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Short Communication

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Fluorescent *in situ* hybridization of pre-incubated blood culture material for the rapid diagnosis of histoplasmosis

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Abstract

Fluorescence *in situ* hybridization (FISH) has been shown to be useful for the detection of *Candida* and *Cryptococcus* species in blood culture materials. FISH procedures for the detection of *Histoplasma capsulatum* var. *capsulatum* have not been reported so far. This study describes the development and evaluation of fluorescently labeled rRNA-targeting FISH probes to detect and identify *H. capsulatum* in blood cultures. All three analyzed *H. capsulatum* reference strains and clinical isolates showed positive signals with the newly designed specific oligonucleotide probes for *H. capsulatum*, whereas negative reactions were observed for all three nontarget yeast species and the two nontarget bacteria. The assay was also successfully applied for detections of *H. capsulatum* cells in pre-incubated blood culture samples of patients with clinical suspicion of histoplasmosis ($n = 33$). The described FISH-based assay was shown to be easy to apply, sensitive, and specific (compared to polymerase chain reaction) for the detection and identification of *H. capsulatum* in this proof-of-principle analysis. Larger multicentric assessments are recommended for a thorough diagnostic evaluation of the procedure.

Key words: development, FISH, histoplasmosis, molecular probes.

Introduction

Histoplasmosis is a widespread systemic infection that is frequently associated with respiratory symptoms and is caused by the fungus *Histoplasma capsulatum*. *H. capsulatum* can produce disseminated infections associated with high morbidity and mortality in patients with human immunodeficiency virus (HIV)/AIDS. Traditional

microbiological diagnosis is based on microscopic assessment of fluids and tissues, serological techniques, and microbial cultures including blood cultures [1–3].

Blood culture diagnosis is typically performed by continuously reading, automated computed systems that detect microorganism growth. If the microcomputer flags samples as positive, gram stain examination is performed followed by subculture on selective and non-selective agars [4,5]. This traditional assessment (subcultures) is slow in disseminated fungal infections. After the detection of microbial growth by the continuously monitoring blood culture systems, species identification can typically be achieved in 1–3 days for *Candida* spp., 3–5 days for *Cryptococcus* spp., and 10–25 days for *H. capsulatum*. More rapid procedures for the detection of *H. capsulatum* in positive blood culture materials are desirable for an early initiation of an appropriate therapy.

Molecular techniques, typically based on polymerase chain reaction (PCR), have been developed to detect fungal species in a short period of time; these techniques include nested PCR, multiplex PCR, real-time PCR, and microarrays, all of which have shown reliable and fast results [1,3,6–9]. Another alternative molecular diagnostic approach is fluorescent *in situ* hybridization (FISH). Targeting the fungal ribosomal RNA of target cells, FISH is a rapid technique that is easy to apply, requiring no more than standard laboratory equipment including an incubator and a fluorescence microscope [10].

FISH from blood culture materials has been shown to provide reliable results for the detection of *Candida* and *Cryptococcus* spp. [11–14]. However, no FISH procedure for the detection of *H. capsulatum* has been evaluated so far. The aim of this study was the design of a FISH probe targeting *H. capsulatum* and its evaluation with pre-incubated blood culture materials of histoplasmosis patients.

Materials and methods

Blood samples

A total of 33 blood samples from HIV-positive patients with clinical diagnosis of invasive mycosis were investigated. Additionally, blood samples of healthy donors were inoculated with three *H. capsulatum* strains (FMT 1400, FMT 2279, FMT 2283) and other clinically relevant fungal and bacterial species (see Table 1 in the Results and Discussion section) to be used as positive and negative controls, respectively.

Microbial reference strains

The following microorganisms were purchased from the American Type Culture Collection (ATCC; Manassas, VA) or from the National Institute for Amazon Research (INPA) microbial collection and were used for the evaluation of the specificity of oligonucleotide probes: *Candida albicans* ATCC 3623, *Cryptococcus neoformans* (formerly *Cryptococcus neoformans* var. *neoformans*) FMT1420, *Cryptococcus gattii* (*Cryptococcus neoformans* var. *gattii*) FMT1170, *Histoplasma capsulatum* (FMT 1400, FMT 2279, FMT 2283), *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923.

Blood culture assessment

Aerobic blood culture bottles were inoculated with 10 ml blood and incubated in an automated BACTEC 9120 blood culture system (Becton-Dickinson, New Jersey, USA). Incubation was performed according to the manufacturer's recommendations at 35°C. Aliquots of the blood culture suspensions were taken aseptically (needle syringe) from the bottles considered positive by the automated system. One aliquot was used for conventional investigation, one aliquot for PCR and one aliquot for FISH staining.

Conventional methods

Two hundred microliters of the blood culture was transferred to the surface of Sabouraud agar (Becton-Dickinson, Sparks, USA). Colonies growing on Sabouraud agar were identified by standard laboratory methods, including micromorphological and physiological tests (germ tube production, growth on Agar Niger and CHROMagar^{TM} Candida (Becton-Dickinson, Sparks, USA), incubation at 42°C, and testing for carbohydrate and nitrogen assimilation and carbohydrate fermentation) [17].

