International Journal of Infectious Diseases 86 (2019) 65–72

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

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

Histoplasma capsulatum and *Pneumocystis jirovecii* coinfection in hospitalized HIV and non-HIV patients from a tertiary care hospital in Mexico

Laura E. Carreto-Binaghi[a,b], Fernando R. Morales-Villarreal[c], Guadalupe García-de la Torre[d], Tania Vite-Garín[a], Jose-Antonio Ramirez[a], El-Moukhtar Aliouat[e], Jose-Arturo Martínez-Orozco[c], Maria-Lucia Taylor[a,*]

[a] *Laboratorio de Inmunología de Hongos, Unidad de Micología, Departamento de Microbiología-Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), CDMX, 04510, Mexico*
[b] *Departamento de Investigación en Microbiología, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" (INER), CDMX, 14080, Mexico*
[c] *Departamento de Microbiología Clínica, INER, CDMX, 14080, Mexico*
[d] *Departamento de Salud Pública, Facultad de Medicina, UNAM, CDMX, 04510, Mexico*
[e] *Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 – CIIL – Centre d'Infection et d'Immunité de Lille, Lille, France*

ARTICLE INFO

Article history:
Received 30 January 2019
Received in revised form 10 June 2019
Accepted 11 June 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Histoplasmosis
Pneumocystosis
Coinfection
Molecular diagnosis
Bronchoalveolar lavage

ABSTRACT

Background: Histoplasma capsulatum and *Pneumocystis jirovecii* are respiratory fungal pathogens that principally cause pulmonary disease. Coinfection with both pathogens is scarcely reported. This study detected this coinfection using specific molecular methods for each fungus in the bronchoalveolar lavage (BAL) of patients from a tertiary care hospital.
Materials and methods: BAL samples from 289 hospitalized patients were screened by PCR with specific markers for *H. capsulatum* (Hcp100) and *P. jirovecii* (mtLSUrRNA and mtSSUrRNA). The presence of these pathogens was confirmed by the generated sequences for each marker. The clinical and laboratory data for the patients were analyzed using statistical software.
Results: The PCR findings separated three groups of patients, where the first was represented by 60 (20.8%) histoplasmosis patients, the second by 45 (15.6%) patients with pneumocystosis, and the last group by 12 (4.2%) patients with coinfection. High similarity among the generated sequences of each species was demonstrated by BLASTn and neighbor-joining algorithms. The estimated prevalence of *H. capsulatum* and *P. jirovecii* coinfection was higher in HIV patients.
© 2019 The Authors. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Histoplasma capsulatum is the causative agent of histoplasmosis, one of the most important systemic mycoses in humans; this disease is particularly related to endemic regions in America, and autochthonous outbreaks have been described from latitude 54° north (Alberta, Canada) (Anderson et al., 2006) to 38° south (Neuquén, Argentina) (Calanni et al., 2013). The epidemic form of histoplasmosis commonly implies an occupational risk, as reported in some Latin American countries, but currently, it represents a human immunodeficiency virus (HIV)-defining condition (Centers for Disease Control and Prevention, 2008). Thus, *H. capsulatum* could be considered a primary fungal pathogen because it infects healthy people, although it also shows an opportunistic behavior since it causes more severe disease in immunocompromised individuals.

H. capsulatum is a dimorphic fungus with two morphotypes, a mycelial infective phase found mainly in bat and bird guano and a yeast virulent phase, which can survive within macrophages and depicts a chronic granulomatous inflammation in host tissues (Köhler et al., 2017; Pomerville, 2018). Infection occurs by inhalation of aerosolized mycelial propagules (mainly microconidia and small hyphal fragments), which become yeasts upon entering the host's respiratory tract.

* Corresponding author.
E-mail addresses: lauraelena_c@yahoo.com (L.E. Carreto-Binaghi), fernando62fmv@gmail.com (F.R. Morales-Villarreal), gsgarciadelatorre@gmail.com (G. García-de la Torre), tania.vite.garin@gmail.com (T. Vite-Garín), anton100559@hotmail.com (J.-A. Ramirez), elmoukhtar.aliouat-3@univ-lille2.fr (E.-M. Aliouat), jarturoinfectologia@iner.gob.mx (J.-A. Martínez-Orozco), emello@unam.mx (M.-L. Taylor).

https://doi.org/10.1016/j.ijid.2019.06.010
1201-9712/© 2019 The Authors. Published by Elsevier Ltd on behalf of International Society for Infectious Diseases. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Pneumocystis jirovecii, which was first described by Frenkel (1976), is currently accepted as the specific fungal agent of human pneumocystosis (Morris and Norris, 2012). During the acquired immunodeficiency syndrome (AIDS) epidemic in the 1980s, this fungal species was associated with the primary cause of mortality in HIV/AIDS patients, despite the use of antiretroviral therapy. Pneumocystosis is distributed worldwide, and it can also be diagnosed in other immunocompromised patients and in chronic obstructive pulmonary disease exacerbations (Morris et al., 2008). In immunocompetent human hosts, *P. jirovecii* has been reported as an asymptomatic infection or colonization, suggesting it can be the source of infection transmission for other individuals (Morris and Norris, 2012). All *Pneumocystis* spp. depict two morphotypes: the trophic and the asci forms. The asci typically contain ascospores, which are rounded or elongated and can be aerosolized, acting as probable transmission propagules from one individual to another (Hauser and Cushion, 2018). Each *Pneumocystis* sp. is host-specific for one mammal species (a characteristic known as stenoxenism) and cannot be grown in laboratory artificial media (Akbar et al., 2012; Hauser and Cushion, 2018).

H. capsulatum and *P. jirovecii* coinfection was previously reported in a few studies (Baughman et al., 1994; Gago et al., 2014; Huber et al., 2008; Le Gal et al., 2013; Velásquez et al., 2010; Wheat et al., 1985). Most of these studies were developed in samples from hospitalized AIDS patients, where *Histoplasma* was diagnosed by culture growth and *Pneumocystis* by Grocott staining of bronchoalveolar lavage (BAL) or pulmonary biopsies. Three studies have described *H. capsulatum* and *Pneumocystis* sp. coinfection using molecular methods: Gago et al. (2014), who used a multiplex PCR assay in human BAL or biopsy samples; González-González et al. (2014), who used specific nested PCRs in lung samples of randomly captured *Tadarida brasiliensis* bats from Argentina, Mexico, and French Guyana; and Almeida-Silva et al. (2016), who reported an HIV patient with several opportunistic fungal infections detected by nested PCR and multiplex qPCR.

