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CONCISE ARTICLE

A. Imhof · C. Schaer · G. Schoedon · D. J. Schaer · R. B. Walter · A. Schaffner · M. Schneemann

Rapid Detection of Pathogenic Fungi from Clinical Specimens Using LightCycler Real-Time Fluorescence PCR

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Abstract In the study presented here a LightCycler real-time PCR system was used for the diagnosis of fungal infections from clinical tissue samples. Nine specimens were investigated from six patients with suspected or proven invasive fungal infections. Seven of nine samples were positive in a broad-range fungal PCR assay. In four samples, *Aspergillus fumigatus* was detected both by a species-specific hybridization assay as well as by sequencing of amplification products. In addition, the broad-range fungal PCR assay and PCR sequencing detected and identified, respectively, the following organisms in the specimens noted: *Candida albicans* in a culture-negative liver biopsy, *Histoplasma capsulatum* in a bone marrow sample, and *Conidiobolus coronatus* in a facial soft tissue specimen. Real-time PCR is a promising tool for the diagnosis of invasive fungal infections in human tissue samples and offers some advantages over culture methods, such as rapid analysis and increased sensitivity.

A. Imhof
Program in Infectious Disease, Clinical Research Division,
Fred Hutchinson Cancer Research Center,
1100 Fairview Avenue N, D3-100, Seattle, WA 98109-1024, USA

R. B. Walter
Pediatric Oncology, Clinical Research Division,
Fred Hutchinson Cancer Research Center,
1100 Fairview Avenue N, D2-373, Seattle, WA 98109-1024, USA

C. Schaer · G. Schoedon · A. Schaffner · M. Schneemann (✉)
Department of Medicine, Clinical Mycology, Medical Clinic B,
University Hospital Zurich,
Rämistrasse 100, 8091 Zurich, Switzerland
e-mail: markus.schneemann@usz.ch
Tel.: +41-1-2554038
Fax: +41-1-2554172

D. J. Schaer
Department of Anesthesiology,
University Hospital Zurich,
Rämistrasse 100, 8091 Zurich, Switzerland

Introduction

Invasive fungal infections have become a major cause of infection-related morbidity and mortality after aggressive chemotherapy, organ transplantation, or in patients with immunodeficiency syndromes. Early diagnosis, prompt initiation of appropriate therapy, and restoration of immunocompetence are crucial for the successful management of these life-threatening conditions [1]. However, the timely and accurate diagnosis of fungal infections using conventional microbiologic and histopathologic techniques is time-consuming and difficult due to the suboptimal sensitivity and/or specificity of these methods [2, 3].

Therefore, efforts are ongoing to develop rapid and sensitive non-culture-based diagnostic strategies such as the detection of fungal antigens, fungal metabolic products, and amplification of specific fungal nucleic acid sequences by PCR [4, 5]. Recently, a variety of PCR methods based on the detection of fungal DNA in otherwise sterile human fluids or tissue samples have been described [6, 7, 8, 9, 10], and these appear promising for improving the sensitivity and diagnostic accuracy of invasive fungal infection testing methods. PCR assays that amplify fungi-specific and highly conserved sequences of multi-copy genes (e.g. the 18S rRNA gene) are clinically useful for the detection of fungal infections, whereas assays targeting species-specific sequences are used to identify the pathogen. However, species identification often requires time-consuming blotting procedures with a sequential hybridization reaction [5]. Recently, a quantitative PCR assay utilizing the LightCycler amplification and detection system (Roche Diagnostics, Switzerland) was developed that allowed species determination of fungal DNA in human blood samples, thereby significantly reducing the amount of time and labor required for diagnosis [9].

In this report, we show that this convenient diagnostic approach is also applicable to the analysis of human tissue samples from deep-seated fungal infections. Furthermore, we adapted a PCR assay with broad-range fungal primers

that uses SYBR green for amplification of DNA. Subsequent sequencing of the amplicon allowed identification of the pathogen.

Materials and Methods

A total of nine tissue specimens from liver, lung, brain, bone marrow, or facial soft tissue, were obtained from six patients with suspected or proven invasive fungal infections (Table 1). Underlying diseases were aggressive chemotherapy for hematological malignancies in three patients (no. 1, 2, and 4), long-term therapy with corticosteroids for lupus nephritis in one patient (no. 3), and diabetes mellitus in one patient (no. 6). Patient number 5 had reactivation of histoplasmosis.

