Folia Microbiol (2017) 62:111–116
DOI 10.1007/s12223-016-0477-4

Usefulness of molecular markers in the diagnosis of occupational and recreational histoplasmosis outbreaks

María Guadalupe Frías-De-León[1] · José Antonio Ramírez-Bárcenas[2] · Gabriela Rodríguez-Arellanes[2] · Oscar Velasco-Castrejón[3] · Maria Lucia Taylor[2] · María del Rocío Reyes-Montes[2]

Received: 22 February 2016 / Accepted: 29 September 2016 / Published online: 10 October 2016

Abstract Histoplasmosis is considered the most important systemic mycosis in Mexico, and its diagnosis requires fast and reliable methodologies. The present study evaluated the usefulness of PCR using *Hcp100* and 1281–1283$_{(220)}$ molecular markers in detecting *Histoplasma capsulatum* in occupational and recreational outbreaks. Seven clinical serum samples of infected individuals from three different histoplasmosis outbreaks were processed by enzyme-linked immunosorbent assay (ELISA) to titre anti-*H. capsulatum* antibodies and to extract DNA. Fourteen environmental samples were also processed for *H. capsulatum* isolation and DNA extraction. Both clinical and environmental DNA samples were analysed by PCR with *Hcp100* and 1281–1283$_{(220)}$ markers. Antibodies to *H. capsulatum* were detected by ELISA in all serum samples using specific antigens, and in six of these samples, the PCR products of both molecular markers were amplified. Four environmental samples amplified one of the two markers, but only one sample amplified both markers and an isolate of *H. capsulatum* was cultured from this sample. All PCR products were sequenced, and the sequences for each marker were analysed using the Basic Local Alignment Search Tool (BLASTn), which revealed 95–98 and 98–100 % similarities with the reference sequences deposited in the GenBank for *Hcp100* and 1281–1283$_{(220)}$, respectively. Both molecular markers proved to be useful in studying histoplasmosis outbreaks because they are matched for pathogen detection in either clinical or environmental samples.

Introduction

Histoplasmosis is an endemic mycosis in the Americas, particularly in the USA. However, it frequently occurs as the epidemic form of the disease, such as in local outbreaks in North, Central and South America, where histoplasmosis has been reported between parallels 54° North and 38° South (Anderson et al. 2006; Calanni et al. 2013). In Mexico, the disease has been reported across the entire republic, with predominance in the central states, followed by the South Pacific and Gulf of Mexico (Contreras et al. 1998; Vaca-Marín et al. 1998; Velasco-Castrejón 1998; Taylor et al. 2005a).

This disease is caused by the inhalation of mycelial propagules (mainly microconidia and hyphae fragments) of the dimorphic fungus *Histoplasma capsulatum*, which is found in moist soils, mainly in those containing bird and/or bat guano (Taylor et al. 1999).

Histoplasmosis can remain asymptomatic or present different clinical forms, from mild or moderate primary lung infection to widespread severe infection in immunocompromised or in immunocompetent patients who inhale a massive dose of the infectious propagules. The most common clinical form in Mexico is the primary pulmonary histoplasmosis (PPH), which is commonly observed in epidemic outbreaks (Vaca-Marín et al. 1998; Velasco-Castrejón 1998; Muñoz et al.

Maria Lucia Taylor and María del Rocío Reyes-Montes were equally involved in the design and coordination of this study, as well as in the writing of this manuscript.