H. capsulatum and *Pneumocystis* sp. share different characteristics that support the possibility of coinfection in the same individual. They are ascomycete fungi that infect through the host's respiratory tract. The lung is the main target organ for both fungi, causing the pulmonary disease representative of their most common clinical form. However, some biological, pathogenic and clinical differences are remarkable between both parasites. For *H. capsulatum*, yeasts are preferentially found in the host's intracellular space (primarily within macrophages). For *Pneumocystis* sp., the two morphotypes are particularly found in the host extracellular space, and the trophic forms develop strong interactions with alveolar epithelial cells. *H. capsulatum* is found in natural habitats, whereas *Pneumocystis* spp. have only been described in infected hosts. *H. capsulatum* does not colonize healthy or immunocompetent individuals, and no possible respiratory transmission from one individual to another has been reported, whereas both features are common for *Pneumocystis* sp. (Köhler et al., 2017; Pomerville, 2018; Skalski et al., 2015). Overall, histoplasmosis patients can develop disseminated disease (rarely seen in pneumocystosis).

The aim of this study was to detect the frequency of *H. capsulatum* and *P. jirovecii* coinfection, using specific molecular methods for each fungus, in BAL samples of patients from a tertiary care hospital focused on respiratory diseases in Mexico.

Materials and methods

Patients

This study was developed in two periods: from June to October 2014, and from May to September 2016. We considered a total of 289 patients hospitalized for acute pulmonary diseases at the Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" (INER), in Mexico City, CDMX, Mexico, who required a bronchoscopic diagnostic procedure due to hypoxemic pneumonia. We also analyzed eight samples collected in 2011 from healthy volunteers, which were kindly donated by the team of Dr. Eduardo Sada Díaz, from the "Departamento de Investigación en Microbiología" at INER, as noninfected controls for molecular diagnosis of *H. capsulatum* and *P. jirovecii*. No samples were obtained ex professo for this study.

Clinical data processed from the medical record of each patient were sex, age, place of birth, lactate dehydrogenase (LDH) levels, smoking status, guano exposure, travel history, and main outcome at hospital discharge. Laboratory data evaluated included complete blood count, HIV status (including viral load and $CD4^+$ cell count), and BAL sample laboratory procedures, such as bacterial and fungal cultures (for *H. capsulatum*), Grocott staining (for *P. jirovecii*), as well as multiplex viral PCR and other specific tests for the diagnosis of infectious diseases, which were performed according to physician requests.

BAL samples

From all patients considered for this study, we analyzed one BAL sample. After BAL collection, samples were centrifuged at 2850 × g for 20 min, the supernatant was aliquoted in 600-μl microtubes (Eppendorf, Inc., Enfield, CT, USA) and frozen at −80 °C in less than one hour for other studies, and the pellet was processed for DNA extraction to search for *H. capsulatum* and *P. jirovecii*.

DNA extraction

The pellet of each BAL sample was processed for DNA extraction using a commercial kit (Molecular Biology Kit, Bio Basic Inc., Toronto, ON, CA), according to manufacturer's instructions. DNA samples were quantified in an Epoch microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at 260–280 nm and then stored at −80 °C until required. To avoid contamination during molecular screening, all DNA samples were processed in specialized cabinets for each step of the molecular assays performed.

Molecular screening for H. capsulatum *in DNA samples*

Fungal presence was investigated in each extracted DNA sample using a nested PCR for a fragment of the *Hcp100* gene encoding a 100-kDa protein, a molecular marker highly specific for this pathogen (Bialek et al., 2002; Taylor et al., 2005). Two sets of primers were used; the outer primer set included HcI (5′-GCG-TTC-CGA-GCC-TTC-CAC-CTC-AAC-3′) and HcII (5′-ATG-TCC-CAT-CGG-GCG-CCG-TGT-AGT-3′); the inner primers were HcIII (5′-GAG-ATC-TAG-TCGCGG-CCA-GGT-TCA-3′) and HcIV (5′-AGG-AGA-GAA-CTG-TAT-CGG-TGG-CTT-G-3′), delimiting a 210-bp fragment unique to *H. capsulatum*. Details of the amplification are provided in González-González et al. (2012). DNA from the EH-53 *H. capsulatum* strain from a Mexican clinical case was used as a positive control, and milli-Q water (Milli-Q water purifier, Merck KGaA, Darmstadt, DE) was always processed as a negative control.

Molecular screening for P. jirovecii *in DNA samples*

We used nested PCR for the amplification of two genes that are reliable markers for *Pneumocystis* detection: the mitochondrial ribosomal large subunit (mtLSUrRNA) and the mitochondrial ribosomal small subunit (mtSSUrRNA) (Wakefield et al., 2003). For the mtLSUrRNA locus, we used the outer primer set pAZ102-H

(5′-GTG-TAC-GTT-GCA-AAG-TAG-TC-3′) and pAZ102-E (5′-GAT-GGC-TGT-TTC-CAA-GCC-CA-3′). The inner primers, pAZ102-X (5′-GTG-AAA-TAC-AAA-TCG-GAC-TAG-G-3′) and pAZ102-Y (5′-TCA-CTT-AAT-ATT-AAT-TGG-GGA-GC-3′), delimit a 267-bp fragment specific for *Pneumocystis* sp. Nested PCR for the mtSSUrRNA locus was performed with the outer primers, pAZ112-10F (5′-GGG-AAT-TCT-AGA-CGG-TCA-CAG-AGA-TCA-G-3′) and pAZ112-10R (5′-GGG-AAT-TCG-AAC-GAT-TAC-TAG-CAA-CCC-3′). The inner primers, pAZ112-13RI (5′-GGG-AAT-TCG-AAG-CAT-GTT-GTT-TAA-TTC-G-3′) and pAZ112-14RI (5′-GGG-AAT-TCT-TCA-AAG-AAT-CGA-GTT-TCA-G-3′), delimit a 300-bp fragment specific for *Pneumocystis* sp. (González-González et al., 2014). A DNA sample obtained from a pulmonary biopsy from a patient diagnosed by Grocott staining with pneumocystosis was used as a positive control. Milli-Q water (Merck) was always used as a negative control for both *Pneumocystis* molecular markers.

