INTRODUCTION

As most of you are already aware, the inaugural meeting of the ISHAM working group on Fungal Respiratory Infections in Cystic Fibrosis was held in Angers, France in June of this year. This highly successful meeting, which was organised by Jean-Philippe Bouchara, permitted many of the current members of the WG to familiarise themselves with the current research and clinical interests of the WG members and also to propose a number of ways in which the WG activities can move forward. During the general discussion at the end of the inaugural meeting, Jean-Philippe expressed a need for aid in running the working group, and contacted Françoise Symoens, from the Scientific Institute of Public Health in Brussels (Belgium) and myself (Andy Borman, HPA Mycology Reference Laboratory, Bristol, UK) to act as co-convenors.

The planned roles of the convenors will include the preparation of an annual report of the activities of the working group (indicating the collaborative works that have been initiated, the list of publications on the topic by members of the working group, the new members of the working group, and the organization of future meetings or special sessions in large congresses.

We also decided to prepare an official newsletter for the members of the working group. The format for the newsletter will be flexible from one edition to another, and is intended to provide up-to-date information relevant to the scientific, political and social activities of the working group.

This first edition has been compiled predominantly in response to the questionnaires completed by participants of the Angers meeting, and includes a list of the current members of the WG (with full contact details), the clinical/research interests stated by the participants and importantly, also a full list of collaborators/collaborations wanted with (where available) details of materials that the various collaborations require. Future editions will include requests for collaborators and materials, research projects and funding proposals which require partners, and updates on working group activities and progress. Material for inclusion in future newsletters (and remarks/corrections to any errors in the current newsletter) can be sent electronically to Andy.Borman@uhBristol.nhs.uk.

Finally, on behalf of all members of the working group, I would like to thank Jean-Philippe Bouchara for his exceptional past and continued efforts in initiating and continuing to organise this working group.

Andy Borman
The following short text presenting our network and objectives has been prepared by Tom Coenye and Christopher Thornton. May we ask you to address this text to your national contact for the European Community. It's only if they are contacted by a large number of teams from several European countries that we will have a chance to see our research topic selected for a next call for proposal.

**Proposal to include the topic “Fungal respiratory infections in cystic fibrosis: from basic research to improvement of patient management” in the next call of the Health theme within FP7**

1. Background
Cystic fibrosis (CF) (also called “mucoviscidosis”) is the most common hereditary disease in Europe. It is an autosomal recessive hereditary disease caused by one or more mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. In Europe, the carrier frequency is appr. 1/25 and the frequency of the disease is appr. 1 in 2000 live births. Patients with CF suffer from a wide range of symptoms, most notably related to the respiratory and gastrointestinal tract. Over recent decades, considerable attention has been paid to (i) improved early diagnosis of CF, (ii) the prevention and treatment of bacterial respiratory infections (most notably these caused by *Pseudomonas aeruginosa*) and (iii) nutritional status of people with CF. Because of this, life expectancy of CF patients has improved dramatically.

2. Fungal respiratory tract infections in CF
Although there have been significant improvements in terms of preventing and treating bacterial respiratory tract infections in people with CF, relatively little progress has been made with regards to respiratory tract infections caused by fungi. Colonization of the airways with fungi, facilitated by the frequent and prolonged use of antibiotics and by the use of corticosteroids, lead to chronic respiratory infections. As life expectancy of CF patients is increasing, due to increased standards of care, fungal infections are increasing in frequency. Historically, attention has been focused on infections with *Aspergillus fumigatus*, but more recent research indicates that a wide range of fungi can colonise the respiratory tract of patients with CF, including the *Scedosporium apiospermum* species complex, *Aspergillus terreus*, *Exophiala dermatitidis* and *Scedosporium prolificans*, and that these infections often lead to a deterioration of lung function and ultimately result in increased morbidity and mortality.

3. Proposed areas for future research within the FP7 framework
Below, we have identified four areas of collaborative research that require urgent attention. We propose that these areas be considered for funding in the next call of the Health theme within FP7.

3.1. Improving diagnosis
Many of the fungal species recovered from respiratory secretions of CF patients are not (or are only rarely) recovered from clinical samples and appropriate tools for their rapid detection and identification are lacking. These tools include appropriate selective media and culture
conditions, novel PCR and antibody-based diagnostic tests and other culture-independent approaches for rapid detection.

