MiniReview

Molecular diagnosis of Pneumocystis pneumonia

Isabelle Durand-Joly *, Magali Chabé, Fabienne Soula, Laurence Delhaes, Daniel Camus, Eduardo Dei-Cas

Parasitology–Mycology (EA-3609), Lille-2 University Hospital Centre, 59037 Lille, France and Ecology of Parasitism, Lille Pasteur Institute, 1 rue du Prof-Calmette BP245, 59019 Lille, France

Received 11 June 2005; accepted 20 June 2005
First published online 18 July 2005

Abstract

The detection of Pneumocystis DNA in clinical specimens by using PCR assays is leading to important advances in Pneumocystis pneumonia (PcP) clinical diagnosis, therapy and epidemiology. Highly sensitive and specific PCR tools improved the clinical diagnosis of PcP allowing an accurate, early diagnosis of Pneumocystis infection, which should lead to a decreased duration from onset of symptoms to treatment, a period with recognized impact on prognosis. This aspect has marked importance in HIV-negative immunocompromised patients, who develop often PcP with lower parasite rates than AIDS patients. The specific amplification of selected polymorphous sequences of Pneumocystis jirovecii genome, especially of internal transcribed spacer regions of the nuclear rRNA operon, has led to the identification of specific parasite genotypes which might be associated with PcP severity. Moreover, multi-locus genotyping revealed to be a useful tool to explore person-to-person transmission. Furthermore, PCR was recently used for detecting P. jirovecii dihydropteroate synthase gene mutations, which are apparently associated with sulfa drug resistance. PCR assays detected Pneumocystis-DNA in bronchoalveolar lavage fluid or biopsy specimens, but also in oropharyngeal washings obtained by rinsing of the mouth. This non-invasive procedure may reach 90%-sensitivity and has been used for monitoring the response to treatment in AIDS patients and for typing Pneumocystis isolates.

© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Pneumocystosis diagnosis; Pneumocystis pneumonia; Pneumocystis jirovecii; PCR; mtLSUrRNA; ITS; DHPS; SSCP; PcP epidemiology

1. Introduction

The standard method for laboratory diagnosis of Pneumocystis pneumonia (PcP) is currently assessed on the basis of the microscopic demonstration of Pneumocystis organisms in samples of alveolar material like bronchoalveolar lavage fluid (BALF), induced sputum (IS) or lung biopsy samples. Dried smears from these respiratory samples can be stained with Gomori-methenamine silver or toluidin blue O stains, which dye Pneumocystis cystic or sporocyte forms, and methanol-Giemsa assay, or other Giemsa-like panoptic method, which stain all the Pneumocystis life cycle stages [1,2]. A higher detection sensitivity can be reached by using commercialized fluorescein- or enzyme-labeled monoclonal or polyclonal Pneumocystis antibodies on BALF smears [3]. BAL sampling procedure is often considered to be an invasive method. For this reason it is only rarely used for monitoring the response to the treatment. Moreover, treatment efficacy cannot be assessed in cultures because no in vitro system for obtaining routinely Pneumocystis isolates from patients is available.

Several PCR assays were developed for the last 15 years. PCR tool revealed highly efficient to detect Pneumocystis DNA in clinical specimens [4,5], in lung samples from domestic or wild mammals [6,7] as well as in
ambient air [8,9] or water [10]. Moreover, sequencing of selected *Pneumocystis*-DNA amplified fragments is used to identify *Pneumocystis* species or genotypes [11]. Genotyping of clinical or environmental *Pneumocystis* isolates constitutes at present the unique way for identifying and tracking them in both human populations and ecosystems. *Pneumocystis jirovecii* genotyping should permit to associate specific molecular types with virulence, drug susceptibility or other medically important biological properties of parasite strains.

*P. jirovecii* molecular diagnostic methods were fully reviewed recently [12]. For many years we performed PcP diagnosis by using microscopy and PCR assays at the Parasitology–Mycology Service of Lille-II University Hospital Center. In this paper, we will examine basically some practical aspects linked to the application of PCR and oligoblotting methods to the diagnosis of PcP.