To enhance disruption of fungal cell walls, tissue specimens were freeze-thawed four times rapidly by freezing the samples in liquid nitrogen for 1 min followed by thawing at 95°C for 2 min. Samples were then incubated with recombinant lyticase (Sigma, Switzerland) and treated with proteinase K. The lyticase step turned out to be unnecessary for DNA extraction (data not shown) and was omitted in specimens from patients 1, 3 and 6 (Table 1). Instead of the lyticase step, samples were sonicated for 15 min at 47 kHz at room temperature in a sonicator bath (Branson, model 2510). DNA isolation was carried out using the high pure PCR template purification kit (Roche Diagnostics) according to the manufacturer's instructions. As negative controls, ddH_2O and sterile spleen tissue were processed in parallel in each assay. Purified DNA samples were stored at −20°C until analysis.

The LightCycler PCR and detection system (Roche Diagnostics) was used for amplification and real-time quantification of DNA with previously described broad-range fungal primers and a species-specific probe for *Aspergillus fumigatus* [9]. Briefly, for detection of fungal species (broad-range fungal PCR), a primer set (forward primer: 5′-ATT GGA GGG CAA GTC TGG TG; reverse primer: 5′-CCG ATC CCT AGT CGG CAT AG; Microsynth, Switzerland) was used that binds to a highly conserved region of the fungal multicopy 18S rRNA gene [9]. The human beta-globin gene served as an internal control (forward primer: 5′-GAA GAG CCA AGG ACA GGT AC-3′; and reverse primer: 5′-CCA CTT CAT CCA CGT TCA CC-3′). PCR for beta-globin was performed in separate reactions within the same run with SYBR green mix as described previously [11]. For amplicon detection, the LightCycler DNA master SYBR green kit was used according to the manufacturer's instructions. For identification of *Aspergillus fumigatus*, oligonucleotides that hybridize to an internal species-specific sequence of the 18S rRNA gene (probe 1: labeled at the 5′ end with the LightCycler Red 640 fluorophore-TGA GGT TCC CCA GAA GGA AAG GTC CAG C; probe 2: labeled at the 3′ end with fluorescein-GTT CCC CCC ACA GCC AGT GAA GGC; both from TIB MOLBIOL, Germany) were used with the LightCycler DNA master hybridization probe kit as described by the manufacturer.

Amplification reactions were performed in a total volume of 20 µl containing either 1× FastStart SYBR green reaction mix and enzyme or LightCycler hybridization mix and enzyme, 4 mM $MgCl_2$, and 1 µM of each flanking primer for broad-range fungal PCR or additional 1 µM hybridization probes and 2 µl of sample DNA. Preincubation at 95°C for 10 min was followed by 40 cycles of repeated denaturation (1 s at 95°C), annealing (18 s at 62°C), and enzymatic chain extension (30 s at 72°C). Thirty-two samples were run in parallel. Melting-curve analysis was performed in the range of 70–98°C to confirm that a single DNA PCR product was generated from the DNA template. Amplicons obtained with the broad-range fungal PCR were sequenced (Microsynth), and the sequences were compared to 18S rRNA genes available in the GenBank database.

Results and Discussion

Table 1 summarizes the results from microscopy, culture, PCR assays, and sequencing of amplification products for all nine specimens from six patients. Broad-range fungal PCR revealed a positive result in seven of the nine specimens tested. The two PCR-negative samples were also negative in either direct microscopy or culture (Table 1).

