Pneumocystis jirovecii detection and genotyping from HIV-positive and HIV-negative Mexican patient samples

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Abstract

Introduction: *Pneumocystis jirovecii* is an atypical fungus particularly detected in HIV-positive or transplanted patients. **Objective:** To detect and genotype *Pneumocystis jirovecii* in patient samples from two hospitals in Mexico City. **Method:** Eighty-nine respiratory tract samples, corresponding to 53 patients (30 HIV-positive and 23 HIV-negative) with respiratory symptoms and to 11 healthy individuals included as negative control, were processed. DNA was extracted and amplified by nested polymerase chain reaction from the internal transcribed spacer, with one fragment being obtained at each round (693 and 550 bp). Genotypes and their phylogenetic relationship were determined by sequencing the 550 bp fragment. **Results:** Forty-eight samples from 30 HIV-positive patients were received from a single hospital, out of which 11 (36.6 %) were positive for *Pneumocystis jirovecii*. No sample was positive in HIV-negative patients or healthy subjects. The most frequently detected haplotypes were Eg and Em. **Conclusions:** The frequency of *Pneumocystis jirovecii* infection was high in the studied Mexican population. The most common genotype was different from those reported in other countries. It is necessary to address this health problem through early detection of this infection.

KEY WORDS: *Pneumocystis jirovecii*. Pneumonia. Human immunodeficiency virus. Mycosis.

Introduction

Pneumocystis jirovecii is an opportunistic fungus whose in vitro culture has not been achieved. It causes pulmonary infection in immunocompromised individuals, particularly in those infected with the human immunodeficiency virus (HIV) who have developed acquired immunodeficiency syndrome (AIDS), who are malnourished or have undergone transplants.¹ *Pneumocystis jirovecii* is acquired via the respiratory tract at early stages in life and often it is found as a commensal in immunocompetent individuals. During immunosuppression, the microorganism multiplies in the

alveoli, inducing an inflammatory process.² *Pneumocystis jirovecii* pneumonia (PCP) is manifested by nonspecific symptoms, although respiratory failure is the most common complication and is associated with elevated mortality.³

To detect *Pneumocystis jirovecii*, stains (Gomori-Grocott and Giemsa) and molecular procedures are commonly used.^{4,5} Polymerase chain reaction (PCR) has been commonly directed towards ribosomal DNA inter-transcriber spacer (ITS) region.^{2,6} This ITS region is the “barcode” to identify the fungus. The comparison of ITS region sequences is widely used in molecular taxonomy and phylogeny because it is very easy to

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amplify, even with small amounts of DNA, and has a high degree of variation, even between tightly close species. This marker exists in multiple copies in most fungal cells and is retrievable with relatively robust oligonucleotides with a high degree of reliability.^{7,8}

For each target gene, type-sequences have been reported, and differences detected in similar studies have determined numerous genotypes.^{2,9,10} For the ITS1 region, more than 30 genotypes are known, and for ITS2, over 40.¹¹

Globally, PCP represents the first or second most common opportunistic infection in patients with HIV infection.¹² In Mexico, it has been reported in up to 51-55 % of HIV infection cases, and its diagnosis has been based on stains.^{13,14} The purpose of this investigation was to determine the frequency of *Pneumocystis jirovecii* and its genotypes in samples of HIV-infected/AIDS patients or with any other immunosuppressive pathology, by means of nested PCR of the ITS region followed by sequencing.

Method

This was a prospective, cross-sectional, prolective study. Respiratory specimens from hospitalized patients with pulmonary symptoms at the National Institute of Respiratory Diseases and the “General Ignacio Zaragoza” Regional Hospital of the Institute of Social Security and Services of State Workers, Mexico City were requested.

Hospitalized patients of any age, of either gender, with respiratory symptoms, without anti-*Pneumocystis* treatment were included. Informed consent was not requested because the study was carried out with part of the sample obtained for other analyses. Negative control included oropharyngeal washing fluid from healthy subjects, after informed consent was obtained.

For the DNA extraction, the conditions described by Lu et al.¹⁵ were considered and the High Pure PCR Template Preparation® kit (Roche, Germany) was used. The DNA was stored at -20 °C and then used for PCR.

