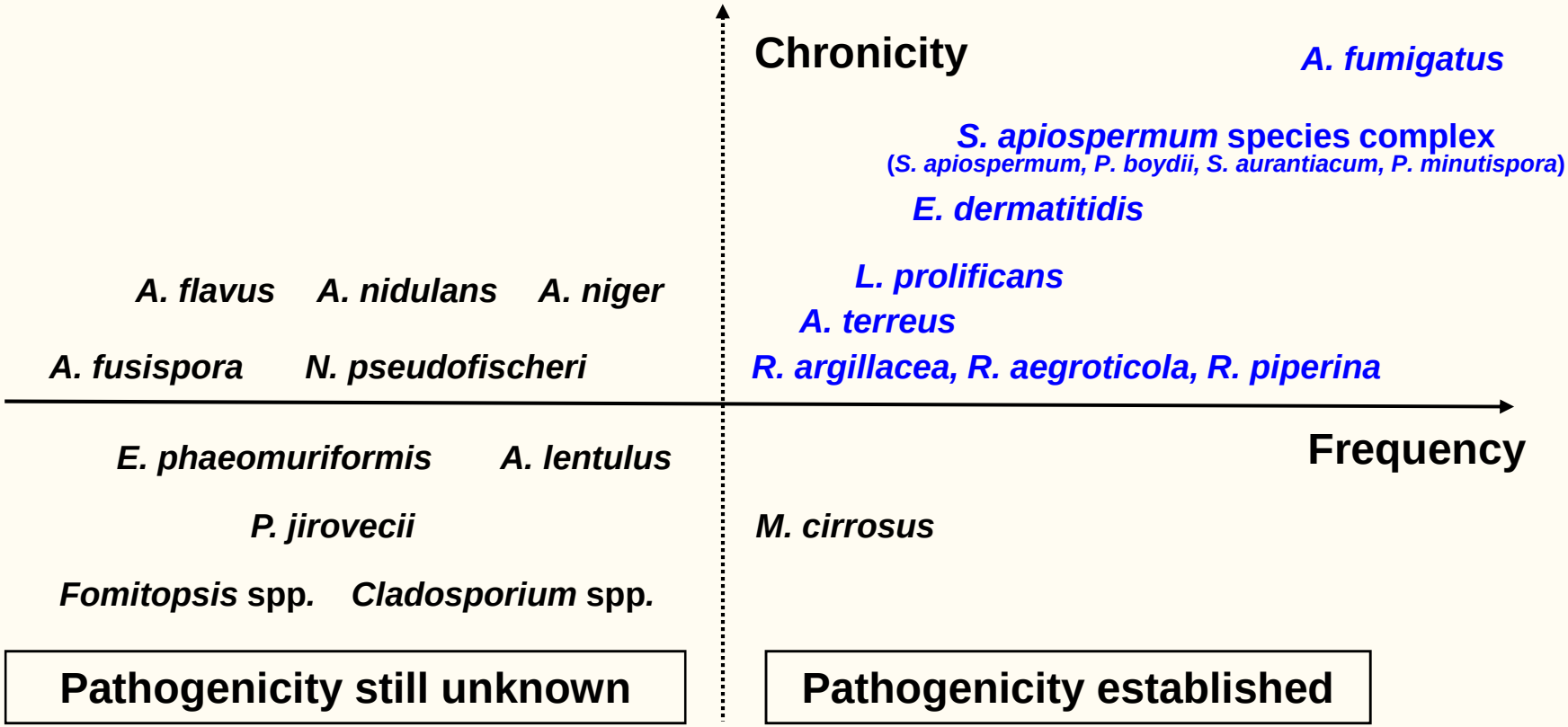
Nagano *et al*., Med Mycol, 2010 ; Delhaès *et al*., PLoS One, 2012

***T. mycotoxinivorans*: recently recognized as a pathogen with a propensity for CF. 5 cases in USA in non transplant patients. 4 were patients with CF (for one patient, repeated isolation from D1 to D11 from sputum, BAL or tracheal aspirates, and from lung biopsy at autopsy)**

Hickey *et al*., 2009

Yeasts and moulds in CF

Fungal biota in CF: a great diversity



DNA detection : *Fusarium culmorum, Acremonium strictum, …*

Nagano *et al*., Med Mycol, 2010

But largely more complex as revealed by microbiome studies

Delhaès *et al.*, PLoS One, 2012

Detecting fungi in CF

Non cultural methods

Detection of fungal metabolites from sputum samples

- **2-pentylfuran (*A. fumigatus*)** — **Syhre *et al*., Med Mycol, 2008**
- **methylcoprogen B (*S. apiospermum*)** — **Bertrand *et al*., Med Mycol, 2010**

PCR-based methods targeting a unique species or a limited number of species

- ***Exophiala dermatitidis*** — **Nagano *et al*., J Cyst Fibros, 2008**
- **Species of the *S. apiospermum* complex** — **Lu *et al*., Mycoses, 2011**
- ***Rasamsonia argillacea* species complex** — **Steinman *et al*., 2014**

Detection and direct identification of the different fungal species that may be encountered in CF

Development of a DNA chip

(Collaboration with T.C. Chang from Tainan, Taiwan)

