JCM
Journals.ASM.org

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

Sara Gago,[a] Cristina Esteban,[b] Clara Valero,[a] Óscar Zaragoza,[a] Jorge Puig de la Bellacasa,[b] María José Buitrago[a]

Mycology Department, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain[a]; Department of Clinical Microbiology, Hospital Clinic de Barcelona, Barcelona, Spain[b]

A molecular diagnostic technique based on real-time PCR was developed for the simultaneous detection of three of the most frequent causative agents of fungal opportunistic pneumonia in AIDS patients: *Pneumocystis jirovecii*, *Histoplasma capsulatum*, and *Cryptococcus neoformans*/*Cryptococcus gattii*. This technique was tested in cultured strains and in clinical samples from HIV-positive patients. The methodology used involved species-specific molecular beacon probes targeted to the internal transcribed spacer regions of the rDNA. An internal control was also included in each assay. The multiplex real-time PCR assay was tested in 24 clinical strains and 43 clinical samples from AIDS patients with proven fungal infection. The technique developed showed high reproducibility (r^2 of >0.98) and specificity (100%). For *H. capsulatum* and *Cryptococcus* spp., the detection limits of the method were 20 and 2 fg of genomic DNA/20 μl reaction mixture, respectively, while for *P. jirovecii* the detection limit was 2.92 $\log_{10}$ copies/20 μl reaction mixture. The sensitivity *in vitro* was 100% for clinical strains and 90.7% for clinical samples. The assay was positive for 92.5% of the patients. For one of the patients with proven histoplasmosis, *P. jirovecii* was also detected in a bronchoalveolar lavage sample. No PCR inhibition was detected. This multiplex real-time PCR technique is fast, sensitive, and specific and may have clinical applications.

HIV infection remains a threat for many people, as it is estimated that almost 34 million adults and children worldwide are infected with the virus, according to WHO/UNAIDS (1). Although mortality associated with HIV infection has decreased in recent years due to the development of highly active antiretroviral therapy (HAART), the incidence and mortality rates of non-AIDS-associated infections remain high, particularly in nondeveloped countries (1). Opportunistic pneumonias are one of the major causes of pulmonary complications among AIDS patients. In fact, almost 70% of people infected with HIV suffer at least one pulmonary complication during the course of the infection (2). Although in early HIV infection the agents that cause pneumonia are similar to those found in the general population, when the number of $CD4^+$ T cells decreases, opportunistic pneumonias are associated with high morbidity and mortality (3–5). Tuberculosis and *Pneumocystis jirovecii* pneumonia (PCP; the abbreviation PCP reflects the previous nomenclature for the *Pneumocystis* species causing opportunistic pneumonia in humans, *P. carinii*; *P. jirovecii* is now recognized as the species that infects humans) are the most commonly reported AIDS-defining illnesses (6). Moreover, opportunistic pneumonias due to *Histoplasma capsulatum* and *Cryptococcus* spp. in immunocompromised patients involve high morbidity and mortality in areas where the virus is not endemic or in those regions where the infection is not very common. *P. jirovecii* has been described as the main cause of pulmonary complication in AIDS patients in developed countries; in fact, in the United States it has been estimated that 75% of HIV-positive persons suffer PCP during their lifetime (7, 8). Clinical diagnosis of PCP is based mainly on microscopic procedures performed directly on the clinical sample and usually involves immunofluorescence microscopy (IFA) or use of Grocott's methenamine silver stain (9, 10). Recently, some reports have described the utility of real-time PCR (rt-PCR) protocols to increase the sensitivity and specificity of PCP diagnosis (11–17), and some of these tests have been commercialized (18). Moreover, a recent study showed the utility of rt-PCR assays for the early diagnosis and treatment of non-HIV-infected patients diagnosed with PCP, for whom a definitive diagnosis could be performed using rt-PCR techniques that detected the low fungal burden present in the patients (19).

Histoplasmosis is a mycosis that is endemic in the United States and Africa that can produce disseminated infection associated with high morbidity and mortality in HIV patients (20–22). Due to travel and immigration, this infection has increased in recent years in areas where *H. capsulatum* mycosis is not endemic (23). Although culture is the reference procedure for the diagnosis of histoplasmosis, it is time-consuming (requiring 3 to 4 weeks of incubation) (24). In fact, the sensitivity and specificity of microscopy-based approaches are very low, and fungal structures are only observed when the infection is advanced. Moreover, serology has shown significant limitations in AIDS patients; it may be negative in 50% of such patients (25). Antigen

Received 16 October 2013 **Returned for modification** 13 December 2013
Accepted 24 January 2014
Published ahead of print 29 January 2014
Editor: D. W. Warnock
Address correspondence to María José Buitrago, buitrago@isciii.es.

doi:10.1128/JCM.02895-13

detection could be useful for these patients, although it is only available in the United States (26). Several rt-PCR protocols with varied sensitivities and specificities have been described for detection of DNA from *Histoplasma capsulatum* in clinical samples (27–29). Recently, a comparison of different protocols for detection of *H. capsulatum* DNA by PCR showed that the sensitivity of the assay depended on both the selected target region and the type of amplification assay (conventional or rt-PCR) (24). Herein, the most sensitive and specific protocols were based on the amplification of the internal transcribed spacer (ITS) by rt-PCR (30), and the less sensitive protocols were based on the amplification of monocopy genes (Hc100p and $SCAR^{220}$) by conventional PCR (31, 32).

In Africa, cryptococcosis, caused by *Cryptococcus neoformans* var. *neoformans* or *C. neoformans* var. *grubii*, is one of the main causes of mortality associated with HIV infection (33). However, in the northwest United States, *Cryptococcus gattii* is the primary causative agent of cryptococcosis and can also affect immunocompetent people; it has been classified endemic in some regions of the eastern United States, and several cases of cryptococcosis outside that area where the disease is endemic have been reported recently (34–36). This infection can produce both meningitis and pulmonary complications in these patients (37, 38). Routine clinical diagnosis of cryptococcosis is based on culture and detection of a capsular antigen, with varied sensitivity and specificity (39, 40). However, molecular methods based on PCR and new technologies for antigen detection also have been described in recent years, although validation of these methods is pending (41–44).

Fungal pneumonias are clinically and radiologically similar to each other, and definitive diagnosis must be reached to initiate the appropriate therapy (45). Moreover, multiplex real-time PCR (Mrt-PCR) protocols allow the detection of mixed infections that cannot be detected by routine protocols. Mixed infections involving *H. capsulatum*, *C. neoformans*, and *P. jirovecii* have been described (46–48).

The aim of this study was the development of a Mrt-PCR for the early detection of fungal opportunistic pneumonia due to *H. capsulatum*, *C. neoformans*, C. *gattii*, and *P. jirovecii* in clinical samples from HIV-positive patients.

MATERIALS AND METHODS

Control strains and plasmids. DNA from different strains was used to standardize the Mrt-PCR assay. All the strains belonged to the Collection of the Mycology Reference Laboratory, Spanish National Center of Microbiology. To standardize the technique, we used the strains *C. neoformans* var. *neoformans* CNM-CL 2132, *H. capsulatum* CNM-CM 2721, and a plasmid that included the sequence target of *P. jirovecii* (pSG1).