Nested PCR

- First round: 20 µL of DNA was used in a final volume of 50 µL containing 1x of PCR-buffer, 1.5 mM MgCl₂, 1.0 mM deoxyribonucleotide triphosphate, 0.2 µM oligonucleotide 1724F (AAG TTG GTC AAA TTT GGT C), 0.2 µM oligonucleotide ITS2R (CTC GGA CGA GGA TCC TCG

CC), 1.25 U Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). Temperature: 94 °C, 5 minutes; 35 cycles at 94 °C for one minute, 47 °C for one minute and 72 °C for two minutes; finally, at 72 °C for five minutes. The expected product size was 693 base pairs (bp).¹⁵ As negative PCR control, 20 µL of sterile milliQ water and as positive control, 3 µL of DNA from a sample markedly positive for *Pneumocystis jirovecii* by Gomori-Grocott staining and by PCR were used.

- Second round: 2 µL of the first round amplification product was used, at a final volume 50 µL. The oligonucleotides were ITS1F (CGT AGG TGA ACC TGC GGA AGG ATC) and ITS2R1 (GTT CAG CGG GTG ATC CTG CCT G). Concentrations of the reaction mixture were similar to those of the first round. Temperatures: 94 °C, 10 minutes; 35 cycles at 94 °C for one minute, 58 °C for one minute, 72 °C for minutes. A final extension was applied at 72 °C for 10 minutes. The expected product size was 550 bp.¹⁵

Amplicons were run on 1.5 % agarose gel, stained with ethidium bromide, purified and sent for sequencing to the Institute of Biology of the National Autonomous University of Mexico, Mexico City, Mexico, with the chain-termination inhibition method.¹⁶ All sequences were carried out in duplicate in 5'-3' and 3'-5' directions.

Sequence analysis

Sense and antisense sequences were analyzed and edited with the ChromasPro program and compared with three databases: Basic Local Alignment GenBank Search Tool (<https://www.ncbi.nlm.nih.gov/>), The European Molecular Biology Laboratory (<http://www.embl.de/>) and Kyoto Encyclopedia of Genes and Genomes (<http://www.genome.jp/kegg/>).

The sequences were also analyzed with the MEGA program, version 6.0, to build a phylogenetic tree with the neighbor-joining method, using the two-parameter Kimura model with a 1000-repetition bootstrap.¹⁷ Multiple alignment was carried out using ClustalW. A matrix of genetic distances was constructed.

Genotype determination

The sequences reported by Lee et al.,⁹ who considered 15 genotypes for the ITS1 region (A-O) and 14 genotypes for ITS2 (a-n) were taken as reference.

Results

Forty-eight samples from 30 patients (23 males and 7 females) were received from the National Institute of Respiratory Diseases. Age ranged from five to 76 years, with an average of 35.7. Most samples (30) were bronchoalveolar lavage fluid and tissue (18). In 26 patients (86.6 %), the main opportunism factor was HIV/AIDS infection, most of them confirmed (Table 1).

Thirty samples, corresponding to 23 patients (15 males and eight females), were received from the "General Ignacio Zaragoza" Regional Hospital. Age ranged from one to 86 years, with an average of 50.6. Most samples (25) were sputum. Patients had various opportunism factors, predominantly pulmonary tuberculosis (seven, 29.16 %), which was probable or confirmed the time of sample reception (Table 2).

The negative control group consisted of 11 oropharyngeal lavage fluid samples from healthy adults, whose average age was 34.2 years, without history of smoking or respiratory symptoms at the moment of sampling.

Pneumocystis jirovecii detection

Figure 1 shows the parasitic structures stained with Gomori-Grocott and the amplicon corresponding to *Pneumocystis jirovecii* obtained from bronchoalveolar lavage fluid, which was used as a positive PCR control. The fragment was purified and sequenced to corroborate its identity with *Pneumocystis jirovecii*.

Of the 30 patients from the National Institute of Respiratory Diseases, 11 were *Pneumocystis jirovecii*-positive (Figures 1B and 2A), all of them had HIV infection as opportunism factor, nine were males and two females. In five patients, PCP had been previously diagnosed by Gomori-Grocott staining, and all were confirmed by PCR. *Pneumocystis jirovecii*-positive patients accounted for 36.6 % of cases at this institution.

In sample 12a, which corresponded to a patient of the National Institute of Respiratory Diseases, a 620-bp amplicon was obtained, which is larger than the expected size (Figure 2B). This fragment was sequenced and analyzed.