Detecting fungi in CF

Non cultural methods

Amplification of the ITS regions of rDNA genes by nested-PCR, followed by hybridization on species-specific oligonucleotide probes (23) immobilized on glass slides

Acfus2a+2b	Asfla4	Asfum2a	M	Asnid2	Asnig2
Aster2	CAB5	CDU1a	M	CGL1	CLUS1
CP6	CP8	CP10	M	CT3c	Exder1
M	M	M	NC	M	M
Fopin1	Palil4	Pavar2	M	Psboy3	Scbre3
Scpro4	Taeme4	Taeme6	M	NC	PC

M : orientation of the chip (Dig-ITS4 primer)

NC: negative control

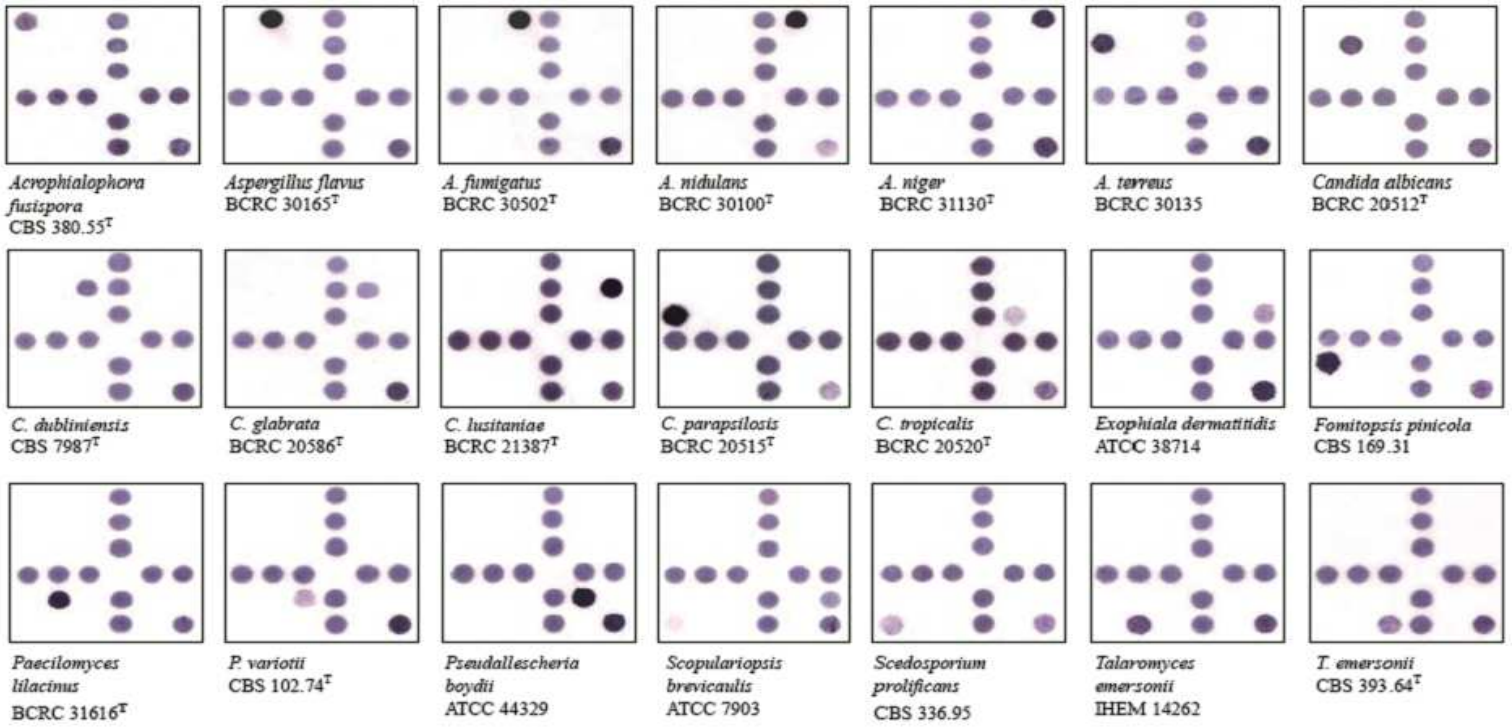
PC: positive control (rDNA 5.8S)

Bouchara *et al*., J Clin Microbiol, 2009

Detecting fungi in CF

Non cultural methods

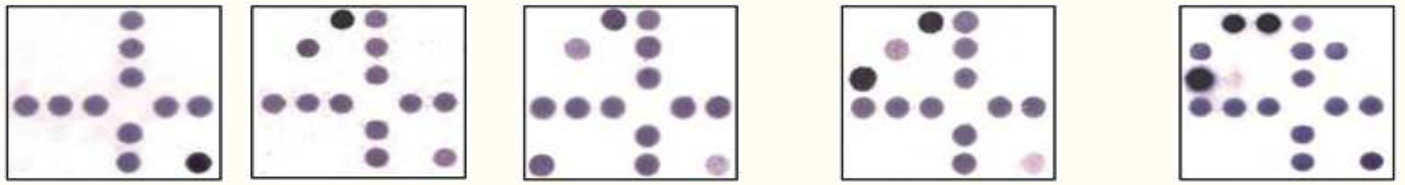
Validation of the DNA chip (specificity of the probes) using DNA extracts from pure cultures of reference strains and clinical or environmental isolates of target (182 strains) and non target species (142 isolates, 135 species)



