Second Meeting
of the ECMM/ISHAM Working Group
Fungal respiratory infections in Cystic Fibrosis (Fri-CF)

Angers (France), 2011, September 1st – 2nd
Faculty of Pharmaceutical Sciences, Angers University

Organizing Committee:

Jean-Philippe Bouchara, Dominique Chabasse, Raymond Robert,
Sandrine Giraud, Agnès Marot, Gérald Larcher, Laurence Delhaès and Françoise Symoens

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Program

Thursday September 1st

Greeting participants from 13 h 30 to 14 h 00

14 h 00 Introduction talk. **Jean-Philippe Bouchara** on behalf the Host-Pathogen Interaction Study Group.

14 h 10 ECMM and promotion of research in medical mycology. **Jacques Meis**, President of the European Confederation of Medical Mycology.

14 h 20 – 16 h 20: Clinical surveillance (Chair: Stéphane Ranque)

14 h 20 Fungal infection/contamination in the sputum of CF patients. **David Shoseyov,** Malena Cohen-Cymberknoh, Alexander Gileles-Hillel and Eitan Kerem.

14 h 40 Frequency of fungi in respiratory samples from Turkish CF patients. **Özge Güngör**, Zeynep Tamay, Nermin Guler and Zayre Erturan.

15 h 00 Isolation of filamentous fungi in the sputum of cystic fibrosis patients in Italy: state of the art and epidemiology. **Esther Manso,** Lisa Cariani, Ersilia Fiscarelli, Novella Ravenni, Vita Dora Tula, Gianfranco De Intinis, Marina Bussetti, Sara Rapagnani and Tatiana Borio.

15 h 20 Filamentous fungi in CF - prevalence and laboratory practice in Danish clinics. **Niels Nørskov-Lauritsen.**

15 h 40 – 16 h 00: Coffee break.

16 h 00 – 19 h 00: Clinical surveillance (Chair: David Shoseyov)

16 h 00 Fungal colonization in Cystic Fibrosis (CF): Epidemiology and antifungal resistance in a French cohort of CF patients – Focused on *Aspergillus fumigatus* colonization. **Laurence Delhaes**, Anne-Sophie Jourdain, Mahussi d'Almeida-Fourquet, Emilie Fréalle, Sylvie Leroy, Claudine Pinel, Catherine Llerena, Loic Favennec, Stéphane Dominique, Odile Vagner, Anne-Lise Fanton, Jean-Philippe Bouchara, Claudine Person, Isabelle Durand-Joly, Gilles Loeuille, Philippe Domblides, Isabelle Accoceberry, Amaury Farce, Patrick Chavatte and Benoit Wallaert.

16 h 20 Epidemiology of filamentous fungi in Cystic Fibrosis patients in the Netherlands. **Paul Terpstra,** Mireille V. Westreenen, Peter Croughs, Paul Verweij, Ronald Brimicombe, Jan-Bart Yntema, Corné Klaassen, Peter Merkus and Jacques Meis.

16 h 40 *Pneumocystis jirovecii* and cystic fibrosis in France. **Solène Le Gal,** Geneviève Héry-Arnaud, Sophie Ramel, Michèle Virmaux, Céline Damiani, Anne Totet and Gilles Nevez.

17 h 00 *Pneumocystis jirovecii* colonization in lung transplant recipients with cystic fibrosis. **Enrique J. Calderón,** Vicente Friaza, Nieves Respaldiza, Francisco J. Dapena, Ruben Morilla, Elena Campano, Esther Quintana, Francisco J. Medrano, José M. Varela and Carmen de la Horra.

17 h 20 *Trichosporon mycotoxinivorans*: An emerging respiratory pathogen in patients with cystic fibrosis. **Patrick W. Hickey**, Andrew J. Lipton, Howard J. Schmidt, Rees L. Lee and Thomas J. Walsh

17 h 40 *Exophiala dermatitidis* agent of colonization in cystic fibrosis in Belgium: prevalence, serology and antifungal susceptibility. **Françoise Symoens**, Ann Packeu, Anca Boeras, Daniel Huang, Hector Rodriguez-Villalobos, Anissa Léonard and Patrick Lebecque.

18 h 00 The influence of moulds in the patho-physiology of Cystic Fibrosis and the place of functional diagnostics in patient care. **Craig Williams**, Elaine McCulloch, Ranjith Rajendran and Gordon Ramage.

18 h 20 Short and long-term outcomes with pulmonary fungal infections in pediatric lung transplant recipients. **Lara Danziger-Isakov**, Michael Liu, Sarah Worley, Susana Arrigan, Stuart Sweet, Al Faro, Peter Michelson, George Mallory Jr, Marc Schecter, Okan Elidemir, Paul Aurora, Manfred Ballmann, Debra Boyer, Gary Visner, Carol Conrad, Irmgard Eichler, Samuel Goldfarb, Marian Michaels, Peter Mogayzel Jr, Daiva Parakininkas and Melinda Solomon.

19 h 00 Departure for the Cointreau museum (visit of the museum and dinner at La Romanerie)

Friday, September 2nd

8 h 20 – 10 h 20: Diagnosis (Chair: Christopher Thornton)

8 h 20 Routine moulds' identification in the clinical laboratory by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). **Stéphane Ranque**, Carole Cassagne, Anne-Cécile Normand, Patrick Fourquet, Sandrine Thiebault, Chantal Planard, Marijke Hendrickx and Renaud Piarroux.

8 h 40 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Scedosporium* species. Emilie Sitterlé, Jean-Philippe Bouchara, Brunilde Dauphin, Jean Luc Berretti, Gilles Quesne, André Paugam, Noura Hassouni, Xavier Nassif and **Marie-Elisabeth Bougnoux.**

9 h 00 *Scedosporium* spp.: detection, identification and antifungal susceptibility profile. **Ana Alastruey-Izquierdo**, Maria Jose Buitrago, Juan Luis Rodriguez-Tudela and Manuel Cuenca-Estrella.

9 h 20 ABPA: biomarkers for diagnosis and disease staging. **Carlos E. Milla.**

9 h 40 Role of real-time PCR and galactomannan in the classification of *Aspergillus* disease in CF. **Caroline Baxter**, Kevin Webb, Andrew Jones and David Denning.

10 h 00 Monoclonal antibodies specific to *Scedosporium apiospermum* and *Scedosporium prolificans* - a resource for ISHAM CF research. **Christopher R. Thornton.**

10 h 20 – 10 h 40: Coffee break

10 h 40 – 12 h 30: Genotype studies (Chair: Françoise Symoens)

10 h 40 Genetic diversity and antifungal susceptibility of *Aspergillus fumigatus* from chronically colonised Portuguese patients. **Ricardo Araujo**, Adelina Amorim, António Amorim and Leonor Gusmão.

11 h 00 Real-time typing of *Aspergillus fumigatus*. **Corné H. Klaassen.**

11 h 20 Different colonization patterns of *Aspergillus terreus* in patients with cystic fibrosis. **Amandine Rougeron**, Sandrine Giraud, Jacques Meis, Jean-Philippe Bouchara and Corné H. Klaassen.

11 h 40 The spectrum of *Scedosporium* isolates from the respiratory tract in CF-patients – multi locus sequence typing (MLST) and documented clinical relevance. Anne Bernhardt, Ludwig Sedlacek, **Carsten Schwarz**, Benjamin Würst, Sonja Wagner and Kathrin Tintelnot.

12 h 00 Update of Australian *Scedosporium* studies, with emphasis on the development of an MLST scheme for the emerging species *S. aurantiacum.* **Wieland Meyer**, Azian Harun, Christopher Blyth, Peter Middleton and Sharon C-A. Chen.

12 h 30 – 13 h 40: Lunch at the Faculty

13 h 40 – 15 h 20: Physiopathology (Chair: Jean-Pierre Gangneux)

13 h 40 Role of PTX3 in cystic fibrosis-associated infections. **Yveline Hamon**, Christine Person, Jean-Louis Giniès, Jean-Philippe Bouchara and Yves Delneste.

14 h 00 *Aspergillus*-induced inflammation in the cystic fibrosis lung: response to anti-fungal therapy. Sanjay H. Chotirmall, Catherine A. Coughlin, Emer P. Reeves, Philip Murphy, Julie Renwick, Cedric Gunaratnam and **Noel Gerard McElvaney.**

14 h 20 Lectins from pathogenic fungi: new therapeutical target. Josef Houser, Gianluca Cioci, Michaela Wimmerova, Anne Imberty and **Annabelle Varrot.**

14 h 40 Ecology of the airway microbiome in cystic fibrosis. **Carlos E. Milla.**

15 h 00 Polymicrobial infections of the Cystic Fibrosis lung: what can we learn from combining culture and molecular approaches? **Francoise Botterel**, Franziska Stressmann, Damian Rivett, Odile Cabaret, Marie Odile Husson, Geraint Rogers, Benoit Wallaert, Laurence Delhaes and Kenneth Bruce.

15 h 20 – 17 h 40: Antifungals and therapy (Chair: Jacques Meis)

15 h 20 Pharmacodynamic effects of antifungal agents on biofilms of *Aspergillus fumigatus* strains isolated from cystic fibrosis patients. **Maria Simitsopoulou**, Efthymia Tasina, Kalliopi Tsampali, Elpis Hatziagorou and Emmanuel Roilides.

15 h 40 *Aspergillus fumigatus* susceptibility to itraconazole in adult patients with cystic fibrosis**.** Pierre-Régis Burgel, Marie-Thérèse Baixench, Mickael Amselem, Etienne Audureau, Jeanne Chapron, Reem Kanaan, Isabelle Honoré, Jean Dupouy-Camet, Daniel Dusser, Dominique Hubert and **André Paugam.**

16 h 00 Clinical and microbiological efficacy of micafungin on *Geosmithia argillacea* infection in a french cystic fibrosis patients. **Loïc Favennec**, Laure Couderc, Olivier Mattray, Sandrine Giraud, Rachid Zouhair, Sébastien Bertout, Gilles Gargala, Christophe Marguet and Jean-Philippe Bouchara.

16 h 20 *Scedosporium* spp. colonization in cystic fibrosis lung transplant patients. A report of 8 cases. Perrine Parize, Catherine Amrein, Mohammed Jelassi, Vianney Poinsignon, Sandrine Lefeuvre, Véronique Boussaud, Romain Guillemain, **Eric Dannaoui** and Eliane Billaud.

16 h 40 – 17 h 00: Coffee break

17 h 00 – 18 h 00: General discussion

Guidelines for mycological examination of sputum samples

Towards standardization for mycological examination of sputum samples from Cystic Fibrosis patients: From the French multicenter experience to an international study. **Laurence Delhaes**, Andrew Borman, Françoise Symoens and Jean-Philippe Bouchara, for the ECMM/ISHAM Working group on *Fungal respiratory infections in Cystic Fibrosis*.

Microbial metagenome in the CF airway

Study of the metagenome present in broncho-alveolar fluids of CF patients diagnosed through new born screening. **Gaudenz M. Hafen** and Philippe M. Hauser.

Azole therapy in Cystic Fibrosis

The ATCF Study. **Jean-Pierre Gangneux.**

18 h 30: Departure for the castle of Brissac-Quincé (visit of the castle and dinner in the castle).

ABSTRACTS

FUNGAL INFECTION/CONTAMINATION IN THE SPUTUM OF CF PATIENTS

David Shoseyov, Malena Cohen-Cymberknoh, Alexander Gileles-Hillel and Eitan Kerem

CF Center –Hadassah Mt Scopus Medical Center, Jerusalem, Israel.

Over the last decade there has been a significant increase in the prevalence of filamentous fungi in CF respiratory cultures, with increases in both *Aspergillus* and non-*Aspergillus* fungal species. However, the effects of fungi on lung function are not completely understood. *Aspergillus fumigatus* (AF) can cause Allergic Bronchopulmonary Aspergillosis (ABPA); an immune mediated sensitization to *Aspergillus*, but can cause also *Aspergillus* bronchiitis, a different entity than that may response to antifungal therapy. *Candida albicans* is the most frequent member of the *Candida* genus isolated from CF patients, but its clinical relevance in CF is still controversial. Other *Candida* spp. are of increasing prevalence in CF but their effect on the course of disease is unknown.