Amplified products (amplicons)

Amplicons from each nested PCR were electrophoresed on a 1.8% agarose gel in 0.5X Tris-borate-EDTA buffer. Electrophoresis was conducted at 120 V for 50 min using a 100-bp DNA ladder (Gibco Laboratories, Grand Island, NY, USA) as a molecular size marker. The amplicons were visualized using a UV transilluminator after GelRed Nucleic Acid Gel Stain (Thermo Fisher Scientific Inc., Waltham, MA, USA) staining (0.5 μg/100 ml). The amplicons were purified using the Nucleotrap PCR Purification Kit (BD Biosciences, Palo Alto, CA, USA) and sent to the High-Throughput Genomics Center (University of Washington, Seattle, WA, USA) for sequencing of the sense and antisense DNA strands; a consensus sequence for each amplified sample was generated.

Sequence analyses

The generated sequences were aligned and manually edited using MEGA software, version 7, http://www.megasoftware.net (Kumar et al., 2016), and their alignments are provided in the electronic supplementary material (Supplementary files 1, 2 and 3) for the readers' reference.

Sequences were analyzed to confirm their high homology for each marker, which was the main criterion for the molecular identification of each fungus in BAL samples, considering for *P. jirovecii* at least one of the two markers used. First, the sequences were analyzed using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search in the GenBank database for all homologous sequences corresponding to the nested PCR products of *H. capsulatum* and *P. jirovecii* gene fragments. Afterward, sequence analysis with the neighbor-joining (NJ) method was used to construct the respective genetic relationship trees for each marker using MEGA7 (Kumar et al., 2016). To infer the NJ trees, genetic distances were conducted using the Kimura two-parameter model, considering gaps/missing data and mutation rates between the analyzed nucleotide sites. Bootstrap values (bt) for NJ analyses were based on 1000 replicates (Kumar et al., 2016). To construct the NJ trees, Hcp100 sequences were aligned with the sequence of the G-217B reference strain from Louisiana, USA (GenBank accession no. AJ005963); mtLSUrRNA sequences were aligned with the reference sequence of *P. jirovecii* EH1-PAZ102E (GenBank accession no. JF733748); and mtSSUrRNA sequences were aligned with the reference sequence of *P. jirovecii* (GenBank accession no. HQ228547).

Statistical analyses

The total infection rate was estimated considering all patients. The Chi-Square (χ^2) test was used to detect significant differences between the groups of patients infected with *H. capsulatum*, *P. jirovecii* or both fungi. The prevalence odds ratio test and its corresponding 95% confidence interval (95% CI) were calculated to evaluate the possible strength of the association between each different analyzed variable and the occurrence of histoplasmosis, pneumocystosis or their coinfection. Both statistical analyses were performed using SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered significant.

Results

We analyzed BAL samples from 289 patients: 84 (29.1%) were from HIV patients and 205 (70.9%) were from non-HIV patients. Seventy-one HIV patients were male and 13 were female with a median age of 34 years, showing a median HIV viral load of 246 852 copies/ml and a median $CD4^+$ count of 23 cells/ml of peripheral blood. One hundred and ten non-HIV patients were male and 95 were female with a median age of 54 years. Most individuals (66.4%) lived in Mexico City and the surrounding conurbation, whereas the rest of patients (33.6%) came from rural areas.

Out of 289 patient BAL samples, the amplified Hcp100 marker diagnosed the presence of *H. capsulatum* infection in 60 patients (20.8%), which included four patients with positive cultures for *H. capsulatum*. The amplification of either mtLSUrRNA or mtSSUrRNA locus diagnosed the presence of *P. jirovecii* infection in 45 cases (15.6%) (Table 1). Regarding *P. jirovecii* detection using two independent molecular markers (mtLSUrRNA and mtSSUrRNA) for its identification, it is important to remark that both markers were amplified in 34 samples, whereas the mtLSUrRNA marker generated more sequences (47) than the mtSSUrRNA (33). None of these markers was amplified in any of the samples from noninfected controls (healthy volunteers). Milli-Q water was always negative in each assay performed.

The BLASTn analysis for Hcp100 sequences showed a range from 94 to 100% similarity between them and the GenBank reference sequence from *H. capsulatum*, whereas mtLSUrRNA and mtSSUrRNA sequences showed a range from 98 to 100% similarity between them and the reference sequences from *P. jirovecii*.

All *H. capsulatum* Hcp100 sequences analyzed by NJ are represented in Figure 1, and the cluster of these sequences supports a close genetic relationship among them. Figure 2 shows the sequence trees analyzed by NJ for the *P. jirovecii* mtSSUrRNA (Figure 2a) and mtLSUrRNA (Figure 2b) markers, respectively, and the topologies of these trees sustained a close genetic relationship among the sequences.

The molecular tools used in this study revealed that 12 (4.2%) patients presented both *H. capsulatum* and *P. jirovecii* infections simultaneously. Out of these 12 coinfected patients, nine samples belonged to HIV patients (10.7% of 84 HIV patients) and the other three to non-HIV patients (1.5% of 205 non-HIV patients) (Table 1).

The main data for all studied patients, such as sex, age, HIV status, LDH levels, smoking status, guano exposure, mechanical ventilation, and lethality, are provided in Table 1. The last column shows the results of the χ^2 test for each variable to detect significant differences among the groups of patients infected with *H. capsulatum*, *P. jirovecii* or both fungi, and *P* values are shown in Table 1. Demographic and clinical data such as sex, LDH levels, mechanical ventilation, and lethality did not show significant differences between groups. Two important risk factors were assessed for the fungal infections studied, such as smoking status (for *H. capsulatum* and *P. jirovecii*) or guano exposure (for *H. capsulatum* only). Significant differences were found between smoking and nonsmoking patients ($P = 0.002$), considering the *H. capsulatum*, *P. jirovecii* and coinfected groups of patients. No significant differences ($P = 0.270$) were found among patients exposed to guano in the environment in regard to *H. capsulatum*

Table 1
Main data from histoplasmosis, pneumocystosis and coinfected patients.