Bouchara *et al*., J Clin Microbiol, 2009

Detecting fungi in CF

Non cultural methods



Cultures:	**Sterile**	**AF + CA**	**AF + Spro + CA**	**AF + CA**	**Afla + AF + Ater + Cpara**
DNA chip:	**Negative**	**AF + CA**	**AF + Spro + CA**	**AF + CA + Cpara**	**Afla + AF + Ater + Cpara + Cgla**

Aspergillus flavus
Aspergillus fumigatus
Aspergillus nidulans
Aspergillus niger
Aspergillus terreus
Emericella nidulans* var. *echinulata
Candida albicans
Candida dubliniensis
Candida glabrata
Candida lusitaniae
Candida parapsilosis
Candida tropicalis

Acrophialophora fusispora
Exophiala dermatitidis
Fomitopsis pinicola
Paecilomyces lilacinus
Paecilomyces variotii
Penicillium emersonii
***S. apiospermum* species complex**
Scedosporium prolificans
Scopulariopsis brevicaulis

Bouchara *et al*., J Clin Microbiol, 2009

Detecting fungi in CF

Non cultural methods

Pyrosequencing **Delhaès et al., PLoS One, 2012**

Next generation automated sequencing equipments based on PCR-electrospray ionization-time-of-flight/mass spectrometry (PCR-ESI-TOF/MS)

Multiplex PCR targeting the ITS 1 or 2 regions of rDNA genes, *TUB* or *CAL*

Separation of the amplified products after positive electrospray ionization

Final identification by mass spectrometry

PLEX-ID (Abbott-Ibis Biosciences), now stopped for IRIDICA (currently under development) : smaller and easier to use, and thus more conducive to a clinical laboratory setting

Detecting fungi in CF
Cultivation of sputum samples

Nagano *et al*., Med Mycol, 2010

Mycological cultures with antibiotics dramatically increased the number of fungi that could be detected

Masoud-Langraf *et al*., Med Mycol, 2013

….. we recommend homogenizing CF sputa with a mucolyticum, to prepare serial dilutions and to use appropriate fungal culture media with added antibiotics

What culture media should be used ?

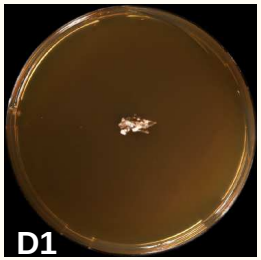
Horre *et al*., Respiration, 2008

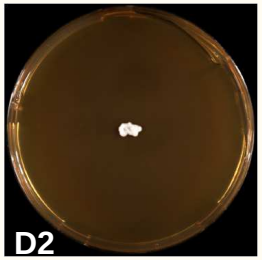
…. standard microbiological media and procedures are not sufficient to detect colonization of the respiratory tract by *Pseudallescheria / Scedosporium* in CF patients.

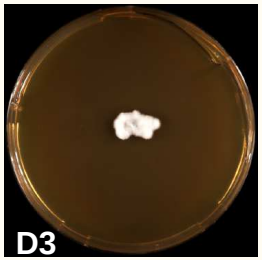
Detecting fungi in CF
Cultivation of sputum samples

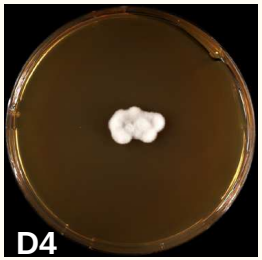
Species of the *S. apiospermum* complex may not be detected because of its usual association with *A. fumigatus* (more rapidly and more extensively growing)

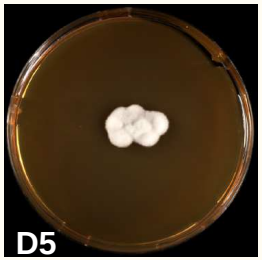
S. apiospermum



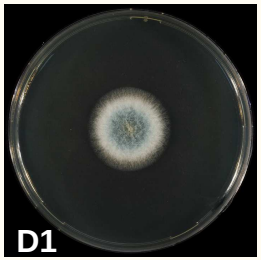


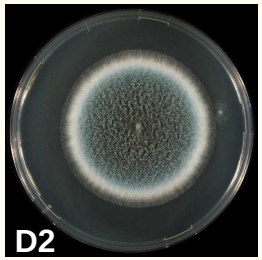


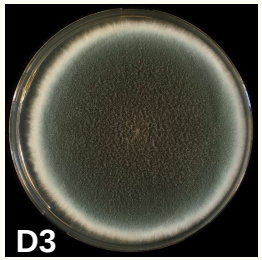


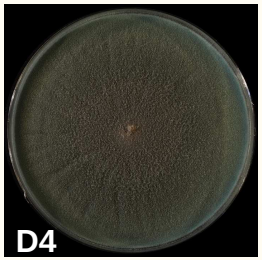


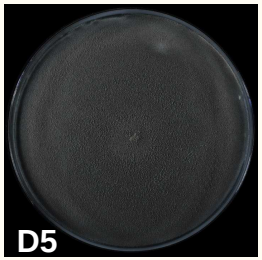
A. fumigatus











A prolonged incubation time (≥ 7 days) and the use of a semi-selective culture medium (YPDA supplemented with cycloheximide) greatly facilitate the detection of species of the *S. apiospermum* complex.

