Pneumocystis jirovecii multilocus genotyping in pooled DNA samples: a new approach for clinical and epidemiological studies

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Abstract

Specific single-nucleotide polymorphisms (SNPs) are recognized as important DNA sequence variations influencing the pathogenesis of *Pneumocystis jirovecii* and the clinical outcome of *Pneumocystis* pneumonia, which is a major worldwide cause of illness among immunocompromised patients. Genotyping platforms for pooled DNA samples are promising methodologies for genetic characterization of infectious organisms. We have developed a new typing strategy for *P. jirovecii*, which consisted of DNA pools prepared according to clinical data (HIV diagnosis, microscopic and molecular detection of *P. jirovecii*, parasite burden, clinical diagnosis and follow-up of infection) from individual samples using quantitative real-time PCR followed by multiplex-PCR/single base extension (MPCR/SBE). The frequencies of multiple *P. jirovecii* SNPs (*DHFR312*, *mt85*, *SOD215* and *SOD110*) encoded at three distinct loci, the dihydrofolate reductase (*DHFR*), the mitochondrial large-subunit rRNA (*mtLSU rRNA*) and the superoxide dismutase (*SOD*) loci, were estimated in seven DNA pooled samples, representing a total of 100 individual samples. The studied SNPs were confirmed to be associated with distinct clinical parameters of infection such as parasite burden and follow-up. The MPCR/SBE-DNA pooling methodology, described in the present study, was demonstrated to be a useful high-throughput procedure for large-scale *P. jirovecii* SNPs screening and a powerful tool for evaluation of clinically relevant SNPs potentially related to parasite burden, clinical diagnosis and follow-up of *P. jirovecii* infection. In further studies, the candidate SNPs *mt85*, *SOD215* and *SOD110* may be used as molecular markers in association with MPCR/SBE-DNA pooling to generate useful information for understanding the patterns and causes of *Pneumocystis* pneumonia.

Keywords: DNA pools, immunocompromised patients, molecular diagnosis/epidemiology, multilocus genotyping, *Pneumocystis* pneumonia
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Introduction

Pneumocystis jirovecii is recognized as a worldwide pathogenic fungus that parasitizes the human alveolar epithelium causing life-threatening interstitial pneumonia, known as *Pneumocystis* pneumonia, or pneumocystosis (PcP). This opportunistic infectious disease inflicts severe morbidity and mortality in immunocompromised patients, especially in those with AIDS and is therefore of growing importance among non-HIV-infected subjects who are undergoing immunosuppressive treatments related to malignancies, connective tissue diseases or organ transplantation [1–6].

In the absence of a culture system to isolate and maintain live organisms, efforts to type and characterize *P. jirovecii* have relied on PCR-based approaches [7–10]. Single-nucleotide polymorphisms (SNPs) are increasingly recognized as the markers of choice to study the population genetics, geographical distribution, modes of transmission and drug susceptibility or resistance of specific *P. jirovecii* genotypes [7,9,11–23].

Multiplex amplification of genomic DNA associated with single base extension (SBE) is a suitable high-throughput methodology for large-scale SNP screening [16,24,25]. Current genotyping surveys in a large number of samples are still time consuming methods. Alternatively, DNA pooling is a reliable genotyping method, in which equal amounts of DNA from a large number of individual samples are pooled and the SNP allele frequencies are estimated [26–29].

Dihydrofolate reductase (*DHFR*), superoxide dismutase (*SOD*) and mitochondrial large-subunit rRNA (*mtLSU rRNA*) are important genomic regions involved in basic metabolic mechanisms (folic acid synthesis and drug resistance, protection against free oxygen radicals and translation) that carry SNPs reported previously to be associated with clinical data and outcome of PcP [10,13,14,30–32].

The goals of the present study were to: (i) develop robust high-throughput methodologies for large-scale *P. jirovecii* genetic characterization; and (ii) correlate clinical parameters of infection such as parasite burden, clinical diagnosis of PcP and follow-up with genotyping results, using four *P. jirovecii* candidate SNPs (*DHFR312*, *mt85*, *SOD110* and *SOD215*).