Method: A retrospective study assessed the correlation of fungal infection to the change in PFTs in two years (2002-203) and routine laboratory data. Two hundred fifty three patients' records were reviewed for this trial (Age 6 months to 51 year old and mean age 17.1). In total, 1229 positive sputum cultures were collected, from three major CF centers in Israel.

Results: We found significant correlation between fungal infection caused by *Candida* species and a lower FEV1%. We also found a significant correlation to antibiotic treatment. No correlation was found between laboratory results and fungal infection (CBC immunoglobulins, ESR etc) nor genotype.

Conclusion: Fungal infection prevalence is increased in CF patients who require more antibiotic treatments. Further studies and prospective studies are required in order to understand their role in the respiratory deterioration.

FREQUENCY OF FUNGI IN RESPIRATORY SAMPLES FROM TURKISH CYSTIC FIBROSIS PATIENTS

Özge Güngör[1], Zeynep Tamay[2], Nermin Güler[2] and Zayre Erturan[1]

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The frequency of airway colonization by fungi was investigated prospectively in 48 patients (age range 2-38; mean 11.63 years) with cystic fibrosis (CF) over a nine month period. Between August 2010- May 2011, 184 respiratory tract specimens (33 sputa, 151 deep throat swabs) were investigated. Each patient enlisted into the study had at least 3 specimens examined, which were collected at least one month apart.

Sputum samples were processed using a quantitative laboratory procedure. In addition to routine and selective media for CF specific bacteria, all specimens were inoculated onto YPDA medium supplemented with antibiotics and CHROMagar *Candida*.

Among the 184 clinical samples, 75 (40.76%) were positive for fungi. *Candida albicans* was the predominant fungus isolated (30 patients (62.5%), 56 specimens (30.43%)). Second to this yeast, *C. parapsilosis* was isolated from the specimens of 6 (12.5%) patients. *Candida dubliniensis* and *Aspergillus fumigatus* were isolated from the specimens of 5 (10.4%) patients each. Other fungi which we detected at low frequency were *C. kefyr*, *C. tropicalis* (3 patients each), *C. glabrata, C. guilliermondii, C. krusei* (2 patients each), *C. lusitaniae, C. norvegensis, Rhodutorula mucilaginosa, Saccharomyces cerevisiae, A. flavus, A. niger, Penicillium* sp. (1 patient each) and nonidentified *Aspergillus* species (3 patients).

Mixed fungal populations accounted for 41.3% (31/75**)** of positive samples. Two or more yeast species, two mould species and yeasts together with filamentous fungi were detected in 9.2% (17/184) , 0.54% (1/184) and 7.06% (13/184) of total specimens, respectively.

Several studies suggest that *Pseudomonas aeruginosa* and *C. albicans* interact with each other *in vivo* and that they are commonly found together in mixed infections. In our study, *C. albicans* and *P. aeruginosa* were detected together in 48.21% of *C. albicans* positive samples. The most frequent bacteria detected as co-colonizer with *C. albicans* was *Staphylococcus aureus* (53.57% of *C. albicans* positive samples). *Aspergillus fumigatus* and *P. aeruginosa* or *A. fumigatus* and *S. aureus* were detected together in 75% of *A. fumigatus* positive samples each.

Not only the presence or absence of fungi, but also the fungal burden is an important contributor to lung disease severity. The quantitative analysis of sputa with *C. albicans* or *A. fumigatus* showed a fungal concentration between $2x10^1$-$3x10^3$ CFU/ml and $2x10^1$-$1.2x10^3$ CFU/ml, respectively.

Although *C. albicans* was the predominant species, this yeast is a commensal frequently isolated from the oropharynx. A high colonization rate of *C. dubliniensis* in CF patients was reported in the literature. This fungus was the third most isolated yeast recovered from the specimens of our patients. *Aspergillus fumigatus* was the most common filamentous fungus and three patients (6.25%) were chronic colonized with this mould. *Exophiala dermatitidis* and *Scedosporium apiospermum* were not isolated during the investigation period. During the study, no patient was diagnosed with allergic bronchopulmonary aspergillosis or other fungal infections.

ISOLATION OF FILAMENTOUS FUNGI IN THE SPUTUM OF CYSTIC FIBROSIS PATIENTS IN ITALY: STATE OF THE ART AND EPIDEMIOLOGY

Esther Manso[1], Lisa Cariani[2], Ersilia Fiscarelli[3], Novella Ravenni[4], Vita Dora Iula[5], Gianfranco De Intinis[6], Marina Busetti[7], Sara Rapagnani[8] and Tatiana Borio[9]

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Approximately 6,000 patients in Italy are estimated to suffer from Cystic Fibrosis. Filamentous fungi may contribute to the local inflammatory response and the progressive deterioration of the lung functions, but colonization rate differs from study to study, depending on patient's age and use of microbiological methods. In 2009 and 2010, the microbiology of 20.346 sputum from 1837 and 1882 patients, respectively, was studied in nine laboratories adhered to the Società Italiana per la Fibrosi Cistica (SIFC). The number of patients aged 20 years old or more was: 690 (37.5%) in 2009 and 711 (37.7%) in 2010. Out of nine laboratories, two looked for filamentous fungi only from 2010. Research of filamentous fungi was done in all the laboratories in Sabouraud dextrose agar + chloramphenicol + gentamicin plates. No other selective medium for specific fungi was used. Seven of them incubated the medium for 7-9 days, another one only for 48 hours and the other for 3 weeks. Most of them incubated the plates at 37°C for 48 h and then at 30°C or at room temperature. The colonization was defined as: sporadic, in the case filamentous fungus was isolated in only one sample per year and other samples were negative; intermittent, if two samples of the same fungus species complex were isolated in a year, separated by other samples negatives, and chronic, if the same fungus species complex was isolated in three samples taken at least with an interval of one month along the year. One laboratory did not identify the species of *Aspergillus* and so we have not taken it into account.

In 2009 and 2010, *Aspergillus fumigatus* was isolated in 241 (13.0%) and 222 (11.8%) patients, respectively: as sporadic in 115 (6.2%) and 90 (4.7%) patients, as intermittent in 49 (2,6%) and 61 (3.2%) patients and as chronic in 77 (4.2%) and 71 (3.7%) patients, respectively; *Aspergillus terreus* was isolated in 38 (2.0%) and 45 (2.4%) patients, respectively: as sporadic in 21 (1.1%) and 24 (1.2%) patients, as intermittent in 8 (0.4%) and 11 (0.5%) patients and as chronic in 9 (0.5%) and 10 patients (0.5%), respectively; *Aspergillus flavus* was isolated in 55 (3.0%) and 58 (3.0) patients, respectively: as sporadic in 41 (2.2%) and 26 (1.3%) patients, as intermittent in 7 (0.4%) and 24 (1.3%) patients and as

chronic in 7 (0.4%) and 8 (0.4%) patients, respectively; *Aspergillus niger* has been rarely isolated, only in one patient as intermittent and in 10 patients as sporadic; *Scedosporium apiospermum* was isolated in 38 (2.0%) and 37 (1.9%) patients, respectively: as sporadic in 15 (0.8%) and 14 (0.7%) patients, as intermittent in 7 (0.4%) and 12 (0.6%) patients and as chronic in 16 (0.8%) and 11 (0.6%) patients, respectively. *Geosmithia argillaceae* has been isolated only in one laboratory in 4 patients: 2 as chronic, 1 as intermittent and 1 as sporadic. *Exophiala dermatitidis* has been isolated only from one patient as a chronic colonization.

FILAMENTOUS FUNGI IN CF - PREVALENCE AND LABORATORY PRACTICE IN DANISH CLINICS

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The CF centre at Aarhus University Hospital provides care for approximately 140 paediatric and adult CF patients. Microbiological surveillance of respiratory sectetions is intensive, but by tradition with little focus on mycological culture (plated on Sabouraud agar for a minimum of 24 h and reported as "moulds"). To further characterize the fungal microbiota of our patients, 187 moulds cultured during 2009 from 47 patients have been kept and are presently being identified by three separate approaches: morphologic characterisation, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and internal transcribed spacer (ITS) sequencing. Furthermore, during one month, all Sabouraud plates were incubated at 35^{o}C for prolonged time (three weeks); only a single culture from one patient yielded growth of *Exophiala dermatitidis*.

FUNGAL COLONIZATION IN CYSTIC FIBROSIS (CF): EPIDEMIOLOGY AND ANTIFUNGAL RESISTANCE IN A FRENCH COHORT OF CF PATIENTS – FOCUSED ON *ASPERGILLUS FUMIGATUS* COLONIZATION.

Laurence Delhaes[1], Anne-Sophie Jourdain[1], Mahussi d'Almeida-Fourquet[2], Emilie Fréalle[1], Sylvie Leroy[1], Claudine Pinel[3], Catherine Llerena[3], Loic Favennec[4], Stéphane Dominique[4], Odile Vagner[6], Anne-Lise Fanton[6], Jean-Philippe Bouchara[7], Claudine Person[7], Isabelle Durand-Joly[5], Gilles Loeuille[5], Philippe Domblides[2], Isabelle Accoceberry[2], Amaury Farce[8], Patrick Chavatte[8] and Benoit Wallaert[1]

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Introduction: Cystic fibrosis (CF) is the major genetic inherited disease in the European Caucasian population, with an average of 1 in 3000 living births in France. Prognostic depend essentially on the lung impairments. While considerable attention therefore has been paid over recent decades to prevent and treat bacterial respiratory infections, we observed emergence of fungi colonization in CF respiratory tract. In particular, *Aspergillus fumigatus* represents the most common causative agent colonizing the airways of CF patients; it can be responsible for Allergic Bronchopulmonary Aspergillosis (ABPA). Since oral corticosteroids and itraconazole represent the mainstay of ABPA treatment, long-term therapy may increase the risk of acquired resistance to azoles that is mainly associated with amino acid substitutions in the *CYP51A* gene of *A. fumigatus*.

Objective: First, we managed to have exhaustive epidemiological data on species of filamentous fungi able to colonize the airway tract of 300 CF patients followed-up in our national prospective study ("MucoFong" study – PHRC1902). Second, CF patients being chronically exposed to azole (especially to itraconazole), our study aimed to evaluate the prevalence of azole resistance in isolates prospectively collected from CF patients followed-up in seven French hospitals involved in our national prospective study. Third, we focused on the most prevalent species: *Aspergillus fumigatus,* studying the azole resistance at molecular level. To our knowledge, it is the first multicenter study focused on azole resistance of *A. fumigatus* in CF.

Methods: A total of 243 sputa were analyzed using the same protocol in each centre. The MICs of antifungal drugs were evaluated for each isolate using the E-test® strips. Focusing on *A. fumigatus*, a total of 87 isolates was collected in 85 patients. These isolates were characterized at the molecular level by targeting ITS, ß-tubulin and MAT-A/α genes. The *CYP51A* gene as well as its promoter was sequenced; a 3D Cyp51A protein homology model was built.

Results and discussion: 300 patients were enrolled in this study. At inclusion time, most of them were adults colonized with *A. fumigatus* (about 35% of the patients). *Scedosporium* was isolated in 5%, and *Exophiala* in about 2%. Regarding antifungal susceptibility, isolates of *Scedosporium* and *Exophiala* exhibited antifungal resistance comparable with published data. Regarding *A. fumigatus*, a majority of isolates (88.1%) were found sensitive to itraconazole (MIC≤ 2µg/ml), and 2 new mutations were identified and localized within 3-dimensional Cyp51A protein model. To obtain insight into azole resistance of *A. fumigatus*, the results are analyzed taking into account clinical data, itraconazole exposition, and the potential correlation between the identified CYP5IA mutations and azole resistance is discussed based on the Cyp51A protein homology model.