### 2. Molecular PcP diagnosis and treatment monitoring: targeted genes

In order to develop PCR assays for *Pneumocystis* detection, several genes of the parasite have been targeted. Comparative evaluation studies are not numerous. PCR assays targeting the mitochondrial 23S rRNA region (mtLSUrRNA), that is present in many copies in each organism [13], were found to have higher sensitivity than methods that targeted cytoplasmic 5S rDNA and dehydrofolate reductase (DHFR) regions [12]. Another study compared *Pneumocystis* gene regions of internal transcribed spacers (ITS), 18S rRNA, mt rRNA, 5S rRNA, thymidylate synthase (TS), and DHFR and concluded that an ITS-based PCR assay was the most effective test for the detection of *P. jirovecii* in BAL specimens from AIDS patients [14]. However, more recently, the detection of *P. jirovecii* by nested PCR at the mtLSUrRNA locus was found to be more sensitive than nested PCR at the ITS regions [13,15] of the rRNA operon, which are thought to be present in the *Pneumocystis* genome in only one copy [16]. Thus, in patients treated for PcP, ITS PCR assay has failed to detect *P. jirovecii* DNA in samples still positive with mtLSUrRNA PCR assay [13]. In the same work, mtLSUrRNA PCR test became negative in 8 out of 12 patients 7–21 days after the start of treatment, and an overall reduction of *P. jirovecii* DNA load, estimated by a simple dilution method, was recorded in all the patients by day 7–21 [13]. Results should however be prudently interpreted as fluctuations in the amount of amplification product were observed in samples of all patients during their treatment course [13]. Although, important effort were made [15,17], it is not known whether a given ITS genotype is associated to more pathogenic, drug resistant or more easily transmitted parasite strains. Unfortunately, culture systems allowing routine isolation of *P. jirovecii* clinical specimens in order to evaluate replication capacity, drug susceptibility or other biological properties of parasite isolates, are unavailable. The problematic was approached by investigating potential association among defined *P. jirovecii* ITS sequence types and clinical presentations of PcP [18]. Interestingly, an ITS sequence type (B2 a1), the commonest type identified in this and other studies, was associated with mild disease, while another type (B1 a3), was rather associated with moderate/severe disease. A third type (A2 c1) was found only in moderate/severe disease [18]. Consistently, a more recent paper [17], found that the type A2 c1 is associated with PcP and it was absent in colonized patients. On the whole, Nevez et al. [17], found that diversity of ITS types, partial communality of types and occurrence of mixed infections can be observed in both colonized individuals and patients with PcP.

### 3. PCR may amplify *P. jirovecii* DNA from non-invasive samples

Until recently, PCR was primarily used with success to amplify *P. jirovecii* DNA from BALF samples [4,5,19–22]. But in practical terms, molecular techniques may play a more significant role if applied to non-invasive specimens [12] as IS, oropharyngeal washings (OW) [23–25] and nasopharyngeal aspirates (NPA) [26]. As PCR detection was estimated to be about 100-fold more sensitive than microscopy [12], it may be used with success for amplifying *P. jirovecii* DNA from non-invasive respiratory samples like OW specimens obtained by simple rinsing of mouth [13,23,27]. If target genes and DNA sequences used as primers or probe have been adequately selected, the specificity of *Pneumocystis*-DNA PCR detection assay applied to non-invasive or invasive samples, including oligoblotting, should usually be of 100% [12,24]. With regard to sensitivity, PCR coupled with dot-blot hybridization using primers and probe from the mitochondrial 23S rDNA region (mtLSUrDNA) showed high sensitivity for the diagnosis of PcP by PCR on BALFs from AIDS patients, with a detection threshold of 0.5–1 organism µl⁻¹ (using *Pneumocystis* organisms from humans and rats, respectively) [12]. PCR detection sensitivity in OW or other non-invasive samples is certainly lower than that of PCR coupled with Southern- or dot-blot applied to BALF samples, which is usually higher than 95% [12]. Thus, a 78% sensitivity of a PCR nested technique applied to OW samples was reported [13,23]. In OW from HIV-positive patients, *P. jirovecii* DNA amplification yielded a sensitivity of 70–80% using a standard PCR protocol [23] and some technical improvements allowed to obtain even higher sensitivities [24,28]. PCR analysis detected *P. jirovecii* DNA in 93% of nasal, and 75% of pharyngeal
aspirates from corticosteroid-treated rats with PcP [29]. On the whole, OW, like other non-invasive sampling procedures, can be easily repeated in order to monitor the evolution of infection and, potentially, the therapeutic response [13].

