Case Report: Occurrence of *Paracoccidioides lutzii* in the Amazon Region: Description of Two Cases

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Abstract. Paracoccidioidomycosis (PCM), the most important human systemic mycosis in Latin America, is known to be caused by at least four different phylogenetic lineages within the *Paracoccidioides brasiliensis* complex, including S1, PS2, PS3, and Pb01-like group. Herein, we describe two cases of PCM in patients native from the Amazon region. The disease was originally thought to have been caused by *P. brasiliensis*. Despite the severity of the cases, sera from the patients were negative in immunodiffusion tests using the standard exoantigen from *P. brasiliensis* B-339. However, a positive response was recorded with an autologous preparation of *Paracoccidioides lutzii* exoantigen. A phylogenetic approach based on the gp43 and ARF loci revealed high similarity between our clinical isolates and the Pb01-like group. The occurrence of PCM caused by *P. lutzii* in the Brazilian Amazon (Pará State) was thus proven. The incidence of PCM caused by *P. lutzii* may be underestimated in northern Brazil.

INTRODUCTION

Paracoccidioidomycosis (PCM) is the most prevalent human systemic mycosis in Latin America, with high incidences in Brazil, Argentina, Colombia, and Venezuela.^{1–3} The disease was discovered in 1908 by Lutz and Splendore and since then it has been attributed to the thermomorphous fungus *Paracoccidioides brasiliensis*. Acquisition of the disease occurs through inhalation of conidia of *P. brasiliensis*, which are dormant in soil. Consequently, rural workers dealing with soil on a daily basis are at risk. The lungs reportedly are the initial focus of the disease; PCM can develop into a disseminated infection in some patients. There is a clear gender bias for males.^{3,4}

Recently it was shown that the monotypic taxon created to accommodate the etiological agent of PCM, *P. brasiliensis*, in fact is a complex of cryptic species, comprising at least four different phylogenetic, potentially human-pathogenic lineages. Three of these lineages differing in geographic distribution and prevalence, were identified as S1, PS2, and PS3 by Matute and others.⁵ Group S1 comprised clinical and environmental isolates from Brazil, Argentina, Paraguay, Peru, and Venezuela and so far seems to be the most prevalent and widespread genotype in Latin America. Group PS2 contained only five isolates from Brazil and one from Venezuela. Lineage PS3 consists of clinical and environmental strains originating thus far exclusively from Colombia.

Carrero and others⁶ recognized the presence of a fourth group, phylogenetically distinct from those identified by Matute and others.⁵ Theodoro and others⁷ and Teixeira and others⁸ referred to this entity as the “Pb01-like group,” because of similarity of the strains with isolate Pb01. Isolates belonging to this phylogenetic group were mostly recovered from diseased patients living in the central-west region of Brazil, although a Pb01-like strain was also detected in Ecuador (iso-

late 7455). A new phylogenetic species *Paracoccidioides lutzii* was proposed for Pb01-like isolates.⁸ Several authors have encountered “atypical” isolates of *P. brasiliensis* s.l.^{6,9,10} Molecular and morphological data suggest that such isolates may bear close relationship with the Pb01-like group. This study aims to describe two “atypical” *Paracoccidioides* isolates from two patients symptomatic for PCM in the Brazilian Amazon (Pará State).