✉ María del Rocío Reyes-Montes
remoa@unam.mx

[1] División de Investigación, Hospital Juárez de México, Av. Instituto Politécnico Nacional 5160, Col. Magdalena de las Salinas, 07760 Ciudad de Mexico, Cd.Mx., Mexico

[2] Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria No. 3000, Ciudad de México, Cd.Mx. 04510, Mexico

[3] Hospital General de México, Dr. Balmis No. 148, Col. Doctores, 06726 Ciudad de Mexico Cd.Mx., Mexico

2010b). The severe form of PPH is associated with the inhalation of high level of fungal infective propagules due to constant and high-effort activities that enhance the risk of infection. In most circumstances, severe cases are linked to occupational factors associated with studies and work-related activities in closed spaces, such as those related to biology, geology, anthropology, archaeology, mining and guano collection, in addition to speleologist and ecotourism activities in caves (Taylor et al. 1996). In Mexico, histoplasmosis is considered an occupational risk disease and is registered as such in the Federal Work Law (2012). In recent years, outbreaks have been reported in non-endemic areas or in areas considered to be at a low risk of exposure to the pathogen (Taylor et al. 1996, 2005a; Corcho-Berdugo et al. 2011); for example, in a hotel in Acapulco, Guerrero, Mexico, several tourists were infected with the fungus, which was present in the compost of ornamental plants (Taylor et al. 2005a). These events demonstrate the importance of epidemiological surveillance to avoid the onset of new outbreaks with clinical dissemination cases, which usually lead to death of the patient if not treated in a timely manner.

The conventional diagnosis of this disease is performed by the culture and microscopic examination of samples from the respiratory tract, biopsy and corporal fluids. However, these techniques yield positive results in only 50 % of cases. In the last two decades, several molecular methods using different molecular markers have been developed based on the PCR technique for the detection of *H. capsulatum* in clinical samples (Haynes et al. 1995; Bialek et al. 2002; Bracca et al. 2003; Guedes et al. 2003; Martagon-Villamil et al. 2003; Ueda et al. 2003; Tang et al. 2006; Frías De León et al. 2012; Dantas et al. 2013; Gago et al. 2014). Due to its high sensitivity and specificity, one of the mostly used markers is a unique fragment of the gene encoding a 100-kDa coactivator protein of *H. capsulatum* (*Hcp100* marker) (Bialek et al. 2002). This marker was standardised in a nested PCR by Bialek et al. (2002) and carefully validated in clinical samples by Muñoz et al. (2010a), who reported a sensitivity of 100 % and a specificity of 95.2 %. These data suggest that nested PCR with *Hcp100* could be useful for the diagnosis of histoplasmosis. Recently, another marker for the detection of *H. capsulatum* was described and amplifies a 220-bp fragment (1281–$1283_{(220)}$ marker), which was designed from the randomly amplified polymorphic DNA (RAPD)-PCR patterns of several fungal isolates from different countries in the Americas. This marker has been tested by simplex PCR in clinical and environmental samples with good specificity (Frías De León et al. 2012). Buitrago et al. (2013) published a multicenter study in which nested and simplex PCR assays were used to validate the *Hcp100* and 1281–$1283_{(220)}$ markers, respectively. Besides, the authors also used real-time quantitative PCR (qPCR) with a marker of the ITS1 region of the rDNA. According to the authors (Buitrago et al. 2013), the marker used in qPCR was the most sensitive in detecting the pathogen, followed by *Hcp100* (nested PCR) and 1281–$1283_{(220)}$ (simplex PCR). However, for intra-hospital laboratories with few resources, where samples from patients with histoplasmosis are concentrated, it is more feasible to perform nested and simplex PCR assays. Therefore, the aim of this study was to test the usefulness of the markers *Hcp100* and 1281–$1283_{(220)}$ for the rapid and timely diagnosis of *H. capsulatum* in clinical and environmental samples collected during histoplasmosis outbreaks.

Methods

In this study, clinical (serum) and environmental samples (moist soil containing organic matter) were analysed. These samples were collected during three histoplasmosis outbreaks between 2012 and 2013 in the Mexican states of Morelos, San Luis Potosí and Chiapas. The probable clinical diagnosis of the seven patients involved in the outbreaks was PPH.

Outbreak 1

This outbreak was reported in the state of Morelos, where a private company was performing maintenance work on particular sewer systems. Two workers (patient 1 and patient 2) showed atypical pneumonia with general malaise and fever.