Detecting fungi in CF
Cultivation of sputum samples

Prevalence of the *S. apiospermum* species complex in CF:
8.6% in France - 4.5% and 6.5% in Austria - 5.3% in Germany- 11.6% in Australia

Cimon *et al*., Eur J Clin Microbiol Infect Dis, 2000
Rainer *et al*., Med Mycol, 2008 ; Masoud-Langraf *et al*., Med Mycol, 2013
Horre *et al*., Respiration, 2009
Harun *et al*., Med Mycol, 2010

Lack of standardization of the procedures used for mycological examination of respiratory secretions from CF patients

- **inoculation of the samples on agar slants**
- **number and nature of the culture media used (absence of semi-selective culture media)**
- **too short incubation time**

Borman *et al*., Med Mycol, 2011

Detecting fungi in CF
Cultivation of sputum samples

French Society for Microbiology – French Society for Medical Mycology – patient organization

Guidelines for microbiological examination of sputum samples in CF

Prior homogenization of the samples with a mucolyticum

Plating in parallel on :

- **chromogenic medium for cultivation of yeasts and easy detection of mixed populations**
- **yeast extract-peptone-dextrose-agar (YPDA) containing antibiotics (Cmp + gentamicin)**
- **YPDA with Cmp (0.5 g/L) and cycloheximide (0.5 g/L)**

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Detecting fungi in CF
Cultivation of sputum samples

Other semi-selective culture media can be more suitable

- **B^+ culture medium** — **Nagano *et al*., J Cyst Fibros, 2008**
- **Sce-Sel^+ culture medium** — **Rainer *et al*., Antonie Van Leeuwenhoek, 2008**
- **Dichloran-rose Bengal agar + Cmp + Benomyl (DRBC-benomyl) , developed for isolation of molds from food** — **King *et al.,* Appl Environ Microbiol, 1979**

	2006 n = 251 (78)	2007 n = 253 (84)	2008 n = 178 (71)
YPDA	23 (7)	15 (7)	12 (3)
YPDA + cycloheximide	35 (8)	24 (7)	13 (4)
DRBC + Benomyl	41 (8)	35 (10)	20 (6)

Detecting fungi in CF
Cultivation of sputum samples

MFIP study (coordinated by L. Delhaès and J.P. Bouchara)

Objectives:

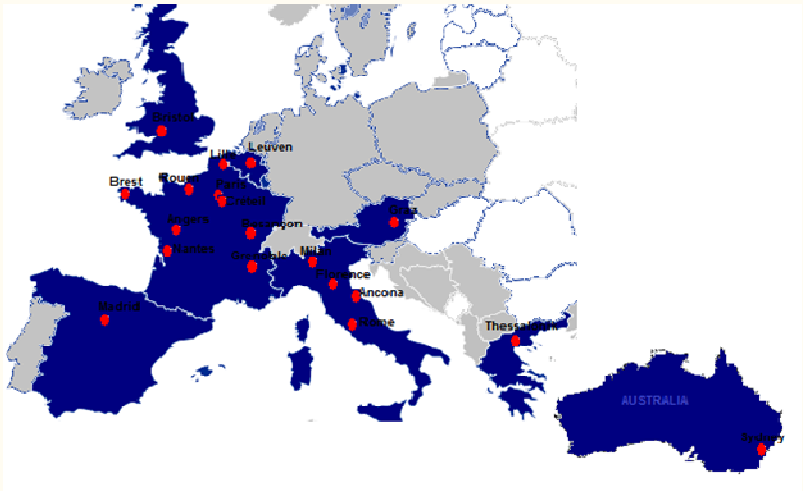
Compare culture media and different temperatures or durations of incubation to determine the best combination of culture media to be used for mycological examination of sputum samples from CF patients

Study design:

Multicenter international study conducted prospectively

19 labs:

France	9
Italy	4
Spain	1
UK	1
Belgium	1
Austria	1
Greece	1
Australia	1



Detecting fungi in CF
Cultivation of sputum samples

Pretreatment of the samples

Digestion of the sample (equal volume of mucolytic agent)

15 s homogenization
15 à 30 min à 37° C

Quantitative inoculation: 20 µl per plate

1. Chromogenic culture medium	**37° C**
2. YPDA + ATB	**37° C**
3. DRBC-benomyl	**37° C**
4. Sce-Sel+ medium	**37° C**
5. YPDA + Cmp (0.5 g/L) and cycloheximide* (0.5 g/L)	**37° C**
6. B+ medium*	**20-25° C**
7. Erythritol Cmp agar	**30° C**

1:10 dilution for early detection of fungal colonization
[A. Borman unpublished data]

