Diagnostic Microbiology and Infectious Disease 74 (2012) 119–124

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Circulation of *Pneumocystis* dihydropteroate synthase mutants in France ☆,☆☆

Solène Le Gal [a,b,*], Céline Damiani [c,d], Maëla Perrot [a], Amélie Rouillé [e], Michèle Virmaux [a], Dorothée Quinio [a,b], Elodie Moalic [a,b], Philippe Saliou [f], Christian Berthou [g,h], Yann Le Meur [g,i], Anne Totet [c,d], Gilles Nevez [a,b]

[a] UEB, University of Brest, LUBEM EA 3882, SFR 148, Brest, France
[b] Laboratory of Parasitology and Mycology, Brest University Hospital, Brest, France
[c] Department of Parasitology and Mycology, Amiens University Hospital, Amiens, France
[d] University of Picardy-Jules Verne, EA 4285 UMI INERIS 01, Amiens, France
[e] Department of Biochemistry and Pharmacology, Brest University Hospital, Brest, France
[f] Department of Public Health, Brest University Hospital, Brest, France
[g] UEB, University of Brest, EA 2216, SFR 148, Brest, France
[h] Department of Haematology, Brest University Hospital, Brest, France
[i] Department of Nephrology and Renal Transplantation Unit, Brest University Hospital, Brest, France

ARTICLE INFO

Article history:
Received 26 April 2012
Accepted 4 June 2012
Available online 15 July 2012

Keywords:
Pneumocystis jirovecii
DHPS
Mutants

ABSTRACT

Data on the prevalence of *Pneumocystis jirovecii* (*P. jirovecii*) dihydropteroate synthase (DHPS) mutants in France are still limited. In this study, mutant prevalence in the Brest region (western France) was determined. Archival pulmonary specimens from 85 patients infected with *P. jirovecii* and admitted to our institution (University Hospital, Brest) from October 2007 to February 2010 were retrospectively typed at the DHPS locus using a polymerase chain reaction–restriction fragment length polymorphism assay. Type identification was successful in 66 of 85 patients. Sixty-four patients were infected with a wild type, whereas mutants were found in 2 patients (2/66, 3%). Medical chart analysis revealed that these 2 patients usually lived in Paris. Another patient usually lived on the French Riviera, whereas 63 patients were from the city of Brest. Thus, the corrected prevalence of mutants in patients who effectively lived in our geographic area was 0% (0/63). Taking into account that i) Paris is characterized by a high prevalence of mutants from 18.5% to 40%, ii) infection diagnoses were performed in the 2 Parisians during their vacation <30 days, iii) infection incubation is assumed to last about 2 months, the results provide evidence of mutant circulation from Paris to Brest through infected vacationers. The study shows that the usual city of patient residence, rather than the city of infection diagnosis, is a predictor of mutants and that *P. jirovecii* infections involving mutants do not represent a public health issue in western France.

1. Introduction

The transmissible fungus *Pneumocystis jirovecii* (*P. jirovecii*) is the causative agent of severe pneumonia (*Pneumocystis* pneumonia [PCP]) in immunocompromised patients (Walzer and Cushion, 2005). However, PCP represents only part of the clinical presentations of the fungal infection, while mild infections such as pulmonary colonization in patients with diverse levels of deficiency and lung diseases account for the main part (Morris et al., 2008; Nevez et al., 1999). *P. jirovecii* is widespread within human communities, while no exosaprophytic form of *Pneumocystis* sp. has been identified so far. Since *Pneumocystis* organisms infecting each mammalian species are host specific (Stringer et al., 2002), an animal reservoir for *P. jirovecii* is excluded. For these reasons, *P. jirovecii* infection in humans is considered to be an anthroponosis, with humans as potential infectious sources of the fungus.

The dihydropteroate synthase (DHPS) is the enzymatic target of sulfonamides, which are the main drugs used in PCP prophylaxis or treatment. *P. jirovecii* organisms with mutations at the DHPS locus, mainly at nucleotide positions 165 and 171, have been detected in patients developing *P. jirovecii* infections (Lane et al., 1997) (reviewed in Matos and Esteves, 2010; Totet et al., 2004). The first mutation corresponds to a transition from A to G and an amino acid substitution from threonine to alanine at codon 55. The second one corresponds to

☆ Conflict of interest: None of the authors has any conflicts of interest to declare.