3.2. Ecology and epidemiology
So far, little is known about the natural environmental reservoirs and the modes of acquisition of fungal pathogens in CF patients. Clearly, more research is needed to determine the source of infective propagules and their modes of transmission (environment-to-patient and potentially also patient-to-patient), to establish evidence-based guidelines for infection control.

3.3. Physiopathology of airway colonization
Very little is known about the interaction of fungal cells with the airway epithelium and/or their interaction with other micro-organisms (bacteria, *Candida albicans*). Similarly, much remains to be learned about the virulence factors of species like *Scedosporium apiospermum/Pseudallescheria boydii*.

3.4. Clinical aspects
Clearly, more (preferably large-scale) studies are required to determine the clinical impact of these infections on CF patients. In addition, systematic studies on antifungal susceptibility (both *in vitro* and *in vivo*) are required to address the question of treatment.

4. Established networks within Europe
Recently, the International Society for Human and Animal Mycology (ISHAM) has launched two working groups, one on “Fungal infections in CF” and one on “*Pseudallescheria/Scedosporium* infections”. Within the framework of these working groups, informal networks (mainly composed of European research groups) have evolved, addressing some of the topics mentioned above.

With sufficient funding, a large group of scientists could be assembled in order to address the problems described. To illustrate this point, we have included a list of research groups active in this field (see appendix). This is by no means a complete list and merely serves to show that the topic of “Fungal infections in cystic fibrosis” has attracted a lot of attention in the EU. Other stakeholders include the European Cystic Fibrosis Society (ECFS) and various national patient organisations e.g. Vaincre la mucoviscidose (FR), Cystic Fibrosis Trust (UK), Fonazione per la ricerca sulla fibosi cistica) (IT), Mukoviszidose eV (GE), Belgische Vereniging voor Strijd tegen mucoviscidose (BE) etc.

5. Contact
- Prof. Dr. Tom Coenye, Laboratory of Pharmaceutical Microbiology, Ghent University, Gent, Belgium (Tom.Coenye@ugent.be)
- Dr. Jean-Philippe Bouchara, Groupe d'Etude des Interactions Hôte-Pathogène, UPRES-EA 3142, Laboratoire de Parasitologie-Mycologie, CHU, Angers, France (Jean-Philippe.Bouchara@univ-angers.fr)
- Dr. Christopher Thornton, Hybridoma Laboratory, School of Biosciences, University of Exeter, United Kingdom (C.R.Thornton@exeter.ac.uk)

Appendix : European research groups active in this field

**Belgium**
- Ghent University (Laboratory for Pharmaceutical Microbiology, Tom Coenye)
- Scientific Institute of Public Health (Mycology Section, Francoise Symoens)
- Catholic University of Leuven (UZ Department of Microbiology, Katrien Lagrou)
- Université Catholique de Louvain (Unité de Pneumologie Pédiatrique et Mucoviscidose, Patrick Lebecque)

Denmark
- Statens Serum Institut (Department of Bacteriology, Mycology and Parasitology, Maiken Cavling Arendrup)

France
- Institut Pasteur (Paris)
  Unité de Défense Innée et Inflammation (Vivianne Balloy)
  Unité des Aspergillus (Jean-Paul Latgé, Anne Beauvais)
- Host-Pathogen Interaction Study Group, UPRES-EA 3142, CHU d’Angers (Jean-Philippe Bouchara)
- Ecology of Parasitism, UPRES-EA 3609, CHRU de Lille (Laurence Delhaes)
- Laboratoire de Parasitologie-Mycologie, CHU Rangueil, Toulouse (Sophie Cassaing).
- Laboratoire de Parasitologie-Mycologie, UPRES-EA 4427, CHU de Rennes (Jean-Pierre Gangneux)
- Laboratoire de Parasitologie-Mycologie, AP-HM Timone, Marseille (Stéphane Ranque)

Greece
- University of Thessaloniki (3rd Dept Pediatrics Hippokration Hospital, Emmanuel Roilides, Maria Simitsopoulou)

Germany
- University of Heidelberg (Department of Pediatrics, Frank-Michael Müller)
- Robert Koch-Institut (FG 16 Mykologie, Kathrin Tintelnot)
- University Hospital RWTH Aachen (Institute of Medical Microbiology, Gerhard Haase)
- Federal Institute for Drugs and Medical Devices (Regine Horré)