8. YPDA + ATB	**37° C**

Incubation of the plates during 15 days
Identification of fungi by conventional procedures

Results to be analyzed using the Chi-squared Automatic Interaction Detector method to determine the best combination of culture media to be used

Detecting fungi in CF
Identification methods

Accurate identification at the species level required:

- **better knowledge on the epidemiology of fungal colonization of the airways/respiratory infections in CF**
- **may provide information about the origin of the contamination of the patient**
- **to guide the antifungal therapy**

Species identification within the *S. apiospermum* complex

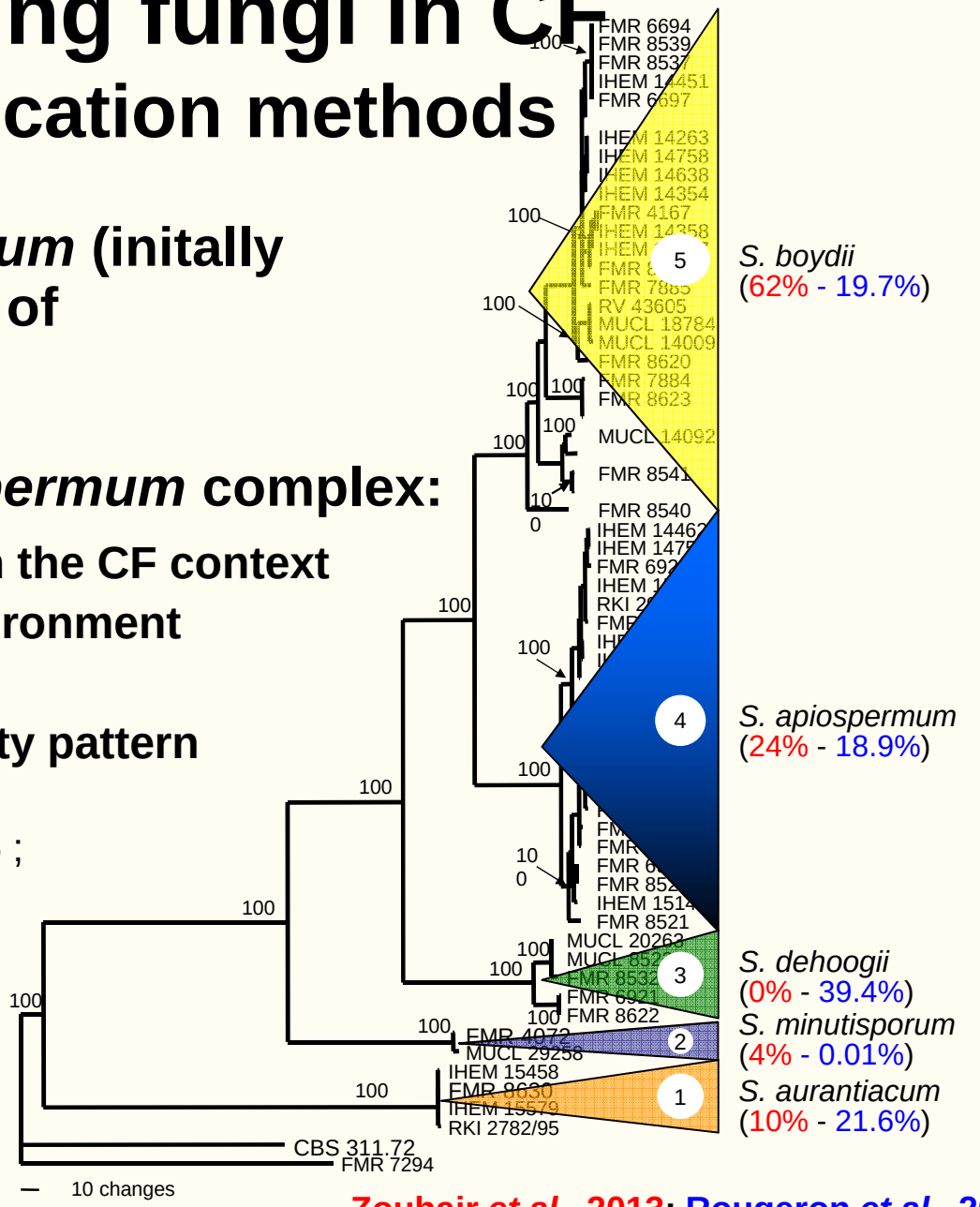
Detecting fungi in CF
Identification methods

***Scedosporium apiospermum* (initally considered the anamorph of *Pseudallescheria boydii*)**

The five species *S. apiospermum* complex:

Differences in their frequency in the CF context
and their distribution in the environment
as well as in their virulence
and their antifungal susceptibility pattern

Gilgado *et al.*, J Clin Microbiol, 2005 ;
J Clin Microbiol, 2008;
Med Mycol, 2010



Zouhair *et al.*, 2013; Rougeron *et al.*, 2014

Detecting fungi in CF
Identification methods

Species identification within the *Scedosporium apiospermum* complex cannot be achieved by phenotypic methods

- **Morphological examination**
- **Physiological tests (resistance to cycloheximide, maximal growth temperature, …)**
- **Conventional biochemical studies (maltose assimilation)**