☆☆ Financial support: This study was supported by the Cellule Régionale d'Epidémiologie Nosocomiale (CRENO), the Agence Française de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET) (contract number EST-2006/1/41), and the Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses) (contract number 2011/1/053). The results of this study were presented in part at the 5th Trends in Medical Mycology, Valencia, Spain, 2–5 October 2011, abstract P230.

* Corresponding author. Tel.: +33-(0)2-98-14-51-02; fax: +33-(0)2-98-14-51-49.
E-mail address: solene.legal@chu-brest.fr (S. Le Gal).

0732-8893/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2012.06.002

a transition from C to T and an amino acid substitution from proline to serine at codon 57. These changes, which are located within the putative sulfa-binding site, have been associated with failure of sulfonamide prophylaxis (Armstrong et al., 2000; Kazanjian et al., 1998; Mei et al., 1998; Nahimana et al., 2003; Visconti et al., 2001).

Prior exposure to sulfonamides has been identified as the main factor for developing infection involving *P. jirovecii* DHPS mutants (Crothers et al., 2005; Huang et al., 2000; Kazanjian et al., 2000; Ma et al., 2002; Miller et al., 2003; Nahimana et al., 2003). In addition, the city of patient residence has also been identified as a predictor of mutants in patients with or without prior exposure to sulfonamides (Beard et al., 2000, Huang et al., 2000). These factors support the hypothesis that *P. jirovecii* is transmitted from infected treated patients to susceptible untreated patients. For these reasons, analysis of the DHPS locus may serve as a marker of *P. jirovecii* circulation within human communities (Beard et al., 2000; Hauser et al., 2010; Huang et al., 2000).

Geographical variations of mutant prevalence have been reported worldwide (reviewed in Matos and Esteves, 2010; Totet et al., 2004). In French cities, this prevalence varies from 18.5% to 40% in Paris (Diop-Santos et al., 1999; Magne et al., 2011); from 33% to 36% in Lyon, southeastern France (Hauser et al., 2010; Nahimana et al., 2003); and 8% in Amiens, northern France (Totet et al., 2004). Data on mutant prevalence in France are still limited, and no information on this topic from our city, Brest, in western France is available. Brest is a city of 160,000 people (in a metropolitan area of 303,000), located at the western tip of France, in the Brittany region on the coasts of the Atlantic Ocean and the English Channel, 660 km from Paris and Amiens, and 900 km from Lyon. The region is characterized by a low incidence of AIDS and AIDS-related PCP (Cazein et al., 2010), which represent particular geographic features of *P. jirovecii* epidemiology. In this context, to determine the frequency of mutants in our region, archival *P. jirovecii* specimens from 85 patients admitted to Brest University Hospital were typed using amplification of the DHPS locus, followed by a restriction fragment length polymorphism (RFLP) assay.

2. Materials and methods

2.1. Patients and specimens

Ninety-three archival *P. jirovecii* DNA specimens from 85 patients were examined for DHPS type identification. The patients were admitted to Brest University Hospital in the period from October 3, 2007, through February 10, 2010. The patients' median age was 63 years (range, 3–87 years). The male/female sex ratio was 53:32. The BAL specimens had initially tested positive for *P. jirovecii* based on microscopic examination using Wright–Giemsa and Toluidine blue O stains and a real-time polymerase chain reaction (PCR) assay. After DNA extraction using NucliSENS easyMag system (bioMérieux, Marcy l'Etoile, France), the PCR assay was performed with specific probe and primers of the gene encoding the mitochondrial large subunit rRNA (mtLSUrRNA) as reported elsewhere (Meliani et al., 2003; Totet et al., 2003). Risk factors for *P. jirovecii* infection were hematologic malignancies (24 patients), transplantation (18 patients), cancers (14 patients), immunosuppressive therapies (13 patients), chronic bronchopulmonary diseases (7 patients), HIV infection (4 patients), malnutrition (2 patients), congenital immunodeficiency, alcoholism, and adrenocortical carcinoma (1 patient each). Forty-four patients developed PCP, whereas 41 patients were colonized by the fungus. The fungus was detected by both microscopic techniques and the PCR assay in 22 patients who developed PCP, whereas it was detected by only the PCR assay in the remaining 22 PCP patients and in 41 colonized patients. The 22 patients were considered to have developed PCP despite the absence of microscopic detection of *P. jirovecii*, because they presented clinical and radiological signs compatible with PCP, combined with improvement after specific treatment and absence of alternative diagnoses of PCP. The 41 patients were considered to be colonized since they presented alternative diagnoses of PCP. These alternative diagnoses were bacterial pneumonia (23 patients), bronchial carcinoma (5 patients), drug-induced pneumonia (4 patients), pulmonary aspergillosis (2 patient), and viral pneumonia, sarcoidosis, tuberculosis, exacerbation of chronic bronchitis, Eisenmenger syndrome, pulmonary edema, pulmonary fibrosis (1 patient each). Extracted DNA samples of the 93 BAL fluids were stored at −80°C until they were typed.