Outbreak 2

This outbreak was reported in San Luis Potosí during ecotourism activities in caves, where two individuals (patient 3 and patient 4) developed fever, profuse sweating and weakness.

Outbreak 3

This outbreak occurred in Chiapas, in an area with caves often visited by archaeological workers and ecotourists. Three members of a family (patient 5, patient 6 and patient 7) were affected with atypical pneumonia and fever.

Clinical and environmental samples

Treating physicians from the *Hospital General de México*, Mexico City, provided the patients' serum samples. These samples were sent to the Mycology Unit, Department of Microbiology and Parasitology, School of Medicine, Universidad Nacional Autónoma de México (UNAM), to perform the necessary immunological and molecular diagnoses. The samples of all patients were obtained prior to antifungal treatment. Further, serum samples were obtained 1 month after the treatment but only from four patients (patients 1, 2, 3 and 4), because patients 5, 6 and 7 did not return to the physician anymore. Additionally, 14 environmental samples of outbreak

1 were collected from different sites of the sewer system associated with infected workers from a private company.

Immunological diagnosis

To obtain the anti-*H. capsulatum* antibody titre in the serum samples, the indirect enzyme-linked immunosorbent assay (ELISA) was used, as previously described by Voller et al. (1979).

Molecular diagnosis

The DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, NRW, Germany) was used for circulating DNA extraction from the serum samples, and the Fast DNA Spin Kit (Qbiogene, Inc., Irvine, CA, USA) was used for the DNA extraction from the environmental samples. In both cases, the manufacturer's instructions were followed.

The total DNA obtained from the serum and environment samples was amplified using nested PCR with the *Hcp100* marker according to the method reported by Bialek et al. (2002) and modified by Taylor et al. (2005a, b), as well as by simplex PCR for the marker 1281–1283$_{(220)}$ according to the conditions described by Frías De León et al. (2012). Nested PCR was performed with DNA concentrations ranging from 5, 50 and 100 ng/μL from either serum or soil samples. Simplex PCR used different DNA volumes (6 and 12 μL). Each PCR was processed once, but when a negative reaction was obtained, other DNA concentrations were tested. In the PCR reactions, negative (Milli-Q water) and positive (DNA from EH-53 *H. capsulatum* strain) controls were processed.

The amplicons of both markers were run through a 1.5 % agarose gel in electrophoresis at 100 V in 0.5× TBE buffer using the 100-bp DNA Ladder (Invitrogen™, Carlsbad, CA, USA) as a molecular size marker. The bands obtained in the gels were observed in a transilluminator UV-MultiDoc-It™ Digital Imaging System (UVP, Inc., Upland, CA, USA). The bands amplified in each PCR assay corresponded to 210 bp (*Hcp100* marker) and 220 bp (1281–1283$_{(220)}$ marker). Finally, the amplified products were sequenced at the High-Throughput Genomics Center, University of Washington, USA. The sequence of each amplified product was the main criterion used to confirm the presence of *H. capsulatum* in clinical and environmental samples.

The alignment of the sequences was analysed with the Basic Local Alignment Search Tool (BLASTn) algorithm (Altschul et al. 1997) to confirm the similarities with the reference sequences for *Hcp100* (accession no. AJ005963) and for 1281–1283$_{(220)}$ (accession no. JN089378) deposited in GenBank.

Isolation of *H. capsulatum* in the environmental samples

The isolation of *H. capsulatum* from environmental samples was only performed for the samples of outbreak 1, which was amplified by PCR one or both molecular markers used. For the isolation, 1 g of the soil sample was used and 10 mL of 150 mM (pH 7.2) phosphate-buffered saline (PBS) was added and left to settle and, finally, soil samples were supplemented with 50 mg/mL streptomycin and 100 IU/mL of penicillin (Lakeside®, Mexico City Cd.Mx., MX). From this suspension, 0.5 mL was intra-peritoneally inoculated in BALB/c syngeneic male mice (4 weeks old), with three mice per sample. At the same time, three mice were inoculated with PBS as negative controls. Mice were maintained under optimal conditions at the animal station of the Laboratory of Fungal Immunology, Department of Microbiology and Parasitology, School of Medicine, UNAM.