Demographic and clinical data	*H. capsulatum* infection only n = 60	*P. jirovecii* infection only n = 45	Coinfection n = 12	*P*
Age[a] (years)	46 [32–66]	37 [30–47]	34 [30–44]	
Sex				0.212
Female	21 (35%)	15 (33.3%)	2 (16.7%)	
Male	39 (65%)	30 (66.7%)	10 (83.3%)	
HIV patients	16 (26.7%)	32 (71.1%)	9 (75%)	<0.0001
Viral load[a] (copies/ml)	293 479 [24 064–848 244]	427 572 [34 951–851 138]	462 080 [228 922–933 417]	
CD4+ count[a] (cells/ml)	42 [10–56]	21 [7–42]	25 [7–42]	
Non-HIV patients	44 (73.3%)	13 (28.9%)	3 (25%)	
LDH[a](UI/l)	186 [140–273]	227 [152–341]	279 [140–396]	0.070
Smoking				0.002
Active	26 (43.3%)	19 (42.2%)	3 (25%)	
Passive	12 (20%)	4 (8.9%)	0 (0%)	
None	16 (26.7%)	16 (35.6%)	8 (66.7%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Guano exposure				0.270
Yes	17 (28.3%)	11 (24.5%)	2 (16.7%)	
No	37 (61.7%)	28 (62.2%)	9 (75%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Mechanical ventilation				0.129
Yes	17 (28.3%)	14 (31.1%)	6 (50%)	
No	37 (61.7%)	25 (55.6%)	5 (41.7%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	
Lethality				0.088
Yes	10 (16.7%)	6 (13.3%)	4 (33.3%)	
No	44 (73.3%)	33 (73.4%)	7 (58.4%)	
No data	6 (10%)	6 (13.3%)	1 (8.3%)	

LDH: Lactate dehydrogenase.
Percentages in parentheses were estimated considering the number of patients in each infected group.
[a] Median values of these clinical data are shown, with the corresponding interquartile range (IQR 25–75) in brackets.

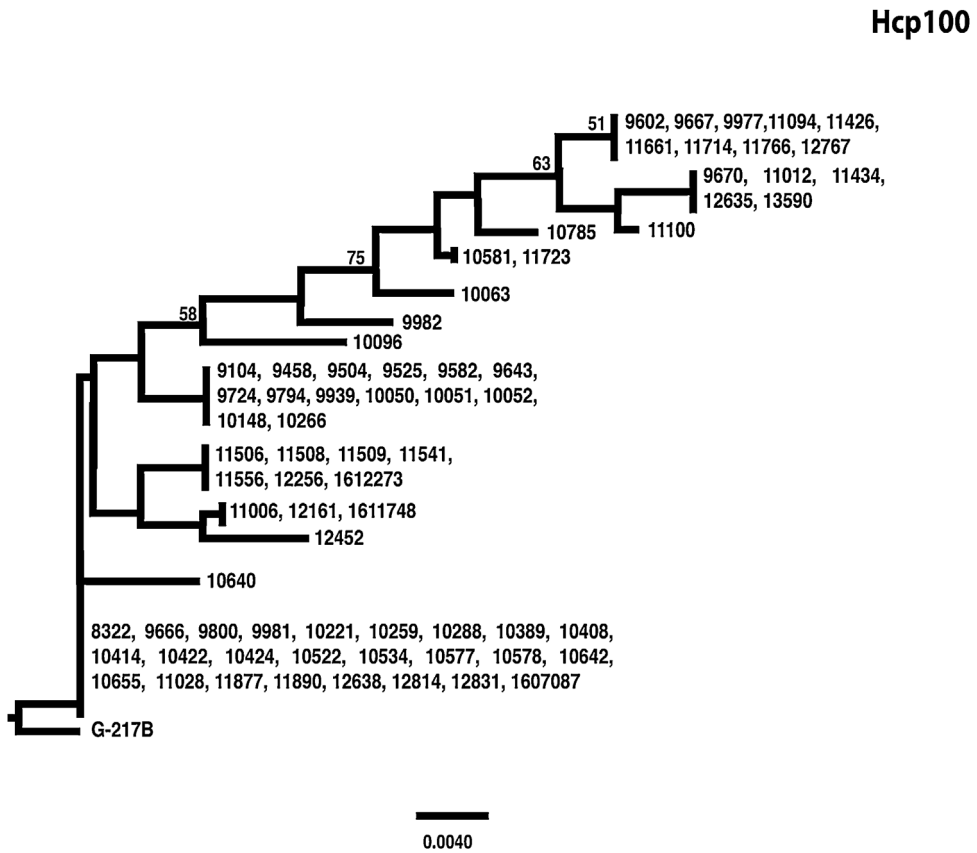

Figure 1. NJ analysis of the *H. capsulatum* sequences. The NJ tree was constructed using a matrix of 142-nt of the Hcp100 gene fragments. The NJ analysis was conducted as described in Materials and methods. Supporting bt values ≥50% are indicated on their corresponding tree branches.

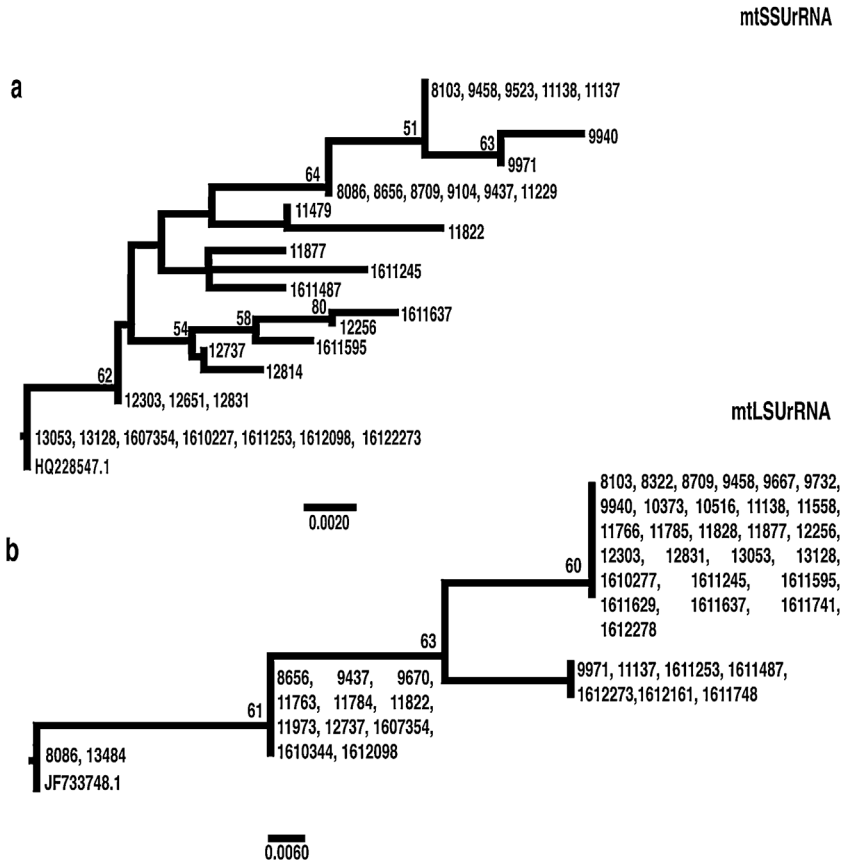

Figure 2. NJ analyses of the *P. jirovecii* sequences. (a) For the mtSSUrRNA sequences, the NJ tree was constructed using a matrix of 274-nt. (b) For the mtLSUrRNA sequences, the NJ tree was constructed using a matrix of 176-nt. The NJ analyses were conducted as described in Materials and methods. Supporting bt values ≥50% are indicated on their corresponding tree branches.