To assess the specificity of the technique, the following strains of yeast and molds were used: *Candida albicans* (CNM-CL 5719), *Candida parapsilosis* (CNM-CL5683), *Candida tropicalis* (CNM-CL 5742), *Candida glabrata* (CNM-CL 5533), *Candida guilliermondii* (CNM-CL 7127), *Candida krusei* (CNM-CL 7057), *Coccidioides posadasii* (CNM-CM 2912), *Paracoccidioides brasiliensis* (CNM-CM 2908), *Aspergillus fumigatus* (CNM-CM AF237), *Aspergillus terreus* (CNM-CM 3508), *Aspergillus flavus* (CNM-CM 3509), *Aspergillus niger* (CNM-CM 3551), *Fusarium verticillioides* (CNM-CM 2975), *Fusarium oxysporum* (CNM-CM 2914), *Fusarium solani* (CNM-CM 3035), *Scedosporium prolificans* (CNM-CM1627), *Scedosporium apiospermum* (CNM-CM 3169), *Rhizopus oryzae* (CNM-CM 3020), and *Rhizopus microsporum* (CNM-CM 4244), *Mucor circinelloides* (CNM-CM 2437). In addition, we employed mouse genomic DNA (Promega, Madrid, Spain) and human genomic DNA (Promega, Madrid, Spain).

TABLE 1 Sequences of primers and probes designed for the multiplex real-time PCR assay

Species and its primers and probe	Sequence[a]
Pneumocystis jirovecii	
OliPJMB1 (f)	5′-CCCTAGTGTTTTAGCATTTTTC-3′
OLIPJMB2 (r)	5′-CTGCAATTCACACTACTTATCG-3′
Probe PJ-MB1	5′-HEX-CGCGATACCTTTGGCGAGGCAAG CAAATCGCG-BHQ1-3′
Histoplasma capsulatum[b]	
Cryptococcus neoformans	
OLI CRYPTO 1 2 (f)	5′-CCTGTTGGACTTGGATTTGG-3′
OLI CRYPTO 2 (r)	5′-AGCAAGCCGAAGACTACC-3′
Probe MB CRYPTO	5′-Cyan 500-CGCGATCATTACGCCGGGCT GACAGGTAATCAGATCGCG-BHQ1-3′
Jellyfish (internal control)	
Oli1-icjf1 (f)	5′-GCCTGGTGCAAAAATTGCTTATC-3′
Oli1-icjf2 (r)	5′-CTAAGACAAGTGTGTTTATGGTATTG-3′
Probe CIJF-MB	5′-Cy5-CGCGATGCTGTTCTTCCGCCACTT CCAATCGCG-BHQ2-3′

[a] BHQ1, black hole quencher 1.
[b] Sequences for the primers and probe for *H. capsulatum* are included in patent PCT/ES2009070340.

Design of primers and probes. Primers and molecular beacon probes were designed to specifically amplify a region of the ITS of the rDNA from *Cryptococcus neoformans*/*C. gattii*, *Histoplasma capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*, and *Pneumocystis jirovecii* by Mrt-PCR. Beacon Designer 5.0 software (Premier Biosoft, Palo Alto, CA) was used for primer and probe design. An internal control was included, as described previously (49). The primers and probes designed were subjected to a BLAST search within the GenBank sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and in the database of the Department of Mycology of the Spanish National Center for Microbiology (which contains more than 8,000 distinct sequences), to avoid cross-homology with other microorganisms. The primer and probe sequences are shown in Table 1.

Multiplex real-time PCR assay. The amplification was carried out in a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). PCRs were performed in a 20-μl final volume containing 2× SensiMix DNA (Quantance; Ecogen, Madrid, Spain) used according to the manufacturer's instructions, 0.5 μM each primer for each one of the species, 0.25 μM internal control primer, 0.2 μM each species-specific probe, 0.1 μM internal control probe, 2 fg of the internal control plasmid (pICJF), and 2 μl of DNA extracted from the sample, used as the template. The PCR conditions were as follows: an initial step of 95°C for 10 min, followed by 50 cycles of 95°C for 25 s, 50°C for 30 s, and 72°C for 5 s, with a cooling cycle at 40°C for 30 s. Results were considered positive when the product amount increased until its fluorescence intensity exceeded the background, as determined by second-derivate analysis; these results were expressed in terms of the quantification cycle (C_q). Each experiment was performed in duplicate and included quantification standards as well as negative controls. Subsequently, a color compensation experiment was performed to prevent cross talk between dyes.

Standardization. Standard curves for *H. capsulatum* and *C. neoformans* were constructed based on the results of five PCR repetitions with dilutions of genomic DNA ranging from 20 ng to 2 fg/20 μl of reaction mixture. The strains CNM-CL 2132 and CNM-CM 2721 were used to construct the standard curves. For *P. jirovecii*, a fragment of 175 bp con-

taining the ITS1 target region was cloned into a pGEMT-Easy plasmid (Promega, Madrid, Spain) according to the manufacturer's instructions. A 10-fold serial dilution of the plasmid clone (pSG1), from 20 ng (2.92×10^8 $\log_{10}$ copies/20-μl reaction mixture) to 2 fg of pDNA/20-μl reaction mixture (2.92 $\log_{10}$ copies/20-μl reaction mixture), was used to construct the standard curve. C_q values for each dilution series were determined in duplicate in three different experiments.

Regression lines were constructed by plotting the logarithm of the initial template concentration versus the corresponding C_q value. If this line exhibited a linear regression coefficient value of >0.980, the standard curve was then used to determine the sensitivity, primer efficiencies, and reproducibility of the assay. In order to evaluate the specificity, 2 ng of DNA/20 μl of reaction mixture from each of the other mold and yeast species, as well as human and mouse genomic DNA, was included in the PCR assay (control strains).

DNA extraction. DNA extraction from cultures of *H. capsulatum* was performed under biosafety level 3 conditions in compliance with Spanish law (Real decreto 664/1997) and following the method described by Buitrago et al. (23). DNA extraction from *C. neoformans* was performed as previously described by Tang et al. (50). Finally, genomic DNA of *P. jirovecii* was extracted from bronchoalveolar lavage (BAL) samples from 16 patients with proven *P. jirovecii* pneumonia. DNA extraction was performed following the recommendations from reference 15 with modifications. For that purpose, 1.5 ml of BAL fluid was centrifuged at 10,000 rpm for 10 min, the supernatant was discharged, and then the pellet was diluted in 200 μl of supernatant. After that, DNA was extracted using a Qiamp DNA minikit (Qiagen) according to manufacturer's instructions in a 50-μl elution volume. Two microliters of this DNA was used for the Mrt-PCR assays. DNA extractions from other specimens were also performed by using the Qiamp DNA minikit, without modifications. DNAs from paraffin-embedded tissues were isolated as described previously (51).

The DNA extraction efficiency was also determined. For that purpose, 5 μl of the plasmid clone or genomic DNA, containing from 10 ng to 1 fg, was added to 200 μl of serum from healthy patients. Then, DNA extraction was performed by using the Qiamp DNA minikit (Qiagen), and 2 μl was used for the Mrt-PCR protocol. The percentage of DNA recovered was determined for each of the dilutions. The experiments were run in triplicate.