For 4 years, in the Lille University Hospital, we used OW sampling, associated with PCR, for PcP diagnosis, especially in hematological patients with PcP suspicion housed in air-controlled area. Touch down-single round PCR (TD-PCR), targeting the mtLSUrRNA region, followed by oligoblotting hybridization was used to detect Pneumocystis in both OW and BALF samples. Interestingly, we found an excellent correlation between PCR results on BALF and OW in infected patients [30]. The specificity of the mtLSUrRNA TD-PCR assay on OW was 100% and the sensitivity was 90% [30].

In patients with negative microscopic test, when TD-PCR results where positive in BALF and negative in OW, the absence of PCP was clinically confirmed and colonization was concluded [30]. In these kinds of patients, nested – PCR on OW was usually positive. On the practical terms, in order to decrease the risk of cross-contamination, we use TD-PCR for PcP diagnosis on OW or BALF samples, and we apply nested-PCR on OW samples to epidemiological research in order to detect subclinical colonization [27,31].

Other authors use TD-PCR and more recently quantitative touch down (QTD) real-time PCR targeting the multicopy major surface glycoprotein (MSG) gene family to detect P. jirovecii in OW and BALF samples [32–34]. The latest studies optimized the technique by using both TD-PCR and quantitative real time PCR procedure [33,34]. Authors applied this technique to OW samples in PCP and non-PCP patients and found high significative differences in the number of copies of MSG gene per tube in PcP patients [33]. So, the authors suggested of applying an arbitrary cutoff value of 50 MSG gene copies/tube for distinction of infection versus colonization within 1 day of initiation of treatment [33]. In practical terms, quantification of PCR results seems to us difficult to use for PcP diagnosis. The main problem is the inability to control the variability of respiratory sample volume (OW or BALF samples). Another difficulty is linked to the category of patients. Probably, a same cutoff could not be applied to AIDS patients with PcP, patients developing PcP in others contexts or patients submitted to chemophrophylaxis.

4. P. jirovecii PCR assay in serum or blood samples

Blood or serum samples might represent also specimens in which P. jirovecii DNA could be detected for approaching PcP diagnosis. Many teams have attempted to detect the parasite in either blood cells or serum samples from experimental rodents or from HIV infected or uninfected patients. The results, which were well summarized recently [35], are contradictory. Some teams reported blood positive results with negative results in BALF samples [35] and data from different teams are difficult to compare as they have used PCR assays with different DNA extraction methods and/or targeting different DNA genes. On the whole, despite efforts of many teams, P. jirovecii DNA detection in blood did not seem to be of value for PcP diagnosis [36]. Positive results reported by some groups may reveal transient blood passages of Pneumocystis organisms, infection with highly virulent parasite strains or the presence of residual parasite material in phagocytic cells [12,35].

5. Detecting and typing P. jirovecii in molecular epidemiological studies

On the epidemiological field, highly sensitive and specific PCR assays were used to identify P. jirovecii isolates responsible for recurrent PcP episodes, for exploring person-to-person Pneumocystis transmission as well as for investigating P. jirovecii prevalence differences potentially related to the geographic origin of parasite isolates [37,38]. Molecular identification by using different markers suggested that recurrences of PcP could result from both reactivation of a previously acquired infection and exogenous reinfection [39–41]. In these studies, finding of mixed infections, i.e. of patients carrying more than one molecular type of P. jirovecii was not rare.