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Conflict of interest: None to declare

Keywords: *Aspergillus fumigatus*, *Scedosporium apiospermum*, *Scedosporium prolificans*, *Exophiala* spp., antifungal resistance, cystic fibrosis

EPIDEMIOLOGY OF FILAMENTOUS FUNGI IN CYSTIC FIBROSIS PATIENTS IN THE NETHERLANDS

Paul D. Terpstra[1,2], Mireille V. Westreenen[3], Peter Croughs[4], Paul Verweij[1], Ronald Brimicombe[5], Jan-Bart Yntema[6], Corné Klaassen[2], Peter Merkus[6] and Jacques F. Meis[2]

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Objectives: The clinical significance of fungal infections in Cystic Fibrosis (CF) are poorly understood. Infections with viruses and bacteria are the main cause of deterioration of lung function in these patients. However there is also evidence of an increasing prevalence of fungi in sputum but the reported results vary widely between countries and centers (9-56%). Very little is known about the epidemiology, genotypes and resistance patterns of filamentous fungi in Dutch patients with CF. Here we describe the first results of a recently initiated large prospective study of fungal colonization in CF patients in the Netherlands.

Methods: Standardized routine sputum samples were collected in four CF centers. All samples were cultured on mould- selective plates and incubated at two temperatures. All non-*Candida* fungi were collected and stored centrally. After initial morphologic identification, molecular confirmation was sought using AFLP and ITS + D1/D2 sequencing. All *Aspergillus fumigatus* isolates were additionally analyzed for presence of the most common mutations in the *CYP51* gene.

Results: A total of 756 filamentous fungi were isolated from 337 patients in the period May 2010 - January 2011. The main species found was *A. fumigatus* (n = 456). In addition, a variety of other *Aspergillus* species were found: *A. flavus, A. terreus, A. sydowii, A. protuberus* and *A. versicolor*. Next to these *Aspergillus* spp., *Penicillium* spp. (n = 117) and *Scedosporium* spp. (n = 15) were the most common fungi. *Exophiala dermatitidis*, an already known potential pathogen in CF, was isolated 17 times. Interestingly we also report the first isolations of *Geosmithia argillacea* in Dutch CF patients. At least 10% of the samples showed a discrepancy between morphologic and molecular identification. A total of 4.2% of the tested *A. fumigatus* appeared to contain the TR/L98H mutation while no other mutations (G54, G138, M220) responsible for azole resistance where found.

Conclusion: These results suggest that the Dutch epidemiology of filamentous fungi in CF patients is comparable to our neighboring countries. The main fungus was *A. fumigatus*, but also other fungal species such as *Scedosporium* spp. and *Exophiala* spp. were identified including the newly described species *Geosmithia argillacea*. In addition 4.2% of *A. fumigatus* isolates appeared to contain mutations reported to confer azole resistance. These are the first epidemiological data of azole resistance in *A. fumigatus* from CF patients in the Netherlands.

PNEUMOCYSTIS JIROVECII AND CYSTIC FIBROSIS IN FRANCE

Solène Le Gal[1, 2], Geneviève Hery-Arnaud[1], Sophie Ramel[3], Michèle Virmaux[1], Céline Damiani[4], Anne Totet[4] and Gilles Nevez[1, 2]

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Pulmonary colonization with *Pneumocystis jirovecii* may occur in patients with cystic fibrosis. Cases have been reported in European patients, particularly from Seville, Spain, among whom the fungus has been detected with a prevalence of 21.6%. Conversely, there are no data on *P. jirovecii* in cystic fibrosis patients from France. The aim of this study was to obtain information on *P. jirovecii* in cystic fibrosis patients from France, and precisely from Brittany, the French region which is characterized by the highest prevalence of this autosomal recessive disorder. The study was carried out in Brest University Hospital. Seventy-six patients were retrospectively enrolled. They initially underwent sputum procedure for routine bacteriological and mycological surveillance from July 2005 to August 2007. None of the patients received long-term sulfonamide treatment. A hundred and forty-six sputum specimens were examined for *P. jirovecii* presence using a nested-PCR assay and a real time PCR assay, both amplifying the mitochondrial large subunit rRNA gene of the fungus. *Pneumocystis jirovecii* was detected in one patient (one sputum specimen) using the real time PCR assay whereas it was not detected using the nested-PCR assay. The results show that the prevalence of pulmonary colonization with *P. jirovecii* in cystic fibrosis patients in Brest is low (1.3%) compared to that reported in Seville (21.6%). Differences in features of *P. jirovecii* epidemiology in the two cities may be the cause of these divergent results. Brest (population, 220,000) is characterised by a low incidence of AIDS and *Pneumocystis* pneumonia, in contrast with Seville (population, 700,000) in which the incidence of these conditions is 20 times higher. Encounters between potentially infected and non-infected persons are more likely in Seville, predisposing to incidental acquisition and detection of *P. jirovecii*. Climatic factors, as non-independent factors, might also affect the circulation and transmission of the fungus in the Spanish city (Mediterranean climate) and in Brest (humid, temperate climate). Finally, our results may reflect a low level of circulation and transmission of *P. jirovecii* in human populations in Brittany.

PNEUMOCYSTIS JIROVECII COLONIZATION IN LUNG TRANSPLANT RECIPIENTS WITH CYSTIC FIBROSIS

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Pneumocystis jirovecii is an atypical opportunistic fungus with lung tropism and worldwide distribution that causes pneumonia (PcP) in immunosuppressed individuals. The development of sensitive molecular techniques has led to the recognition of a colonization or carrier state of *P. jirovecii*, in which low levels of the organism are detected in persons who do not have PcP. *Pneumocystis* colonization has been described in patients with various lung diseases, including individuals with cystic fibrosis who have prevalence ranges from 1.3% to 21.6%. Cystic fibrosis is one of the most common indications for lung transplantation worldwide and certainly the most common indication for all pediatric lung transplants and for bilateral lung transplantation irrespective of age. *P. jirovecii* is a major cause of fatal pneumonia in immunosuppressed lung transplant patients. However, there is no data about *P. jirovecii* colonization in lung transplant recipients with Cystic fibrosis. The aim of this study was to analyze the frequency and dynamic evolution of *P. jirovecii* colonization among patients with Cystic fibrosis after lung transplantation. The study included 11 cases (3 males, 8 females; median age 21.9 ± 6.9 years, range 13–32 years) who attended a specialized Cystic fibrosis unit. All of them received chemoprophylaxis with trimethoprim-sulfamethoxazole. Patients were followed for a 1-year period and underwent a clinical and biological examination every 6 months in conjunction with use of a standardized questionnaire. A patient colonized with *P. jirovecii* was defined as an individual, with no symptoms or thorax radiography signs of PcP, whose respiratory specimen contained *P. jirovecii* DNA detectable by nested PCR. At baseline, *P. jirovecii* colonization was detected in five (45.5%) patients, but everyone except for one were colonized sometime during the follow-up period. Molecular identification of *P. jirovecii* polymorphisms in the mt LSU rRNA gene was determined by direct sequencing. Three different genotypes were detected: 10% genotype 1 (85C ⁄ 248C); 20% genotype 2 (85A ⁄ 248C) and 70% genotype 3 (85T ⁄ 248C). Plasma samples from patients were assayed for ß-D-glucan, with standard assay reference values defining > 80 pg/mL as positive for PcP. Median ß-D-glucan was 87.6 ± 37.7 pg/mL in *P. jirovecii* colonized patients, compared with 80 ± 29.3 pg/mL in patients without colonization (P = 0.59). In these cases, other possible fungal infections were explored by 18S-rDNA PCR-DGGE. Our results demonstrate that *P. jirovecii* colonization is common and may appear at any time after lung transplantation in patients with Cystic fibrosis despite use of chemoprophylaxis. Serum level of **ß**-D-glucan is not useful for detecting *P. jirovecii* colonization in Cystic fibrosis patients. Therefore, frequent monitoring of this patients using molecular techniques is thus necessary for detecting *P. jirovecii* colonization.

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TRICHOSPORON MYCOTOXINIVORANS: AN EMERGING RESPIRATORY PATHOGEN IN PATIENTS WITH CYSTIC FIBROSIS

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We review the recently described molecular epidemiology, in vitro susceptibility, colonial and microscopic morphology, and biochemical features of *Trichosporon mycotoxinivorans*, a newly recognized pathogen that has a propensity for patients with cystic fibrosis. The index patient died with histologically documented *Trichosporon* pneumonia complicating cystic fibrosis. Five additional cases, three from archived isolates previously identified as other members of the genus, were subsequently recognized. Four of these five cases also were patients with cystic fibrosis. All isolates demonstrated resistance to amphotericin and echinocandins ≥ 16, but displayed varying susceptibilities to the triazoles. The clinical course and long term management challenges of two patients with cystic fibrosis and *T. mycotoxinivorans* pulmonary infection are described. This case series highlights that further investigation regarding the ecological niche of *T. mycotoxinivorans*, diagnostic challenges, and the pharmacokinetics of treatment agents in cystic fibrosis patients is warranted.

EXOPHIALA DERMATITIDIS AGENT OF COLONIZATION IN CYSTIC FIBROSIS IN BELGIUM: PREVALENCE, SEROLOGY AND ANTIFUNGAL SUSCEPTIBILITY

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Exophiala dermatitidis is a black yeast absent in the outdoor environment (soil, organic matter) in temperate regions. Agent of cerebral mycoses in South East Asia in immunosuppressed patients, this fungus in temperate region is mainly isolated from cystic fibrosis (CF) patients.

At the first meeting of this Working Group, a prospective study of the prevalence of *E. dermatitidis* in CF Belgian patients was presented, and risk factors were evaluated. In this study Ed was isolated from 5.8% (9 out of 154 patients); *Aspergillus fumigatus* (Af) colonization and genotype (F508del mutation) seems to be predisposing factors for *E. dermatitidis* colonization (Lebecque *et al.*, 2010).

The aim of this study was to evaluate the presence of precipitins for *E. dermatitidis* in the patients with positive culture and to study the antifungal susceptibility of the *E. dermatitidis* isolates.

Presence of precipitins. The serological study for presence of precipitins was realized blindly with 20 sera (11 from patients with a positive culture for *E. dermatitidis* and 9 from patients with a negative culture for *E. dermatitidis*) using in house protein extracts of *E. dermatitidis* and *A. fumigatus*. The table hereunder summarizes the results obtained.

Concordance of the presence of *precipitins for* E. dermatitidis *and positivity of the culture: 20 patients (11 with positive and 9 with negative culture for* E. dermatitidis*)*

Concordance/ (nb patient)	%	Culture /Serology	Remark
Full (n = 13)	65%	+ culture / > 2 prec. lines, (n = 7) - culture / no prec. line (n = 6)	
Intermediate (n = 2)	10%	+ culture / 1 prec. line	1 patient with + culture Ed since 1 month 1 patient with + culture Ed since 3 years
Not concordant (n = 2)	10%	- culture /1 prec. line	
Not concordant (n = 2)	10%	+ culture / no prec. line	+ culture for Ed since 3 years for both patients, + culture for Af, no prec. line for Af for both patients
Not concordant (n = 1)	5%	- culture /2 prec. lines	

Ed: *Exophiala dermatitidis*, Af: *Aspergillus fumigatus*+: positive culture, -: negative culture, prec. lines: precipitating lines

A concordance of 75% between the presence of precipitins and positivity of the culture for *E. dermatitidis* was observed. Two patients with a positive culture for *E. dermatitidis* had no precipitins for *E. dermatitidis*, although they are colonized since more than 3 year. One out of these two patients, after colonization by *E. dermatitidis*, became also colonized by the related

species *Exophiala phaerumiformis* (identified only by molecular methods), However, he had also no precipitins against this fungus. We could demonstrate the cross-reaction between both species, patients with precipitins for *E. dermatitidis* had also precipitins for *Exophiala phaerumiformis*.

Antifungal susceptibility of isolates of *E. dermatitidis*. Susceptibility to VRZ (voriconazole), ITZ (itraconazole) and AmB (Amphotericin B) of strains from 11 patients was evaluated by CLSI 38A. All the strains from 10 out of 11 patients had low MICs for AmB (medium MIC 0.83 µg/ml); VRZ (medium MIC 0.62 µg/ml), ITZ (medium MIC 0.52 µg/ml). 9 out of the 10 strains isolated from one patient showed a decreased sensitivity for VRZ (MIC > 4 µg/ml) and showed CMI = 2 µg/ml for AmB; this decreased of sensitivity for VRZ could be acquired due to the discontinuous treatment with VRZ, the first isolate from this patient had a MIC = 0,55 µg/ml.