and coinfected groups of patients. Based on additional information from the hospital records, we found that *H. capsulatum* was detected in 25.5% of the patients from urban areas and in 23.7% of the patients from rural areas, whereas *P. jirovecii* was detected in 18.2% of the patients from urban areas and in 22.6% of the patients from rural areas, and no significant differences were detected. Other laboratory data (hemoglobin, hematocrit, total leukocyte count, and platelet count) for each group of patients studied revealed no significant differences among patients with histoplasmosis, pneumocystosis and coinfection (data not shown).

According to laboratory reports, *H. capsulatum* cultures were positive only in four BAL samples of HIV/AIDS patients, corresponding to 5.6% of all the *H. capsulatum*-positive samples detected by PCR, whereas *P. jirovecii* was observed by Grocott staining only in 14 BAL samples, representing 24.6% of all the *P. jirovecii*-positive samples detected by PCR.

Microorganisms distinct from the studied fungi were detected only in 44 out of 289 patients, by culture (for bacteria and other fungi) and by multiplex PCR (for viruses), as reported in the clinical records. Eight patients were infected with *M. tuberculosis*, 14 with Gram-negative bacteria and four with Gram-positive bacteria. Mixed infections with different microorganisms (virus-bacteria, bacteria-bacteria, bacteria-fungi or virus-fungi) were found in 19 patients; 11 patients presented more than two types of microorganisms. Interestingly, out of the 12 patients coinfected with *H. capsulatum* and *P. jirovecii*, three were infected with other microorganisms that could be part of the normal microbiota of the upper respiratory tract: one patient had *Pseudomonas aeruginosa*, the second had *P. aeruginosa* and *Klebsiella pneumoniae*, and the third had *P. aeruginosa*, parainfluenza 2 virus and rhinovirus. Details about the different microorganisms found in the studied groups are available in the Supplementary Table.

Figure 3 shows a detailed description of the statistical analyses for histoplasmosis, pneumocystosis and coinfection in HIV vs. non-HIV patients.

The prevalence of histoplasmosis infection in the whole studied population was 20.8% (60 of 289 patients), whereas the prevalence in HIV patients was slightly lower (19%) than in non-HIV patients (21.5%); however, this difference was not significant ($P = 0.64$) (Figure 3a).

The prevalence of pneumocystosis infection in the whole studied population was 15.6% (45 of 289 patients), and it was significantly higher in HIV patients with $P < 0.0001$ (38.1%) when compared to non-HIV patients (6.3%). Moreover, the estimated prevalence odds ratio for pneumocystosis was 9.08 (95% CI, 4.45–18.56), which suggested that infection with *P. jirovecii* was 9.08-fold more possible in HIV patients (Figure 3b).

The prevalence of coinfection with both fungi was 4.2% (12 of 289 patients), and the frequency of coinfection in HIV patients (10.7%) was significantly higher than in non-HIV patients (1.5%) with $P = 0.001$. Therefore, the prevalence odds ratio estimated for coinfection was 8.08 (95% CI, 2.13–30.6), revealing that HIV patients had an 8.08-fold greater probability of becoming infected with both fungi (Figure 3c).

In particular, it is remarkable that the lethality rate for patients in the histoplasmosis-pneumocystosis coinfection group (33.3%, 4 of 12 patients) was higher than the lethality rate for patients with only histoplasmosis infection (16.6%, 10 of 60 patients) or only pneumocystosis infection (13.3%, 6 of 45 patients), even though these results were not statistically significant (see Table 1).