All samples from the "General Ignacio Zaragoza" Regional Hospital and from the negative control group (healthy individuals) were negative for *Pneumocystis jirovecii*.

Sequence similarity analysis

Identity with *Pneumocystis jirovecii* ranged from 90 to 100%. The analysis in all three databases was similar. All amplified fragments corresponded to *Pneumocystis jirovecii* ITS region. In sample 12a, electropherogram was highly reliable and the comparative analysis indicated 87 % identity with *Cryptococcus* sp.

With the sequencing data, a genetic distance matrix was constructed (data not shown), and correlations were explored by analyzing the origin of the samples (Fig. 3). Sample 12a remained on a separate branch with regard to the others. Samples 11a and 11b corresponded to the same patient, whose genetic distance value was 0.000; both were grouped in the same branch, which indicates that it is the same *Pneumocystis jirovecii* strain. Samples 10a and 10b also belonged to a single patient, with a genetic distance of 0.152, and were grouped into different phylogenetic branches, which suggested there was a difference between the *Pneumocystis jirovecii* strains.

Genotype determination

Of the 14 *Pneumocystis jirovecii*-positive samples, in 11 the genotype was determined in both directions. In three sequences, determining the genotype was not possible due to the large number of insertions and deletions. In these samples, the procedure was repeated twice more, from DNA extraction to sequence analysis, and the results were similar. For ITS1, genotype E was predominant (seven samples) and for ITS2, genotype g (five samples). The predominant haplotypes were Em (four cases) and Eg (three cases). In samples 10a and 10b, which corresponded to a single patient, the genotypes were different. In samples 11a and 11b, which also corresponded to a single patient, the same genotype was found.

Discussion

Due to the impossibility to retrieve *Pneumocystis jirovecii* in cultures, molecular procedures have been particularly useful for detecting this organism in samples of patients in whom PCP is suspected. The frequency of *Pneumocystis jirovecii* detection reported by other authors is variable, depending on various factors such as the type of sample, fungal load, the molecular procedure used and the selected target gene. Nested

Table 1. General data of patients with pulmonary symptoms hospitalized at the National Institute of Respiratory Diseases

Sample number	Gender	Age (years)	Specimen	Risk factor	PCR <i>Pneumocystis jirovecii</i>
1	M	—	BALF	HIV/AIDS	Positive
2a	F	28	BALF	HIV	—
2b	F	28	LBx	—	—
3a	M	25	BALF	HIV	—
3b	M	25	BALF	HIV, PTb	—
4a	M	43	BALF	HIV/AIDS	—
4b	M	43	LBx	HIV/AIDS	—
5	F	59	BALF	HIV	Positive
6a	M	32	BALF	HIV	Positive
6b	M	32	LBx	HIV	—
7	M	25	BALF	HIV/AIDS, PTb	—
8a	M	26	BALF	HIV/MC	—
8b	M	26	BALF	HIV/MC	—
9a	M	33	BALF	HIV	Positive
9b	M	33	LBx	HIV	—
10a	M	26	LBx	HIV/PCP	Positive
10b	M	26	BALF	HIV/PCP	Positive
11a	M	28	BALF	HIV/PCP	Positive
11b	M	28	LBx	HIV/PCP	Positive
12a	M	25	LBx	HIV/AIDS	<i>Cryptococcus</i>
12b	M	25	BALF	HIV/AIDS	—
13a	F	33	LBx	HIV/PCP	—
13b	F	33	BALF	HIV/PCP	Positive
14	F	57	BALF	HIV	—
15	M	76	BALF	HIV	—
16	M	53	BALF	Pneumonia	—
17	F	5	LBx	Unknown	—
18a	M	44	LNBx	HIV/KS	—
18b	M	44	BALF	HIV/KS	Positive
19	M	31	LBx	HIV	—
20a	M	28	BALF	Pb HIV/PCP	—
20b	M	28	LBx	Pb HIV/PCP	—
21a	M	44	BALF	HIV/pneumonia	Positive
21b	M	44	BALF	HIV/pneumonia	—
22a	F	31	BALF	HIV/PCP	—
22b	F	31	LBx	HIV/PCP	—

(Continued)

Table 1. General data of patients with pulmonary symptoms hospitalized at the National Institute of Respiratory Diseases (*Continued*)