PCR

The generation of PCR products was performed as described by Sampaio [16]. DNA was extracted from samples (200 μl blood culture) using the QIAamp Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Twenty nanograms of the extracted DNA served as template for PCR amplification. PCR was performed in a total volume of 25 μl containing PCR buffer (final concentration: 1×, 10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 200 nM of primers HcIII (5-GAGATCTAGTCGCGGCCAGGTTCA-3) and HcIV (5-AGGAGAGAACTGTATCGGTGGCTTG-3) (both primers were described by Bialek et al. [18]), 50 μM dNTPs, and 1 U AmpliTaq DNA polymerase. PCR was performed using a thermocycler (Kyratec SuperCycler, Seoul, South Korea) with the following conditions: initial denaturation for 5 min at 94°C, 40 cycles for

Table 1. Blood cultures analyzed by conventional diagnostic methods, PCR, and FISH (probes Hca1 and Hca2).

Blood Culture	Different methodologies applied for *H. capsulatum* detection (Time)*		
	Sabouraud culture†	PCR	FISH/Hca1 and Hca2
Inoculated with *H. cap* FMT 1400	Positive (10 days*)	Positive (**)	+++ (***)
Inoculated with *H. cap* FMT 2279	Positive (7 days*)	Positive (**)	++ (***)
Inoculated with *H. cap* FMT 2283	Positive (12 days*)	Positive (**)	++ (***)
Inoculated with *C. alb* ATCC 3623	Negative	Negative	Negative
Inoculated with *C. neo* FMT1420	Negative	Negative	Negative
Inoculated with *C. gat* FMT1170	Negative	Negative	Negative
Inoculated with *S. aur* ATCC 25923	Negative	Negative	Negative
Inoculated with *E. col* ATCC 25922	Negative	Negative	Negative
Inoculated with *H. cap* + *C. alb* + *C. neo*	Negative	Positive (**)	++ (***)
Patient 14/102	Positive (11 days*)	Positive (**)	++ (***)
Patient 14/504	Positive (11 days*)	Positive (**)	++ (***)
Patient 14/647	Positive (12 days*)	Positive (**)	+ (***)
Others (= 30)	Negative	Negative	Negative

FISH, Fluorescence *in situ* hybridization; *H. cap*, *Histoplasma capsulatum* (FMT 1400, FMT 2279 and FMT 2283); *C. Alb*, *Candida albicans* ATCC 3623; *C. Neo*, *Cryptococcus neoformans* FMT1420; *C. Gat*, *Cryptococcus gattii* FMT1170; *S. Aur*, *Staphylococcus aureus* ATCC 25923; *E. Col*, *Escherichia coli* ATCC 25922; PCR, polymerase chain reaction.

†Colonies investigated by a microscopist especially trained for the identification of H. capsulatum.

*Time necessary for diagnosis after detection of microbial growth by the continuous-monitoring blood culture system.

**Time consumed between 8 and12 h.

***Time consumed between 3 and 5 h.

+bright and intense fluorescence signals of labeled cells;

nd: not determined.

30 s at 94°C (denaturation), 30 s at 70°C (annealing), 90 s at 72°C (extension), and a final extension for 10 min at 72°C. PCR products were visualized by electrophoresis on a 2% agarose gel and stained with SYBR® Green (SYBR Safe DNA Gel Stain, Invitrogen, Carlsbad, USA). A DNA Ladder Mix (SM0331, MBI Fermentas, St. Leon-Rot, Germany) served as the size marker.

FISH

We developed two *Histoplasma capsulatum* specific FISH probes, 5′-labeled with the red sulfoindocyanine dye Cy3, targeting the ribosomal 18S subunit Hca1 (5′-AGTCGAGGCTTTCAGCATGT-3′) and Hca2 (5′-CTGACGACCATTAAGCCAGC-3′). The probes were designed using the Primer 3 software (www.primer3.com) with data extracted from NCBI nucleotide. *In silico* evaluation was performed with the software probeCheck (www.microbial-ecology.net/probecheck/) using the sequence collection program SILVA [20].

The FISH reactions were carried out by using the probes Hca1 and Hca2 separately. Some experiments were carried out by using both probes simultaneously in order to investigate the influence of the interaction on the fluorescent signal. In addition to the probes, all the samples were counter-stained with DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride).

For the investigation of pre-incubated blood cultures, 0.5 ml acetic acid (100%) was added to 5 ml blood culture medium to lyse the erythrocytes. The suspension was centrifuged at 10,000 g for 5 min; the pellet containing cells was washed with 500 μl of phosphate-buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and fixed for 4 h with 4% w/v paraformaldehyde in PBS at 4°C. After fixation, cells were washed twice with PBS and suspended in one volume of PBS and one volume of cold absolute ethanol and stored at −20°C until use.