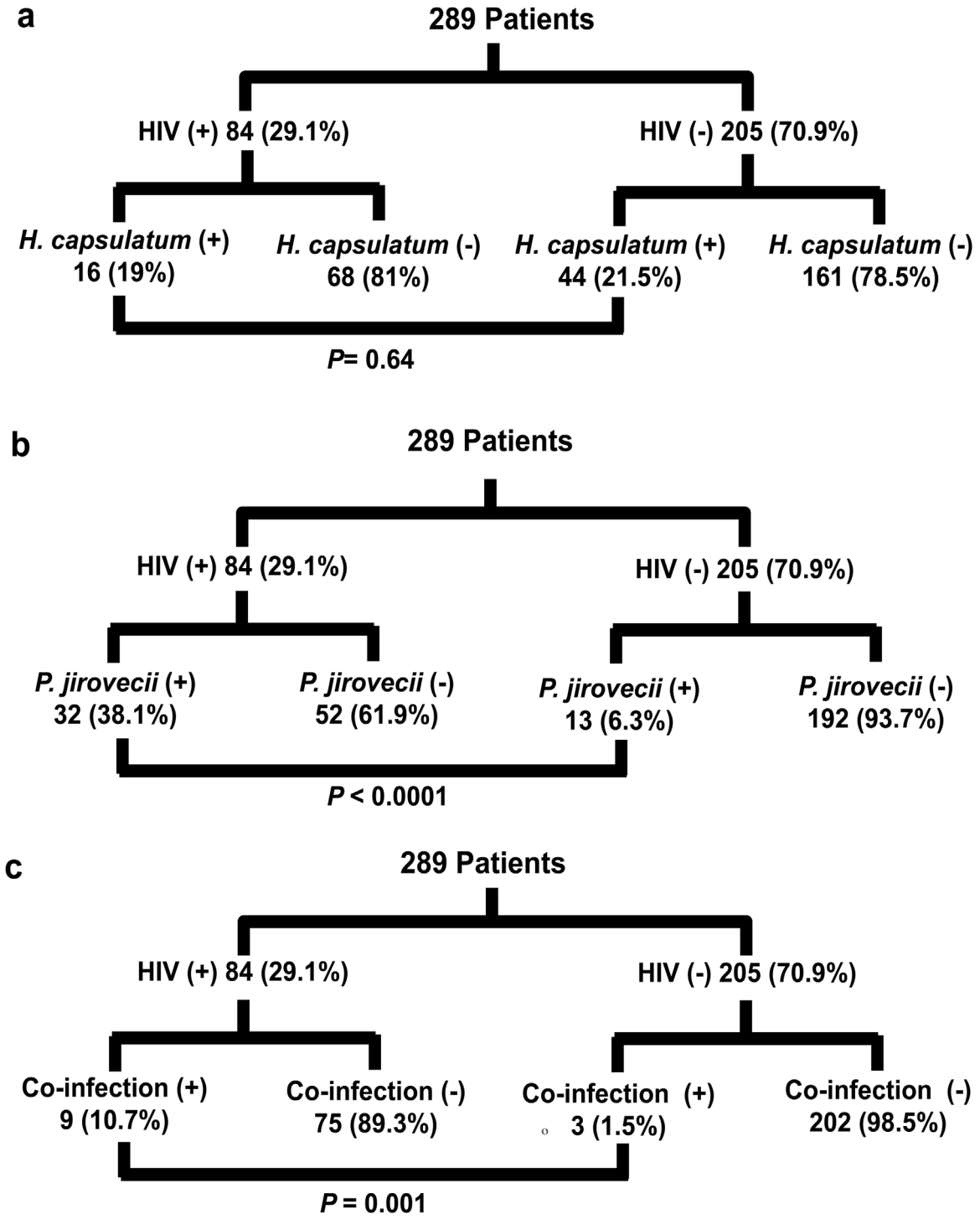

Figure 3. Statistical analyses. Within the studied population, χ^2 and prevalence odds ratio tests were calculated to compare the prevalence of (a) histoplasmosis, (b) pneumocystosis, and (c) coinfection of *H. capsulatum* and *P. jirovecii*, in HIV versus non-HIV patients.

Discussion

The present manuscript highlights the importance of coinfection with two respiratory fungi (*H. capsulatum* and *P. jirovecii*) in a tertiary care hospital, where most individuals were HIV patients, although coinfection was also found in three non-HIV patients without an immunocompromised condition or any other remarkable data in their clinical records. The diagnosis of this coinfection is critical for the establishment of adequate therapeutic strategies to eliminate both fungi.

In previous years, the diagnosis of coinfection with *H. capsulatum* and *P. jirovecii* has been unintentionally neglected because it has not specifically been evaluated previously. The real frequency of this coinfection is unknown, but in a few studies, coinfection has been serendipitously reported, mostly while describing opportunistic infections in HIV patients. When the HIV epidemic started, Wheat et al. (1985) described the first 15 cases of AIDS in Indianapolis, where seven patients developed disseminated histoplasmosis, and two of them were also diagnosed with pneumocystosis; based on these authors' data,

we estimated a coinfection rate of 13.3%. Later, Baughman et al. (1994) screened opportunistic infections in 894 BAL samples from AIDS patients; they found 420 (46.97%) patients with *Pneumocystis,* five of whom were coinfected with *H. capsulatum* (0.55% according to our estimation). In French Guiana, Huber et al. (2008) described 200 cases of AIDS-associated histoplasmosis over 25 years; seven were coinfected with *Pneumocystis* (3.5% according to our estimation). Velásquez et al. (2010) studied 44 HIV/AIDS patients with histoplasmosis and found concomitant pneumocystosis in 11.4% of the cases. Caceres et al. (2018) reported three cases of pneumocystosis in 45 HIV patients with histoplasmosis (6.7% with coinfection, according to our estimation). Interestingly, Le Gal et al. (2013) described the intracellular coexistence of *H. capsulatum* and *P. jirovecii* in an alveolar macrophage from a BAL sample of one AIDS patient.

The abovementioned reports were entirely focused on HIV patients, and most of the frequencies of coinfection that we estimated from their results were similar to our reported rate of 10.7% (9 of 84 HIV patients). Coinfection could be explained by the virus-related immunocompromised condition, which makes the patients more susceptible to concomitant infections.

In regard to the LDH levels, which is a marker associated with the prognosis of patients with disseminated histoplasmosis (Butt et al., 2002; Ramos et al., 2018) and more frequently used to distinguish bacterial from *Pneumocystis* pneumonia (Sun et al., 2016), our results showed that the serum LDH levels were not significant to support the diagnosis of either histoplasmosis or pneumocystosis.

Other risk factors assessed in this study were smoking and guano exposure. Smoking has been previously reported as a risk factor for complications in the ocular histoplasmosis syndrome (Chheda et al., 2012; Ganley, 1973). Additionally, smoking is a presumed risk factor for presenting *P. jirovecii* pneumonia in HIV patients (Blount et al., 2013; Miguez-Burbano et al., 2005) and in non-HIV patients (Santos et al., 2017). Regarding the lack of significance in the analysis concerning guano exposure related to *H. capsulatum* infection, it is important to emphasize that histoplasmosis outbreaks might occur both in rural and urban areas because the fungus has been found in several niches where people could be inadvertently exposed. (Corcho-Berdugo et al., 2011; Muñoz et al., 2010; Taylor et al., 2005). However, other risk factors associated with histoplasmosis or pneumocystosis, such as previous treatment with immunosuppressive drugs, were scarcely referred for the studied patients.

It would be fascinating to identify the influence of one pathogen in regard to the other on the coinfection outcome; however, it was not possible to detect this association because some patients' medical records were incomplete. It is crucial to underline the higher lethality rate associated with coinfection, suggesting a contribution of both pathogens in the fatal clinical course of the patients (Table 1). In general, most of the causes of death referred for histoplasmosis, pneumocystosis and coinfected patients were pneumonia with sepsis and/or septic shock.

An important feature to consider from most studies is the diagnostic procedure used for histoplasmosis (culture) and pneumocystosis (microscopic observation), which require specialized supplies and technical experience for successful diagnosis. Therefore, new methods should be implemented to detect coinfection with these fungi. To date, only a few studies have used molecular methods. Gago et al. (2014) reported one of 14 HIV patients with *H. capsulatum* and *P. jirovecii* coinfection (7.14% according to our estimation) while developing a multiplex PCR assay for fungi. González-González et al. (2014) described coinfection with *H. capsulatum* and *Pneumocystis* spp. in 122 randomly captured bats (35.2% of cases), supporting a high frequency of both pathogens in this particular host (González-González et al., 2014). Thus, we chose molecular methods for histoplasmosis and pneumocystosis diagnosis due to their rapid, specific and accurate detection, emphasizing that these methods were very useful to reveal *H. capsulatum* and *P. jirovecii* in BAL samples. We would like to emphasize that the four HIV/AIDS patients with positive *H. capsulatum* cultures also amplified the specific marker Hcp100, which highlights the efficiency of the molecular method used. Fungal sequences generated by PCR, using DNA extracted from BAL samples, reflected active mycotic disease, and the specificity of the molecular markers used in the present study makes misdiagnosis very improbable. In contrast, immunological diagnostic procedures sometimes are unable to differentiate past from present infection. For both fungi, we selected nested PCR protocols, which have been demonstrated to be very sensitive, reproducible for the identification of pathogens in different clinical samples, and able of avoiding molecular contamination under well-controlled laboratory conditions. Moreover, nested PCR is a useful tool for epidemiological studies, where this method serves as a great screening technique when compared to real-time PCR (Seo et al., 2014; Sharifdini et al., 2015).