After the onset of the signs of murine histoplasmosis (weight loss, hirsute hair, immobility and spine curvature), the mice were sacrificed. Fragments of the lung and spleen were obtained and immediately plated in mycobiotic agar plates (Bioxon, Becton Dickinson, Mexico City, MX) and incubated for 3–4 weeks at 28 °C. Subsequently, the fungi were identified by routine methods in the laboratory, as previously described by Taylor et al. (1999).

Animal manipulations were in accordance with the suggestions of the Ethics Committee of the School of Medicine, UNAM, and following the recommendations of the Animal Care and Use Committee of the UNAM and the Mexican Official Guide (NOM 062-ZOO-1999).

Results

The immunological diagnosis with the serum samples from the seven individuals studied revealed the detection of anti-*H. capsulatum* antibodies, with titres between 1:320 and 1:2560 (Table 1). Individuals from outbreaks 2 (patients 3 and 4) and 3 (patients 5, 6 and 7) developed the highest antibody titres (Table 1). These results confirm the presumptive clinical diagnosis of histoplasmosis in the seven individuals involved in the outbreaks.

According to the molecular diagnosis, the circulating DNA extracted from the serum samples amplified both markers used in their respective PCR, when serum samples of six out of the seven patients studied were analysed (Table 1). The sample from one patient (patient 3) from outbreak 2 did not amplify the expected PCR products for each marker (Table 1).

The DNA extracted from the sera obtained prior to the medical treatment of the patients from outbreak 1 generated the products expected from PCR for the markers *Hcp100* and 1281–1283$_{(220)}$, while PCR with the DNA of the sera obtained after treatment did not amplify any of the markers (Table 1). Of both patients from outbreak 2, only one sample (patient 4)

Table 1 Detection of *Histoplasma capsulatum* in the serum samples using ELISA and PCR with specific markers

Serum	ELISA	Nested PCR *Hcp100* Before/After Clinical Treatment	Simplex PCR 1281–1283$_{(220)}$ Before/After Clinical Treatment
Patient 1[a]	1:640	(+)/(−)	(+)/(−)
Patient 2[a]	1:320	(+)/(−)	(+)/(−)
Patient 3[a]	1:2560	(−)/(−)	(−)/(−)
Patient 4[a]	1:2560	(+)/(−)	(+)/(−)
Patient 5	1:1280	(+)/ND	(+)/ND
Patient 6	1:2560	(+)/ND	(+)/ND
Patient 7	1:2560	(+)/ND	(+)/ND

(+) = amplified; (−) = not amplified

ND not determined

[a] Patients with clinical treatment follow-up

showed that both markers were amplified before treatment but not after (Table 1). The sera from the three patients infected in outbreak 3 taken before treatment showed the amplification of the expected products in the respective PCR for each marker. However, the subsequent molecular tests (after treatment) were not performed because these patients did not return for their doctor's visit (Table 1).

Of the 14 environmental samples collected in areas close to outbreak 1, the *Hcp100* marker was amplified in samples 2 and 5, while the 1281–1283$_{(220)}$ marker was amplified in samples 2, 3 and 4 (Table 2). The analysis of the sequences of the amplicons obtained revealed 95–98 % similarity for marker *Hcp100* and 98–100 % similarity for marker 1281–1283$_{(220)}$ when compared with the corresponding sequences of each marker deposited in GenBank.