PCR associated to sequencing of amplified DNA fragments was also used for studying Pneumocystis human-to-human transmission. Thus, each member of three HIV-infected couples with PcP was found to be infected by distinct genotypes of P. jirovecii, defined on the basis of polymorphic sequences of ITS regions [42]. These data suggested that the six patients did not acquire their Pneumocystis infection from their consort. Likewise, a report exploring P. jirovecii transmission in a hospital suggested that person-to-person transmission of Pneumocystis may occur but did not account for the majority of cases [43]. However, on the one hand, a patient can harbor mixed genotypes in his lungs [44] and on the other hand, P. jirovecii strains with mutations in dihydropteroate synthase (DHPS) gene (potentially associated to sulfa resistance) in patients previously unexposed to sulfa drugs was report. In the same way, the rapid increase of P. jirovecii DHPS gene mutations over the past 10 years suggests that person-to-person transmission is not rare [45,46]. Therefore, single-strand conformation polymorphism (SSCP) molecular typing was successfully used recently to explore the hypothesis of inter-human transmission [47,48]. The method associates PCR amplification of four variable DNA regions of
P. jirovecii, with the detection of SSCP polymorphisms [49,50].

Available molecular markers for P. jirovecii strains have been used to investigate prevalence differences related to geographic origin of patients. Different prevalence of parasite molecular types related to geographic location (United States, Italy and Denmark) was reported [51]. More recently, frequency differences in two P. jirovecii independent genetic loci were found among samples from five cities placed in different regions of the Unites States [37]. In contrast, other studies had not found significant genomic differences among P. jirovecii isolates from two European countries [52] or from countries from three continents [11,53,54].

6. PCR tool for detecting Pneumocystis drug resistance

PCR was recently used to detect mutations in P. jirovecii therapeutic target genes potentially responsible for drug resistance. While only a synonymous mutation, unrelated to exposure to antifolinic treatment, was detected in the P. jirovecii dihydrofolate reductase (DHFR) gene [55], the P. jirovecii dihydropteroate synthase (DHPS) gene showed mutations associated with impaired prognosis, which apparently resulted from exposure to sulfa drugs [46,56]. Homologous DHPS gene mutations were associated with sulfa drug resistance in other microorganisms. Nonsynonymous mutations potentially associated to atovaquone resistance, were also reported in the P. jirovecii cytochrome b gene [57]. In so far as no routine in vitro drug assay is available for Pneumocystis, the assessment of P. jirovecii potential drug resistance by using molecular methods has an obvious interest. However, recent experiments of in vitro site-directed mutagenesis suggest P. jirovecii DHPS gene mutations induce sulfa resistance [58–60].

7. Pneumocystis PCR assay in clinical practice

On the whole, PCR associated to specific probe hybridization blot has represented a significant advance in PcP laboratory diagnosis. The assay takes usually more than 48 h but real-time PCR systems should lead to a significant decrease of this time. In our experience, the PCR assay sometimes revealed PcP in patients with negative microscopy test. For instance, for the last five years, more than 2000 BALF samples, most from HIV-negative individuals, were tested in our laboratory for P. jirovecii detection. About 16% of samples were PCR-positive (mtLSU rRNA fragment), but parasites were observed microscopically in only 5% of samples [30]. No divergence in the opposite direction, i.e. negative PCR results with positive microscopy results, was found.

Many patients with positive-PCR and negative-microscopy results developed PcP with typical clinical and/or radiological signs. However, most patients with positive-PCR and negative-microscopy results were considered colonized by the parasite, as they did not develop PcP during a follow up period of many months. Similar results were reported previously [20,61]. Furthermore, many immunocompromised HIV negative patients may develop PcP with lower parasite burden than HIV positive patients [62,63], a situation that lead to a decreased sensitivity of microscopic testing. In addition, in HIV–infected or infected PcP patients, chemoprophylaxis could decrease the number of parasites rendering uneasy the microscopic detection. PCR assay can be especially helpful in these cases.

Practical problems raised by Pneumocystis PCR derive essentially from divergence between PCR and microscopic results. These problems have to be resolved on the basis of a careful clinical, radiological and laboratory assessment of the patient pathological condition, like we do in other infectious diseases, especially when their agents are opportunistic pathogens.

Acknowledgements

This work was developed in the framework of research programs supported by the European network ‘Eurocarinii’ (QLK2-CT-2000-01369), French Ministry of Research (EA-3609, ‘Projet quadriennal’), Lille Pasteur Institute (IFR-17) and ANRS (‘Agence Nationale de Recherche sur le SIDA’, contract no. 98016). Thanks are given to Michèle Wauquier, Nadine François and Filomena Nagy from the LilleII University Regional Hospital Center for their technical assistance.

References