CASE REPORTS

Paracoccidioides isolate IEC-2005 was initially recovered from a male patient, 44-year-old rural worker from Pará State (Castanhal City, 01°17'49"S 47°55'19"O) in the Brazilian Amazon, suffering from chronic PCM infection. He was admitted to our service on August 2005, with a 6-month history of fever, cough, emaciation (weight loss 34 kg), bloody sputum, dyspnea on exertion, and fistulous cervical lymphadenopathy. The patient was previously treated for unproven tuberculosis. Chest x-ray revealed nodules in the upper lobes of the lungs and pulmonary opacity. Tomography showed consolidation, bronchiectasis, and fibrotic lesions in the posterior segments of both lungs. Despite the severity of the disease, results of routine serological tests (double immunodiffusion, DID) were negative for *P. brasiliensis*, *Histoplasma capsulatum*, and *Aspergillus fumigatus* antigens. The PCM diagnosis was established by visualization of birefringent multibudding yeast cells compatible with *P. brasiliensis* in a lymph node biopsy and in bronchoalveolar lavage. Cultures were positive for *Paracoccidioides*. Treatment was then started with 100 mg itraconazole, combined with sulfamethoxazole and trimethoprim, 400 mg + 80 mg, respectively, for 1 month, with good response. After a month of follow-up an improvement of symptoms (clinical and radiological) was noted and the patient was discharged from the hospital with a prescription of maintenance treatment in a basic regional health unit.

Isolate IEC-1744 was recovered from a 55-year-old male rural worker (chainsaw operator) from Pará State (Paragominas City, 02°59'42"S 47°21'10"O) in the Brazilian Amazon. He was admitted to our service on June 2010 with a 15-month history emaciation (weight loss 15 kg), and with

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lesions of the oral and nasal mucosa, palate, and lips (muriform lesions), with swelling. The patient presented cervical palpable ganglia on the left and was without cough and fever. Initially, the patient was treated for leishmaniasis (Montenegro reaction was positive), even in the absence of confirmatory diagnosis. Results of a routine DID test was negative for *P. brasiliensis*, *H. capsulatum*, and *A. fumigatus* antigens. Diagnosis was established by direct examination of scrapings from the oral mucosa with visualization of birefringent multilateral budding yeast cells similar to those of *P. brasiliensis*, and by isolation of the fungus in culture. The PCM treatment was started receiving sulfamethoxazole + trimethoprim (400 and 80 mg, respectively, for 2 months). After 2 months of treatment, the patient still had oral lesions reminiscent. He was discharged from the hospital and returned to his original city with a prescription of maintenance treatment in a basic health unit of the region.

MATERIAL AND METHODS

Paracoccidioides culture and DNA extraction.

Paracoccidioides isolates IEC-2005 and IEC-1744 were allowed to grow at 25°C on Fava-Netto's semi-solid medium,¹¹ and sub-cultured every 15 days. Genomic DNA was extracted and purified directly from fungal mycelial colonies by following the Fast DNA kit protocol (MP Biomedicals, Solon, OH).

Identification of the Pb01-like genotype. The identities of isolates IEC-2005 and IEC-1744 were evaluated in polymerase chain reaction (PCR) reactions containing the primers HSPMMT1 (5'AACCAACCCCTCTGTCTTG) and PLMMT1 (5'GAAATGGGTGGCAGTATGGG) that anneal to an indel region in the HSP70 gene that are exclusive of the Pb01-like isolates as previously described by Teixeira and others.⁸ As a positive control we included the isolate 351, which had already been shown to belong to the Pb01-like group.⁸ Amplified products were analyzed on 1% agarose gels and visualized by ethidium bromide staining using UV transillumination.

Molecular characterization. The 43 kDa glycoprotein (gp43) and ADP-ribosylation factor (ARF) loci were amplified directly from the genomic DNA by PCR, as described by Teixeira and others,⁸ using the primers gp43-E2F (CCAGGAGGCGTGCAGGTGTCCC-3') and gp43-E2R (GCCCC TCCGTCTTCCATGTCC-3'), which generated a 450-bp corresponding to exon 2¹² and ARF-F (TCTCATGGTTGGCC TCGATGCTGCC-3') and ARF-R (GAGCCTCGACGACA CCGTCACGATC-3'), which generated a 400-bp amplicon corresponding to exon 2 through 3.¹³

Amplified products were gel-purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), following the manufacturer's instructions. DNA samples were completely sequenced with a MegaBACE 1000 DNA Sequencer (Amersham Sunnyvale, CA) using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase II DNA Polymerase, Amersham, Piscataway, NJ). Fragments were sequenced on both strands to increase the quality of sequence data (phred > 30). Sequence alignment was performed using the ClustalW algorithm¹⁴ implemented in BioEdit software¹⁵; retrieved alignments were manually corrected to avoid mispaired bases. Sequences were exported as FASTA files for BLASTN search at <http://www.ncbi.nlm.nih.gov/BLAST>.