Environmental samples 2, 3, 4 and 5, whose amplified PCR sequences corresponded to *H. capsulatum* (Table 2), were tested for fungal isolation in inoculated mice and did not show any apparent signs of the disease. However, a fungal isolate compatible with the typical macroscopic and microscopic characteristics of *H. capsulatum* was obtained from a mouse inoculated with sample 2.

Discussion

The laboratory tests for the identification of *H. capsulatum* are crucial for the confirmation of sole cases and outbreaks of histoplasmosis as well as for locating the source of infection. To date, the conventional diagnostic tools are still in use despite taking longer. Three weeks are required for fungal growth in order to isolate the pathogen, which delays and complicates for an accurate diagnosis. Furthermore, identification of the microorganism can only be performed in biosafety level 3 laboratories (Frías De León et al. 2012). In addition, other confirmatory tests are needed for microorganisms suspected to be *H. capsulatum*, because certain saprobe microbes mimic the morphological mycelial phase of this fungus. In regard to serological tests used as diagnostic tools,

Table 2 Isolation and detection of *Histoplasma capsulatum* in the environmental samples collected from outbreak 1 in the state of Morelos, Mexico

Samples	Source	PCR *Hcp100*	PCR 1281–1283$_{(220)}$	Fungal isolation
1	Sewer A Zone 1 (dry soil)	(−)	(−)	No
2	Sewer A Zone 2 (moist soil)	(+)	(+)	*H. capsulatum*
3	Sewer A Zone 3 (dry soil)	(−)	(+)	No
4	Sewer A Zone 4 (dry soil)	(−)	(+)	No
5	Sewer A1 Zone 1 (moist soil)	(+)	(−)	No
6	Sewer A2 Zone 1 (moist soil)	(−)	(−)	No
7	Sewer A3 Zone 1 (moist soil)	(−)	(−)	No
8	Sewer A4 (dry soil)	(−)	(−)	No
9	Sewer B (dry soil)	(−)	(−)	No
10	Sewer C (dry soil)	(−)	(−)	No
11	Sewer D (dry soil)	(−)	(−)	No
12	Sewer E (fertilised soil)	(−)	(−)	No
13	Trolley 1 (dry soil)	(−)	(−)	No
14	Trolley 2 (dry soil)	(−)	(−)	No

(+) = amplified; (−) = not amplified

such as ELISA, cross reactions with *Mycobacterium tuberculosis* and some fungi (*Blastomyces dermatitidis*, *Paracoccidioides* spp. and *Coccidioides* spp.) are critical inconveniences. Taking these facts into account, there is a need for alternative tools and the use of molecular tests is an adequate option. Molecular tests, besides being specific to each microorganism, allow detection from small amounts of sample, provide results in a short time period and can be applied to any type of sample. Many of the molecular markers reported for the detection of *H. capsulatum* have not been validated in clinical samples, and even fewer have been employed for environmental samples. However, the markers *Hcp100* and 1281–1283$_{(220)}$ have been validated with clinical samples from some laboratories (Muñoz et al. 2010a; Frías De León et al. 2012; Buitrago et al. 2013). Therefore, they were used to assess the fungal presence in both clinical and environmental samples from epidemic histoplasmosis outbreaks.

The results obtained in this study showed that both the *Hcp100* and 1281–1283$_{(220)}$ markers were equally sensitive in detecting the pathogen in the samples analysed, although *Hcp100* has been reported to be highly sensitive in the detection of as little as 1 fg of DNA in different samples of human tissues (Bialek et al. 2002).

Nested and simplex PCR assays have amplified the same clinical samples (Table 1) and have confirmed the specificity of both markers used. Likewise, the molecular diagnosis with the sequences of the PCR products obtained with *Hcp100* and 1281–1283$_{(220)}$ from DNA extracted from the sera of patients involved in the three histoplasmosis outbreaks coincided with the serological diagnosis established by the high antibody titres detected by ELISA, which suggested the existence of an active infectious process.

