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Molecular analysis based on *mtLSU-rRNA* and *DHPS* sequences of *Pneumocystis jirovecii* from immunocompromised and immunocompetent patients in Italy

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ABSTRACT

Pneumocystis jirovecii is an opportunistic fungus predominantly reported in immunocompromised individuals, who develop severe interstitial pneumonia (PcP). However, it is known that asymptomatic or mild pulmonary infections, defined as colonization, are widely observed in the general adult population. So far, genetic and epidemiological data of *P. jirovecii* infections in Italy are rather scarce and limited to defined geographical regions, mainly regarding isolates from HIV-infected patients. The aim of this study was to evaluate the polymorphisms at the *mtLSU-rRNA* and the *DHPS* loci by the screening and genotyping of a cohort of patients from two major hospitals in Rome (Italy). The study included 263 patients divided into two groups, all enrolled consecutively from January 2006 to December 2010: (i) 38 immunocompromised subjects including 25 HIV-infected; (ii) 225 immunocompetent patients. Sixty-seven out of 263 patients (25.5%) were found positive after PCR amplification of the *mtLSU-rRNA* gene. Overall, genotyping at *mtLSU-rRNA* locus revealed that the genotype 2 was the most frequent. Sequences of the *DHPS* gene were obtained from 21 patients, 9 from immunocompromised patients (6 from HIV infected individuals), 12 from immunocompetent ones. Considering the most common *DHPS* mutations usually detected at amino acid positions 55 and 57 and potentially related to drug resistance, all samples analyzed showed the wild-type signatures. These are the first data in Italy on prevalence and genotypes of *P. jirovecii* regarding colonized immunocompetent adults. Further multicenter analyses on *P. jirovecii* infection will be necessary to better define the specific epidemiology of the disease in the Italian populations.

1. Introduction

Pneumocystis jirovecii (previously known as *Pneumocystis carinii* f. sp. *hominis*) is an ascomycetous fungus causing opportunistic infections, specifically interstitial pneumonia (PcP) in the lower respiratory tract of patients with impaired immunity (Huang et al., 2006).

Human PcP remains an important cause of morbidity and mortality, especially in HIV/AIDS patients (Calderon-Sandubete et al., 2002; Santamauro et al., 2002). With the advent of highly active antiretroviral therapy (HAART), *pneumocystosis* in HIV-positive immunodeficient patients deserves to be revisited. In developing countries, PcP remains a common cause of severe pneumonia and a major cause of illness and death. In industrialized countries, PcP still occurs, despite the availability of HAART and anti-*Pneumocystis* prophylaxis. However, a significant decline in rates in HIV-infected patients has been observed, as reported in Europe by the EuroSIDA study (Mocroft et al., 2003) and in the United States (Huang et al., 2011; Morris et al., 2004).

Pneumocystis has been also isolated from HIV-negative immunocompromised patients, such as subjects affected by cancer and transplant recipients, and recently from individuals with rheumatologic pathologies and inflammatory bowel disease (Kaneko et al., 2006; Morris and Norris, 2012; Poppers and Scherl, 2008; Sepkowitz, 2002).

Moreover, *P. jirovecii* DNA has been repeatedly detected in immunocompetent individuals with primary respiratory disorders, and regarded as colonization (Morris and Norris, 2012). Recently, Ponce and colleagues have shown that a mild *P. jirovecii* pulmonary infection was prevalent in their cohort from Chile in more than half of the general adult population, strengthening the concept that immunocompetent adults may develop recurrent self-limiting

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re-infections throughout life and participate in the circulation of *P. jirovecii* as infective reservoirs for susceptible individuals (Ponce et al., 2010).

Several drugs are so far available for the treatment of the infection. Sulfonamides, usually combined with trimethoprim as in trimethoprim-sulfamethoxazole (TMP-SMX) and dapsone are commonly used for the treatment and prophylaxis of PcP (Morris and Norris, 2012).

Elucidating the epidemiological patterns of transmission is crucial to improve prevention of the disease. Multilocus genotyping has led to major advances in the understanding of the genetic diversity of this pathogen, showing that specific polymorphisms could determine distinct epidemiological profiles of the pathogen, including geographical distribution, drug resistance, virulence and routes of transmission (Esteves et al., 2011, 2012).

In Italy several studies have been conducted, mainly on isolates from HIV patients and mostly focused on the genetic diversity of *Pneumocystis* isolates at *DHPS* and *ITS* regions (Agostoni et al., 2000; Ma et al., 2002; Margutti et al., 1998; Valerio et al., 2007; Visconti et al., 2001; Volpe et al., 2001).

The objective of the present study was to investigate the presence of *P. jirovecii* in immunocompromised and immunocompetent patients from two major hospitals in Rome, Italy, and to infer the genetic variability of the recovered isolates, by analysing polymorphisms at two independent loci: the mitochondrial large subunit ribosomal RNA (*mtLSU-rRNA*) and the dihydropteroate synthase (*DHPS*) gene.