Multiplex real-time assay of cultured clinical strains. The Mrt-PCR assay was initially validated by using cultures from 13 clinical strains of *Cryptococcus* spp. complex (*C. neoformans* var. *neoformans* [$n = 3$], *C. neoformans* var. *grubii* [$n = 3$], *C. gattii* [$n = 4$], and hybrids [$n = 3$]) and 10 *H. capsulatum* strains belonging to the Mold Collection of the Mycology Department of the National Center for Microbiology. All the strains included in the study were previously identified by sequencing the ITS region from the rDNA (52). For *P. jirovecii* validation, clones from 4 different BAL samples were evaluated in triplicate. Two microliters of the extracted DNA was employed to perform the Mrt-PCR assay.

Mrt-PCR assay of clinical samples. The utility of the Mrt-PCR was also evaluated by using clinical samples. Forty-three clinical specimens from 40 HIV-positive patients with proven infection (according to the EORTC/MSG criteria) were evaluated by Mrt-PCR. Because the EORTC/MSG criteria do not include infection caused by *Pneumocystis jirovecii*, proven PCP was considered when the fungus was visualized by methenamine silver stain and the patient showed clinical signs of pneumonia. Patients and clinical specimens are summarized in Table 2. Two microliters of DNA from each sample was used for the Mrt-PCR.

Twenty-nine specimens from the following groups of patients were included to assess the specificity of the technique: HIV^+ patients from areas where histoplasmosis is endemic, HIV^+ patients from areas of endemicity and with viral or bacterial pneumonia, and HIV^+ patients with invasive fungal infection (IFI) caused by other fungal species; patients with IFI and other underlying diseases were included as negative controls (Table 3).

RESULTS

Assay standardization *in vitro*. The Mrt-PCR assay developed was able to reliably detect *C. neoformans*, *H. capsulatum*, and *P. jirovecii* DNA. The sizes of the amplicons generated were 175 bp for *P. jirovecii*, 106 bp for *H. capsulatum*, and 139 bp for *C. neoformans*.

No cross-reactivity to other fungi or either human or mouse DNA was detected (control materials). In addition, no cross-reactivity between them was observed. The detection limits of the assay were 2 and 20 fg of genomic DNA per 20 μl of PCR mixture for *C. neoformans* and *H. capsulatum*, respectively, and 2.69 $\log_{10}$ copies/20 μl of reaction mixture for *P. jirovecii* (Fig. 1). Quantification was linear for all the fungal species included in the assay, and the standard curve generated showed a high coefficient of determination ($R^2 = 0.98$ to 0.99). The average coefficient of variation was 2.18% for *H. capsulatum* DNA, 3.93% for *C. neoformans* DNA, and 3.35% for 2 replicates from two different plasmids of *P. jirovecii* (Table 4). Each of the species was detected in their corresponding fluorophore channels (Fig. 2). The mean crossing point (C_p) value for the internal control was 33 ± 0.6. The calculated average DNA extraction efficiencies from clinical samples were 79.8%, 78%, and 96.5% for *H. capsulatum*, *C. neoformans*, and *P. jirovecii*, respectively.

Results with clinical cultured strains. Results were positive for all clinical isolates tested. For the four *P. jirovecii* clones, the mean (± standard deviation) C_q obtained was 14.03 ± 0.71; the mean C_q was 26.27 ± 2.04 for all the clinical strains of *H. capsultaum* and 20.47 ± 1.34 for the *C. neoformans*/*C. gattii* isolates. No differences among C_q values for the different *Cryptococcus* spp. were detected.

Results with clinical samples. A total of 43 clinical samples from 40 patients with HIV as the underlying disease were analyzed by Mrt-PCR. The source of the clinical samples was very heterogeneous (Table 2). Our Mrt-PCR assay was positive for 37/40 patients (92.5% sensitivity) and 39/43 clinical samples (90.7% sensitivity); the estimated clinical specificity was 100% for both analyses. The assay was negative in 3 cases: in 2 patients diagnosed with histoplasmosis and in 1 patient diagnosed with cryptococcosis. All patients with proven PCP showed a positive Mrt-PCR result. All the samples from patients included as controls showed negative PCR results. The average amount of DNA in each of the clinical samples is shown in Table 2. No inhibition of the PCR was detected with any of the clinical samples tested.

Although proven histoplasmosis was demonstrated by Mrt-PCR for case number 3, *P. jirovecii* infection was also detected (Fig. 1). Results were verified by sequencing the PCR products.

DISCUSSION

Opportunistic fungal pneumonias are one of the most frequent pulmonary complications in HIV-positive patients (4). In this study, we evaluated the utility of a single-tube Mrt-PCR assay for the diagnosis and identification of four of the most frequent causes of opportunistic fungal pneumonia in AIDS patients. Clinical symptomatology of these infections is based on the observation of abnormalities in chest X-rays, but in patients with proven or suspected HIV infection such findings are also related to *Mycobacterium tuberculosis* infection (45). Therefore, tuberculosis could overshadow other opportunistic infections, such as crypto-

TABLE 2 Mrt-PCR results for clinical samples from patients with proven opportunistic fungal pneumonia

Patient no.	Proven infection	Sample	PCR result	C_p(s)	Amt of DNA[c]
1	Histoplasmosis	BAL	Positive	27.54	7×10^{-3}
2	Histoplasmosis	Bone marrow	Negative		
		Blood	Positive	29.46	1×10^{-3}, 8×10^{-3}
3[a]	Histoplasmosis	Bronchiaspirate	Positive	29.18, 28.8[b]	2.2×10^{-3}, 3.37 $\log_{10}$
4	Histoplasmosis	Bone marrow	Positive	29.33	1.9×10^{-3}
		Serum	Negative		
5	Histoplasmosis	Blood	Positive	29.53	1.7×10^{-3}
		BAL	Positive	29	2.5×10^{-3}
6	Histoplasmosis	Biopsy	Positive	27.2	8.9×10^{-3}
7	Histoplasmosis	Biopsy	Positive	29.3	2×10^{-3}
8	Cryptococcosis	Blood	Positive	40.97	2.5×10^{-3}
9	Cryptococcosis	Biopsy	Negative		
10	PCP	BAL	Positive	27.5	5×10^{2} $\log_{10}$
11	PCP	BAL	Positive	36.75	1.42 $\log_{10}$
12	PCP	BAL	Positive	29.93	1×10^{2} log10
13	PCP	BAL	Positive	27.7	4.4×10^{2} log10
14	PCP	BAL	Positive	27.51	5×10^{2} log10
15	PCP	BAL	Positive	25.61	1.7×10^{3} $\log_{10}$
16	PCP	BAL	Positive	33.28	12 $\log_{10}$
17	PCP	BAL	Positive	33.05	14 $\log_{10}$
18	PCP	BAL	Positive	32.92	16 $\log_{10}$
19	PCP	BAL	Positive	33.16	13 $\log_{10}$
20	PCP	BAL	Positive	33.15	14 $\log_{10}$
21	PCP	BAL	Positive	33.83	9 $\log_{10}$
22	PCP	BAL	Positive	33.57	10 $\log_{10}$
23	PCP	BAL	Positive	33.37	12 $\log_{10}$
24	PCP	BAL	Positive	32.44	22 $\log_{10}$
25	PCP	BAL	Positive	33.22	13 $\log_{10}$
26	Cryptococcosis	BAL	Positive	28.12	8.6×10^{-3}
27	Cryptococcosis	BAL	Positive	28.94	5.1×10^{-3}
28	Histoplasmosis	Biopsy	Positive	19.35	2.1×10^{-3}
29	Histoplasmosis	Biopsy	Positive	27.14	9.3×10^{-3}
30	Histoplasmosis	Sputum	Positive	25.46	3×10^{-2}
31	Histoplasmosis	Sputum	Positive	26.45	1.5×10^{-2}
32	Histoplasmosis	Sputum	Positive	26.79	1.1×10^{-2}
33	Histoplasmosis	Serum	Positive	26.97	1×10^{-2}
34	Histoplasmosis	Bone marrow	Negative		
35	Cryptococcosis	BAL	Positive	26.9	1.8×10^{-2}
36	PCP	BAL	Positive	28.02	3.65×10^{2} $\log_{10}$
37	PCP	BAL	Positive	27.69	4.50×10^{2} $\log_{10}$
38	PCP	BAL	Positive	27.26	5.9×10^{2} $\log_{10}$
39	PCP	BAL	Positive	25.28	2×10^{3} $\log_{10}$
40	PCP	BAL	Positive	26.7	8.410×10^{2} $\log_{10}$