Italy
- University of Genoa (Laboratory for Cystic Fibrosis Microbiology, Grazianno Manno)

Spain
- Instituto de Biomedicina de Sevilla (CIBER de Epidemiología y Salud Pública, Enrique J. Calderon Sandubete)
- Universitari la Fe (Unidad de Micología, Javier Péman, Amparo Solé)

The Netherlands
- Canisius Wilhelmina Hospital (Department of Medical Microbiology and Infectious Diseases, Corne Klaassen, Jacques Meis)

United Kingdom and Northern Ireland
- Health Protection Agency (Mycology Reference Laboratory, Andrew Borman)
- King’s College London (Pharmaceutical Science Division, Kenneth Bruce, Geraint Rogers)
- Belfast City Hospital (Adult CF Centre, Stuart Elborn - John Moore)
- University of Exeter (Hybridoma Laboratory, Christopher Thornton)
RESULTS of QUESTIONNAIRES COMPLETED AT THE ANGERS MEETING

Research/ Clinical interests of the working group members:

- Clinicians involved in the follow-up of paediatric patients
  Jacqueline Carrère – Laurent Mély (240 patients)
  Patrick Lebecque (84 patients)
  Jan-Bart Yntema (35 patients)
  Frank-Michael Müller (65 patients)
  Emmanuel Roilides (100 patients)
  Refika Hamutcu Ersu (180 patients)
  Carlos Milla (180 patients)
  Marina Almeida (130 patients)

- Clinicians involved in the follow-up of adult patients
  Jacqueline Carrère – Laurent Mély
  Perrine Parize (230 patients)
  Sanjay Haresh Chotirmall (100 patients)
  Caroline Baxter
  Patrick Lebecque (85 patients)
  Enrique Calderone (100 patients)
  Luiz Maiz Carro (65 patients)
  Amparo Solé (150 patients)
  Carlos Milla (200 patients)
  Laurie Whittaker (70 patients)
  Ilma Paschoal – Gisele Yonezawa (90 patients)

- Clinicians involved in lung transplantation
  Eliane Billaud (pharmacologist – 10 à 12 patients per year)
  Amparo Solé (6 per year – 82 CF patients among a total number of 366 lung transplant recipients)
  Carlos Milla (10 patients per year)

- Mycologists involved in the follow-up of CF patients
  Jean-Philippe Bouchara – Marc Pihet (50 patients)
  Laurence Delhaes – Emilie Fréalle
  Isabelle Durand-Joly (50 patients)
  Loïc Favennec (100 patients)
  Françoise Botterel
  André Paugam
  Frédéric Dalle
  Florence Persat
  Claudine Pinel
  Jacqueline Carrère
  Stéphane Ranque
  Sophie Cassaing
- Scientists involved in basic research
  Isabelle Durand-Joly: *Pneumocystis* detection
  Anne Beauvais: *Aspergillus* biofilm
  Judith Fillaux: Epidemiology, infectious diseases
  Jean-Pierre Gangneux: Immunopathology, therapeutic options
  Andrew Borman: Taxonomy, diagnosis, identification, antifungal susceptibility
  Christopher Thornton: *Aspergillus, Pseudallescheria and Scedosporium* diagnostic
  Geraint Rogers – Kenneth Bruce: Profiling microbial communities in respiratory disease
  François Symoens: Identification, genotyping, culture collection
  Tom Coenye – Lies Vanhee: Genotype studies, ecology of opportunistic fungi
  Patrick Lebecque: NPD measurements, curative treatment for the F508 mutation in mouse model and CF patients, lung inflammation in the CF mouse (BAL, Histology, IL, …).
  Corne Klaassen: Molecular epidemiology, detection, identification
  Klaus Leth Mortensen: *Aspergillus* in CF patients – clinical significance and antifungal susceptibility
  Nahid Kondori
  Frank-Michael Müller: *In vitro* antifungal susceptibility, *Candida* and *Aspergillus* biofilms
  Kathrin Tintelnot: Ecology, epidemiology of *Scedosporium/Pseudallescheria* species
  Gerhard Haase: Taxonomy of black yeasts
  Regine Horré / Günter Marklein: detection, identification of *Exophiala* and *Scedosporium/Pseudallescheria* species.
  Krzysztof Ulfig: Ecology of opportunistic fungi
  Vladimir Havlicek: Characterization of *Pseudallescheria* and *Scedosporium* strains based on biomolecular profiles
  Maria Simitsopoulou: Immunopathology
  Enrique Calderon: Epidemiology, detection of *Pneumocystis jirovecii*
  Vicente Friaza: Identification of microbial communities
  Rachid Zouhair: Ecology of *Scedosporium* species, genotype studies
  Wieland Meyer: Ecology, epidemiology, molecular identification, genotyping of *Scedosporium/Pseudallescheria* species, virulence study
The following people agreed to be contacted for various projects:

**For clinical surveillance (± epidemiological studies):**
Jean-Philippe Bouchara – Marc Pihet
Laurence Delhaes
Isabelle Durand-Joly
Loïc Favennec
André Paugam
Jacqueline Carrère
Judith Fillaux
Sophie Cassaing
Jean-Pierre Gangneux – Sylviane Chevrier
Sanjay Chotirmall
Andrew Borman
Caroline Baxter
Geraint Rogers – Kenneth Bruce
Patrick Lebecque
Katrien Lagrou
Jacques Meis
Jan-Bart Yntema
Maiken Arendrup – Klaus Leth Mortensen
Nahid Kondori
Frank-Michael Müller
Regine Horre
Astrid Mayr
Emmanuel Roilides
Grazziana Manno
Amparo Solé
Enrique Calderon
Luiz Maiz Carro
Refika Ersu
Carlos Milla
Laurie Whittaker
Ilma Paschoal – Gisele Yonezawa
Marina Almeida
Wieland Meyer

**For determination of genetic risk factors:**
Jean-Philippe Bouchara – Marc Pihet
Laurence Delhaes
André Paugam
Frédéric Dalle
Stéphane Ranque
Judith Fillaux
Andrew Borman
Geraint Rogers – Kenneth Bruce
Caroline Baxter
For evaluation of procedures for mycological examination of sputum samples:
  Jean-Philippe Bouchara – Marc Pihet
  Laurence Delhaes
  Isabelle Durand-Joly
  Loïc Favennec
  André Paugam
  Françoise Botterel
  Frédéric Dalle
  Claudine Pinel
  Jacqueline Carrère
  Stéphane Ranque
  Sophie Cassaing
  Frédéric Gabriel – Isabelle Accocéberry
  Jean-Pierre Gangneux – Sylviane Chevrier
  Andrew Borman
  Geraint Rogers – Kenneth Bruce
  Caroline Baxter
  Tom Coenye
  Patrick Lebecque
  Katrien Lagrou
  Jacques Meis
  Maiken Arendrup – Klaus Leth Mortensen
  Nahid Kondori
  Frank-Michael Müller
  Kathrin Tintelnot
  Gerhard Haase
  Regine Horré / Günter Marklein
  Astrid Mayr
  Gordana Mircevska
  Emmanuel Roilides
  Grazziana Manno
  Javier Peman
  Carlos Milla
  Laurie Whittaker
  Ilma Paschoal – Gisele Yonezawa
  Wieland Meyer

For evaluation of molecular detection from clinical samples:
  Jean-Philippe Bouchara – Sandrine Giraud
  Laurence Delhaes
  Isabelle Durand-Joly
Françoise Botterel
André Paugam
Sophie Cassaing
Stéphane Ranque
Jean-Pierre Gangneux
Andrew Borman
Geraint Rogers – Kenneth Bruce
Caroline Baxter
Patrick Lebeceque
Katrien Lagrou
Tom Coenye – Lies Vanhee
Corne Klaasen
Jacques Meis
Maiken Arendrup – Klaus Leth Mortensen
Frank-Michael Müller
Gunter Marklein
Kathrin Tintelnot
Vladimir Havlicek
Gordana Mircevska
Emmanuel Roilides
Grazziana Manno
Enrique Calderon – Vicente Friaza
Carlos Milla
Laurie Whittaker
Ilma Paschoal
Wieland Meyer