We considered the generated sequence of each marker as a unique criterion for diagnosis because the single visualization of the amplified PCR products could lead to misinterpretation or overdiagnosis of the results and because non-specific products could sometimes be produced. Although our criterion is very strict, it is undoubtedly precise and reliable, as demonstrated by BLASTn and NJ genetic analyses of the generated sequences. Thus, the close genetic relationship among the sequences of Hcp100 for *H. capsulatum* and among the sequences of mtLSUrRNA and mtSSUrRNA for *P. jirovecii* confirmed their respective fungal molecular identification. The scientific literature has reported mtLSUrRNA marker as a better diagnostic tool for *P. jirovecii*; however, based on our previous experience (González-González et al., 2014), we also selected the mtSSUrRNA marker because the fungus was identified in a few samples in which only this marker was amplified.

In conclusion, statistical analyses support the highest prevalence of pneumocystosis and coinfection in HIV patients, and based on our findings, *H. capsulatum* and *P. jirovecii* coinfection is a more common medical problem than expected, not only in immunocompromised patients. Searching for both pathogens at the initial stages of disease should be routinely performed to establish adequate treatments for both fungi to improve patient outcomes and diminish the risk for complications. Basic research studies on this coinfection are still needed to explain the interaction of the two pathogens in vivo.

Funding

This paper was only supported by the internal funding of the Laboratorio de Inmunología de Hongos, Facultad de Medicina, UNAM.

Ethical approval

Patients' and volunteers' BAL samples were obtained in accordance with the ethical standards of the Helsinki Declaration (1964, amended in 2008). Written consent was obtained and kept in the hospital's individual medical records. This work was approved by the School of Medicine Research and Ethics Committee (UNAM, report 132/2015) and by the INER Ethics Research Committee (protocol B13-14).

Conflict of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

LECB and MLT conceived the study, participated in its design and coordination and helped to draft the manuscript. LECB and FRMV collected the samples. LECB and JAR performed the experimental procedures. MLT, LECB, GGT, TVG, and JAR analyzed the data. EMA and JAMO drafted the manuscript and revised it critically for important intellectual content. JAMO and MLT share the academic responsibility for this manuscript. All authors read and approved the final manuscript.

Acknowledgments

LECB thanks the "Programa de Doctorado en Ciencias Biomédicas", Universidad Nacional Autónoma de México (UNAM) and fellowship No. 329884 from "Consejo Nacional de Ciencia y Tecnología Mexico (CONACyT-Mexico)". The authors thank I. Mascher for editorial assistance and Dr. Olivia Sánchez Cabral, Dr. Berenice López González, Dr. Iván Rodolfo García Izquierdo, Dr. Miguelina Altagracia Jaquez Carrasco, and the bronchoscopy team from INER for their valuable help during the BAL collection.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.010.

References

Akbar H, Pinçon C, Aliouat-Denis CM, Derouiche S, Taylor ML, Pottier M, et al. Characterizing *Pneumocystis* in the lungs of bats: understanding *Pneumocystis* evolution and the spread of *Pneumocystis* organisms in mammal populations. Appl Environ Microbiol 2012;78(22):8122–36.

Almeida-Silva F, Damasceno LS, Serna MJ, Valero C, Quintella LP, Almeida-Paes R, et al. Multiple opportunistic fungal infections in an individual with severe HIV disease: a case report. Rev Iberoam Micol 2016;33(2):118–21.

Anderson H, Honish L, Taylor G, Johnson M, Tovstiuk C, Fanning A, et al. Histoplasmosis cluster, golf course, Canada. Emerg Infect Dis 2006;12(1):163–5.

Baughman RP, Dohn MN, Frame PT. The continuing utility of bronchoalveolar lavage to diagnose opportunistic infection in AIDS patients. Am J Med 1994;97(6):515–22.

Bialek R, Feucht A, Aepinus C, Just-Nübling G, Robertson VJ, Knobloch J, et al. Evaluation of two nested PCR assays for detection of *Histoplasma capsulatum* DNA in human tissue. J Clin Microbiol 2002;40:1644–7.

Blount RJ, Djawe K, Daly KR, Jarlsberg LG, Fong S, Balmes J, et al. Ambient air pollution associated with suppressed serologic responses to *Pneumocystis jirovecii* in a prospective cohort of HIV-infected patients with *Pneumocystis* pneumonia. PLoS One 2013;8(11)e80795.

Butt AA, Michaels S, Greer D, Clark R, Kissinger P, Martin DH. Serum LDH level as a clue to the diagnosis of histoplasmosis. AIDS Read 2002;12(7):317–21.

Caceres DH, Tobón AM, Restrepo A, Chiller T, Gómez BL. The important role of co-infections in patients with AIDS and progressive disseminated histoplasmosis (PDH): a cohort from Colombia. Med Mycol Case Rep 2018;19:41–4.

Calanni LM, Pérez RA, Brasili S, Schmidt NG, Iovannitti CA, Zuiani MF, et al. Outbreak of histoplasmosis in province of Neuquén, Patagonia Argentina. Rev Iberoam Micol 2013;30(3)193–9 [in Spanish].

Centers for Disease Control and Prevention. AIDS-defining conditions. 2008. . . [Accessed 15 November 2018] https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5710a2.htm.

Chheda LV, Ferketich AK, Carroll CP, Moyer PD, Kurz DE, Kurz PA. Smoking as a risk factor for choroidal neovascularization secondary to presumed ocular histoplasmosis syndrome. Ophthalmology 2012;119(2):333–8.