[a] Patient number 3 showed a mixed infection with *Histoplasma capsulatum* and *Pneumocystis jirovecii*.
[b] The first and second C_ps correspond to *H. capsulatum* and *P. jirovecii*, respectively.
[c] Values shown are in ng/μl except where specified as "$\log_{10}$," indicating $\log_{10}$ numbers of copies/μl.

coccosis and pulmonary histoplasmosis, delaying the diagnosis and correct treatment (53).

Molecular techniques have been proven to be very useful for the diagnosis of fungal infections (54). However, reports describing Mrt-PCR approaches for the diagnosis of opportunistic pneumonias are scarce (55). In fact, this is the first report to describe an Mrt-PCR assay for the simultaneous detection of *P. jirovecii*, *H. capsulatum*, and *C. neoformans*.

Our assay proved to be species specific (100%), with high reproducibility ($r^2 > 0.98$) and sensitivity (100%) for each of the species. The Mrt-PCR was also validated by using clinical samples from 40 patients with proven infection diagnosed using classical techniques. Although respiratory samples (bronchoalveolar lavage fluid, pulmonary biopsy tissue, sputum, or pleural fluid) are the most appropriate samples for the diagnosis of opportunistic pneumonias, in patients with advanced immunosuppression systemic infection may develop, and other samples, such as blood, serum, or bone marrow biopsy specimens can be used for definitive diagnosis. Our assay was able to detect 92.5% of the infections in patients with proven infection. The Mrt-PCR yielded a negative result for four clinical samples (from serum, bone marrow, and biopsy) for three patients. These negative clinical samples were

TABLE 3 Summary of control populations used to determine background colonization levels and the specificity of the Mrt-PCR assay

Population	Control sample no.	Specimen	PCR result	Species causing IFI
HIV^+ patients from areas of endemicity	1	Bone narrow	Negative	
	2	Biopsy	Negative	
	3	Biopsy	Negative	
	4	Bone narrow	Negative	
	5	Whole Blood	Negative	
	6	Whole Blood	Negative	
HIV^+ patients from areas of endemicity and with viral or bacterial pneumonia	7	BAL	Negative	
	8	BAL	Negative	
	9	BAL	Negative	
	10	Biopsy	Negative	
HIV^+ patients with invasive fungal infection caused by other fungal species	11	Biopsy	Negative	*Lichtheimia corymbifera*
	12	Biopsy	Negative	*Rhizomucor pusillus*
	13	BAL	Negative	*Cryptococcus laurentii*
	14	BAL	Negative	*Cryptococcus humicola*
	15	BAL	Negative	*Aspergillus flavus*, *Penicillium* spp., and *Cryptococcus albidus*
Non-HIV-infected patients with invasive fungal infection	16	Biopsy	Negative	*Coccidioides immitis*
	17	Biopsy	Negative	*Scedosporium apiospermum*
	18	Biopsy	Negative	*Bipolaris* spp.
	19	BAL	Negative	*Aspergillus fumigatus*
	20	BAL	Negative	*Aspergillus fumigatus*
	21	BAL	Negative	*Aspergillus fumigatus*
	22	BAL	Negative	*Aspergillus fumigatus*
	23	BAL	Negative	*Aspergillus fumigatus*
	24	Ascitic fluid	Negative	*Aspergillus fumigatus*
	25	Cerebrospinal fluid	Negative	*Aspergillus fumigatus*
	26	BAL	Negative	*Aspergillus fumigatus*
	27	Biopsy	Negative	*Aspergillus fumigatus*
	28	Biopsy	Negative	*Aspergillus fumigatus*
	29	BAL	Negative	*Aspergillus fumigatus*

stored for more than 5 years at −20°C, which may have decreased the sensitivity of the assay (56).

Interestingly, for one of the patients diagnosed with histoplasmosis, *P. jirovecii* DNA was detected. In fact, coinfections may occur, and accurate diagnosis and prompt appropriate antifungal treatment are required. Several reports of cases of mixed infections have been published, and for all of them the definitive diagnosis was based on microbiological evidence. Bava et al. (47) reported a case in which a patient had mixed infection due to *Cryptococcus* and *Pneumocystis*. Mixed infections of *H. capsulatum* and *C. neoformans* (46), as well as of *P. jirovecii* and *H. capsulatum*, have also been reported in individuals from areas (48). Finally, coinfection with *H. capsulatum* and *M. tuberculosis* is frequent in some areas where histoplasmosis is endemic and may involve important implications for treatment (57).

To our knowledge, only one PCR-based protocol has been previously reported for the detection of tuberculosis and PCP in HIV-infected patients (55). rt-PCR approaches for the detection of *C. neoformans/C. gattii*, *H. capsulatum*, and *P. jirovecii* separately have been widely described based on the amplification of different targets, with varied sensitivity and specificity. Most of the rt-PCR approaches for the diagnosis of cryptococcosis are based on the amplification of monocopy genes (43, 58), which may decrease the sensitivity of the technique. Our technique allows detection of both *C. neoformas* spp. complex and *C. gattii*, but it cannot differentiate species. Although the identification at species level is important for epidemiological studies, clinical signs and treatment are similar for infections caused by either species (59–61). Moreover, a majority of the real-time protocols for the detection of PCP are based on nested PCR, which may decrease the specificity of the technique due to the possibility of false-positive results (58). Although there are several articles describing rt-PCR assays for the detection of *H. capsulatum* based on the amplification of multicopy regions, such as ITS, the use of molecular beacon probes can improve the sensitivity of the technique due to their small size and sequence specificity (23). The technique we have described in this article can detect several fungi in a single-tube reaction. The limit to detection of more fungal species is determined by the real-time PCR equipment.