A retrospective analysis of medical charts was performed to collect data on the clinical outcome, the city of patient residence, and the past history of *P. jirovecii* prophylaxis or treatment with sulfonamides in the 3 months preceding *Pneumocystis* infection diagnoses.

2.2. P. jirovecii DHPS typing

The typing was performed using an original PCR-RFLP assay. First, the DHPS locus was amplified using a nested-PCR assay. The first round was performed using the primer pair F1 (5′-CCT GGT ATT AAA CCA GTT TTG CC-3′) (Beard et al., 2000) and B45 (5′-CAA TTT AAT AAA TTT CTT TCC AAA TAG CAT C-3′) (Lane et al., 1997). The second-round PCR was performed using the primer pair A_{HUM} (5′-GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3′) (Lane et al., 1997) and BN (5′-GGA ACT TTC AAC TTG GCA ACC AC-3′) (Beard et al., 2000; Lane et al., 1997). The same reagent conditions were used for the 2 rounds with 1 μmol/L of each primer, 2 mmol/L $MgCl_2$, 15 mmol/L Tris-HCl, 50 mmol/L KCl, 200 μmol/L dNTPs (dNTP set, Eurogentec, Seraing, Belgium), and 1 U of polymerase (HotGoldstar® DNA Polymerase, Eurogentec) in a final volume of 50 μL. The second-round PCR was performed with 2.5 μL of the first PCR products. The first-round PCR was done with denaturation at 92 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min for 35 cycles (Beard et al., 2000). The second-round PCR was carried out for 35 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min (Beard et al., 2000). The first and the second PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide to visualize the expected bands of 895 and 371 bp, respectively. To avoid contamination due to environmental amplicons, extraction, reagent preparation, and amplification procedures were performed in separate rooms with different sets of micropipettes and using barrier tips. Extraction and reagent preparations were performed in flow cabinets. To monitor for possible contamination, negative controls (ultrapure distilled water) were included in extraction and first- and second-round PCR procedures. Second, the RFLP assay was performed with 2 restriction enzymes, *Acc*I and *Hae*III, according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). The PCR products were divided into 2 parts. One part was digested with *Acc*I and the second part with *Hae*III, making it possible to detect mutations at nucleotide positions 165 and 171, respectively (Diop-Santos et al., 1999). The restriction profiles were visualized using gel electrophoresis of each digested product on a 1.5% agarose gel with ethidium bromide (Fig. 1). The mutations inhibit the restriction enzyme activity. Thus, a wild type (W_{165}/W_{171}) that has no mutations at nucleotide positions 165 and 171 was shown, after digestion with *Acc*I, by 2 fragments of 229 and 142 bp, and after digestion with *Hae*III, by 3 fragments of 221, 131, and 19 bp. A mutant (M_{165}/W_{171}) that has a mutation at nucleotide position 165 was shown, after digestion with *Acc*I, by only 1 uncut fragment of 371 bp, and after digestion with *Hae*III, by the 3 fragments of 221, 131, and 19 bp. A mutant (W_{165}/M_{171}) that has a mutation at nucleotide position 171 was shown, after digestion with *Acc*I, by the 2 fragments of 229 and 142 bp, and after digestion with *Hae*III, by 2 fragments of 352 and 19 bp. A double mutant (M_{165}/M_{171}) that has mutations at 2 nucleotide positions, 165 and 171, was shown after