Lebecque P., Leonard A., Huang D., Reychler G. Boeras A., T. Leal, Symoens F. *Exophiala* (*Wangellia*) *dermatitidis* and cystic fibrosis – prevalence and risk factors. Medical Mycology, 2010; 48 (Suppl I) S4-S9.

THE POTENTIAL USE OF RNA DIAGNOSTIC TESTS IN CYSTIC FIBROSIS PATIENTS WITH *ASPERGILLUS FUMIGATUS*

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Respiratory colonization by *Aspergillus fumigatus* is common in patients with Cystic Fibrosis with reported prevalence ranging widely from 6 to 57% depending on culture frequency, methodology, duration of monitoring, and patient population. There is increasing evidence that infection with this organism causes detectable radiological lesions and may be associated with increased risk of hospitalization for infective pulmonary exacerbations.

The major clinical problem is determining the significance of the isolate in this patient population and it is often considered a contaminant.

Using microarray analysis of our aspergillus biofilm model, we have identified phase dependant transcriptional markers of biofilm growth and maturation. We have identified a number of these markers in respiratory samples from an animal model and confirmed that these markers vary in a phase dependant manner in this model. Further we have identified these markers in clinical samples.

We propose that these biomarkers may be a useful adjunct to clinical decision making in this complex patient group in particular in determining the difference between transient colonization with *A. fumigatus* and organisms which are present as biofilm.

SHORT AND LONG-TERM OUTCOMES WITH PULMONARY FUNGAL INFECTIONS IN PEDIATRIC LUNG TRANSPLANT RECIPIENTS

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BACKGROUND: Risk factors, morbidity and mortality from pulmonary fungal infections (PFIs) within the first year after pediatric lung transplant have not previously been characterized. Further, the epidemiology and impact of fungal colonization and PFIs on long-term outcome in pediatric lung transplant recipients is previously unknown.

METHODS: Two retrospective reviews of pediatric lung transplant recipients were conducted. A multicenter study from 1988 to 2005 was conducted with institutional approval from the 12 participating centers in North America and Europe. Data were recorded for the first post-transplant year. The log-rank test assessed for the association between PFI and survival. Associations between time to PFI and risk factors were assessed by Cox proportional hazards models. For the impact of colonization, a retrospective analysis of 55 pediatric lung transplant recipients from 2002 to 2007 at a single institution was performed. Associations between risk factors and time to post-transplant colonization, PFI, and other outcomes were assessed using Cox proportional hazard models.

RESULTS: Of the 555 subjects transplanted in the first study, 58 (10.5%) had 62 proven (*Candida, Aspergillus* or other) or probable (*Aspergillus* or other) PFIs within the first year post-transplant. The mean age for PFI subjects was 14.0 years vs 11.4 years for non-PFI subjects ($p < 0.01$). *Candida* and *Aspergillus* species were recovered equally for proven disease. Comparing subjects with PFI ($n = 58$) vs those without ($n = 404$), pre-transplant colonization was associated with PFI (hazard ratio [HR] 2.0; 95% CI 0.95 to 4.3, $p = 0.067$). Cytomegalovirus (CMV) mismatch, tacrolimus-based regimen and age >15 years were associated with PFI ($p < 0.05$). PFI was associated with any prior rejection higher than Grade A2 (HR 2.1; 95% CI 1.2 to 3.6). Cystic fibrosis, induction therapy, transplant era and type of transplant were not associated with PFI. PFI was independently associated with decreased 12-month survival (HR 3.9, 95% CI 2.2 to 6.8).

In the evaluation of colonization and its impact, although 29 patients had positive pre-transplant colonization, 33 (60%) were colonized post-transplant and 20% (11 subjects) developed proven or probable PFI. In a multivariate model, post-transplant fungal colonization was associated with older age (hazard ratio [HR] 2.9, 95% confidence interval [CI] 1.1 to 7.6), cytomegalovirus (CMV) prophylaxis (HR 5.6, 95% CI 1.3 to 24.6) and respiratory viral infection prior to fungal colonization (HR 2.9, 95% CI 1.0 to 8.3).

CONCLUSIONS: Risk factors for PFI include Grade A2 rejection, repeated acute rejection, CMV-positive donor, tacrolimus-based regimen and pre-transplant colonization. In addition, neither fungal colonization nor PFI was associated with the development of chronic allograft rejection or death.

ROUTINE MOULDS' IDENTIFICATION IN THE CLINICAL LABORATORY BY MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

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Objective. MALDI-TOF MS analyses the protein content of a microorganism which is materialized by a spectrum similar to a proteomic fingerprint. Unknown organisms are then identified by comparing their spectrum with a reference spectra database. This technique recently emerged as a valuable identification tool of bacteria and yeasts but has not yet been adapted to the identification of moulds that are isolated from cystic fibrosis patients' sputa. Our study aimed to validate a standardized procedure suited for the MALDI-TOF MS-based proteomic identification of moulds in the routine clinical laboratory.

Methods. In a first step, we tested on a wide array of mould's species the influence of protein extraction from colonies using chemical extraction and/or a mecanical lysis and/or a heat shock on the spectra quality. The optimized procedure was used to build a reference spectra database from 146 mould's reference strains.

In a second step, sequential clinical moulds' isolates from routine activity of the mycology laboratory at the teaching hospital of Marseille (France) were prospectively identified using this standardized proteomic identification procedure. Spectra analyses were performed on an UltraFlex III MALDI TOF-TOF mass spectrometer using the MALDI Biotyper 2.1 software (Bruker Daltonics). Each isolate was also identified by conventionnal identification methods available in our laboratory, *i.e* macroscopic and microscopic identification by skilled mycologists and DNA sequencing of the ITS1-ITS2 and D1-D2 variable region of the 28S unit of the rRNA gene. The results of proteomic and conventional identifications were then compared. The proteomic identification of the clinical isolates was considered adequate if it was concordant with the conventional identification. Otherwise, it was considered inadequate.

Results. The standardized protein extraction procedure consisted in a chemical extraction of fungal colonies. From July to November 2010, 177 clinical isolates were cultured and identified in our laboratory and submitted to proteomic identification. Proteomic identification failed in 21 isolates belonging to species that were not included in the reference database. Of the 156 remaining isolates, proteomic identification was adequate in 154 (98.7%). Proteomic identification yielded uninterpretable results for one *Beauveria bassiana* isolate and falsely identified as *Mucor circinelloides* a *Rhizopus oryzae* isolate.

Conclusions. This work's seminal finding is that a standardized procedure can also be used for proteomic-based identification of a wide array of clinically relevant mould species. It thus makes it possible to timely identify moulds in the routine clinical laboratory setting and opens new avenues for the development of a high-throughput proteomic-based solution for the identification of moulds in the respiratory track of cystic fibrosis patients.

MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY FOR FAST AND ACCURATE IDENTIFICATION OF CLINICALLY RELEVANT *SCEDOSPORIUM* SPECIES

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Current identification of *Scedosporium spp.* based on morphological characteristics fails to detect emerging species described using multi-locus sequencing (MLS). Among the 8 recently described phylogenetic species which grouped in five different clades, only *Scedosporium apiospermum* (clade 4) and *Pseudallescheria boydii* (clade 5) are known as common pathogens in humans, while the 6 others are emerging, including for exemple *Scedosporium aurantiacum* (clade 1), *Pseudallescheria minutispora* (clade 2), or *Scedosporium dehoogii* (clade 3). In addition, another *Scedosporium* species, *Scedosporium prolificans*, which is also common human pathogen but is not included in these clades, cannot be differentiated from those belonging to the above-mentioned clades using morphological techniques. However, accurate identification is important because antifungal drug susceptibility patterns may vary with the species. Matrix-assisted laser desorption ionization-time mass spectrometry (MALDI-TOF MS), allows rapid and reliable identification of microorganisms. We postulated that this method, can also discriminate species of *Scedosporium* just as we showed for *Aspergillus* species

A set of 7 reference strains belonging to 5 clinically relevant species *(S. apiospermum, P. boydii, S. aurantiacum, S. prolificans, P. minutispora)* was used to build a reference database Profiles from each referenced strain at different age of the cultures (Days 3, 5, 7) were analyzed to identify species specific discriminating peaks. The spectra of 50 strains (25 *P. boydii,* 19 *S. apiospermum,* 3 *S. aurantiacum,* 2 *P.minutispora*, 1 *S. prolificans*) previously identified with MLS (tubulin and/or ITS) were compared to that of each of the reference strains. This database allowed correct identification of 48/50 strains (96%), with no misidentification. Our results, obtained using a simple protocol with no extraction step showed that MALDI-TOF-MS is a powerful tool for rapid identification of clinically relevant species of *Scedosporium,* including those emerging that cannot currently be identified by micoscopic examination.

SCEDOSPORIUM SPP.: DETECTION, IDENTIFICATION AND ANTIFUNGAL SUSCEPTIBILITY PROFILE.

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INTRODUCTION: *Scedosporium apiospermum* is among the most common filamentous fungi colonizing the lungs of cystic fibrosis patients. *Pseudallescheria boydii* has been described for many years as the sexual state of *S. apiospermum*, however the use of molecular techniques showed that *P. boydi* and *S. apiospermum* is a complex of species. In the last years, several new species able to cause infections in humans have been described.

OBJECTIVE: The aim of this work was to describe the prevalence of these new species in a collection of clinical samples and to asses their susceptibility profile against the antifungal drugs available.

METHODS: Fifty two clinical isolates were identified by morphological and molecular methods. Susceptibility profile to nine antifungals was determine by EUCAST methodology.

RESULTS: Twenty five strains were identified as *S. apiospermum*, 13 as *P. boydii*, six as *P. ellipsoidea*, five as *S. aurantiacum* and three as *S. dehoogii*. No clear differences in antifungal activity were detected among the species of the complex. Voriconazole was the most active drug followed by posaconazole, whereas amphotericin B and itraconazole showed a limited activity.

CONCLUSIONS: The prevalence of new species of *S. apiospermum* species complex in clinical samples is low. No significant differences were found in the susceptibility profile of these species to the antifungal drugs.

ABPA: BIOMARKERS FOR DIAGNOSIS AND DISEASE STAGING

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Allergic Bronchopulmonary Aspergillosis (ABPA) is a common complication in patients with CF, with a reported prevalence of 4.4% in the US. The colonization of the airways of CF patients with *Aspergillus*, likely a consequence of disrupted mucociliary clearance, leads to a heavy allergen load on the epithelial surface and sensitization of the individual. ABPA develops as a result of an immune mediated response through activation and proliferation of the bronchoalveolar lymphoid tissue (BALT), with TH2 lymphocytes playing a central role. Immunoblasts in the BALT produce large amounts of IgE directed against specific Aspergillus proteins. For reasons that probably include individual genetic susceptibility, affected patients mount a hyperIgE response that is more noticeable in the acute flares of the disease. Additional detectable immunologic parameters in patients with ABPA include peripheral blood and sputum eosinophilia, immediate cutaneous reactivity to *A. fumigatus* antigen, presence of precipitating antibodies to *A. fumigatus*, elevated specific IgE and IgG antibodies to *A. fumigates* antigen, and increased serum concentrations of IL-2 receptor (IL-2R). Thus a combination of these biomarkers has been recommended as diagnostic criteria for the presence and staging of ABPA in the individual patient. However, given the high frequency of colonization with *Aspergillus* among CF patients, it is clearly recognized that in many patients there is great difficulty in making a determination as to the presence and activity of the disease, with confounding factors such as polymicrobial infections, medications use, and other allergic sensitizations. Therefore, there is a growing interest for the development of better biomarkers for ABPA diagnosis and monitoring. At Stanford University, we have had an interest in biomarkers based on the activation of basophils. Basophils are key effectors in allergic responses, following the cross-linking by incoming allergens of membrane-bound IgE molecules. These signal through the basophil high-affinity receptor for IgE, leading to rapid degranulation and release of histamine, tryptase, leukotrienes and cytokines. CD203c is a glycosilated transmembrane protein that is restricted to basophils and mast cells and can be utilized as a specific marker for this cell lineage. Further, upon allergen challenge, a rapid upregulation of CD203c occurs in the basophil and it can be used to monitor their activation state. Taking advantage of this biomarker, by flow cytometry our group has already reported that the mobilization of CD203c upon antigen stimulation can be used to monitor patients with peanut allergy in their response to therapy with IgE blockade (omalizumab). For this study, blood samples were activated *in vitro* with peanut allergen and by flow cytometry surface mobilization of CD203c was monitored. With this assay, treatment responses were clearly demonstrated. We have taken this observations further to investigate their potential utility for the detection of ABPA activity in patients with CF. Our preliminary studies in a small group of CF patients with ABPA demonstrates increased mobilization of CD203c to the surface of basophils upon *in vitro* stimulation with antigen, compared to CF patients without ABPA but colonized with *Aspergillus* and CF patients with no history of *Aspergillus* colonization or ABPA. Our ongoing studies are further validating the mobilization of basophil CD203c upon stimulation as a biomarker of ABPA and its correlation with already established biomarkers of ABPA as well as with clinical parameters of disease severity. In addition, we are evaluating the value of longitudinal monitoring of this parameter in comparison with other biomarkers used for disease monitoring.