In conclusion, this is the first report to describe an Mrt-PCR for the diagnosis of opportunistic fungal pneumonias. This approach could be very useful for differential diagnosis with tuberculosis, as clinical signs are similar, and also to detect mixed infections that could appear in patients with advanced immunosuppression living in areas of endemicity. Moreover, this assay could be useful for the clinical management of other immunocompromised patients at risk for these fungal infections (e.g., patients under corticosteroid therapy or chemo-

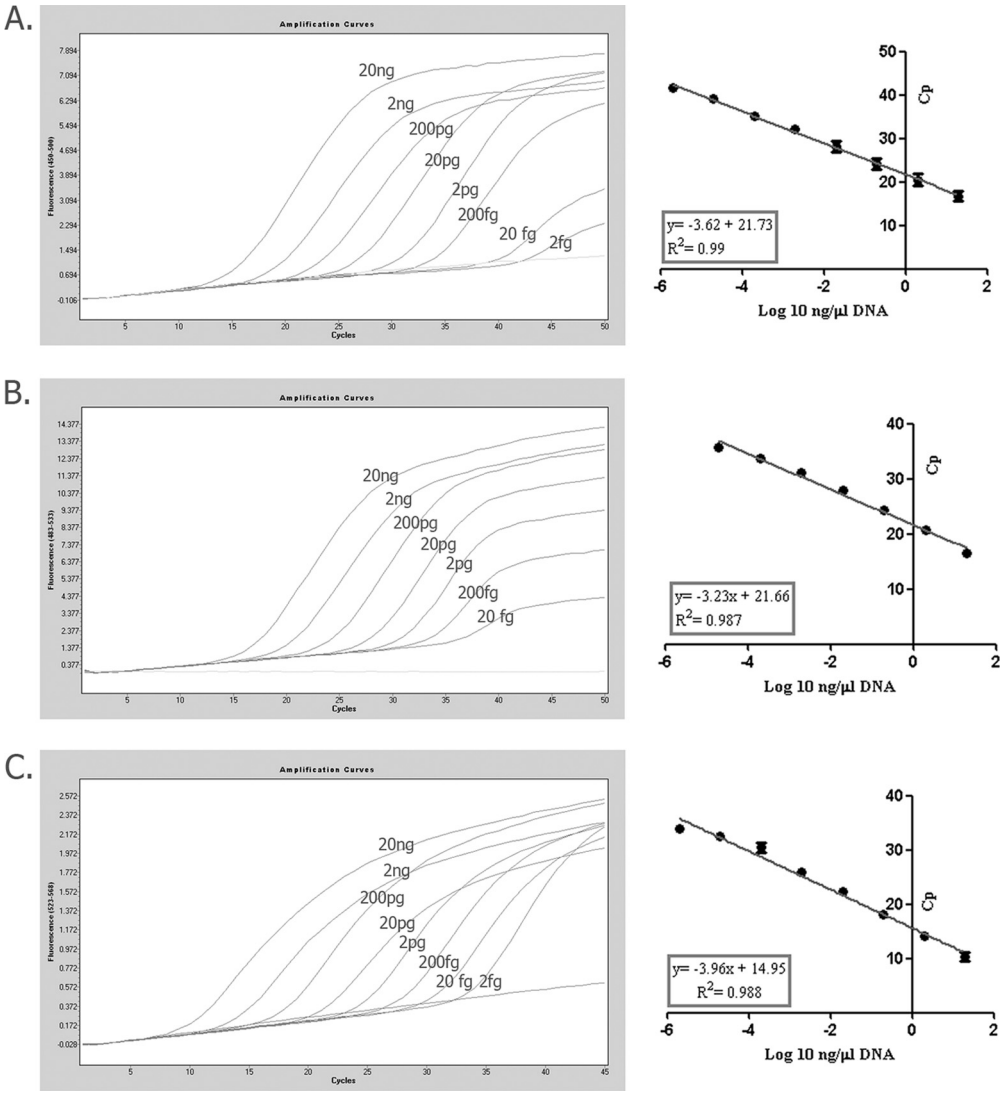

FIG 1 Quantification standard curve obtained by using serial 1:10 dilutions of different concentrations of DNA from *Pneumocystis jirovecii* (A), *Histoplasma capsulatum* (B), and *Cryptococcus neoformans* (C). Error bars represent the percent coefficient of variation. Standard curves were constructed after a color compensation experiment.

therapy or those treated with tumor necrosis factor). Although Mrt-PCR assays are more expensive than conventional approaches (the approximate cost is around 100 € per PCR determination), they provide a fast and specific diagnosis, as they allow the detection of mixed infection and avoid delays in appropriate antifungal therapy. Moreover, our Mrt-PCR could be implemented in clinical settings in developing countries and in countries that receive immigrant populations from those re-

TABLE 4 Overview of standardization of the Mrt-PCR assay with serial dilutions of a plasmid clone containing the target for *P. jirovecii* or with genomic DNA of *Histoplasma capsulatum* or *Cryptococcus neoformans*

Amt of DNA/µl	*H. capsulatum*			*C. neoformans*			*P. jirovecii*		
	Cq	SD	%CV	Cq	SD	%CV	Cq	SD	%CV
10 ng	16.53	0.52	3.15	16.78	1.17	6.97	10.26	0.803	7.834
1 ng	20.656	0.50	2.46	20.494	1.36	6.64	14.205	0.716	5.047
100 pg	24.304	0.59	2.43	24.128	1.21	5.02	18.055	0.733	4.06
10 pg	27.986	0.66	2.36	28.172	1.27	4.53	22.3075	0.769	3.450
1 pg	31.158	0.51	1.66	32.156	0.93	2.89	25.87	0.222	0.858
100 fg	33.758	0.48	1.43	35.058	0.96	2.74	30.4925	0.921	3.022
10 fg	35.644	0.29	0.83	39.1	0.88	2.25	32.47	0.621	1.914
1 fg	16.53	0.52	3.15	41.76	0.16	0.38	Negative	Negative	Negative