For evaluation of serological tests:
Jean-Philippe Bouchara – Marc Pihet – Sandrine Nail
Laurence Delhaes – Emilie Fréalle
Loïc Favennec
André Paugam
Florence Persat
Claudine Pinel
Sophie Cassaing
Catherine Kauffmann-Lacroix
Jean-Pierre Gangneux – Sylviane Chevrier
Andrew Borman
Geraint Rogers – Kenneth Bruce
Caroline Baxter
Patrick Lebeceque
Katrien Lagrou
Maiken Arendrup
Frank-Michael Müller
Kathrin Tintelnot
Gordana Mircevska
Emmanuel Roilides
Grazziana Manno
Laurie Whittaker
For environmental studies:
Jean-Philippe Bouchara – Sandrine Giraud
Laurence Delhaes – Emilie Fréalle
Isabelle Durant-Joly
André Paugam
Claudine Pinel
Judith Fillaux
Jean-Pierre Gangneux
Andrew Borman
Christopher Thornton
Geraint Rogers – Kenneth Bruce
Tom Coenye – Lies Vanhee
Patrick Lebecque
Jan-Bart Yntema
Maiken Arendrup – Klaus Leth Mortensen
Nahid Kondori
Frank-Michael Müller
Kathrin Tintelnot
Regine Horré
Astrid Mayr
Krysztof Ulfig
Emmanuel Roilides
Enrique Calderon – Vicente Friaza
Laurie Whittaker
Ilma Paschoal – Gisele Yonezawa
Wieland Meyer

For genotype studies on fungal isolates:
Jean-Philippe Bouchara
Laurence Delhaes
André Paugam
Françoise Botterel
Stéphane Ranque
Sophie Cassaing
Frédéric Gabriel – Isabelle Accocéberry
Andrew Borman
Geraint Rogers – Kenneth Bruce
Caroline Baxter
Tom Coenye – Lies Vanhee
Patrick Lebecque
Françoise Symoens
Jacques Meis – Corne Klaassen
Klaus Leth Mortensen
Frank-Michael Müller
Kathrin Tintelnot
Luiz Maiz Carro
Grazziana Manno
Enrique Calderon – Vicente Friaza
Laurie Whittaker
Wieland Meyer
The following members are searching for collaborators/partners/materials:

- Vladimir Havlicek: Evaluation of in-house developed ELISA kits for serodiagnosis of Pseudallescheriasis/scedosporiosis (needs sera from patients with proven pseudallescheriasis/scedosporiosis).

- Christopher Thornton would be interested in collaborating with any member of the group who is interested in detecting *P. boydii/Sc. apiospermum and Aspergillus* species in sputum samples using MAb-based diagnostics. He would need sputum samples from CF patients who had colonisation previously proven by plate culture or any other diagnostic technique. Control samples from known un-colonised patients would also be useful. He needs approximately 400 microlitres of fluid, preferably stored at -20°C and with no additional treatment.

- Enrique Calderon and Gisele Yonezawa would be interested in the evaluation of the prevalence of *P. jirovecii* in CF and the clinical relevance of its detection. **A detailed research proposal for a multicentre study on Pneumocystis jirovecii colonization in Cystic Fibrosis is included below.**

- Ilma Paschoal would be interested in similarities and differences in fungal respiratory infections between CF lung disease and non-CF bronchiectasis.

- Frank-Michael Müeller would be interested in the evaluation of new antifungal therapy in CF patients (Pk, PD studies). Similar questions were raised by Jean-Pierre Gangneux and Eliane Billaud (how and when to treat, duration of treatment).

- Grazziana Manno, Gordana Mircevska and Maria Simitsopoulou would be interested in the evaluation of antifungal susceptibility on clinical isolates, or are searching partners to do this.

- Klaus Leth Mortensen is searching partners for studies on antifungal resistance mechanisms.

- Gunter Marklein is interested in rapid and accurate determination of clinical yeasts and moulds by MALDI-TOF-MS.

- Maria Simisopoulou is interested in the host immune response against clinical isolates.

- Patrick Lebecque with T. Leal have developed mouse models of chronic respiratory infections (in CF mice).

- Françoise Botterel proposes a study of the interactions between bacteria and fungi on the airway epithelium.

- Wieland Meyer proposes his help for molecular identification within the *Scedosporium apiospermum* complex.