Materials and Methods

Patients and data

In total, 100 pulmonary specimens (19 induced sputa and 81 bronchoalveolar lavage fluids) tested previously and found to be positive for *P. jirovecii* (2001–2008) were included in the study. The pulmonary specimens were obtained from 85 HIV-positive adult patients (mean age 41 years; range 24–60 years; 56% were men) and 15 HIV-negative patients (four neonates, four patients with neoplasia, three organ recipients, and four children between 2 and 6 years old with no established cause of immunodeficiency), living in Lisbon, Portugal, and were collected, between 2001 and 2008, at two major hospitals (Hospital de Santa Maria and Hospital de Egas Moniz, Lisbon, Portugal) for diagnostic purposes, before the patients started their anti-*P. jirovecii* therapy with trimethoprim–sulfamethoxazole. The BAL samples were retrieved by washing with 150 mL 0.9% NaCl from which 30 mL was used for the study of *P. jirovecii*. The present study had the approval of the Institutional Review Boards/Ethical Committees from the involved institutions and all patients who enrolled gave their informed consent.

Extraction of DNA was performed using a Mini-BeadBeater/guanidinium thiocyanate–silica method, as described previously [33]. Detection of *P. jirovecii* organisms was performed by both indirect immunofluorescence with monoclonal antibodies (IF) (MonoFluo™ kit *P. jirovecii*; Bio-Rad, Marnes la Coquette, France) and nested-PCR. The parasite burden was estimated using the semi-quantitative method of IF and was defined as: low (no cysts identified, but positive by nested-PCR); moderate (one to three cysts in 30 fields at ×1000); and high (four to 30 or more cysts in one field at ×1000), as described previously [10,16,30,33].

A clinical diagnosis of PcP was considered when at least two of the following variables were present: symptoms such as unproductive cough, fever and dyspnoea; arterial partial pressure of oxygen lower than 65 mmHg; and chest radiographs presenting fine bilateral, perihilar interstitial shadowing. Positive follow-up or clinical improvement was considered when the patient showed a favourable response to anti-*P. jirovecii* therapy (trimethoprim–sulfamethoxazole) and survived for at least 4 weeks after the diagnosis of PcP. Follow-up was considered negative either when there was a failure to improve clinically after administration of the anti-*P. jirovecii* therapy (trimethoprim–sulfamethoxazole) for more than 10 days or when the patient died during a PcP episode [1,2,11,30].

DNA quantification

A DNA pooling technique was developed using a quantitative fluorescence-based real-time PCR (qPCR) for *P. jirovecii* DNA quantification that specifically targets the nuclear single-copy gene encoding the kexin-like serine protease (*KEX1*), described as unique for this organism [34,35]. The qPCR assays were performed in the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), as follows: 2 min at 50°C, 10 min at 95°C, and 50 amplification cycles of 15 s at 95°C and 1 min at 60°C using 9 μL DNA sample, 1× reaction buffer (TaqMan® Gene Expression Master Mix; Applied Biosystems), 1× assay mix (TaqMan® MGB probes, FAM™ dye-labelled; Applied Biosystems), in a 20 μL reaction volume. This assay comprises unlabelled PCR primers (forward primer 5′-CAACCCTGTTCCAATGCCTAA-3′ and reverse primer 5′-CAACACCGATTCCACAAACAGT-3′) and a dye-labelled (FAM) minor groove binder (MGB) probe (5′-TGCTGGTGAAGTAGCTGCCGTTCGA-3′), as described previously [34]. The baseline was taken from cycles three to 15 and the threshold was set at 0.02. In the present work The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines were followed for qPCR experiments. The use of MIQE guidelines is intended to standardize the qPCR method, providing practical rules for the incorporation of minimum consistency standards and encompassing the key assay parameters for accurate design, documentation and reporting of the experimental procedure [36,37].

The qPCR was designed for absolute quantification of *P. jirovecii* DNA in pulmonary specimens, so the exact quantities of *P. jirovecii* DNA in each sample were calculated by com-