Phylogenetic analysis. Gp43 exon-2 and ARF nucleotide sequences from other isolates belonging to the *P. brasiliensis* complex were included in this study as reference strains for the phylogenetic analysis. These sequences were previously deposited at GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and described by Matute and others⁵ and Teixeira and others.⁸

Evolutionary analyses were conducted in MEGA5¹⁶ for the combined data set using the maximum likelihood method. The evolutionary distances were computed using the Kimura 2-parameter model,¹⁷ with 1,000 bootstrap replicates.¹⁸

RESULTS AND DISCUSSION

The identity of our clinical isolates IEC-2005 and IEC-1744 that originally were thought to be *P. brasiliensis* was re-evaluated based on the HSP70 marker.⁸ These isolates presented positive amplification of a fragment of 400 bp using the primers HSPMMT1 and PLMMT1, similar to the amplicon found in isolate 351 (positive control). This indicates that they share an indel in the first intron of the HSP70 gene limited to isolates classified as "Pb01-like." As a negative control we used DNA from isolate Pb339 (S1) (Figure 1).

To determine the taxonomic position for both clinical *Paracoccidioides* isolates, the gp43 exon 2 and ARF loci were amplified and sequenced using the primers gp43-E2F and gp43-E2R or ARF-F and ARF-R, respectively. The final data matrix was composed of 80 taxa, including our two clinical isolates. The combined data set of gp43 and ARF were 823 bp long, including 736 invariable characters, 75 variable parsimony-informative (9.1%), and 12 singletons. Positions containing gaps and missing data were eliminated.

The 80 sequences were distributed into three major groups. The first clade included *P. brasiliensis* s. str. (S1) and the Colombian isolates identified as "phylogenetic species 3" (PS3) by Matute and others.⁵ The second group was constituted by isolates from "phylogenetic species 2" (PS2) including strains from Brazil and Venezuela. The third clade comprised isolates "Pb01-like," denominated *P. lutzii*⁸

FIGURE 1. Genotyping of Pb01-like isolates using the HSP70 gene as a marker. 1) *Paracoccidioides lutzii* 351 (positive control); 2) IEC-2005, 3) IEC-1744; 4) *Paracoccidioides brasiliensis* B-339 (negative control); 5) quality control (no DNA).

FIGURE 2. Molecular phylogenetic analysis by the maximum likelihood method of the *Paracoccidioides lutzii* isolates IEC-2005 and IEC-1744 compared with other *P. lutzii* strains and S1, PS2 and PS3 phylogenetic lineages within the *Paracoccidioides brasiliensis* complex. The evolutionary history was inferred by using the maximum likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Evolutionary analyses were conducted in MEGA5. GenBank accessions nos. [GP43/ARF, respectively] are indicated next to the strain code.

(Figure 2). A molecular phylogenetic analysis by the maximum likelihood method based on gp43 and ARF sequences from our clinical isolates IEC-2005 and IEC-1744 revealed that they are genetically similar to the Pb01-like group (*P. lutzii*) with high bootstrap (100%) support. The IEC-2005 and IEC-1744 nucleotide sequences for gp43 (JX065607 and JX065608, respectively) and ARF (JX065609 and JX065610, respectively) were deposited in GenBank. We did not find a hybrid genotype for our isolates using these two loci as previously described by Teixeira and others⁸ for *P. lutzii* isolates 133 and 769. Similar topologies were observed evaluating the separated data set with a congruency index $I_{\text{cong}} = 2.36$ ($P = 4.21\text{E-}13$) indicating that the gp43 and ARF trees are more congruent than expected by chance.¹⁹