ROLE OF REAL TIME PCR AND GALACTOMANNAN IN THE CLASSIFICATION OF *ASPERGILLUS* DISEASE IN CF

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Purpose. Patients with ABPA are routinely treated with azole antifungals but it is not known whether colonised or sensitised patients would similarly benefit from antifungal treatment. The reported prevalence of *Aspergillus* from culture of CF sputum varies widely from 12-57%. To aid treatment decisions and monitor treatment response more accurate methods to detect *Aspergillus* in sputum are needed. This study aimed to validate and then integrate Real time PCR and galactomannan antigen detection, from CF sputum, with serological analysis to classify patients groups that may benefit from antifungal therapy.

Methods. 146 adult CF patients provided a sputum sample and a blood sample. 30 patients provided a second sputum sample within 6 months. Serological tests included total IgE (tIgE), specific *A. fumigatus* IgE (sIgE) and specific *A. fumigatus* IgG (sIgG) performed by Phadia ImmunoCAP® assay. Sputum samples were homogenised with sputasol (Oxoid, UK) and sonication (Sonics® VC505). 10µL was cultured on sabouraud agar with chloramphenicol (Oxoid, UK) for 72 hours. 300µL was used in the Platelia™ *Aspergillus* EIA kit (Bio-rad, Marrnes-La-Cocquette, France) to measure galactomannan (GM) antigen, the cut-off optical index of≥ 0.5 being used to define positivity. The remaining sample was used in a commercial real time PCR assay, MycAssay™ Aspergillus, to detect *Aspergillus* DNA. Latent class analysis was used to define patient groups using M*plus* version 6.1 software.

Results. 37% (39) of the 146 sputum samples were positive for *Aspergillus* species by standard culture whereas 74% were positive for *Aspergillus* species by PCR. Repeatability with 6 months was performed in 30 patients. All PCR positive samples remained positive and 4 negative samples became positive. PCR repeatability within a sample was performed on 10 samples. Repeatability was 100% with a Ct values varying by <1 PCR cycle. 68 of the 146 samples were GM positive. Inter-assay % coefficient of variation (%CV) over 5 days was 12% and intra-assay %CV was 5%. Latent class analysis identified 4 classes based on serological results, PCR and GM. The average latent class probabilities were: class 1: 0.969, class 2: 0.993, class 3: 0.917, class 4: 0.977. Table 1 demonstrates the mean serological data for each class and the probability of positive PCR and GM.

	Mean sIgG (mg/l)	Mean sIgE (KUa/l)	Mean tIgE (KUI/l)	+ve PCR probability	+ve GM probability
Class 1	40	0.5	42	0.5	0.04
Class 2	101	0.6	85	1.0	1.0
Class 3	53	7.4	228	0.6	0.0
Class 4	113	27.0	1300	0.9	0.8

16 patients were already on antifungal therapy and repeat latent class analysis is currently ongoing with these patients excluded.

Conclusion. Real time PCR can more accurately identify CF patients with *Aspergillus* in their sputum than standard culture. GM may help to distinguish those colonised from those with active *Aspergillus* growth. Latent class statistical analysis suggests the presence of 4 distinct groups of patients based on serological, PCR and GM data. These 4 classes can be clinically interpreted as: Class 1 = controls, Class 2 = '*Aspergillus* bronchitis', Class 3 = sensitised and Class 4 = ABPA. A randomised trial of antifungal therapy is required to determine if there is clinical benefit in treating class 2 or 3 patients.

MONOCLONAL ANTIBODIES SPECIFIC TO *SCEDOSPORIUM APIOSPERMUM* AND *SCEDOSPORIUM PROLIFICANS* – A RESOURCE FOR ISHAM CF RESEARCH

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Previously, we reported the development of murine immunoglobulin M (IgM) and IgG1□ - light chain monoclonal antibodies (MAbs) specific to *Pseudallescheria boydii* (Thornton [2009] *Clinical and Vaccine Immunology* **16:** 756-764). The MAbs bind to an immunodominant carbohydrate epitope on an extracellular 120-kDa antigen present in the spore and hyphal cell walls of *P. boydii*, *Scedosporium apiospermum* and *S. aurantiacum*. The MAbs do not react with *S. prolificans*, *Scedosporium dehoogii*, or a large number of clinically relevant fungi including those known to be present in the lungs of cystic fibrosis patients. The MAbs were used in immunofluorescence and double-antibody-sandwich-enzyme-linked immunosorbent assays (DAS-ELISAs) to accurately differentiate *P. boydii* from other infectious fungi and to track the pathogen in environmental samples.

The purpose of this paper is to report the development of a species-specific MAb raised against the related emerging human pathogen *Scedosporium prolificans*. The IgG1 MAb, CA4, binds to a protein epitope on an intracellular antigen. It is highly specific, recognising *S. prolificans* only, and does not cross-react in ELISA or Western blotting studies with the related fungi *P. boydii*, *S. apiospermum*, *S. aurantiacum*, *S. dehoogii*, with other fungi in the *Pseudallescheria* complex, or with other clinically relevant fungi including *Aspergillus*, *Candida*, *Exophiala*, *Fusarium* and *Rhizopus*.

The *P. boydii* and *S. prolificans* specific MAbs represent a practical resource for use by the CF research community and their potential use in collaborative studies will be discussed.

GENETIC DIVERSITY AND ANTIFUNGAL SUSCEPTIBILITY OF *ASPERGILLUS FUMIGATUS* FROM CHRONICALLY COLONISED PORTUGUESE PATIENTS

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The application of molecular methods for genotyping of fungi, particularly *Aspergillus fumigatus*, revealed that cystic fibrosis patients may present chronic colonisation by the same strain. We have been comparing the diversity and persistence of *A. fumigatus* strains in several cystic fibrosis patients with and without the diagnosis of allergic bronchopulmonary aspergillosis (ABPA). Simultaneously, we determined the susceptibility to several antifungal agents of *A. fumigatus* isolates (some of them showing the same genotype) before and after the treatment of cystic fibrosis patients with itraconazole or voriconazole. The genetic diversity of the fungal isolates was assessed following microsatellite genotyping and some strains were found in the patient sputum samples more than 6 years apart. Three out of six patients (50%) matching the modified criteria for diagnosis of ABPA showed *A. fumigatus* strain persistence, as well as two other patients without ABPA diagnosis. Microevolutionary events could be frequently found among *A. fumigatus* strains isolated from the same patient over time, particularly in four patients that received antifungal treatment (itraconazole or voriconazole). No resistant isolates were identified to the antifungals amphotericin B, caspofungin, itraconazole and voriconazole, while a single *A. fumigatus* isolate was identified outside of the epidemiological cutoff of 0.25 µg/mL for posaconazole. Even after azole exposure, several recurrent *A. fumigatus* strains were detected in the following sputum samples. The development of resistance of *A. fumigatus* to the antifungals seems to be rare among cystic fibrosis patients.

REAL TIME TYPING OF *ASPERGILLUS FUMIGATUS*

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Confirmation of suspected outbreaks of fungal infections require high resolution molecular genotyping methods. A large variety of methods have been reported in literature that differ in complexity, discriminatory power, costs, turn-around-time, reproducibility and stability. In recent years there is a growing tendency to use repeated DNA elements based or sequence based typing methods since these allow generation of typing data that is easily stored and exchanged between laboratories. However, these methods are not readily available to laboratories with no access to the specialized equipment needed for these kinds of analyses (usually a capillary DNA analyzer) or with no or only little experience in molecular typing. As a result, such laboratories often refrain from performing such typing analyses.

In an attempt to develop a typing method that is more accessible to non-specialized laboratories as well we chose to develop an entirely new genotyping approach. In this approach we choose not to try to determine if isolates may be identical or clonally related, but instead we aim to show in a definitive manner that they are not. The choice of typing targets allows easy and unambiguous genotyping. The real-time PCR format without the use of expensive fluorescent labeled probes makes this approach far more accessible than methods currently in use. Furthermore, compared to existing genotyping methods, the new approach is extremely simple, fast and economical. The diversity index evaluated on a collection of >200 isolates of various origin was 0.985. This means that there is a chance of only 1.5% that any two randomly tested isolates yield the same genotype. This approach thus provides a rapid simple and accessible screening method to evaluate potential outbreak situations.

DIFFERENT COLONIZATION PATTERNS OF *ASPERGILLUS TERREUS* IN PATIENTS WITH CYSTIC FIBROSIS

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Due to the abnormal viscosity of airway secretions, patients with cystic fibrosis (CF) are at high risk of fungal colonization of the respiratory tract. *Aspergillus fumigatus* is by far the most common fungal specie encountered in the CF context, but other species are increasingly reported such as *Scedosporium* spp. or *Geosmithia argillacea*, as well as other aspergilli including *Aspergillus terreus*. In our experience, this saprophytic fungus ranks the third among the filamentous fungi colonizing the respiratory tract of CF patients. Additionally, although relatively uncommon, infections caused by *A. terreus* present a high mortality rate due to its usually low susceptibility to systemic antifungals. Nevertheless, little is known about the epidemiology of *A. terreus* colonization/infections. In the present study, nine short tandem repeats of *A. terreus* were used to genotype 122 clinical isolates recovered from sputum samples from five patients with CF followed-up in two distinct hospitals in France (Angers and Giens hospitals). Sputum samples were collected over a two-month to seven-year period depending on the patients, and for each sample, all the obtained isolates were studied, with a maximum of five per sample.

Three colonization patterns were observed. The first colonization pattern consisted of a chronic colonization (defined as the presence of the same genotype in at least two successive samples collected over a minimum period of two months) by a largely dominant genotype associated with two or three other genotypes found occasionally (patient 4) or over a short period (patient 3). The second colonization pattern consisted of a chronic colonization by two distinct genotypes simultaneously detected (patients 1 and 2). For the last patient (patient 5), who was followed during four years, 16 isolates recovered from 6 sputum samples were analyzed, corresponding to 8 genetically distinct genotypes which succeeded to each other. Numerous questions therefore raise from these different colonization patterns, regarding (i) the origin of the contamination of the patients by this relatively uncommon environmental fungus, (ii) the differences between genotypes in their ability to chronically colonize the airways of the CF patients, or (iii) the differences between CF patients in their individual susceptibility to same genotypes.

THE SPECTRUM OF *SCEDOSPORIUM* ISOLATES FROM THE RESPIRATORY TRACT IN CF-PATIENTS – MULTI LOCUS SEQUENCE TYPING (MLST) AND DOCUMENTED CLINICAL RELEVANCE

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Within the German network for *Scedosporium* in cystic fibrosis 158 *Scedosporium* isolates from 51 patients have been collected and re-identified between 12/2008 and 11/2010. *S. apiospermum* was found in 53%, *P. boydii* in 22%, *S. prolificans* in 18% and S. *aurantiacum* in 7%. Although many patients are colonized for months or even years without significant clinical relevance, a dramatic decrease of the lung function was documented in patients in association with the isolation of *S. apiospermum* from the airways.

MLST was performed for >110 *P. boydii*- and *S. apiospermum* isolates, including several retrospective isolates of the same patient.

The MLST based on identification of the species by sequencing of the ITS regions, and the five loci actin (*ACT*), calmodulin (*CAL*), RNA polymerase II second largest subunit (*RPB2*), beta-tubulin (*ßTUB*) and Mn-superoxide dismutase (*SOD2*).