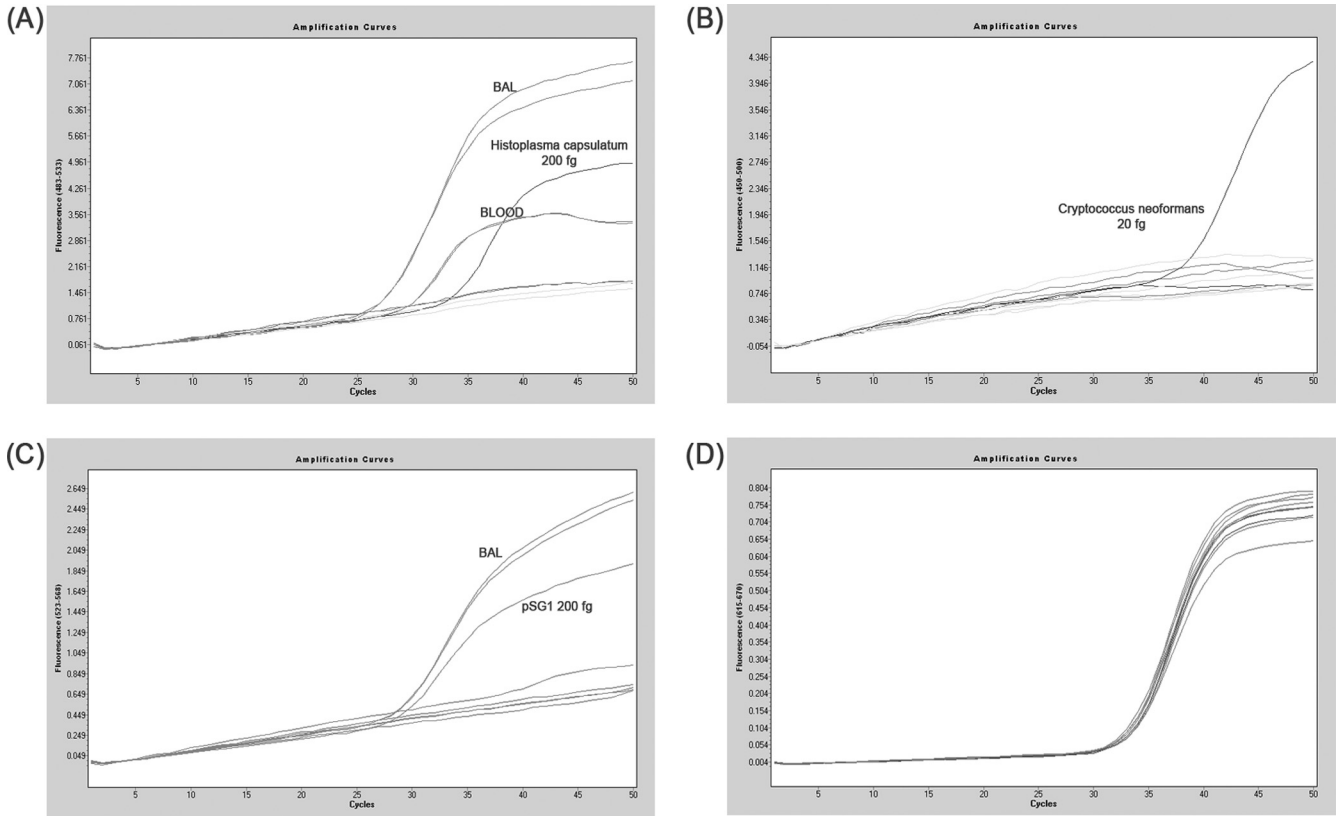

FIG 2 Dependence of fluorescence signal on the number of cycles in the multiplex real-time PCR assay for the patient with mixed infection. (A) Fluorescence detection in the 6-carboxyfluorescein (FAM) fluorescence channel, based on positive signal for the *Histoplasma capsulatum* molecular beacon probe. (B) Fluorescence detection in the Cyan 500 fluorescence channel. The positive signal is for the *Cryptococcus neoformans/C. gattii* molecular beacon probe. (C) Fluorescence detection in the 6-carboxy-hexachlorofluorescein (HEX) fluorescence channel. The positive signal is for the *Pneumocystis jirovecii* molecular beacon probe. (D) Fluorescence detection of Cy5 dye as end label to the specific pICJP molecular beacon probe.

gions. Further studies with a larger number of samples are warranted.

ACKNOWLEDGMENTS

We warmly thank Frannk Hodkings for his careful reading and editing of the manuscript.

This work was supported by research project PI11/00412 from Spanish Fondo de Investigaciones Sanitarias of the Instituto de Salud Carlos III. S.G. and C.V. are supported by research fellowships from the Fondo de Investigaciones Biomedicas of the Spanish Ministry of Science and Innovation (FI10/00464 and FI12/00095, respectively).

We declare no conflicts of interest.

REFERENCES

1. **Huang L, Cattamanchi A, Davis JL, den Boon S, Kovacs J, Meshnick S, Miller RF, Walzer PD, Worodria W, Masur H, International HIV-Associated Opportunistic Pneumonias (IHOP) Study, Lung HIV Study.** 2011. HIV-associated *Pneumocystis* pneumonia. Proc. Am. Thorac Soc. **8:**294–300. http://dx.doi.org/10.1513/pats.201009-062WR.
2. **Gingo MR, Balasubramani GK, Kingsley L, Rinaldo CR, Jr, Alden CB, Detels R, Greenblatt RM, Hessol NA, Holman S, Huang L, Kleerup EC, Phair J, Sutton SH, Seaberg EC, Margolick JB, Wisniewski SR, Morris A.** 2013. The impact of HAART on the respiratory complications of HIV infection: longitudinal trends in the MACS and WIHS cohorts. PLoS One **8**(3):e58812. http://dx.doi.org/10.1371/journal.pone.0058812.
3. **Rosen MJ.** 1992. Pulmonary complications of HIV infection. Mt. Sinai J. Med. **59:**263–270.
4. **Rosen MJ.** 1994. Pneumonia in patients with HIV infection. Med. Clin. North Am. **78:**1067–1079.
5. **Rosen MJ.** 2008. Pulmonary complications of HIV infection. Respirology **13:**181–190. http://dx.doi.org/10.1111/j.1440-1843.2007.01167.x.
6. **Schneider E, Whitmore S, Glynn KM, Dominguez K, Mitsch A, McKenna MT, Centers for Disease Control and Prevention.** 2008. Revised surveillance case definitions for HIV infection among adults, adolescents, and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years—United States, 2008. MMWR Recommend. Rep. **57**(RR-10)**:**1–12.
7. **Hay JW, Osmond DH, Jacobson MA.** 1988. Projecting the medical costs of AIDS and ARC in the United States. J. Acquir. Immune Defic. Syndr. **1:**466–485.
8. **McKinnell JA, Cannella AP, Kunz DF, Hook EW, III, Moser SA, Miller LG, Baddley JW, Pappas PG.** 2012. *Pneumocystis* pneumonia in hospitalized patients: a detailed examination of symptoms, management, and outcomes in human immunodeficiency virus (HIV)-infected and HIV-uninfected persons. Transpl. Infect. Dis. **14:**510–518. http://dx.doi.org/10.1111/j.1339-3062.2012.00739.x.
9. **Calderon EJ, Gutierrez-Rivero S, Durand-Joly I, Dei-Cas E.** 2010. *Pneumocystis* infection in humans: diagnosis and treatment. Expert Rev. Anti Infect. Ther. **8:**683–701. http://dx.doi.org/10.1586/eri.10.42.
10. **Carmona EM, Limper AH.** 2011. Update on the diagnosis and treatment of *Pneumocystis* pneumonia. Ther. Adv. Respir. Dis. **5:**41–59. http://dx.doi.org/10.1177/1753465810380102.
11. **Summah H, Zhu YG, Falagas ME, Vouloumanou EK, Qu JM.** 2013. Use of real-time polymerase chain reaction for the diagnosis of *Pneumocystis pneumonia* in immunocompromised patients: a meta-analysis. Chin. Med. J. **126:**1965–1973. http://dx.doi.org/10.3760/cma.j.issn.0366-6999.20122506.
12. **Tasaka S, Tokuda H.** 2013. Recent advances in the diagnosis of *Pneumocystis jirovecii* pneumonia in HIV-infected adults. Expert Opin. Med. Diagn. **7:**85–97. http://dx.doi.org/10.1517/17530059.2012.722080.