In general, PCR confirmed the clinical diagnosis in most patients in a shorter time than did the immunological tests. Circulating DNA in serum samples has been used for fungal molecular diagnosis (Millon et al. 2015). However, even though the serum sample from one patient from outbreak 2 had high antibody titres, its DNA extracted did not amplify the PCR products for the *Hcp100* and 1281–1283$_{(220)}$ markers. This can be explained by the low amount of circulating DNA in the serum sample of the patient, probably associated with the outcome of the disease, as has been reported by Dantas et al. (2013). Thus, this result could not be corroborated with other clinical specimens, because only serum samples were sent to our laboratory.

A difference in the results obtained with both markers was found in the processing of the environmental samples. The 1281–1283$_{(220)}$ marker detected the pathogen in soil samples 3 and 4 as well as in sample 2, while *Hcp100* detected it in samples 2 and 5. To corroborate that this difference was not due to a false-positive result, the sequences obtained with both markers were analysed by BLASTn, revealing high similarity percentages with the sequences corresponding to *H. capsulatum* deposited in GenBank. This finding supported that the 1281–1283$_{(220)}$ marker could be as sensitive and specific as the *Hcp100* marker.

Molecular markers were also useful for locating the source of infection, as the fungus was isolated from sewer A, where *H. capsulatum* was detected by both markers. The etiological agent was clearly identified utilising macromorphology and micromorphology. Maintenance of this fungus in the neighbouring areas of the sewers could probably be explained by the very important role that bats play in spreading *H. capsulatum* in the environment, because the colonial behaviour of bats in roosting caves, the size of colonies, the ability to fly and the high fidelity to roosts are all important factors that explain the dynamics of fungal dispersion in nature. For example, diseased bats could act as parasite dispersers by incorporating the fungus into new favourable environments, possibly through their carcasses once dead (Taylor et al. 2005b).

It is important to consider that both markers have limitations. The high sensitivity of *Hcp100* in nested PCR assays (Bialek et al. 2002) can sometimes elicit false-positive results due to the poor quality of the samples processed. Likewise, even with good sensitivity, another limitation related to its specificity has been described to be probably associated with a 51-amino acid fragment from its corresponding Hcp100 protein, which shares an identity of 91 and 87 % with a transcription factor in *Ajellomyces dermatitidis* and with a hypothetical protein of *Paracoccidioides brasiliensis*, respectively (González-González et al. 2012).

Moreover, because no cross reaction with other related fungi has been detected, the 1281–1283$_{(220)}$ marker shows a lower sensitivity compared to the *Hcp100* marker, even though it has high specificity (Buitrago et al. 2013).

Based on the present findings, we propose the use of the *Hcp100* and 1281–1283$_{(220)}$ markers with their respective PCR methods for the identification of *H. capsulatum*, as the limitations of each one could be overcome and complemented for the detection of the pathogen.

Conclusion

The markers *Hcp100* and 1281–1283$_{(220)}$ can be useful for the diagnosis and prognosis of histoplasmosis. Additionally, they are excellent tools that can be used simultaneously to determine sources of infection. They can also be used to classify sites with higher or lower risks of infection in places that meet the micro-environmental conditions for the development of the pathogen *H. capsulatum*, particularly those associated with sites of high occupational or recreational risks, which would allow the competent authorities to propose the necessary prevention and control measures.

Compliance with ethical standards Animal manipulations were in accordance with the suggestions of the Ethics Committee of the School of Medicine, UNAM, and following the recommendations of the Animal Care and Use Committee of the UNAM and the Mexican Official Guide (NOM 062-ZOO-1999).

Conflict of interest The authors declare that they have no competing interests.