2. Materials and methods

The study involved 199 respiratory specimens from subjects with suspected pulmonary infection (161 immunocompetent and 38 immunocompromised, including 25 HIV-infected and 13 bone marrow and solid organ transplanted, or affected by haematological malignancy), enrolled consecutively from January-2006 to December-2010 at the Laboratory of Parasitology of the Polyclinic Tor Vergata in Rome, Italy. All these specimens were collected for detection of *Pneumocystis* as requested by the physician.

Moreover, 64 BAL or sputum samples from immunocompetent subjects, investigated for possible respiratory disease as part of their standard management, were collected from the Microbiology Laboratories of two hospitals in Rome (Polyclinic Tor Vergata and I.N.M.I. "L. Spallanzani" Hospital).

Overall, mean age was 51.1 years (range: 9–88 years), 79 were females and 184 were males. The main Departments of admission were: Hematology, Infectious Diseases, Pneumology, and the Intensive Care Unit. The following data were collected for all patients: clinical features, HIV serological status (when available) and immune-condition. CD4+ lymphocyte counts were routinely measured only for HIV infected patients. None of the immunocompetent subjects was known to be HIV positive, although HIV testing was not performed routinely

For all samples, the detection of *Pneumocystis* was based on nested-PCR. The nested-PCR amplification of *P. jirovecii* mitochondrial large subunit (*mtLSU*) rRNA is the most sensitive and commonly used laboratory molecular approach (Robberts et al., 2007; Morris and Norris, 2012). Immunofluorescent procedures (DFA) with monoclonal antibodies (MERIFLUOR® *Pneumocystis*, Meridian Diagnostic, Cincinnati, OH, USA) were performed only on samples from immunocompromised patients.

DNA extraction was performed from biological samples (bronchoalveolar lavage-BAL fluid, sputum, and tracheobronchial aspirate fluid) using a commercially available kit (QIAamp DNA kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The nested PCR of *mtLSU-rRNA* gene (260-bp fragment length) was performed by using the primers pAZ102-H and pAZ102-E for the first amplification round and the primers pAZ102-X and pAZ102-Y in the second round as previously described (Wakefield, 1996). The amplifications were carried out in a 25 μl volume containing 12.5 μl 2X PCR master mix (Promega, Italy), 5 μl template DNA (1 μl of the primary PCR products in the second round of PCR) and 0.6 mM of each primer. Identical conditions were used for the primary and secondary amplification: an activation step at 95 °C for 2 min, 35 cycles of denaturing for 1 min at 95 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C.

All samples resulted positive to the *mtLSU-rRNA* nested PCR were subjected to the hemi-nested PCR for *DHPS* amplification (965 bp fragment length) by the primers PK95 and PK160 in the first round amplification and the primers PK160 and PS876 in the second round as described by Ma et al. (1999). The PCR mixture was as that used for the *mtLSU-rRNA* amplification. The same thermocycler settings for the first and the second round were used: an activation step at 95 °C for 2 min, 35 cycles of denaturing for 1 min at 95 °C, annealing for 90 s at 58 °C, and extension for 2 min at 72 °C, followed by a final extension for 7 min at 72 °C.

Negative controls were included in each DNA extraction and amplification round, to monitor for possible cross contamination of samples. All PCR amplifications were performed in a TProfessional Basic Thermocycler (Biometra GmbH, Germany).

Genotyping for detection of mutations were based on the sequence analysis of the two loci *mtLSU-rRNA* and *DHPS*. The amplified products were purified using the NucleoSpin® Extract kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and directly sequenced on both strands by the Bio-Fab Research s.r.l. (Rome, Italy). Multiple alignments were obtained using ClustalW2 Multiple Sequence Alignments (Larkin et al., 2007) in comparison with other *Pneumocystis* strains available in GenBank and manually edited with the Bioedit software (Hall, 1999).

The research protocols were performed in concordance with the WMA Helsinki Declaration (Edinburgh 2000) and its subsequent modification as well as with the Italian National Law n. 675/1996 on the protection of personal data.

3. Results and discussion

Two hundred sixty-three samples were analyzed for the presence of *Pneumocystis*. Overall, 67/263 (25.5%) were found positive at molecular analysis by *mtLSU-rRNA* PCR. The results obtained from DFA test, although performed only on immunocompromised patients, were consistent with molecular diagnosis. Eighteen (47.4%) were from 38 immunocompromised patients, 49 (21.8%) from 225 immunocompetent individuals. Fourteen out of the 18 immunocompromised positive patients (77.8%) were HIV infected; three of them were showing co-infection with HCV. The CD4+ lymphocytes counts were into the range 6–470/mm^3. Among the 49 immunocompetent individuals, five patients were simultaneous infected by *Candida* spp. (3 patients), *Pseudomonas aeruginosa* (1 patient) and *Haemophilus parainfluenzae* (1 patient). Characteristics of the 67 positive patients are given in Table 1.

3.1. mtLSU-rRNA gene analysis

Among the 18 immunocompromised patients positive for *Pneumocystis*, 15 sequences at *mtLSU-rRNA* locus were successfully obtained. Among these, eleven were from HIV-positive patients. Genotypes were identified on the basis of polymorphisms at nucleotide positions 85 and 248 (Beard et al., 2000). Four sequences types were isolated: genotype 2 (85A/248C) was the most frequent (5/15; 33.4%), while the genotype 4 (85C/248T) was the less