13. **Fillaux J, Berry A.** 2013. Real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* pneumonia. Methods Mol. Biol. **943:**159–170. http://dx.doi.org/10.1007/978-1-60327-4_11.
14. **Fillaux J, Malvy S, Alvarez M, Fabre R, Cassaing S, Marchou B, Linas MD, Berry A.** 2008. Accuracy of a routine real-time PCR assay for the diagnosis of *Pneumocystis jirovecii* pneumonia. J. Microbiol. Methods **75:**258–261. http://dx.doi.org/10.1016/j.mimet.2008.06.009.
15. **Botterel F, Cabaret O, Foulet F, Cordonnier C, Costa JM, Bretagne S.** 2012. Clinical significance of quantifying *Pneumocystis jirovecii* DNA by using real-time PCR in bronchoalveolar lavage fluid from immunocompromised patients. J. Clin. Microbiol. **50:**227–231. http://dx.doi.org/10.1128/JCM.06036-1.
16. **van Oosterhout JJ, Laufer MK, Perez MA, Graham SM, Chimbiya N, Thesing PC, Alvarez-Martinez MJ, Wilson PE, Chagomerana M, Zijlstra EE, Taylor TE, Plowe CV, Meshnick SR.** 2007. *Pneumocystis* pneumonia in HIV-positive adults, Malawi. Emerg. Infect. Dis. **13:**325–328. http://wwwnc.cdc.gov/eid/article/13/2/06-0462/htm.
17. **Dalpke AH, Hofko M, Zimmermann S.** 2013. Development and evaluation of a real-time PCR assay for detection of *Pneumocystis jirovecii* on the fully automated BD MAX platform. J. Clin. Microbiol. **51:**2337–2343. http://dx.doi.org/10.1128/JCM.00616-13.
18. **Orsi CF, Gennari W, Venturelli C, La Regina A, Pecorari M, Righi E, Machetti M, Blasi E.** 2012. Performance of 2 commercial real-time polymerase chain reaction assays for the detection of *Aspergillus* and *Pneumocystis* DNA in bronchoalveolar lavage fluid samples from critical care patients. Diagn. Microbiol. Infect. Dis. **73:**138–143. http://dx.doi.org/10.1016/j.diagmicrobio.2012.03.001.
19. **Asai N, Motojima S, Ohkuni Y, Matsunuma R, Nakashima K, Iwasaki T, Nakashita T, Otsuka Y, Kaneko N.** 2012. Early diagnosis and treatment are crucial for the survival of *Pneumocystis* pneumonia patients without human immunodeficiency virus infection. J. Infect. Chemother. **18:**898–905. http://dx.doi.org/10.1007/s10156-012-0441-4.
20. **Hernandez SL, Lopez De Blanc SA, Sambuelli RH, Roland H, Cornelli C, Lattanzi V, Carnelli MA.** 2004. Oral histoplasmosis associated with HIV infection: a comparative study. J. Oral Pathol. Med. **33:**445–450. http://dx.doi.org/10.1111/j.1600-0714.2004.00200.x-i1.
21. **Huber F, Nacher M, Aznar C, Pierre-Demar M, El Guedj M, Vaz T, Vantilcke V, Mahamat A, Magnien C, Chauvet E, Carme B, Couppie P.** 2008. AIDS-related *Histoplasma capsulatum* var. *capsulatum* infection: 25 years experience of French Guiana. AIDS **22:**1047–1053. http://dx.doi.org/10.1097/QAD.0b013e3282ffde67.
22. **Couppie P, Aznar C, Carme B, Nacher M.** 2006. American histoplasmosis in developing countries with a special focus on patients with HIV: diagnosis, treatment, and prognosis. Curr. Opin. Infect. Dis. **19:**443–449. http://dx.doi.org/10.1097/01.qco.0000244049.15888.b9.
23. **Buitrago MJ, Canteros CE, Frias De Leon G, Gonzalez A, Marques-Evangelista De Oliveira M, Munoz CO, Ramirez JA, Toranzo AI, Zancope-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. http://dx.doi.org/10.1016/j.riam.2013.03.004.
24. **Buitrago MJ, Cuenca-Estrella M.** 2012. Current epidemiology and laboratory diagnosis of endemic mycoses in Spain. Enferm Infecc Microbiol. Clin. **30:**407–413. (In Spanish.) http://dx.doi.org/10.1016/j.eimc.2011.09.014.
25. **Deepe GS.** 2000. *Histoplasma capsulatum*, p 2718–2733. *In* Mandell GL, Bennett JE, Dolin R (ed), Principles and practice of infectious diseases, 7th ed. Churchill Livingston, Philadelphia, PA.
26. **Wheat LJ, Freifeld AG, Kleiman MB, Baddley JW, McKinsey DS, Loyd JE, Kauffman CA, Infectious Diseases Society of America.** 2007. Clinical practice guidelines for the management of patients with histoplasmosis: 2007 update by the Infectious Diseases Society of America. Clin. Infect. Dis. **45:**807–825. http://dx.doi.org/10.1086/521259.
27. **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. http://dx.doi.org/10.1128/JCM.40.5.1644-1647.2002.
28. **Bialek R, Ernst F, Dietz K, Najvar LK, Knobloch J, Graybill JR, Schaumburg-Lever G.** 2002. Comparison of staining methods and a nested PCR assay to detect *Histoplasma capsulatum* in tissue sections. Am. J. Clin. Pathol. **117:**597–603.
29. **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. http://dx.doi.org/10.1128/JCM.41.3.1295-1298.2003.
30. **Buitrago MJ, Berenguer J, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2006. Detection of imported histoplasmosis in serum of HIV-infected patients using a real-time PCR-based assay. Eur. J. Clin. Microbiol. Infect. Dis. **25:**665–668. http://dx.doi.org/10.1007/s10096-006-0207-y.
31. **Porta A, Colonna-Romano S, Callebaut I, Franco A, Marzullo L, Kobayashi GS, Maresca B.** 1999. An homologue of the human 100-kDa protein (p100) is differentially expressed by *Histoplasma capsulatum* during infection of murine macrophages. Biochem. Biophys. Res. Commun. **254:**605–613.
32. **Frias De Leon MG, Arenas Lopez G, Taylor ML, Acosta Altamirano G, Reyes-Montes Mdel R.** 2012. Development of specific sequence-characterized amplified region markers for detecting *Histoplasma capsulatum* in clinical and environmental samples. J. Clin. Microbiol. **50:**673–679. http://dx.doi.org/10.1128/JCM.05271-11.
33. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525–530. http://dx.doi.org/10.1097/QAD.0b013e328322ffac.
34. **Byrnes EJ, Heitman J.** 2009. *Cryptococcus gattii* outbreak expands into the northwestern United States with fatal consequences. F1000 Biol. Rep. **1:**pii. http://dx.doi.org/10.3410/B1-62.
35. **Fyfe M, MacDougall L, Romney M, Starr M, Pearce M, Mak S, Mithani S, Kibsey P.** 2008. *Cryptococcus gattii* infections on Vancouver Island, British Columbia, Canada: emergence of a tropical fungus in a temperate environment. Can. Commun. Dis. Rep. **34:**1–12.
36. **Marr KA.** 2012. *Cryptococcus gattii* as an important fungal pathogen of western North America. Expert Rev. Anti Infect. Ther. **10:**637–643. http://dx.doi.org/10.1586/eri.12.48.
37. **Govender NP, Patel J, van Wyk M, Chiller TM, Lockhart SR, Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA).** 2011. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates obtained through population-based surveillance in South Africa in 2002–2003 and 2007–2008. Antimicrob. Agents Chemother. **55:**2606–2611. http://dx.doi.org/10.1128/AAC.00048-11.
38. **Lortholary O, Sitbon K, Dromer F, French Cryptococcosis Study G.** 2005. Evidence for human immunodeficiency virus and *Cryptococcus neoformans* interactions in the pro-inflammatory and anti-inflammatory responses in blood during AIDS-associated cryptococcosis. Clin. Microbiol. Infect. **11:**296–300. http://dx.doi.org/10.1111/j.1469-0691.2005.01074.x.
39. **Liechty CA, Solberg P, Were W, Ekwaru JP, Ransom RL, Weidle PJ, Downing R, Coutinho A, Mermin J.** 2007. Asymptomatic serum cryptococcal antigenemia and early mortality during antiretroviral therapy in rural Uganda. Trop. Med. Int. Health **12:**929–935. http://dx.doi.org/10.1111/j.1365-3156.2007.01874.x.
40. **Jarvis JN, Percival A, Bauman S, Pelfrey J, Meintjes G, Williams GN, Longley N, Harrison TS, Kozel TR.** 2011. Evaluation of a novel point-of-care cryptococcal antigen test on serum, plasma, and urine from patients with HIV-associated cryptococcal meningitis. Clin. Infect. Dis. **53:**1019–1023. http://dx.doi.org/10.1093/cid/cir613.
41. **Carvalho VG, Terceti MS, Dias AL, Paula CR, Lyon JP, de Siqueira AM, Franco MC.** 2007. Serotype and mating type characterization of *Cryptococcus neoformans* by multiplex PCR. Rev. Inst. Med. Trop. Sao Paulo **49:**207–210. http://dx.doi.org/10.1590/S0036-46652007000400002.
42. **Diaz MR, Fell JW.** 2005. Use of a suspension array for rapid identification of the varieties and genotypes of the *Cryptococcus neoformans* species complex. J. Clin. Microbiol. **43:**3662–3672. http://dx.doi.org/10.1128/JCM.43.8.3662-3672.2005.
43. **Veron V, Simon S, Blanchet D, Aznar C.** 2009. Real-time polymerase chain reaction detection of *Cryptococcus neoformans* and *Cryptococcus gattii* in human samples. Diagn. Microbiol. Infect. Dis. **65:**69–72. http://dx.doi.org/10.1016/j.diagmicrobio.2009.05.005.
44. **Hansen J, Slechta ES, Gates-Hollingsworth MA, Neary B, Barker AP, Bauman S, Kozel TR, Hanson KE.** 2013. Large-scale evaluation of the immuno-mycologics lateral flow and enzyme-linked immunoassays for detection of cryptococcal antigen in serum and cerebrospinal fluid. Clin. Vaccine Immunol. **20:**52–55. http://dx.doi.org/10.1128/CVI.00536-12.
45. **Reynolds JH, Banerjee AK.** 2012. Imaging pneumonia in immunocompetent and immunocompromised individuals. Curr. Opin. Pulm. Med. **18:**194–201. http://dx.doi.org/10.1097/MCP.0b013e328351f953.
46. **Aronis ML, dos Santos RP, Goldani LZ.** 2011. Disseminated *Histoplasma capsulatum* and *Cryptococcus neoformans* co-infection in patients with