References

Altschul S, Madden T, Shäffer A, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Anderson H, Honish L, Taylor G, Johnson M, Tovstiuk C, Fanning A, Tyrrell G, Rennie R, Jaipaul J, Sand C, Probert S (2006) Histoplasmosis cluster, golf course, Canada. Emerg Infect Dis 12:163–165

Bialek R, Feucht A, Aepinus C, Just-Nubling G, Robertson VJ, Knobloch J, Hohle R (2002) Evaluation of two nested PCR assays for detection of *Histoplasma capsulatum* DNA in human tissue. J Clin Microbiol 40:1644–1647

Bracca A, Tosello ME, Girardini JE, Amigot SL, Gomez C, Serra E (2003) Molecular detection of *Histoplasma capsulatum* var. *capsulatum* in human clinical samples. J Clin Microbiol 41:1753–1755

Buitrago MJ, Canteros CE, Frías De León G, González Á, Marques-Evangelista De Oliveira M, Muñoz CO, Ramírez JA, Toranzo AI, Zancopé-Oliveira R, Cuenca-Estrella M (2013) Comparison of PCR protocols for detecting *Histoplasma capsulatum* DNA through a multicenter study. Rev Iberoam Micol 30:256–260

Calanni LM, Pérez RA, Brasili S, Schmidt NG, Iovannitti CA, Zuiani MF, Negroni R, Finguelievich J, Canteros CE (2013) Brote de histoplasmosis en la provincia de Neuquén, Patagonia Argentina. Rev Iberoam Micol 30:193–199

Contreras C, Shibayama H, Gutiérrez P (1998) Aportaciones del Instituto Nacional de Diagnóstico y Referencia Epidemiológica a la histoplasmosis. Rev Inst Nal Enf Resp Mex 11:216–220

Corcho-Berdugo A, Muñoz-Hernández B, Palma-Cortés G, Ramírez-Hernández A, Martínez-Rivera M, Frías-de León M, Reyes-Montes M, Martínez-Valadez E, Manjarrez-Zavala M, Alfaro-Ramos L, Higuera-Iglesisa AL (2011) An unusual outbreak of histoplasmosis in residents of the state of Mexico. Gac Med Mex 147:377–384

Dantas KC, Freitas RS, Moreira AP, Silva MV, Benard G, Vasconcellos C, Criado PR (2013) The use of nested polymerase chain reaction (nested PCR) for the early diagnosis of *Histoplasma capsulatum* infection in serum and whole blood of HIV-positive patients. An Bras Dermatol 88:141–143

Frías De León MG, Arenas López G, Taylor ML, Acosta Altamirano G, Reyes-Montes MR (2012) Development of specific sequence-characterized amplified region markers for detecting *Histoplasma capsulatum* in clinical and environmental samples. J Clin Microbiol 50:673–679

Gago S, Esteban C, Valero C, Zaragoza O, Puig de la Bellacasa J, Buitrago MJ (2014) 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 52:1168–1176

González-González AE, Taylor ML, Curiel-Quesada E (2012) Aspectos relevantes del marcador molecular *Hcp100* de *Histoplasma capsulatum* y su potencial uso terapéutico en la histoplasmosis. Rev Iberoam Micol 29:115–119

Guedes HL, Guimarães AJ, De Medeiros-Muniz M, Pizzini CV, Hamilton AJ, Peralta JM, Deepe GS, Zancopé-Oliveira RM (2003) PCR assay for identification of *Histoplasma capsulatum* based on the nucleotide sequence of the M antigen. J Clin Microbiol 41:535–539

Haynes K, Westerneng T, Fell J, Moens W (1995) Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J Med Vet Mycol 3:319–325

Ley Federal del trabajo (2012) Diario Oficial de la Federación. Distrito Federal, México, 13 de noviembre

Martagon-Villamil J, Shrestha N, Sholtis M, Isada CM, Hall GS, Bryne T, Lodge BA, Reller LB, Procop GW (2003) Identification of *Histoplasma capsulatum* from culture extracts by real-time PCR. J Clin Microbiol 41:1295–1298