The broad-range fungal primer set used herein targets a highly conserved sequence of the fungal 18S rRNA gene [9]. This ribosomal gene was chosen because the rRNA region exists in multiple copies within the fungal genome

Table 1 Results from PCR assays, PCR amplicon sequencing, direct microscopy, and culture performed on nine clinical samples obtained from six patients with suspected or proven invasive fungal infections

Patient no./ sample source	Direct microscopy	SYBR green	Hybridization probe (*A. fumigatus*)	Culture	Sequencing
1					
Brain abscess	mm ++	positive	positive	*A. fumigatus*	*A. fumigatus*
Sinus	mm +	positive	positive	negative	not done
Sinus (not infected)	mm –	negative			
2					
Lung biopsy	mm +++	positive	positive	*A. fumigatus*	*A. fumigatus*
3					
Liver biopsy	ym +, yc ++	positive	not done	negative	*C. albicans*
4					
Eye	negative	positive	positive	*A. fumigatus*	*A. fumigatus*
Lung biopsy	negative	negative			
5					
Bone marrow	yc ++	positive	not done	*H. capsulatum*	*H. capsulatum*
6					
Soft tissue	mm ++	positive	not done	*Conidiobolus* sp.	*C. coronatus*

Mm, mold mycelium; ym, yeast mycelium; yc, yeast cells; +, a few elements; ++, clearly visible structures; +++, crowded samples

[12, 13], thus providing increased amplification of the signal compared to single copy genes. It is important to note that some members of the zygomycetes such as *Rhizopus* and *Rhizomucor* spp. have different 18S rRNA sequences and are not detected with this primer set [14]. Therefore, the term "panfungal" used by others is misleading [6]. Previously, it has been demonstrated that the LightCycler system facilitates the detection of fungal species in human blood with a sensitivity that is comparable to conventional PCR [9]. Herein, we extend these findings by showing reliable detection of fungi in human tissue samples in the clinical setting. This real-time PCR approach further obviates the need for gel electrophoresis, thereby reducing the time required until a result is obtained and minimizing the risk of cross-contamination of amplification products.

Identification of fungal species can be achieved by two different means: (i) by hybridization with species-specific probes or (ii) by direct sequencing of amplicons. Using melting curve analysis, amplicons yielded single products with the following defined melting temperatures: *Aspergillus* spp. 90°C, *Candida* spp. 86°C, *Conidiobolus* spp. 88°C and *Histoplasma* spp. 92°C. Loeffler et al. [8] previously demonstrated accurate identification of fungal species in human blood using the LightCycler-based technique with probes that target an internal species-specific sequence of the 18S rRNA gene. Our data show that this method can successfully be applied to human tissue samples as well. In all three patients with culture-proven *Aspergillus fumigatus* infection, the hybridization assay gave a positive result (Table 1). One sample that was culture-negative gave a positive result by hybridization probe analysis (Table 1).

For six of the nine specimens tested, amplification products were sequenced and the results were compared with the GenBank database. Sequencing results not only confirmed the presence of *Aspergillus fumigatus* infection in these three patients, but was also in agreement with the result of culture in another patient infected with *Histoplasma capsulatum*. Furthermore, the amplicon identified one organism that did not grow in culture (*Candida albicans*), and identified the *Conidiobolus* organism growing in one sample to the species level as *Conidiobolus coronatus*. The zygomycete *Conidiobolus* typically infects mucocutaneous sites to produce sinusitis.

Limitations of PCR include the generation of both false-positive and false-negative results. These issues are addressed in several ways, including standardization of technical procedures and use of internal controls [15]. Impurities in nucleic acid preparations (e.g. ethanol) or in biological samples (e.g. hemoglobin or heparin) can inhibit PCR amplification or reduce its sensitivity. In our study, we used the human beta-globin gene as a positive control to verify successful DNA amplification. False-positive results can stem from contamination of lyticase with fungal DNA, as described previously [9]. In our protocol, lyticase treatment was not necessary for the successful extraction of fungal DNA and could be omitted.

The diagnosis of invasive fungal infections is traditionally established by culture, microscopy of clinical specimens or histology. However, light microscopy of fungal smears is ambiguous in many cases, and culture has a low sensitivity and is time-consuming, requiring up to several weeks until the final diagnosis is reached. Typically, a real-time PCR result is generated within a few hours, which is significantly earlier than culture results. Although the costs of PCR generally exceed those of conventional culture methods and may currently limit its widespread use, earlier and more sensitive diagnosis could eventually decrease the high mortality of fungal infections in immunocompromised patients. Thus, PCR technology, including real-time approaches such as the one presented here, offers valuable options for the rapid detection and identification of fungi.

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