Sequencing four loci in the fungal genome

- **ITS 1 and 2 regions of rDNA genes**
- **Two loci in the β-tubulin gene (β-TUB and TUB2)**
- **One locus in the calmodulin gene (CAL)**

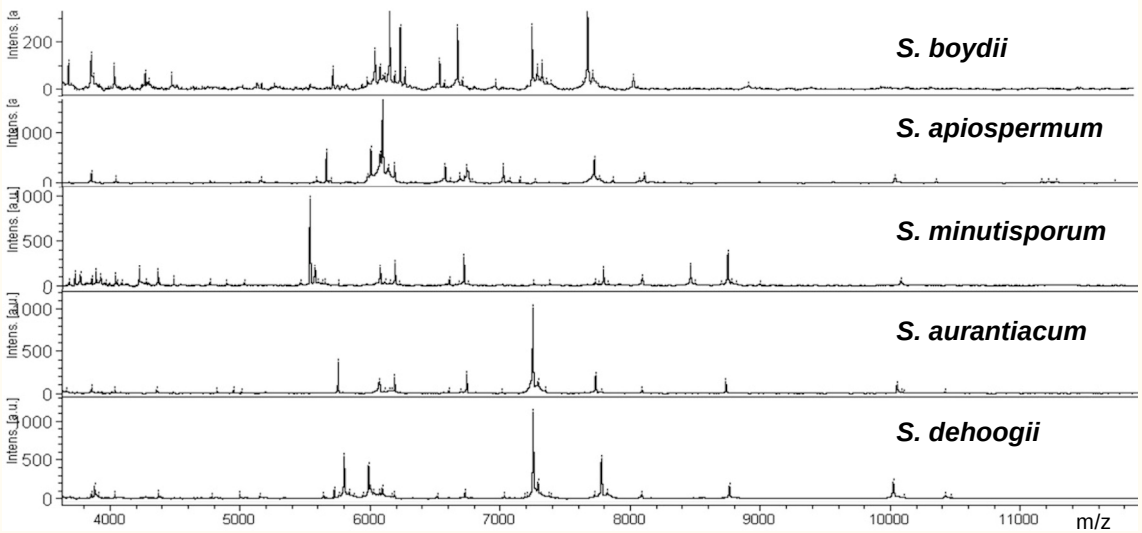
MALDI-TOF/mass spectrometry may be helpful for differenciation of sibling species within species complexes

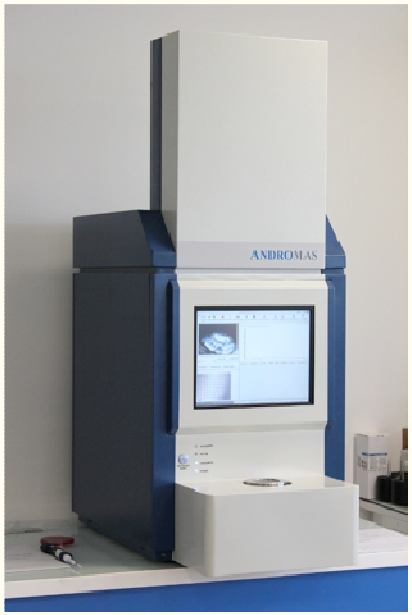
Detecting fungi in CF
Identification methods

MALDI-TOF/mass spectrometry (collaboration with M.E. Bougnoux and the Andromas team)

No prior extraction of proteins - No standardization of the age of the cultures

Reference spectra acquired from 3-, 5- and 7-day-old cultures of reference strains. Superspectra. Validation using a large number of clinical or environmental isolates





Sitterlé *et al*., 2014

Detecting fungi in CF
Clinical significance – serological studies

Clinical and radiological signs and results from bacteriological examination of respiratory secretions

Detection of serum specific antibodies may help to determine the clinical significance of fungal detection

In the absence of serum specific IgE, detection of serum specific IgG allows the differenciation between non diseased colonized CF patients, and patients with bronchitis

Baxter *et al*., J. Allergy Clin. Immunol., 2013

But commercially available only for *A. fumigatus*

Detecting fungi in CF
Clinical significance – serological studies

Regarding *Scedosporium* species:

Antibody detection performed only in a few specialized laboratories by counter-immunoelectrophoresis using home-made crude antigenic extracts – not for epidemiological studies

Development of ELISA for epidemiological studies

Parize *et al*., J. Cyst. Fibros., 2014

But possible cross-reactions with *A. fumigatus* using crude extracts

- **Many polysaccharides common to the different fungal species encountered in CF (mannans, ß-glucans and chitin)**
- **Proteins shared by these fungi (*e.g.* enzymes involved in essential metabolic pathways like ergosterol synthesis, …)**

Detecting fungi in CF
Clinical significance – serological studies

Characterization of catalases produced by *S. boydii* (Cat A1, Cat A2, Cat A2')

Purification of Cat A1, a promising target for development of specific antibody detection assays **Mina *et al.*, CVI, in press**

Development of an ELISA assay which was evaluated with sera from CF patients:

- **No fungi recovered from sputum samples, no serum antibodies (group A)**
- ***A. fumigatus* exclusively and exclusive presence of serum anti-*A. fumigatus* antibodies by CIE (group B)**
- ***S. apiospermum* or *S. boydii* exclusively and exclusive presence of serum anti-*S. boydii* antibodies by CIE (group C)**

High sensitivity and no cross reactions with *A. fumigatus*

Detecting fungi in CF
Clinical significance

In most cases, no specific antibodies

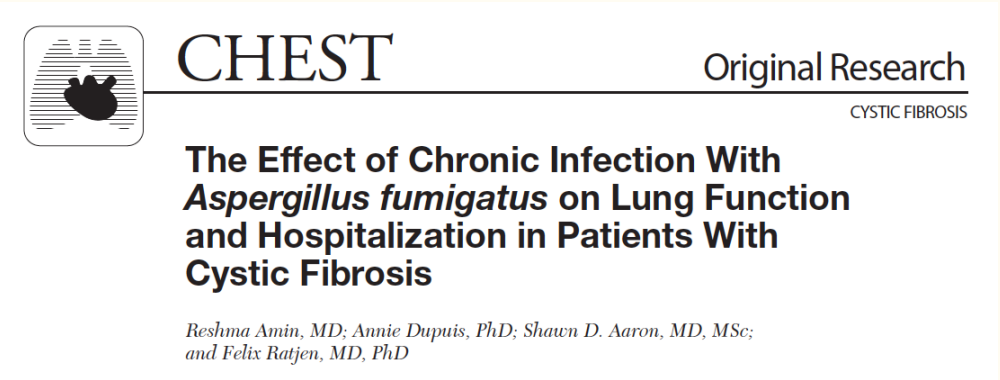
Moulds in the CF airways : not innocent bystanders

- **Usually living in the outdoor environment as saprophytes**
- **Not commensals of the respiratory tract**

Chronic colonization contributes to the inflammatory reaction progressively leading to a clinical or functional deterioration

Amin *et al*., Chest, 2010

Retrospective cohort study
230 CF patients followed-up
in Toronto during 5 years


CHEST Original Research

CYSTIC FIBROSIS

The Effect of Chronic Infection With *Aspergillus fumigatus* on Lung Function and Hospitalization in Patients With Cystic Fibrosis

Reshma Amin, MD; Annie Dupuis, PhD; Shawn D. Aaron, MD, MSc; and Felix Ratjen, MD, PhD

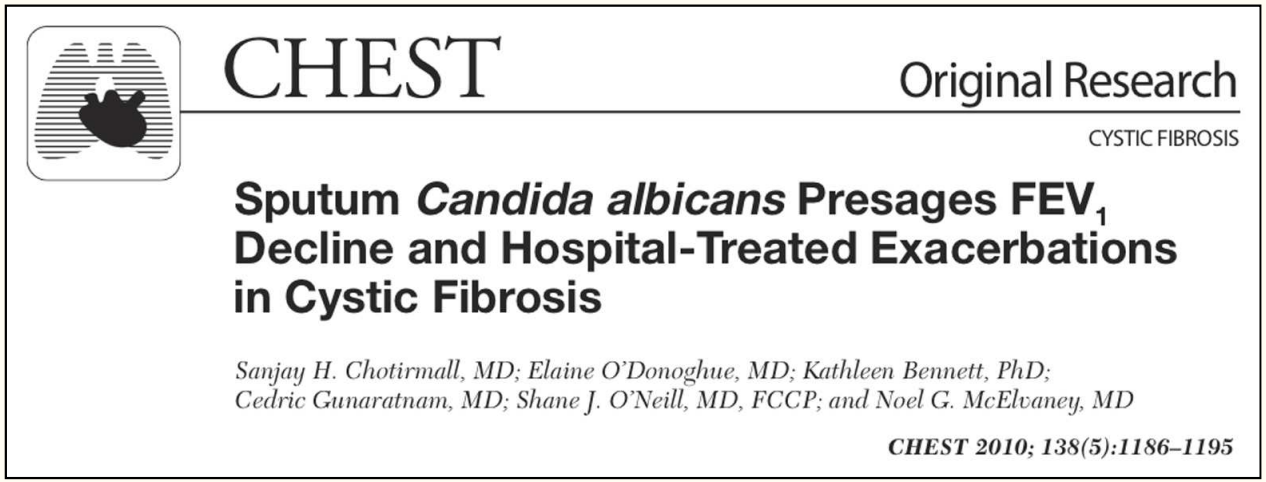
The chronic colonization
of the airways by *A. fumigatus*:
an independent risk factor for hospital admissions in patients with CF

Detecting fungi in CF
Clinical significance

Assessment of *Aspergillus* sensitization or persistent carriage as a factor in lung function impairment in cystic fibrosis patients
Fillaux *et al*., Scand J Infect Dis, 2012

251 patients followed-up in Toulouse from 1995 to 2007

Persistent carriage (persistence of *A. fumigatus*-positive cultures) is associated with lung function decline


CHEST Original Research

CYSTIC FIBROSIS

Sputum *Candida albicans* Presages FEV_1 Decline and Hospital-Treated Exacerbations in Cystic Fibrosis