The FISH assay was carried out as described by Amann [21]. The whole fixed cells were transferred to pre-cleaned microscope slides dried at 37°C for 20 min, resulting in smears. The slides were then covered with the hybridization buffer (0.9 M NaCl, 0.01% w/v SDS, 20 mM Tris-HCl pH 7.2, 30% formamide, and 1 μM probe) and incubated at 46°C for 2 h. After this period, the slides were washed using the wash buffer (20 mM Tris-HCl, pH 8.0, 0.01% w/v SDS, 5 mM EDTA and 150 mM NaCl for 30 min at 46°C). The microscope slides were dried at 37°C for 20 min and were then mounted with Vectashield solution (Vector, Burlingame, CA, USA) and examined with a Zeiss Axioskop 40 microscope (Zeiss, Jena, Germany).

Ethical considerations

Ethical clearance for the study was obtained from the Ethical Committee at the Fundação de Medicina Tropical Heito

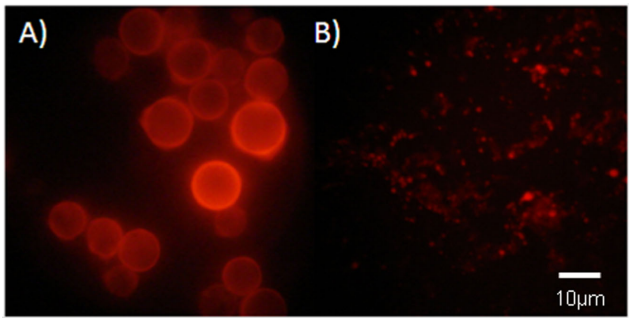

Figure 1. FISH staining of *Histoplasma capsulatum* in a human blood culture sample. Macro conidia (a) and filamentous growth (b) were visualized with the specific probes 5′-labeled with Cy3 (red signal). This Figure is reproduced in color in the online version of *Medical Mycology*.

Viera Dourado, in accordance with Brazilian laws relating to research with human subjects.

Results and Discussion

Artificially inoculated blood culture materials and blood cultures from suspected histoplasmosis patients were examined by culture, PCR, and FISH (Table 1).

The Hca1 and Hca2 probes allowed the identification of *H. capsulatum* in blood cultures. Both probes presented similar fluorescence signals (Table 1). When probes designed to detect the same microorganism present different signals, the complementary target of one of the probes may be located in a low-accessibility region of the respective rRNA region [13]. The utilization of both probes simultaneously increased the fluorescence signal; however, their signal when used separately was sufficient for pathogen detection.

The probes allowed the identification of *H. capsulatum* cells in both pure and mixed samples containing *H. capsulatum* and other fungal species and showed negative reactions with the assessed nontarget microorganisms (Table 1). The detection limit of our Hca1 and Hca2 probe-based FISH assay was estimated to be 10^3 cells/ml by using serially diluted artificially inoculated blood culture samples.

HCa1 and HCa2 were evaluated for the detection of *H. capsulatum* in blood cultures in comparison with culture-based approaches and PCR (Table 1, Fig. 1). Traditional morphological and biochemical identification, PCR targeting a 100 kDa protein and FISH showed identical results in our evaluation (three positive detections between the 33 blood cultures that were investigated). However, the time to diagnosis after the detection of microbial growth by the automated blood culture system was, on average, 4 h, 10 h, and 12 days for the detection by FISH, PCR, and culture-based approaches, respectively. In addition, FISH identification of *H. capsulatum* does not require subcultures, which is a significant advantage for histoplasmosis diagnosis because *H. capsulatum* cultures must be manipulated under high biosafety containment level facilities.

Our results suggest that FISH might be a useful technology for the correct and timely diagnosis of histoplasmosis infections, which is urgently needed [9]. FISH procedures combine the speed, sensitivity, and specificity of molecular diagnostic methods with the direct observation of the morphologic features of *H. capsulatum* in blood culture materials. This combined approach makes FISH particularly valuable for *H. capsulatum* detection from blood culture materials.

Recent publications have demonstrated the reliability of FISH for the diagnosis of *Candida* spp, *C. neoformans* and *C. gattii* [11–14] from blood culture materials. The data from our proof-of-principle assessment suggest that a FISH probe for *H. capsulatum* should be used, at least in endemic areas.

Several limitations reduce the significance of our analyses, requiring further studies. One limitation is the low number of *H. capsulatum*-positive clinical samples. Large multicentric studies for the diagnostic assessment of the Hca1 and Hca2 probes might overcome this problem. Another limitation is the fact that no blood samples were assessed for the detection of *H. capsulatum*. Thus, conclusions regarding the specificity of Hca1 and Hca2 in blood samples cannot be drawn in this way. Accordingly, future studies should include blood samples, other biological samples, and even other detection techniques, including flow cytometry (Flow-FISH). Again, a multicentric approach would be useful to obtain relevant numbers of respective samples.

In conclusion, the present work described how Hca1 and Hca2 probes might contribute to the identification of *H. capsulatum* in blood culture samples, reducing the risk of missing the diagnosis. Due to the lack of information on the specificity of the probe with clinical materials of patients with invasive fungal infections other than histoplasmosis, the results should be interpreted with care. Future multicentric studies might help to close this information gap.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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