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Detection of *Histoplasma capsulatum* DNA in human samples by real-time polymerase chain reaction

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Abstract

The main aim of our study was to determine the added value of real-time polymerase chain reaction (PCR) for the diagnosis of *Histoplasma capsulatum* in routine biologic practice. No amplification signal was observed with the 18 non-*H. capsulatum* strains used to test the specificity of the protocol. The sensitivity threshold of the real-time PCR assay was about 10 fg of *H. capsulatum* DNA per microliter, tested with a 10-fold serial dilution of the positive control. We analyzed 348 human samples submitted for the routine diagnosis of systemic mycosis. Real-time PCR using the TaqMan system was evaluated against direct microscopic examination and culture. Among the 341 samples without PCR inhibition ($n = 7$), 66 tested positive by culture, whereas 74 tested positive by real-time PCR. Sensitivity of the real-time PCR assay was estimated at 95.4% and specificity at 96.0% with respect to culture, widely considered to be the gold standard method; however, the molecular approach in fact produced better sensitivity and specificity results. Moreover, for the 38 samples that tested negative by direct examination but positive by culture, the culture method took a mean of 31 days longer than the PCR method to generate results. The protocol presented here may be very useful for improving routine histoplasmosis diagnosis.

Keywords: *Histoplasma capsulatum*; Diagnosis; Real-time PCR; Culture; Human samples

1. Introduction

First described in 1906 by Darling (1906), histoplasmosis is an infectious disease caused by inhaling the dimorphic fungi *Histoplasma capsulatum* microconidia (Goodman and Larsh, 1967). Two varieties of *H. capsulatum* are pathogenic to humans: *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*. Although histoplasmosis is an endemic tropic systemic mycosis localized in the Americas and parts of Asia and Africa, several cases do occur in nonendemic areas (Wheat, 2006). This disease mostly affects immunodeficient patients, particularly those with HIV. However, some cases of disseminated histoplasmosis in immunocompetent individuals have been described (Benevides et al., 2007; Schestatsky et al., 2006; Washington and Palacio, 2007).

H. capsulatum has been reported in French Guiana since 1952 (Floch, 1953). The number of histoplasmosis cases diagnosed has increased every year since 1990. Sixty-three new cases have been diagnosed in Cayenne Hospital between 2005 and 2007. Histoplasmosis and cerebral toxoplasmosis has become the leading cause of mortality in HIV patients (Couppie et al., 2006; Lewden et al., 2004).

Direct microscopic examination and culture on Sabouraud media are the gold standard method for diagnosis. However, microscopic examination requires a good level of expertise. Moreover, the culture method is very slow (up to 4 months) and is hazardous (due to the presence of spores). Development in molecular biologic and polymerase chain reaction (PCR) techniques has permitted the detection of *H. capsulatum* DNA. Various nested PCR protocols, including the use of a number of target sequences, were

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used to characterize *H. capsulatum* DNA (Bialek et al., 2002a, 2002b; Bracca et al., 2003; Maubon et al., 2007). However, the contamination risk was previously high, particularly with nested PCR. Today, with real-time PCR technology, this risk and reaction times are minimized. Moreover, real-time PCR increases the sensitivity and specificity, and also enables DNA quantification. We developed a real-time PCR protocol using the TaqMan system, as TaqMan probes can be used in all real-time PCR machines, unlike LightCycler® (Roche Molecular Biochemicals, Meylan, France) probes. Also, there are no previous reports of TaqMan probes being used for *H. capsulatum* detection. The primers and the probe chosen were tested with fungal strains and various clinical samples.

2. Materials and methods

2.1. Analytic specificity

We used 20 fungal strains provided by the LHUPM (Cayenne Hospital) and the Pasteur Fungi Collection (Pasteur Institute, Paris) to test for specificity (samples run in triplicate) (Table 1).

2.2. Analytic sensitivity

H. capsulatum var. *capsulatum*-positive cultures were picked up with a tip and placed in a microtube containing 200 μL of sterile water. The DNA was extracted (see DNA extraction below) and standardized at 100 μg/mL by measuring the absorbance at 260 nm with a spectrophotometer (Eppendorf, Hamburg, Germany). Samples were serially diluted 10-fold to obtain solutions ranging from 100 ng to 0.1 fg of genomic *H. capsulatum* DNA per microliter. The sensitivity threshold of real-time PCR (see details below) was analyzed by running these DNA solutions in triplicate.

2.3. Clinical sensitivity and specificity

A total of 348 clinical specimens (bronchoalveolar lavage [n = 43], liver biopsy [n = 30], intestinal biopsies [n = 31], whole blood [n = 48], bone marrow [n = 108], lymphatic nodes [n = 25], cerebrospinal fluid [n = 55], and cutaneous biopsy [n = 8]) collected for the diagnosis of systemic mycosis between 2004 and 2007 at the Unit of Mycology (LHUPM) were included in this study. Samples were directly examined (India ink and May–Grunwald Giemsa staining were used). The samples were divided into 2 groups: those undergoing DNA extraction and thus stored at 4 °C and those undergoing culture. DNA samples were analyzed prospectively for 2005 to 2007; DNAs of year 2004, stored at −80 °C, were run retrospectively. Samples were cultured on Sabouraud agar with chloramphenicol and gentamicin, with and without cycloheximide. Cultures were incubated at 30 or 37 °C for up to 8 weeks.

2.4. DNA extraction

2.4.1. Strains

Fungal colonies were picked up with a tip and placed in a microtube containing 200 μL of sterile water. DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Briefly, after proteinase K incubation, all specimens were subjected to 3 cycles of freezing at −80 °C for 10 min and boiling for 10 min. This step was applied to disrupt cell walls of the fungal organisms. DNA samples were standardized at 2 μg/mL by measuring the absorbance at 260 nm with a spectrophotometer (Eppendorf).

Table 1
Tested strains

Strain	Origin	Collection no.	Real-time PCR result
A. fumigatus	LHUPM, Cayenne		Negative
Candida albicans	LHUPM, Cayenne		Negative
Candida dubliniensis	LHUPM, Cayenne		Negative
Candida parapsilosis	LHUPM, Cayenne		Negative
Candida tropicalis	LHUPM, Cayenne		Negative
Malassezia globosa	LHUPM, Cayenne		Negative
Blastomyces dermatitidis	Pasteur Fungi Collection	UMIP 973.68	Negative
Chrysosporium indicum	Pasteur Fungi Collection	UMIP 1393.82	Negative
C. neoformans var. *neoformans*	Pasteur Fungi Collection	UMIP 1173.78	Negative
Cryptococcus amylolentus	Pasteur Fungi Collection	UMIP 2091.92	Negative
Cryptococcus laurentii	Pasteur Fungi Collection	UMIP 2258.94	Negative
H. capsulatum var. *capsulatum*	Pasteur Fungi Collection	UMIP 632.61	Positive
H. capsulatum var. *duboisii*	Pasteur Fungi Collection	UMIP 638.61	Positive
Malassezia furfur	Pasteur Fungi Collection	UMIP 1634.86	Negative
Penicillium marneffei	Pasteur Fungi Collection	UMIP 560.56	Negative
Penicillium roqueforti	Pasteur Fungi Collection	UMIP 1404.82	Negative
Rhodotorula rubra	Pasteur Fungi Collection	UMIP 2257.94	Negative
Saccharomyces cerevisiae	Pasteur Fungi Collection	UMIP 1432.83	Negative
Sporothrix schenckii	Pasteur Fungi Collection	UMIP 1934.90	Negative
Trichosporon spp.	Pasteur Fungi Collection	UMIP 660.63	Negative