Sample number	Gender	Age (years)	Specimen	Risk factor	PCR <i>Pneumocystis jirovecii</i>
23a	M	20	BALF	Pb HIV	—
23b	M	20	LBx	Pb HIV	—
24a	M	32	LBx	HIV, LNTb	—
24b	M	32	BALF	HIV, LNTb	—
25	M	24	BALF	HIV/PCP	Positive
26	M	37	BALF	HIV/PCP	Positive
27	M	31	LBx	HIV/Pb PTb	—
28	F	25	BALF	PCP	—
29	M	74	BALF	Pb IPF	—
30a	M	42	BALF	HIV/PTb	—
30b	M	42	LNBx	HIV	—
30c	M	42	LBx	HIV/PTb	—

M = male, F = female, BALF = bronchoalveolar lavage fluid, LBx = lung biopsy, LNBx = lymph node biopsy, Pb = probable, HIV = human immunodeficiency virus, AIDS = acquired immunodeficiency syndrome, PTb = pulmonary tuberculosis, MC = Mycobacterium complex, PCP = Pneumocystis pneumonia, LNTb = lymph node tuberculosis, IPF = idiopathic pulmonary fibrosis, KS = Kaposi's sarcoma.

PCR and real-time PCR using mitochondrial DNA fragments, ITS1 and ITS2 as target genes have shown high sensitivity and specificity values.^{2,18}

PCR has revealed sensitivity and specificity values for bronchoalveolar lavage fluid ranging between 84 and 100 % and 58 and 100 %, respectively,^{19,20} which is why it has been the most commonly used and reliable specimen. Gupta et al.²¹ studied 180 samples of immunosuppressed patients with clinical suspicion of PCP; using nested PCR directed to the ITS region they found 24.69 % positivity in 81 bronchoalveolar lavage fluid samples and in 12 % of 50 sputum samples. In other countries, such as Brazil, 44.8 % positivity for *Pneumocystis jirovecii* has been identified by nested PCR in HIV-positive patients.²² In this study, out of 30 bronchoalveolar lavage fluid processed samples, 11 (36.6 %) were positive.

The 25 sputum samples that corresponded to patients of the “General Ignacio Zaragoza” Regional Hospital were negative for *Pneumocystis jirovecii*. In other investigations, negative results have been attributed to the presence of PCR inhibitors.²³ In the case of the patient group of that hospital, it is possible that other factors could have influenced on negative results, such as a lower degree of immunosuppression caused by the underlying disease, which was mainly pulmonary tuberculosis, occasionally reported in coinfection with *Pneumocystis jirovecii*.²⁴ In this study, the two HIV-positive patients of the “General Ignacio Zaragoza”

Regional Hospital showed no evidence of *Pneumocystis*, probably due to an immunosuppression moderate stage, such as the cases of pulmonary tuberculosis.

Medrano et al.²⁵ detected *Pneumocystis jirovecii* DNA in 12 (20 %) of 50 oropharyngeal lavage fluid samples from healthy individuals, and Vargas et al.,²⁶ in 32 % of 74 healthy children. In this work, the absence of *Pneumocystis jirovecii* in healthy individuals possibly occurred because the subjects were not *Pneumocystis jirovecii* carriers, because fungal load was too low or the oropharyngeal lavage procedure was inadequate to detect to the microorganism.

In recent years, genetic databases have exponentially grown and currently there is more information on microorganisms' identity. The analysis of the oligonucleotides herein used with the Basic Local Alignment Search Tool (BLASTn) indicates that they amplify for one *Pneumocystis jirovecii* and one *Cryptococcus* sp DNA fragment. Amplification of a fragment corresponding to *Cryptococcus* can be explained because the oligonucleotides described for the ITS region are universal for fungal species' molecular identification.^{7,8} In addition, it is not surprising to find *Cryptococcus* colonizing or infecting patients with HIV infection, since this yeast (mainly *Cryptococcus neoformans*) is acquired via the respiratory tract, initially infecting the lung and then invading the central nervous system and other tissues.²⁷

Pneumocystis jirovecii genotype mixture (as in samples 10a and 10b) is a common condition.²⁸ It is possible

Table 2. General data of patients from the “General Ignacio Zaragoza” Regional Hospital with pulmonary symptoms