- Loïc Favennec is interested in the evaluation of antifungal susceptibilities of clinical isolates.
Project submitted to the ISHAM Working Group on Fungal infections in Cystic Fibrosis:
Multicentre study on *Pneumocystis jirovecii* colonization in Cystic Fibrosis

Enrique J. Calderon
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**Concept and objectives**

*Pneumocystis jirovecii* (human-derived *Pneumocystis*) is an atypical opportunistic fungus with lung tropism and worldwide distribution that causes pneumonia in immunosuppressed individuals. Basic research on *Pneumocystis* has been hampered by the lack of a reliable in vitro culture system; nevertheless, through the use of molecular techniques and experimental models, progress has been made over the last decades in our understanding of the epidemiological and clinical features of the infection (Calderon, Protist 2002; 153, 303–310). *Pneumocystis* colonization (that is, detection of the organism or its DNA, without signs or symptoms of pneumonia) has recently been described, and accumulating evidence suggests that it may be an important clinical phenomenon (Morris, J Infect Dis 2008; 197:10-7). Because of the absence of *in vitro* culture system, sensitive molecular techniques such as polymerase chain reaction need to be used to identify *Pneumocystis* colonization. Low levels of *Pneumocystis* in the lungs may stimulate pulmonary inflammation and may play a role in the development of lung diseases such as chronic obstructive pulmonary disease. In this sense, one study in human has showed an association between *Pneumocystis* colonization and severity of airflow obstruction in smokers and other recent study shows that patients colonized by *Pneumocystis* have higher pro-inflammatory cytokines levels than non-colonized patients (Morris, Am J Respir Crit Care Med 2004; 170: 408-413; Calderon, Clin Infec Dis 2007; 45: e17-9).

Only a few studies carried out in Europe have evaluated the prevalence of *Pneumocystis* colonization in patients with Cystic Fibrosis (CF), reporting ranges from 7.4% to 22% (Sing, J. Clin. Microbiol.2001; 39: 2717-2718; Respaldiza, Clin. Microbiol. Infect 2005;11:1012-1015), but there are not data about the clinical significance of *Pneumocystis* colonization in Cystic Fibrosis patients. Until recently, the high prevalence of *Pneumocystis jirovecii* colonization among CF patients was unknown. However, it has been suggested that *P. jirovecii* could be involved in the progression of CF by means of its capacity to induce alveolar macrophage activation, proinflammatory interleukin elevation, changes in pulmonary surfactant during very early stages of the infection and possible interactions with other microorganisms.
General objective:
The main goal of this project is to provide new knowledge on the epidemiology and the clinical impact of *P. jirovecii* colonization in Cystic Fibrosis patients.

Goals of the research
1) To improve our knowledge of the epidemiology of colonization by *P. jirovecii* in CF patients.
2) To explore potential correlation between prevalence rates of *P. jirovecii* colonization and geographic, meteorological or other environmental factors.
3) To determine if there is a correlation between prevalence of *P. jirovecii* colonization and CF severity
4) To determine if prevalence of the *Pneumocystis* colonization is conditioned by host genetic factors
5) To improve our knowledge on the potential interactions between *P. jirovecii* and pathogenic microorganisms in the ecological niche of CF patients

Brief study design
This project is the first international multicentre study on *Pneumocystis jirovecii* colonization in CF patients, in which basic and clinical researches will be combined in order to assess its importance for pathogenesis of this disease. It is designed so that following an initial epidemiological study in which all the groups participate, providing information on the real dimensions of the problem, a series of studies may be developed for an in-depth analysis of specific aspects of the colonization in CF subjects. Ideally, each group should include between 30 and 60 cases.

Study population
**Inclusion Criteria**
- Male or female aged > 5 years
- Legally effective consent available
- Well-established diagnosis of Cystic fibrosis

Cystic fibrosis is diagnosed if a patient has a sweat chloride concentration > 60 mEq/l and chronic pulmonary disease of an appropriate character (obstructive pulmonary disease with bacterial infection) or pancreatic insufficiency or both, or a history of CF in a sibling or first cousin.

All the subjects included in the study (or their parents) will be informed of its content and asked to give their informed consent. All of them will provide epidemiological and clinical data, as well as biological samples (sputum and serum) which will be properly preserved for the later development of specific studies.

The epidemiological and clinical data of the patients included in the study will be recorded on previously prepared Case report form (CRF) as well as in a local computerized register. All the epidemiological and clinical data of the subjects included in the study will be handled at all times confidentially.