The genotyping results revealed, that the individual patients can be colonized with an individual genotype for years, rarely harbouring a second clone or species.

UPDATE OF AUSTRALIAN *SCEDOSPORIUM* STUDIES, WITH EMPHASIS ON THE DEVELOPMENT OF AN MLST SCHEME FOR THE EMERGING SPECIES *S. aurantiacum*

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Scedosporium species are clinically important emerging pathogens. Human infections are mainly caused by *Scedosporium prolificans, Scedosporium apiospermun, Pseudallescheria boydii* and *Scedosporium aurantiacum.* Amongst patients with damaged airways, they are the second commonest filamentous fungi after *Aspergillus* spp. isolated from cystic fibrosis (CF) patients. Although invasive infection is rare prior to lung transplantation, fungal colonisation may be a risk factor for invasive disease with attendant high mortality post transplantation.

In a pilot study at the Westmead Hospital CF clinic, 218 sputum specimens from 69 patients were investigated. *Aspergillus fumigatus* was isolated from 46 patients (66.7%), which was followed by *Scedosporium* spp. (12 patients; 17.4%), *Penicillium* spp. (14 patients, 20.3%) and *Aspergillus flavus* (7 patients, 10.1%). Co-colonization of *Scedosporium* and *Aspergillus* species was found in 12 patients. The newly emerging species *S. aurantiacum* was found in equal numbers with *S. prolificans*. *S. aurantiacum* was also found in high numbers in none-CF patients and in the Australian urban environment.

To enable fast and accurate laboratory diagnostic we designed species- or group specific primers based on the ITS1/2 region for *S. aurantiacum, Scedosporium dehoogii, S. prolificans, Pseudallescheria boydii* species complex (former clade 5)*/Pseudallescheria apiosperma* (formerly classified as *S. apiospermum* sensu lato) and *Pseudallescheria minutispora.* A multiplex PCR assay was developed specific for the three most clinically relevant species: *S. aurantiacum, S. prolificans*, and the *P. boydii* species complex/*P. apiosperma.* This multiplex assay was validated using sputum specimens collected from CF patients. The sensitivity and specificity of the multiplex PCR assay was 62.1% and 97.2%, respectively.

To gain overall insight into the population genetic structure of *S. aurantiacum* a multilocus sequence typing (MLST) scheme was developed including six genetic loci: actin (*ACT*), elongation factor-1α (*EF1*α), calmodulin (*CAL*), RNA polymerase subunit II (*RPB2*), manganese superoxide dismutase (SOD2), β-tubulin (TUB). Among the geographically diverse collection of 127 clinical and environmental isolates, between 6-18 variable sites per genetic locus were revealed, resulting in 10-16 defined alleles per genetic locus. A unusual high genetic diversity was observed in the *S. aurantiacum* population with the majority of the strains forming unique sequence types. The Australian strains were clearly genetically separated from non-Australian strains. There was no clustering of Australian strains

originating from the same state. Strains did also not cluster according to their sources, i.e from clinical specimen, veterinary or environmental origin. The high diversity among the Australian strains suggests that *S. aurantiacum* may have originated within the Australian continent and subsequently dispersed to other regions, a fact revealed by the close phlyogenetic relationships between some of the Australian sequence types and those found in other parts of the world. The MLST data are accessible at mlst.mycologylab.org.

ROLE OF PTX3 IN CYSTIC FIBROSIS-ASSOCIATED INFECTIONS

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PTX3, a soluble innate immunity receptor, binds to selected microbes and facilitates their clearance by phagocytes. PTX3 selectively binds to *Pseudomonas aeruginosa* and *Aspergillus fumigatus*, two microorganisms frequently colonizing the airways of patients with cystic fibrosis (CF), and sometimes causing true respiratory infections. $PTX3^{-/-}$ mice are sensitive to *A. fumigatus* infection, highlighting the role of this protein in the protection against this pathogen. We thus hypothesized that PTX3 could be altered in CF patients and that this could be responsible, at least in part, to their susceptibility to some opportunistic pathogens.

Serum and sputum samples from 30 CF patients (20 adults and 15 children) and 7 patients with chronic obstructive pulmonary disease (COPD) as the control group were analyzed for PTX3 expression and integrity by ELISA and Western-blotting, respectively. The role of endogenous or microbial proteases on recombinant human PTX3 was also analyzed.

Results showed that PTX3 level was increased in CF and COPD serum, highlighting their infectious/inflammatory status, while, in contrast, PTX3 concentration was lower or undetectable in CF sputum than in COPD. Western-blotting showed that PTX3 is degraded in sputum samples from most of CF patients, but not in clinical specimens from COPD patients. The degradation of PTX3 was shown to be mediated by serine proteases. More precisely, both the neutrophil elastase and the alkaline proteinase from *A. fumigatus* have the ability to degrade *in vitro* PTX3.

This study which shows that PTX3 is degraded in respiratory secretions from CF patients, provide new insights into the pathogenesis of microbial colonization of the airways and respiratory infections in CF patients, since degradation of PTX3 could be responsible, at least in part, for the sensitivity of CF patients to some opportunistic infections.

ASPERGILLUS-INDUCED INFLAMMATION IN THE CYSTIC FIBROSIS LUNG: RESPONSE TO ANTIFUNGAL THERAPY.

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In this study, we show that vitamin D receptor (VDR) gene expression was significantly down regulated by *Aspergillus* culture media in CFTE and CFBE cells. qRT-PCR confirmed significant down regulation of VDR gene expression *in vivo* in bronchial brushings from *Aspergillus* colonised patients in line with *in vitro* results. The VDR modulator present within culture filtrates of *A. fumigatus* was characterized. The inhibitory activity was stable within cultures stored at -70°C and subsequently used against CFBE cells at 37°C but was significantly abolished by heat inactivation (99°C) for 20 min. Passage of culture filtrates through a 3 kDa size exclusion filter removed approximately 65% of the VDR inhibitory activity, and this was recovered in the filtrate, which suggested the presence of both high- but predominately low-molecular weight (<3kDa) VDR inhibitory factors. Secreted by the hyphal form of *A. fumigatus*, gliotoxin (Gt) has previously been characterised as a small heat inactivated toxin of 326.4 daltons[2] and to confirm Gt as a fungal agent capable of VDR down regulation, the effect of a low Gt producing *A. fumigatus* strain (CaF1) on VDR expression was explored. Strain CaF1 produced minimal levels of Gt compared to the high Gt producing strain ATCC-26933 after 96 h of fungal growth (2 ± 0.05 and 102 ± 23 ng/mg hyphae respectively). Moreover, VDR expression in both CFTE and CFBE cells remained relatively unchanged when treated with 1-4 day culture filtrates of CaF1, in comparison to the ATCC-26933 strain which resulted in significant down regulation of VDR. The clinical relevance of these findings were confirmed as BAL obtained from *Aspergillus* positive patients with CF had significant higher levels of Gt compared to *Aspergillus* negative patients, as quantified by HPLC analysis of chloroform extracts. Moreover, the effect of purified Gt (0.16 µM and 0.8 µM) on VDR expression in both CF and non-CF cells revealed that Gt (0.8 µM) significantly down regulated VDR gene expression in CFTE ($P = 0.002$), CFBE ($P = 0.02$). In addition, it was observed that Gt (0.8 µM) overcame the positive effect of vit D on VDR expression *in vitro*. CFBE cells treated with $1,25D_3$ in the presence of 0.8 µM Gt exhibited significant down regulation of VDR gene expression compared to cells treated with $1,25\ D_3$ only. Collectively these experiments suggest an overwhelming inhibitory effect of Gt on VDR gene expression which was confirmed in vivo by significant decrease in Gt in CF lung BAL post aspergillus therapy with itraconozole.

LECTINS FROM PATHOGENIC FUNGI: NEW THERAPEUTICAL TARGET

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Invasive fungal infections are increasingly common as hospital acquired infections related to the development of immunosuppressive therapies. They are highly dangerous for patients in intensive care units or under immunosuppressive treatments. Lungs are the most common target but other organs (skin, heart, kidneys, brain, etc.) can be affected. Prolonged lung colonization leads to sensitization and sometimes to allergic bronco-pulmonary disease, which accelerate lung deterioration. These infections are difficult to diagnose and cause high morbidity and mortality in particularly in cystic fibrosis patients, which present an altered mucociliary clearance and viscous mucus. The increasing resistance to antifungal treatments required the development of new therapeutical approaches.

Aspergillus fumigatus is the most prevalent airborne fungal pathogen in developed countries and the second most common cause of nosocomomial fungal infections shortly followed by *Scedosporium apiospermum.* Fungi like other pathogenic microorganisms often use glycoconjugates present on the host epithelia for specific recognition and adhesion by receptors called lectins or adhesins. The nature of the glycans vary with the tissue, but also with the individuals (ABO and Lewis histo-blood group phenotypes) resulting in variability in cell surface glycolandscapes. To determine their role in adhesion, we want to explore the lectins of those fungi and hence their glycostrategies. Lectins are promising drug-targets in particularly for antiadhesive therapy which is based on preventing pathogen adhesion - and therefore following infection – by fooling the pathogen receptors with soluble glyco-compounds that are similar to the human glycoconjugate epitopes on the surface of epithelial cells (glycomimetics). The use of glycomimetics has been demonstrated to be an efficient therapeutical route for some viruses and bacteria (*Pseudomonas aeruginosa).1* Since this approach does not affects directly the life processes of the pathogen, the development of resistance is less probable in this case.

Development of glycomimetics requires detailed information on the lectin-ligand interactions at a molecular level. We have already identified ten potential lectins in the genome of *A. fumigatus*. The protein AFL1 displays significant homology to previously studied fungal lectin from *Aleuria aurantia* (AAL).2 The recombinant lectin has been expressed in *Escherichia coli* and purified by conventional chromatography. Its binding activities were determined by hemagglutination studies, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) and demonstrated preference for fucose and fucosylated oligosaccharides. The 3-D structures of AFL1 in complex with several fucosylated ligands were determined by X-ray crystallography. They permitted a thorough analysis of its six sugar binding sites and opened the route to the design of inhibitors.

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ECOLOGY OF THE AIRWAY MICROBIOME IN CYSTIC FIBROSIS

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The airways of CF patients are typically overburdened with microorganisms, oxidants and proteases, as well as by large concentrations of inflammatory mediators. It is now clearly recognized that the presence of infection in the CF airway is a dynamic process that evolves as the patients age. It is traditionally being held that early in life bacterial species such as *Haemophilus influenzae* and *Staphylococcus aureus* predominate and later in life *Pseudomonas aeruginosa* becomes the most prevalent microorganism. Further, bacteria like *Pseudomonas* will adapt to the airway environment and change their phenotype to alginate producing biofilm forms. What is incompletely understood is the evolution of this bacterial presence in the airway and the number of species that might be involved in this process. We have hypothesized that the current paradigm of disease pathogenesis driven by a primary microrganism that is replaced over time by a more virulent species ignores the possibility of an evolving larger microbial population and with an ecologic profile unique to CF. We have taken advantage of the availability of culture independent microbiological identification tools to characterize the microbial ecology of the CF airway and to investigate for any contrasts against healthy controls, as well as for any correlations between microbiologic and clinical characteristics.

Our investigational approach is based on a phylogenetic survey of microbes in induced sputum specimens obtained from 16 CF patients and compared to 8 healthy controls. DNA was extracted from these samples and the 16S ribosomal gene sequences amplified using barcoded and universal fusion primers. PCR products were quantified using real-time and digital PCR and sequenced on the 454 DNA pyrosequencing platform. After eliminating human sequences and reads less than 150 bp long, the number of high-quality microbial reads per sample ranged from 1,389 to 48,525, for a total of 219,804. Each quality-filtered sequence was classified to a phylogenetic group using the ribosomal database project (RDP) naïve Bayesian rRNA classifier and nearly all the reads (219,728) were assigned to one of the RDP phyla. The Firmicutes were found to be the most prevalent in both CF and healthy control samples (60% vs 45%). The prevalence of Bacteroidetes was strikingly different between the two groups, with 22.6% in the healthy group, and only 3.4% in the CF group. Conversely, Actinobacteria were found with much higher prevalence in the CF samples (23.3%) than in the healthy samples (4.5%). A number of microbial phyla with few uncultured representatives were also present with lower prevalence, including TM7, SR1, and Gemmatimonadetes, with some (TM7 & SR-1) differentially represented across the healthy and CF sample groups. A striking difference between CF and controls was also noted in the relative uniformity of representation across microbial phyla and uncultivated groups of microbes in the healthy controls compared to the CF patients. This can be taken as evidence for a lack of microbial diversity in the CF samples compared to the healthy controls. Further, we investigated for any potential correlations between the diversity of the CF samples and the clinical characteristics of the patients. Using a severity index based on pulmonary function we found an inverse correlation between disease severity and microbial diversity ($r=-0.51$), suggesting that diversity is associated with better preserved lung function. Similarly we also found an inverse correlation between inflammatory biomarkers in sputum (elastase, total protein, IL-6, IL-8, MIP-1) and microbial diversity ($r=-0.61$). Our ongoing investigations are focused on the development and longitudinal changes of the microbial ecology of the lung as well as trying to better identify those organisms that are more intimately associated with either the preservation or the deterioration of the lung function.