Millon L, Herbrecht R, Grenouillet F, Morio F, Alanio A, Letscher-Bru V, Cassaing S, Chouaki T, Kauffmann-Lacroix C, Poirier P, Toubas D, Augereau O, Rocchi S, Garcia-Hermoso D, Bretagne S, Group FMS (2015) Early diagnosis and monitoring of mucormycosis by detection of circulating DNA in serum: retrospective analysis of 44 cases collected through the French Surveillance Network of Invasive Fungal Infections (RESSIF). Clin Microbiol Infect. doi:10.1016/j.cmi.2015.12.006

Muñoz C, Gómez BL, Tobón A, Arango K, Restrepo A, Correa MM, Muskus C, Cano LE, González A (2010a) Validation and clinical application of a molecular method for identification of *Histoplasma capsulatum* in human specimens in Colombia, South America. Clin Vaccine Immunol 17:62–67

Muñoz B, Martínez MA, Palma G, Ramírez A, Frías MG, Reyes MR, Taylor ML, Higuera AL, Corcho A, Manjarrez ME (2010b) Molecular characterization of *Histoplasma capsulatum* isolated from an outbreak in treasure hunters. BMC Infect Dis 10:264–269

Tang YW, Li H, Durkin MM, Sefers SE, Meng S, Connolly PA, Stratton CW, Wheat LJ (2006) Urine polymerase chain reaction is not as sensitive as urine antigen for the diagnosis of disseminated histoplasmosis. Diagn Microbiol Infect Dis 53:46–51

Taylor ML, Granados J, Toriello C (1996) Biological and sociocultural approaches of histoplasmosis, in the state of Guerrero, Mexico. Mycoses 39:375–379

Taylor ML, Chávez-Tapia CB, Vargas-Yáñez R, Rodríguez-Arellanes G, Peña-Sandoval GR, Toriello C, Pérez A, Reyes-Montes MR (1999) Environmental conditions favoring bat infection with *Histoplasma capsulatum* in Mexican shelters. Am J Trop Med Hyg 61:914–919

Taylor ML, Chávez-Tapia CB, Rojas-Martínez A, Reyes-Montes MR, Bobadilla del Valle M, Zúñiga G (2005a) Geographical distribution of genetic polymorphism of the pathogen *Histoplasma capsulatum* isolated from infected bats, captured in a central zone of Mexico. FEMS Immunol Med Microbiol 45:451–458

Taylor ML, Ruíz-Palacios GM, Reyes-Montes MR, Rodríguez-Arellanes G, Carreto-Binaghi LE, Duarte-Escalante E, Hernández-Ramírez A, Suárez-Alvarez RO, Roldán-Aragón YA, Romero-Martínez R, Sahaza-Cardona JH, Sifuentes-Osornio J, Soto-Ramírez LE, Peña-Sandoval GR (2005b) Identification of the infectious source of an unusual outbreak of histoplasmosis, in a hotel in Acapulco, state of Guerrero, Mexico. FEMS Immunol Med Microbiol 45:435–441

Ueda Y, Sano A, Tamura M, Inomata T, Kamei K, Yokoyama K, Kishi F, Ito J, Mikami Y, Miyaji M, Nishimura K (2003) Diagnosis of histoplasmosis by detection of the internal transcribed spacer region of fungal rRNA gene from a paraffin-embedded skin sample from a dog in Japan. Vet Microbiol 94:219–224

Vaca-Marín MA, Martínez-Rivera MA, Flores-Estrada JJ (1998) Histoplasmosis en México, aspectos históricos y epidemiológicos. Rev Inst Nal Enf Resp Mex 11:208–215

Velasco-Castrejón O (1998) La histoplasmosis pulmonar primaria en México. Rev Inst Nal Enf Resp Mex 11:221–225

Voller A, Bidwell DE, Bartlett A (1979) The enzyme linked immunosorbent assay (ELISA). A guide with abstracts of microplate applications. Dynatech Europe Laboratories, Inc, London