The following samples will be obtained from each subject included in the study: two sputum samples and 10 ml of serum sample. The samples will be handled according to all universally recommended safety norms. In each case, one sample of sputum will be used for the local microbiological diagnosis and the rest of the samples properly preserved for the subsequent specific studies. Serum samples and DNA extracted from sputum samples will be send to Instituto de Biomedicina de Sevilla for studying *P. jirovecii* colonization and specific anti-*Pneumocystis* antibodies.
*P. jirovecii* colonization will be detected by a nested-PCR assay that amplifies a portion of the gene encoding the mitochondrial large-subunit (mtLSU) rRNA. Genotyping of isolates will be done at two independent gen loci: mtLSU rRNA fragment (assessed by direct sequencing) and dihydropteroate synthase (assessed by restriction fragment-length polymorphism). Serum samples will be examined for antibodies against *Pneumocystis* by immunoblotting.

**Methods for Extraction of DNA**

Divide each sputum sample into aliquots, each of approximately 500 µl volume. Label each sample with patient code, date of collection of sample and aliquot number. Use disposable plastic-ware throughout the procedure. Use sterile racked hydrophobic filter tips for micropipettes (for example Laser, catalogue number LATF-20-R-S). Take all necessary precautions to avoid cross contamination between samples. Stock solutions of reagent (EDTA, SDS, proteinase K, H₂O) should be divided into small volumes in microfuge tubes. For each experiment, a new tube should be used and then discarded.

1. Thaw 1 aliquot of sample to be tested, approximate volume 500 µl.
2. Add EDTA (ethylenediaminetetraacetic acid), pH8.0, to a final concentration of 10mM (for example: 10 µl of 0.5M EDTA, pH8.0, to 500 µl sample). EDTA: Sigma, catalogue number E7889, 0.5M solution, store at room temperature.
3. Add SDS (sodium dodecyl sulphate) to a final concentration of 0.5% (v/v) (for example: 25 µl of 10% SDS to 500 µl sample). SDS: Sigma, catalogue number L4522, 10% solution, store at room temperature.
4. Add proteinase K to a final concentration of 1mg.ml (for example add 20 µl of proteinase K (14mg/ml) to 500 µl sample). Proteinase K: Roche, catalogue number 1-373-196, stock solution at 14-22 mg/ml. Store at 4°C.
5. Incubate sample at 56°C. until complete lysis is obtained (at least 4 h). Vortex occasionally during incubation or use a shaking incubator. (Samples can be incubated overnight as well). minimum: 4 hours (the sample must be completely fluid and homogeneous) maximum: overnight incubation

After PK treatment, DNA extraction can be done (preferable) or sample can be frozen at -20°C up to DNA extraction. DNA extraction can be done according to the protocol from Macherey -Nagel (GMBH & Co, Cat Number 740 952):

Briefly,

1. Add 200ul Buffer B3
2. Add 210ul Ethanol
3. For each sample, place one column into a collection tube.
4. Apply 750 ml of treated sample, and centrifuge for 1 min at 11000 g.
5. Discard flow-through and place the column back into the collection tube.
6. Wash silica membrane with 500 ml BW. and centrifuge for 1 min at 11000 g.
7. Discard flow-through and place the column back into the collection tube.
8. Wash silica membrane with 600 ml Buffer 5, and centrifuge for 1 min at 11000 g.
9. Discard flow-through and place the column into a new collection tube.
10. Dry silica membrane: Centrifuge the column 2 min at 11000 g.
11. To elute pure DNA: Place the column into a clean 1.5 ml eppendorf and add 55 ml highly pure water.
12. Incubate at room temperature for at least 1 min. Centrifuge 1 min at 11000 g.
13. The DNA must be stored at –20°C.

Those interested in participating in this joint project could contact Enrique by e-mail (ecalderon@ibis-sevilla.es) for further details
Current members of the working group

* Dr. Isabelle Accocéberry (UMR - CNRS 5162, Université Victor Ségalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex. Phone: 33-(0)5-56 79 58 37; Télécopie : 33-(0)5-56 79 58 79; E-mail: isabelle.accoceberry@chu-bordeaux.fr) - Clinical surveillance, biological diagnosis.

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