POLYMICROBIAL INFECTIONS OF THE CYSTIC FIBROSIS LUNG: WHAT CAN WE LEARN FROM COMBINING CULTURE AND MOLECULAR APPROACHES?

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Background: Studies are increasingly showing the complexity of the microbes present in the Cystic Fibrosis (CF) airways. However, most of this work currently has focused on the bacterial species present with less work carried out on the fungi present. In addition to developing research on fungi alone, more work is needed to understand the impact of these polymicrobial infections[1] on lung disease progression.

Aim of the study: To analyse the bacteria and fungi present in sputa sampled from adults with CF by using conventional culture-based and culture-independent approaches.

Material and methods: Sputa were collected at Lille Hospital, North of France from individuals with full ethical approval. In addition to respiratory sample collection, a multidisciplinary set of information on clinical parameters, treatment regimes and ABPA status was recorded as previously described[2]. Samples were processed using standard protocols to detect a range of bacterial and fungal species by using routine diagnostic microbiological culture-based approaches. For culture-independent analysis, DNA was extracted from the sputum samples by using a High Pure PCR Template Preparation kit (Roche Applied Science, Germany). To characterise the bacteria in these samples, the culture-independent approach of Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was used to resolve the dominant community members through the resolution of ribosomal gene fragments[3]. To characterise the fungi in these samples, a culture-independent approach of ITS-RFLP was developed along with PCRs specific for *Aspergillus fumigatus*, *Scedosporium* sp. and *Pneumocystis jirovecii*.

Results: Thirty six sputum samples were successively collected from 15 adult CF patients between 2006 and 2009. The medium age was 31 (22-67) years. For each patient, the microbes in sputum samples were studied at several time-points (at least 1 per year between 2006 and 2009). At the bacterial level, we described from 2 to 15 (average of 6.5) bacterial species detectable by T- RFLP in this population of adult CF patients. Among these 15 patients, 9 were colonised with *H. influenzae* confirmed by using specific PCR assays but for which no growth was reported by culture-based assessment. At the fungal level, we were able to identify from 1 to 3 micromycete species, according to the ITS-RFLP method developed. Culture-based findings were compared to the culture-independent data; species richness as well as evenness of fungi and bacteria is discussed taking into account clinical data.

Discussion and conclusion: For the first time, we proposed an overall approach, studying both bacteria and fungi in sputa sampled from individuals with cystic fibrosis. These results reveal the complexity of CF lung microbial ecology as well as give some insight into community dynamics. One of the next steps is to extend the depth of analysis through the application of next generation sequencing technologies to characterise the microbial metagenome. We also anticipate benefits from determining whether the behaviour of bacteria may significantly influence the behaviour of fungi and vice versa. As such, as informed by culture-independent analysis, findings from *in vitro* systems[4] may provide some ecological insight of value to understanding and ultimately treating these polymicrobial infections.

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PHARMACODYNAMIC EFFECTS OF ANTIFUNGAL AGENTS ON BIOFILMS OF ASPERGILLUS FUMIGATUS STRAINS ISOLATED FROM CYSTIC FIBROSIS PATIENTS

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Aspergillus fumigatus is an opportunistic mould that can colonize the respiratory tract of patients with cystic fibrosis (CF) potentially causing allergic bronchopulmonary aspergillosis, mycetoma or invasive pulmonary aspergillosis. Recent studies have shown that *A. fumigatus* can develop biofilms (BF) by producing an extracellular hydrophobic matrix that acts as an interconnecting structure for the development of complex hyphal networks and establishment of fungal niches. Biofilms allow nutrients to flow into the resident cells, but they also alter the antifungal drug susceptibility profiles reducing their efficacy as compared to that of planktonic cells. Consequently, biofilm-related infections are difficult to treat and new therapeutic schemes are needed to be considered.

In this study, we analyzed the BF-forming capacity and antifungal drug resistance of BF of *A. fumigatus* isolated from sputa of seven patients with CF and a non-CF *A. fumigatus* strain (NIH 4215) to three antifungal agents: a polyene, amphotericin B (AMB); an azole, voriconazole (VRC); and an echinocandin, micafungin (MFG). We aimed to determine the *in vitro* activities of the three agents against *A. fumigatus* biofilms from CF patients and compare them to those against *A. fumigatus* planktonic (PL) cells, in order to identify putative differences in drug resistance among the seven CF clinical isolates and the non-CF strain of *A. fumigatus*.

Aspergillus fumigatus BF were produced in polystyrene 96-well plates using $1x10^6$ conidia/mL in MOPS-buffered RPMI, pH 7.2 by static incubation at 37^{o}C for 24 h. BF production was evaluated after 24 h using safranin staining and measuring the optical density at 492 nm by spectrophotometry. Biofilms were incubated in the presence of AMB, VRC, or MFG at doubling dilutions ranging from 0.015-128, 0.007-128, and 0.0035-128 mg/L, respectively for 24 h at 37^{o}C. Drug-free BF containing only RPMI-1640 served as controls. Five replicate BF were included to each condition and three independent experiments were performed. BF MICs were determined at approximately 50% fungal damage of the BF compared to controls, using the XTT reduction assay. PL MICs were determined according to the CLSI M38-A microdilution method as the minimum drug concentration that causes a prominent decrease in turbidity corresponding to 50% inhibition in growth as compared to controls. The concentrations used for the antifungal drugs in evaluating PL MICs were as follows (mg/L): AMB, 0.03-16; VRC, 0.007-128; MFG, 0.0035-32. The absorbance values of CF biofilms vs non-CF biofilms were compared by one-way ANOVA and Bonferroni's post-test. $P < 0.05$ was considered significant.

Planctonic MICs of all isolates were susceptible to all three drugs (PL MIC50 of AMB, VRC and MFG: 0.5 *vs* 0.25 mg/L, 0.06 *vs* 0.015 mg/L, and 0.03 *vs* 0.007 mg/L for CF *vs* non-CF isolates, respectively). BF of both CF and non-CF strains appeared to be susceptible to AMB with MIC50 ($\geq$ 50% fungal damage) comparable to those of the planktonically grown *A. fumigatus* (1-2 mg/L for BF of CF strains and 0.25-0.5 mg/L for BF of the non-CF strain).

At concentrations >2 mg/L, AMB achieved almost complete sterility of all BF isolates (>93% fungal damage); however, at 0.5 and 1 mg/L of AMB the CF strains exhibited significantly lower fungal damage as compared to the non-CF strain (4.80% *vs* 72% and 33.1% *vs* 95.4%, respectively; $P < 0.05$). By comparison, VRC and MFG did not appear to appreciably affect BF of any strain. VRC seemed to have a slightly better antifungal activity than MFG against BF of CF strains, but its activity did not cause > 30% of fungal damage.

We present preliminary data suggesting that PL cells of *Aspergillus* strains isolated from CF patients are as susceptible as the non-CF strain to all antifungal agents. By comparison, BF of both CF- and non-CF strains are markedly less susceptible to VRC and MFG; moreover, BF of CF-isolated strains are less susceptible (lower fungal damage) than the non-CF strain to AMB. These pharmacodynamic differences among BF and PL cells of *A. fumigatus* and among isolates derived from CF patients compared to non-CF strain may be important in the antifungal management of these patients.

ASPERGILLUS FUMIGATUS SUSCEPTIBILITY TO ITRACONAZOLE IN ADULT PATIENTS WITH CYSTIC FIBROSIS

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Objective: We hypothesized that *A. fumigatus* isolates in the sputum of cystic fibrosis (CF) patients with previous exposure to itraconazole may have higher prevalence of azole resistance than *A. fumigatus* isolates of CF patients with no exposure to itraconazole.

Methods: From June 2010 to April 2011 (10 months), adult CF subjects at Cochin University Hospital (France) had systematic examination of sputum samples for the detection of *A. fumigatus*. Minimum inhibitory concentrations (MICs) of *A. fumigatus* isolates against azoles were determined using the Etest method.

Results: *A. fumigatus* was isolated in the sputum of 131/249 (52.6%) adult CF subject and 47/131 (35.9%) subjects had previous exposure to itraconazole. Reduced *A. fumigatus* susceptibility to itraconazole (MICs≥ 2 mg/L) was found in 16/131 (12.2%) subjects; 6 of these 16 subjects also had reduced susceptibility to posaconazole (MIC≥ 0,5 mg/L), whereas all isolates were susceptible to voriconazole (MICs < 2 mg/L). Itraconazole exposure within the previous 3 years was associated with increased prevalence of azole resistant *A. fumigatus* isolates, with up to 32% and 20% subjects harboring itraconazole and posaconazole non-suceptibles isolates, respectively.

Conclusion: Unexpected high rates of azole-resistant *A. fumigatus* isolates were found in adult CF subjects and were significantly associated with prior itraconazole exposure. Although clinical implications of these findings will require further studies, cautious use of itraconazole in adult CF subjects may be recommended.

CLINICAL AND MICROBIOLOGICAL EFFICACY OF MICAFUNGIN ON *GEOSMITHIA ARGILLACEA* INFECTION IN A FRENCH CYSTIC FIBROSIS PATIENT

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Cystic fibrosis are at risk of colonization by a number of fungi, including *Geosmithia argillacea* which appears to be an emerging pathogen in these patients. This pathogen has been recently reported as a cause of invasive/systemic mycosis in immunocompromized patients such as colonized patients who are immunosuppressed for lung transplantation. In this context, we report here a case of clinical and microbiological efficacy of micafungin in a French cystic fibrosis patient chronically colonized with *G. argillacea*.

O.D., a female F508Del-CFTR homozygous patient was diagnosed at birth with cystic fibrosis in January 1996. She was found chronically colonised with multi-resistant *Staphylococcus aureus* (MRSA) from 1997 to 2011, and with *Aspergillus fumigatus* from 2001 to 2006. She was treated alternatively with oral voriconazole and itraconazole from 2004 to 2008, and with posaconazole since february 2008. *Geosmithia argillacea* was first diagnosed in May 2007, and chronic colonisation was persistent from this date to August 2010 with 23/28 fungus positive sputum samples, in spite of posaconazole therapy. For an isolate obtained in October 2008, minimal inhibitory/effective concentrations (MIC/MEC, mg/ml) determined using the Eucast method were 2.0, 2.0, 16.0, 2,0, 0.25 and 0.015 for amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin and micafungin, respectively. The FEV1 predicted value was 73% at the time of first fungus isolation and was decreased to 47% in October 2009. She then was given caspofungin for 21 days ((70 mg/day, later reduced to 50 mg) which resulted in clinical improvement (FEV1 = 64% in January 2010) without eradication of *G. argillacea*. In June 2010, treatment with micafungin (75 mg, 21days) was realized owing to deterioration of the respiratory function (FEV1 = 56%),without clinical improvement (FEV1 = 47% in August 2010). O.D. was then treated from September, 23 to November 3, 2010 with micafungin (100mg bid for 21 days and 100mg/day for the following 21 days) which resulted in clinical and microbiological improvement. FEV1 predicted ranged 67-68% in October and December 2010, and February and May 2011, and from the end of treatment to December 2010, 5/6 sputum samples were found negative for *G. argillacea*. The positive sample contained fungus of the same genotype as previous isolates.