Sanjay H. Chotirmall, MD; Elaine O'Donoghue, MD; Kathleen Bennett, PhD; Cedric Gunaratnam, MD; Shane J. O'Neill, MD, FCCP; and Noel G. McElvaney, MD

CHEST 2010; 138(5):1186–1195

Detecting fungi in CF
Clinical significance - genotype studies

Future studies dealing with the clinical relevance of fungi in CF should include the genotype analysis of multiple and sequential isolates of the fungus

- **Multiple : collected from the same sputum sample**
- **Sequential : collected from successive sputum samples from the same patient**

Repeated isolation of the same fungal species does not imply a chronic colonization

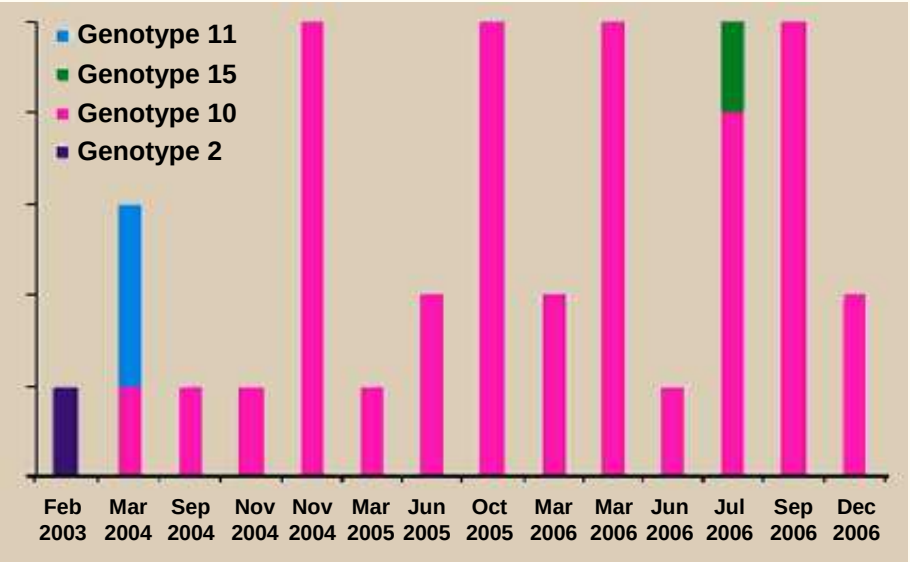
Genotyping needed to discriminate between

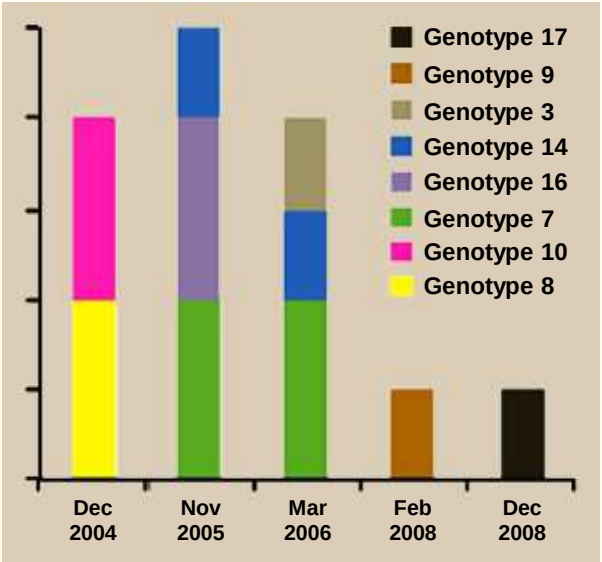
- **Regular, but transient carriage of always distinct genotypes**
- **Chronic colonization of the airways (repeated isolation of the same genotype which reveals the development of the fungus within the respiratory tract)**

Detecting fungi in CF
Clinical significance - genotype studies

Epidemiological study of the airway colonization by *A. terreus* (collaboration with J. Meis, Nijmegen, The Netherlands)

- **5 patients with CF followed-up for 2 months to 7.5 years**
- **115 isolates (45 samples - from 1 to 5 isolates per sample) investigated by microsatellite analysis (9 di-, tri, or tetra-nucleotide markers)**
- **17 genotypes identified**



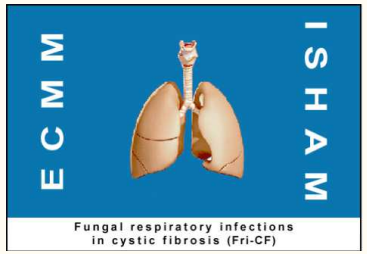


Rougeron *et al*., Clin Microbiol Infect, 2013

Weird moulds
Working together for faster progress

Despite significant improvements, progress are still needed in the biological diagnosis and treatment of these infections, as well as in our understanding of the pathogenic mechanisms or ecology of these fungi

ECMM/ISHAM working group on
***Fungal respiratory infections in Cystic Fibrosis* (*Fri-CF*)**
Convenors: J.P. Bouchara, A. Borman and F. Symoens



1st Meeting in Angers, on 2009, June 7-8th
2nd Meeting in Angers, on 2011, September, 1st-2nd
3rd Meeting in Angers, on 2014, June, 5th-6th

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