AIDS. Mycopathologia **172:**233–236. http://dx.doi.org/10.1007/s11046-011-9422-x.
47. **Javier B, Susana L, Santiago G, Alcides T.** 2012. Pulmonary coinfection by *Pneumocystis jirovecii* and *Cryptococcus neoformans*. Asian Pac. J. Trop. Biomed. **2:**80–82. http://dx.doi.org/10.1016/S2221-1691(11)60195-0.
48. **Tschudy J, Michail S.** 2010. Disseminated histoplasmosis and pneumocystis pneumonia in a child with Crohn disease receiving infliximab. J. Pediatr. Gastroenterol. Nutr. **51:**221–222. http://dx.doi.org/10.1097/MPG.0b013e3181c2c10d.
49. **Bernal-Martinez L, Buitrago MJ, Castelli MV, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2013. Development of a single tube multiplex real-time PCR to detect the most clinically relevant Mucormycetes species. Clin. Microbiol. Infect. **19:**E1–E7. http://dx.doi.org/10.1111/j.1469-0691.2012.03976.x.
50. **Tang CM, Cohen J, Krausz T, Van Noorden S, Holden DW.** 1993. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect. Immun. **61:**1650–1656.
51. **Buitrago MJ, Aguado JM, Ballen A, Bernal-Martinez L, Prieto M, Garcia-Reyne A, Garcia-Rodriguez J, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2013. Efficacy of DNA amplification in tissue biopsy samples to improve the detection of invasive fungal disease. Clin. Microbiol. Infect. **19:**E271–277. http://dx.doi.org/10.1111/1469-0691.12110.
52. **White TJ, Bruns S, Lee S, Taylor J.** 2011. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics, p 315–324. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.
53. **Kyeyune R, den Boon S, Cattamanchi A, Davis JL, Worodria W, Yoo SD, Huang L.** 2010. Causes of early mortality in HIV-infected TB suspects in an East African referral hospital. J. Acquir. Immune Defic. Syndr. **55:**446–450. http://dx.doi.org/10.1097/qai.0b013e3181eb611a.
54. **Cuenca-Estrella M, Bassetti M, Lass-Florl C, Racil Z, Richardson M, Rogers TR.** 2011. Detection and investigation of invasive mould disease. J. Antimicrob. Chemother. **66**(Suppl. 1)**:**i15–i24. http://dx.doi.org/10.1093/jac/dpkq438.
55. **Boondireke S, Mungthin M, Tan-ariya P, Boonyongsunchai P, Naaglor T, Wattanathum A, Treewatchareekorn S, Leelayoova S.** 2010. Evaluation of sensitivity of multiplex PCR for detection of *Mycobacterium tuberculosis* and *Pneumocystis jirovecii* in clinical samples. J. Clin. Microbiol. **48:**3165–3168. http://dx.doi.org/10.1128/JCM.00323-10.
56. **Lau A, Halliday C, Chen SC, Playford EG, Stanley K, Sorrell TC.** 2010. Comparison of whole blood, serum, and plasma for early detection of candidemia by multiplex-tandem PCR. J. Clin. Microbiol. **48:**811–816. http://dx.doi.org/10.1128/JCM.01650-09.
57. **Agudelo CA, Restrepo CA, Molina DA, Tobon AM, Kauffman CA, Murillo C, Restrepo A.** 2012. Tuberculosis and histoplasmosis coinfection in AIDS patients. Am. J. Trop. Med. Hyg. **87:**1094–1098. http://dx.doi.org/10.4269/ajtmh.2012.12-0292.
58. **Qishui O, Ling J, Ni L, Bin Y, Wen L.** 2012. Comparison of real-time florescence quantitative PCR measurements of VAD1 mRNA with three conventional methods in diagnosis and follow-up treatment of *Cryptococcus neoformans* infection. Mycoses **55:**326–332. http://dx.doi.org/10.1111/j.1439-0507.2011.02100.x.
59. **Antinori S.** 2013. New insights into HIV/AIDS-associated cryptococcosis. ISRN AIDS **2013:**471363. http://dx.doi.org/10.1155/2013/471363.
60. **Bratton EW, El Husseini N, Chastain CA, Lee MS, Poole C, Sturmer T, Weber DJ, Juliano JJ, Perfect JR.** 2013. Approaches to antifungal therapies and their effectiveness among patients with cryptococcosis. Antimicrob. Agents Chemother. **57:**2485–2495. http://dx.doi.org/10.1128/AAC.01800-12.
61. **Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC.** 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin. Infect. Dis. **50:**291–322. http://dx.doi.org/10.1086/649858.