The present case is to our knowledge the first description of *G. argillacea* eradication in a chronically colonized cystic fibrosis patient. Similar to previous studies, *G. argillacea* colonization was detected in the presence of chronic MRSA after *A. fumigatus* eradication. Since no change in bacterial colonization was observed before, during, and after *G. argillacea* colonization, the present case is consistent with a pathogenic role of the fungus in cystic fibrosis patients. *In vitro* antifungal susceptibility assays suggested that echinocandins are most effective agents against this fungus with a lowest MEC for micafungin (7 isolates studied, MEC range: 0.015-0.03), although eradication could only be obtained with high dose micafungin for a long time (6 weeks).

SCEDOSPORIUM SPP. COLONIZATION IN CYSTIC FIBROSIS LUNG TRANSPLANT PATIENTS. A REPORT OF 8 CASES.

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Background: *Scedosporium* spp. are filamentous fungi with low susceptibility to conventional antifungal drugs which are responsible for colonization and severe infections in cystic fibrosis lung transplant patients (CFLTx). The main antifungal drugs used for long term treatment are voriconazole and posaconazole.

Objectives: To retrospectively analyze clinical and mycological characteristics, therapeutic strategies, and outcome of CFLTx patients with *Scedosporium* spp. colonisation over a 10 years period at a single center.

Patients and Methods: All cases of *Scedosporium* spp. colonization in CFLTx patients in our center from 2000 to 2010 were reviewed. Recorded data included mycology, immunosuppressive regimen, antifungal treatments and clinical outcome. *Scedosporium* spp. colonization was assessed on respiratory samples by routine culture on mycological media. Identification to the species level was done by morphology and *in vitro* antifungal susceptibility testing was performed by Etest®. Therapeutic drug monitoring (TDM) of both azole and immunosuppressive drugs were routinely performed using analytical liquid chromatographic methods.

Results: Out of 134 CFLTx patients, 8 (4 males and 4 females) had a *Scedosporium* spp. colonization or infection. Mean age and weight were 19.9 ± 4.4 yrs, and 48.4 ± 9.3 kg, respectively which was not different from our usual CFLTx patients. Six out of the eight patients were colonized before transplantation and five of them received an antifungal treatment. One patient was excluded from further analysis because he developed an invasive *S. apiospermum* infection and died 2 months after transplantation despite intensive antifungal treatment (combination of voriconazole and caspofungin). The nine isolates exhibited usual susceptibility profile to voriconazole and posaconazole.

After transplantation, six patients were colonized with *S. apiospermum* and 1 with both *S. apiospermum* and *S. prolificans*.

The seven CFLTx patients colonized were initially treated with VRZ (n = 5) or PSZ (n = 2). VRZ was subsequently stopped in 3 cases. Mean VRZ and PSZ maintenance doses were 572 ± 207 (+ 43%) and 1550 ± 638 mg/d (+ 200%), respectively. Mean plasma levels were acceptable at 1.4 ± 0.7 mg/L for VRZ and 0.8 ± 0.6 mg/L for PSZ. Immunosuppressive treatments were steroids, tacrolimus and adjuvant. Survival ranged from 14 to 114 months after transplantation. Three patients died early (< 24 months) from bronchiolitis obliterans syndrome. Four are still alive, 2 of them currently treated with PSZ (with 2.25 and 1.4 g/day, respectively, to obtain serum levels of >1 mg/L).

Conclusions: *Scedosporium* spp. colonization may be controlled in CFLTx patients, using de novo, probably lifelong, treatment with VRZ or PSZ. VRZ is more appropriate if an IV route is needed, but exposes to adverse effects. Due to azole pharmacokinetic variability in CFLTx patients, the stady-state is longer to reach, and higher dosages with careful TDM are needed to achieve appropriate therapeutic efficacy and safe immunosuppressive drug-drug interactions.

TOWARDS STANDARDIZATION FOR MYCOLOGICAL EXAMINATION OF SPUTUM SAMPLES FROM CYSTIC FIBROSIS PATIENTS: FROM THE FRENCH MULTICENTER EXPERIENCE TO AN INTERNATIONAL STUDY.

Laurence Delhaes[1], Andrew Borman[2], Françoise Symoens[3], Jean-Philippe Bouchara[4], for the ECMM/ISHAM Working group on *Fungal respiratory infections in Cystic Fibrosis*

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Introduction: Prognosis of cystic fibrosis (CF) essentially depends on impairment of the lung function. While considerable attention therefore has been paid over recent decades to the prevention and treatment of bacterial respiratory infections, leading to a marked increase in life expectancy of the patients, prevalence of colonization of the airways by filamentous fungi or yeasts, sometimes leading to true respiratory infections, has been regularly increasing. However, recently, Borman *et al*. (2010) reported wide variations in the range of the causative fungal pathogens and in their prevalence, related at least to a lack of standardization in the methods used to detect these microorganisms.

Here, our aims are: (i) to analyze the impact of the culture conditions used on the detection of specific fungal pathogens throughout the French multicenter experience, and (ii) to discuss the methods used in various European or Australian laboratories, in order to carry out an international prospective study that will make possible a standardized protocol for efficient detection of the whole fungal biota that can be encountered in respiratory secretions of CF patients.

Results: First, from January 2007 to the end of 2009, a multicenter study was conducted in France encompassing 7 university or general hospitals which agreed to use the same procedure for mycological analysis of sputum saples from CF patients, including prior digestion of the sample with dithiolbutane and inoculation of the digested sample on 6 semi-selective agar-based media ("MucoFong" study – PHRC1902). Data obtained during one year were analyzed using the CHAID (Chi-squared Automatic Interaction Detector) method, which is a statistical approach able to give best association of media to detect a specific pathogen. CHAID is a type of decision tree technique, based upon adjusted significance testing that we apply to our data in order to define the best set of semi-selective media able to isolate 99.99% of the fungal pathogens that were detected in our CF population. Second, the major data of the international survey will be presented, and discussed with the purpose of developing a standardized approach for mycological examination of respiratory secretions from CF patients.

Conclusion: Defining the optimal method for mycological analysis of the fungal components of CF lungs microbiome through a large international study is becoming a major requirement. This will make possible not only to analyze the role of some "rare" filamentous species in CF exacerbation or the existence of geographic variations in the fungal species that colonize the airways, but also to study the complexity of the CF lung microbiome as well as its dynamics.

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Conflict of interest: None to declare

Keywords: Cystic fibrosis, filamentous fungi, yeasts, respiratory samples, culture methods

STUDY OF THE LUNG MICROBIOME OF NEWBORN CYSTIC FIBROSIS PATIENTS

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Cystic fibrosis (CF) patients suffer from chronic lung colonization as well as acute infections due to numerous bacterial and fungal infectious agents. There is more and more evidence that their bronchial microbiome is a complicated and dynamic association of pathogens, benign commensals, and/or synergetic organisms. Some of these microorganisms are believed to influence the pathogenic capacity of the microbiome through alteration of the microbe-microbe or polymicrobe-host interactions. Moreover, an increasing number of potentially emerging pathogens within CF airways are being described, both bacterial (e.g. *Stenotrophomonas maltophilia*, non-tuberculous mycobacteria, *Alcaligenes xylosoxidans*) and fungal (*Scedosporium* spp.). The clinical impact of the latter organisms on the evolution of the CF lung disease remains unclear, and therefore the clinicians are left with the questions if and when to treat them. In addition, the microbiological analysis of all these microbes with the classical culture methods remains to a certain degree unreliable. In particular, the possible importance of uncultivable microorganisms in CF lung disease remains to be determined.

The peculiarity of the respiratory system is the ongoing exposition to the environmental microbes at every breath. Some reports proposed a composition of the “normal” bronchial microbiome of CF patients. However, no data have been published concerning newborns with CF who have not been exposed for years to the environment. The latter microbiome seems of particular interest as its knowledge will allow studying the evolution overtime of CF lung microbiome, and investigating if a "normal" microbiome is a valuable concept.

The aim of the present research project is to characterize the bronchial microbiome of CF newborns using the metagenomics approach. Whole genomic DNA purified from broncho-alveolar lavages of newborns aged four to six weeks will be sequenced using high throughput technology. Although the vast majority of the DNA will be from the patient, the recent developments of sequencing technologies and bioinformatics will allow gathering sufficient numbers of ribosomal sequences to ensure reliable identification and quantification of the species composing the microbiome. This approach will avoid the bias introduced by the PCR approach and will also permit the analysis of non-ribosomal sequences. The use of a combination of extraction procedures designed specifically for bacteria and fungi will provide a good representation of the whole microbiome.

ATCF (AZOLE THERAPY IN CYSTIC FIBROSIS): EFFICACY OF ITRACONAZOLE AND OF VORICONAZOLE IN PATIENTS WITH CYSTIC FIBROSIS AND PRESENTING WITH PERSISTENT POSITIVE SPUTUMS FOR *ASPERGILLUS*.

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Context : The efficacy of azole therapy for patients presenting with persistent positive sputums for *Aspergillus* is still unknown. And when the indication of azole therapy is established, the choice alternately turns to itraconazole or voriconazole. The position of itraconazole and voriconazole in this indication have never been evaluated in a large prospective controlled clinical trial, even though many teams have already used it.

Primary objective : To evaluate the rates of converting sputum cultures for *Aspergillus* from positive to negative, for each antifungal agent and to reject a weak strategy, i.e. a response rate lower than 50%. The negativisation is defined by two negative sputum cultures for *Aspergillus* on two consecutive samples, confirmed one month after the end of therapy.

Method : A prospective, multicenter, randomised, open-label, controlled phase II trial of voriconazole and itraconazole, with blind evaluation of the primary evaluation criterion on two parallel groups of patients.

International study for standardization in the procedures for mycological examination of sputum samples from CF patients

Feedback form to design the study
Reply to laurence.delhaes@pasteur-lille.fr

Dr Laurence Delhaes, on behalf of the ECMM/ISHAM Fri-CF working group

1- Are you involved in mycological analysis of sputum samples from CF patient

☐ **Yes** ☐ **No**

2- Please identified your team

Name of the investigator

Department

Town

Country

E-mail

3- Current protocol used routinely in your lab for mycological analysis of sputum samples

***Pretreatment of sputum** ☐ **Yes** **No**
If yes, specify - incubation time:.....min
- incubation temperature:.....°C
- reagent used (commercial name, provider, and chemical name):
.....

***Cultures** ☐ **Agar slants** ☐ **Agar plates**
- How many plates or tubes are used (number) ? .....
- Bacteriological media only ☐ Yes ☐ No
- Mycological media used ☐ Yes ☐ No
- Maximum incubation time (in days): .....
- Analysis closed when *Aspergillus fumigatus* is recovered
☐ Yes ☐ No
- Plates or tubes are kept until the maximum of incubation time, even if *Aspergillus* is recovered ☐ Yes ☐ No

*** For those using agar plates, specify the media currently used, the temperature and time of incubation:**

- Chromogenic agar (Specify name and provider): .....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

- Sabouraud agar: ☐ In-house prepared

☐ Commercial, name and provider:

.....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

- YPDA: ☐ In-house prepared

☐ Commercial, name and provider:

.....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

- DRBC-benomyl agar: ☐ In-house prepared

☐ Commercial, name and provider:

.....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

- Erythritol agar: ☐ In-house prepared

☐ Commercial, name and provider:

.....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

- Other medium: Name: .....

☐ In-house prepared

☐ Commercial, name and provider:

.....

☐ With ATB? Name and Concentration

.....

Incubation temperature.....°C

Incubation time .....days

4- Would like to be involved in this international study? □ **Yes** □ **No**

5- If yes, please answer to the following questions

*** How many media (the maximum) are you able to plate and to analyze per sputum?**

□ About 6 media at 1 temperature □ About 6 media at 2 temperatures

□ About 10 media at 1 temperature □ About 10 media at 2 temperatures

***Is it possible for you to prepare and use in-house media?** □ Yes □ No

*** Is there any medium you would like to be included in this study?**

.....

***How much sputa are you expected to include if this study will be realized during 3 to 6 months?** □ <10 sputa □ 10-25 sputa □ 25-50 sputa □ >50 sputa

***Do you agree to add your results into a common database accessible only to study members (with control access) and shared by internet?**
You will spend about 5 min per sputum □ Yes □ No

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