Serological diagnosis of PCM has been successfully performed employing the major antigen secreted by the fungus *P. brasiliensis*, a 43 kDa glycoprotein.^{20–22} However, both clinical cases reported here proved to be negative, indicating the absence of antibodies against gp43 produced by the fungus B-339 (lineage S1). Nevertheless, in both cases, the presence of yeast cells and isolation of the fungus in culture provided proof for PCM that should enable adequate management of the patients. Subsequent molecular characterization of the strains proved evidence that these cases of PCM were caused by *P. lutzii*.

Lower levels of antibodies against fungal antigens in PCM have been reported in patients with immune deficiencies such as HIV^{23,24} or in patients receiving antifungal therapy.^{25,26,27} However, neither of these conditions were noted in our patients. The negative results obtained in serology may be a consequence of antigenic variation in the *P. brasiliensis* complex.

Batista and others²⁸ reported the importance of diagnosis of PCM using the reference antigen B-339 (lineage S1) and the antigen of strain 550B (isolated in the central-west region of Brazil and not identified because of low viability of the isolate *in vitro*). According to these authors the results of immunodiffusion tests were heavily impacted by the antigen used. Serum from patients in the Midwest region, where the phylogenetic species *P. lutzii* occurs with high frequency, seem to react very well against antigenic preparations from autochthonous strains such as isolate 550B. On the other hand, Batista and others²⁸ reported weak or negative reactions against the reference antigen B-339. Our initial serological tests (immunodiffusion-ID) were performed with the reference antigen B-339, providing a negative result. After molecular characterization of the fungi, we investigated the serological response (ID) of both patients against the exoantigen produced by *P. lutzii* (IEC-1744, IEC-2005, and Pb01) as described by Camargo and others.²⁹ Surprisingly, the patient showed a strong response against the *P. lutzii* antigen in the ID test with antibody titer 1:16 (data not shown). Our serological results are in agreement with those found by Batista and others²⁸ and suggest an antigenic diversity in the *P. brasiliensis* complex. The serological finding reported here, indicates that *P. brasiliensis* and *P. lutzii* vary in their antigen composition, and that the use of antigens from regional strains in the PCM diagnosis must be considered. Additional studies have to be conducted to better understand the role of proteins, polysaccharides, and lipids in the immune response development against the cryptic species of the *Paracoccidioides* complex.

Similar antigenic polymorphism has been reported by several authors^{5,8,30,31} in the nucleotide coding region of the main antigen of reference from the fungus B-339 (AY005437) and Pb01 (EU870196). Herein, we identified the same variation in the isolates IEC-2005 (JX065607) and IEC-1744 (JX065608), resulting in different amino acid compositions of gp43, which strongly supports the hypothesis of antigenic variability. However, further studies are needed to prove immunological identity of *P. lutzii* isolates.

Clinical manifestations observed in the two patients reported here, with diagnosis of PCM by *P. lutzii*, are similar to those caused by *P. brasiliensis*. Compared with each other the patients presented variations in the intensity, extent, dissemination, and characteristics of the lesions. However, PCM is a dynamic and polymorphic disease, and thus clinical comparisons are difficult.

The incidence of PCM may be underestimated in the northern region of Brazil. In our laboratory in Ananindeua, the previous use of antigen from *P. brasiliensis* B-339 may have led to numerous cases of false-negative results. In addition, the negligence of this antigenic variability in the diagnosis of PCM in specific areas such as Central-west and North region may underestimate the incidence of the disease, cause delay in the treatment of the patient, leading to a significant worsening in clinical status and consequent difficulty in the therapeutic of the disease. A retrospective study using different antigenic preparations from autochthonous strains may elucidate the real incidence of PCM in the Amazon region.

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