Sample number	Gender	Age (years)	Specimen	Risk factor
1	M	50	BALF	Unknown
2	F	64	BALF	Unknown
3a	M	38	Sputum	Pulmonary tuberculosis
3b	M	38	Sputum	Pulmonary tuberculosis
3c	M	38	Sputum	Pulmonary tuberculosis
3d	M	38	Sputum	Pulmonary tuberculosis
4	F	52	Sputum	Unknown
5	M	57	Sputum	Probable PTb
6a	M	37	Sputum	Probable PTb
6b	M	37	Sputum	Probable PTb
7a	F	45	Sputum	Pulmonary tuberculosis
7b	F	45	Sputum	Pulmonary tuberculosis
8	M	21	Sputum	HIV, probable PTb
9a	M	63	Sputum	Pulmonary neoplasm
9b	M	63	Sputum	Hemoptysis in study
10	M	43	Sputum	Pulmonary tuberculosis
11	M	31	Sputum	HIV
12	M	67	Sputum	Unknown
13	F	50	Pleural fluid	Probable PTb
14	M	80	Pleural Fluid	Pulmonary neoplasm
15	F	23	BALF	Epilepsy
16a	M	57	BALF	Pneumonia
16b	M	57	Sputum	Septic shock
17	F	37	Sputum	Septic shock
18	M	76	Sputum	Respiratory failure
19	F	62	Sputum	Respiratory failure
20	M	86	Sputum	COPD, pneumonia
21	F	64	Sputum	Pneumonia
22	M	1	Sputum	Sepsis, pneumonia
23	M	61	Sputum	Septic shock

M = male, F = female, BALF = bronchoalveolar lavage fluid, PTb = pulmonary tuberculosis, HIV = human immunodeficiency virus, COPD = chronic obstructive pulmonary disease.

that the high genetic variability and difficulty in determining genotypes in some samples of this study were due to contact of patients with different sources of infection and strains.²⁹ Regarding the detected genotypes, the Em haplotype (four samples) has not been reported as being predominant, in contrast with haplotype Eg (three samples), which was found to be the most

common in another report.³⁰ This research is not sufficient to affirm that the Mexican population is carrier of a different genotype than other analyzed populations.

Although sputum is used in most studies as specimen, it is possible for the form of obtaining it to be a decisive factor to detect the fungal parasite. Casanova et al.¹³ found *Pneumocystis carinii* (*Pneumocystis*

Figure 1. Study of the sample used as positive control (sample 1 of patients from the National Institute of Respiratory Diseases). **A:** Gomori-Grocott staining, whereby *Pneumocystis jirovecii* typical cystic forms are observed (scale, 5 μm). **B:** Nested PCR product run on 1.5% agarose gel, stained with ethidium bromide. The 550-bp amplicon corresponds to Figure 1A sample. M = 100-bp molecular weight marker.

Figure 2. *Pneumocystis jirovecii* ITS region amplicons. **A:** Samples that amplified a fragment of the expected size (550 bp). **B:** Sample that amplified a larger-than-expected fragment (620 bp). M = 100-bp molecular weight marker. The numbers at the top indicate the sample number.

Figure 3. Phylogenetic tree, constructed with the neighbor-joining method. The numbers correspond to each processed sample and the haplotype is indicated in parenthesis. Samples 10a and 10b, as well as 11a and 11b, correspond to a single patient, respectively. *Cryptococcus neoformans* was identified in sample 12a and was used as external control.

jirovecii) in 51 % of 128 patients after obtaining sputum by induced expectoration and with Giemsa staining.

The HIV-infected population continues to be at high risk for developing PCP due to its low level of CD4 lymphocytes.³¹

PCP is a pathology that has a preponderant place in the population subjected to any immunosuppression factor. Globally, the annual prevalence of pneumonia due to *Pneumocystis jirovecii* is estimated to be 500,000 cases.³² To apply measures for the control of infectious diseases, in particular opportunistic infections such as PCP, it is necessary to know their epidemiological status at each country.

In this work, high positivity for *Pneumocystis jirovecii* and its genotypes was identified in HIV-positive Mexican patients through nested PCR targeting the gene of the ITS region. It is necessary to surveil the Mexican population at risk for developing PCP by applying molecular procedures to determine with certainty the fungal load and, if possible, detecting strains resistant to therapeutically used medications in order to start specific treatment.

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