Corcho-Berdugo A, Muñoz-Hernández B, Palma-Cortés G, Ramírez-Hernández A, Martínez-Rivera M, Frías-de León M, et al. [An unusual outbreak of histoplasmosis in residents of the state of Mexico]. Gac Med Mex 2011;147(5)377–84 [in Spanish].

Frenkel JK. *Pneumocystis jirovecii* n. sp. from man: morphology, physiology, and immunology in relation to pathology. Natl Cancer Inst Monogr 1976;43:13–30.

Gago S, Esteban C, Valero C, Zaragoza O, Puig de la Bellacasa J, Buitrago MJ. A multiplex real-time PCR assay for identification of *Pneumocystis jirovecii*, *Histoplasma capsulatum*, and *Cryptococcus neoformans*/*Cryptococcus gattii* in samples from AIDS patients with opportunistic pneumonia. J Clin Microbiol 2014;52(4):1168–76.

Ganley JP. Epidemiologic characteristics of presumed ocular histoplasmosis. Acta Ophthalmol Suppl 1973;119:1–63.

González-González AE, Aliouat-Denis CM, Carreto-Binaghi LE, Ramírez JA, Rodríguez-Arellanes G, Demanche C, et al. An Hcp100 gene fragment reveals *Histoplasma capsulatum* presence in lungs of *Tadarida brasiliensis* migratory bats. Epidemiol Infect 2012;140(11):1955–63.

González-González AE, Aliouat-Denis CM, Ramírez-Bárcenas JA, Demanche C, Pottier M, Carreto-Binaghi LE, et al. *Histoplasma capsulatum* and *Pneumocystis* spp. co-infection in wild bats from Argentina, French Guyana, and Mexico. BMC Microbiol 2014;14:23, doi:http://dx.doi.org/10.1186/1471-2180-14-23.

Hauser PM, Cushion MT. Is sex necessary for the proliferation and transmission of Pneumocystis?. PLoS Pathog 2018;14(12)e1007409.

Huber F, Nacher M, Aznar C, Pierre-Demar M, El Guedj M, Vaz T, et al. AIDS-related *Histoplasma capsulatum*var. *capsulatum* infection: 25 years experience of French Guiana. AIDS 2008;22(9):1047–53.

Köhler JR, Hube B, Puccia R, Casadevall A, Perfect JR. Fungi that infect humans. In: Heitman J, Howlett B, Crous P, Stukenbrock E, James T, Gow N, editors. The fungal kingdom. Washington: ASM Press; 2017. p. 813–43.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol 2016;33(7):1870–4.

Le Gal S, Damiani C, Virmaux M, Schmit JL, Totet A, Nevez G. Photo quiz: a 39-year-old man with human immunodeficiency virus infection presenting with an alveolo-interstitial pulmonary syndrome. J Clin Microbiol 2013;51(9)3165 2809.

Miguez-Burbano MJ, Ashkin D, Rodriguez A, Duncan R, Pitchenik A, Quintero N, et al. Increased risk of *Pneumocystis carinii* and community-acquired pneumonia with tobacco use in HIV disease. Int J Infect Dis 2005;9(4):208–17.

Morris A, Norris KA. Colonization by *Pneumocystis jirovecii* and its role in disease. Clin Microbiol Rev 2012;25(2):297–317.

Morris A, Sciurba FC, Norris KA. *Pneumocystis*: a novel pathogen in chronic obstructive pulmonary disease?. COPD 2008;5(1):43–51.

Muñoz B, Martínez MA, Palma G, Ramírez A, Frías MG, Reyes MR, et al. Molecular characterization of *Histoplasma capsulatum* isolated from an outbreak in treasure hunters. BMC Infect Dis 2010;10:264.

Pomerville JC. Eukaryotic microorganisms: the fungi. Fundamentals of microbiology. 11th ed. Burlington: Jones & Bartlett Learning; 2018. p. 621–2.

Ramos IC, Soares YC, Damasceno LS, Libório MP, Farias LABG, Heukelbach J, et al. Predictive factors for disseminated histoplasmosis in AIDS patients with fever admitted to a reference hospital in Brazil. Rev Soc Bras Med Trop 2018;51(4):479–84.

Santos CR, de Assis ÂM, Luz EA, Lyra L, Toro IF, Seabra JCC, et al. Detection of *Pneumocystis jirovecii* by nested PCR in HIV-negative patients with pulmonary disease. Rev Iberoam Micol 2017;34(2):83–8.

Seo AN, Park HJ, Lee HS, Park JO, Chang HE, Nam KH, et al. Performance characteristics of nested polymerase chain reaction vs real-time polymerase chain reaction methods for detecting *Mycobacterium tuberculosis* complex in paraffin-embedded human tissues. Am J Clin Pathol 2014;142(3):384–90.

Sharifdini M, Mirhendi H, Ashrafi K, Hosseini M, Mohebali M, Khodadadi H, et al. Comparison of nested polymerase chain reaction and real-time polymerase chain reaction with parasitological methods for detection of *Strongyloides stercoralis* in human fecal samples. Am J Trop Med Hyg 2015;93(6):1285–91.

Skalski JH, Kottom TJ, Limper AH. Pathobiology of *Pneumocystis* pneumonia: life cycle, cell wall and cell signal transduction. FEMS Yeast Res 2015;15(6) pii: fov046.

Sun J, Su J, Xie Y, Yin MT, Huang Y, Xu L, et al. Plasma IL-6/IL-10 ratio and IL-8, LDH, and HBDH level predict the severity and the risk of death in AIDS patients with *Pneumocystis* pneumonia. J Immunol Res 2016;2016:1583951.

Taylor ML, Ruíz-Palacios GM, Reyes-Montes MR, Rodríguez-Arellanes G, Carreto-Binaghi LE, Duarte-Escalante E, et al. Identification of the infectious source of an unusual outbreak of histoplasmosis, in a hotel in Acapulco, state of Guerrero, Mexico. FEMS Immunol Med Microbiol 2005;45:435–41.

Velásquez G, Rueda ZV, Vélez LA, Aguirre DA, Gómez-Arias RD. Histoplasmosis in AIDS patients. A cohort study in Medellín, Colombia. Infectio 2010;14:S99–S106.

Wakefield AE, Lindley AR, Ambrose HE, Denis CM, Miller RF. Limited asymptomatic carriage of *Pneumocystis jirovecii* in human immunodeficiency virus-infected patients. J Infect Dis 2003;187:901–8.

Wheat LJ, Slama TG, Zeckel ML. Histoplasmosis in the acquired immune deficiency syndrome. Am J Med 1985;78(2):203–10.