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Application of Real-Time PCR to the Diagnosis of Invasive Fungal Infection

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Abstract

The management of invasive fungal infections has been hampered by the inability to make a diagnosis at an early stage of the disease. Molecular diagnosis by PCR appears very promising since fungal DNA can be detected in the blood of infected patients earlier than when using conventional methods. Recently, interest in the diagnosis of invasive fungal infections by real-time PCR has increased. Real-time methods also have quantitative properties and are useful both for initial diagnosis and to assess the response to treatment. Many recent studies have combined serological tests with measurement of fungal DNA by using real-time PCR. Real-time PCR helps early diagnosis and arrangement of treatment protocols for patients with high risk of fungal infection. Here real-time PCR methods for diagnosis of invasive fungal infections are described and discussed.

Introduction

Rapid tests with high specificity and sensitivity are needed for early diagnosis of invasive fungal infections, which have non-specific clinical signs. The major groups of organisms involved are the *Aspergillus* and *Candida* spp. Blood culture and serology are the conventional methods used for diagnosis of these fungi but they have limited sensitivity, specificity and do not give a rapid indication of current infection. Consequently, ELISAs for detection of antigens (*e.g.* galactomannan and glucan) and nucleic acid detection techniques have come into use for the differential diagnosis and follow-up of fungal infections.

Tests for fungal infection are especially important for transplant recipients and patients with hematological malignancies, who have high mortality and morbidity ratios. New advances in cancer treatment and intense supportive treatment provide longer life for cancer patients but morbidity and mortality ratios increase due to invasive fungal infections. Fungal infection is now the cause of death in about half of the patients with acute leukemia. The most important factor for successful antifungal treatment in cancer patients, is to make a positive diagnosis but this may be difficult. Atypical clinical findings, difficulty in taking samples and insufficient diagnostic methods are basic problems. Although certain clinical signs and symptoms found in appropriate patient groups might indicate fungal infection the correlations are not strong. Different fungal and bacterial infections and even non-infectious conditions may cause the same clinical appearance. For many years culture has been the gold standard for diagnosis of fungal infection but taking samples for culture is problematic. Deep tissue biopsies for culture from patients with thrombocytopenia or pulmonary infiltration are particularly difficult to take. Failure to make an accurate diagnosis may prevent the administration of optimal treatment (Anaissie, 1992; Einsele *et al.*, 1997; Denning, 1998; Van Burik *et al.*, 1998).

Although the use of NASBA for detection of 18S rRNA is promising, most of the nucleic acid-based methods use PCR for detection of fungal DNA and the results have been encouraging (Kami *et al.*, 2001; Loeffler *et al.*, 2001). Often the primers are directed to conserved genes

so that a wide range of fungal pathogens may be detected in a single PCR reaction (Skladny *et al*., 1999). Several studies have shown that PCR has greater sensitivity and specificity than measurement of the plasma (1→3)-β-D-glucan (BDG), latex agglutination tests, ELISA or culture. The main difficulties have been the non-quantitative nature of the PCR results and contamination in reactions that use primers targeting conserved genes (Loeffler *et al*., 1999). In comparison with the conventional PCRs the antigen detection tests are quantitative within a narrow concentration range (Becker *et al*., 2000) and may therefore be superior for monitoring invasive disease.

The PCR based-methods are now being improved by the development of sensitive new assays based on real-time monitoring of amplification products (Kami 2001; Costa *et al*., 2002; Loeffler *et al*., 2002). Potential advantages of real-time PCR for detection of fungal agents are its speed, convenience and accuracy. Furthermore, the quantitative data provided assists in identification of false positives and may be useful clinically. Real-time PCR technology combines rapid *in vitro* amplification of DNA with real time detection and quantification of the target molecules present in a sample. For example, assays based on the LightCycler®, which include identification of the product and quantification, are completed in approximately 45 minutes (Schnerr *et al*., 2001). Real-time PCR appears to be highly sensitive for the diagnosis of invasive aspergillosis (IA) and quantitation has been found to be accurate (Ohgoe *et al*., 1997; Kawamura *et al*., 1999; Latge, 1999; Kami 2001). The sensitivities of real-time PCR, EIA and the BDG assay were 79, 58 and 67%, respectively, with specificities of 92, 97 and 84%, respectively. The real-time assays should assist early diagnosis and the selection of optimal treatment regimens, so reducing the mortality and morbidity associated with invasive fungal infection.

Sample Preparation

Contamination Control

Contamination of PCR reactions with fungal spores and both fungal and bacterial DNA is a recognized problem (Loeffler *et al.*, 1999; Corless *et al.*, 2000). When primers that target conserved sequences are used residual fungal DNA in the reagents used for extraction may lead to significant background problems. Particular caution is required in selecting enzymes derived from fungal sources (Loeffler *et al.*, 1999). It is recommended that separate rooms be used for extraction of fungal DNA, preparation of PCR reagents, setting-up the PCR reactions and for handling the PCR amplicons. The use of aerosol barrier tips and the use of one-way laboratory coats, disposable gloves and masks can also reduce the incidence of contamination. Methods of eliminating amplicons from the environment are also helpful, for example, by UV illumination either with or without 8-methoxypsoralen pre-treatment. Alternatively, amplicons may be eliminated from the PCR reaction before cycling by using the uracil-DNA-glycosylase system. Fungal cultures should be handled in exhaust protective cabinets to avoid the dissemination of spores in the laboratory. It is essential to include multiple negative controls in each run and it has been suggested that one control for every five samples is appropriate (Loeffler *et al.*, 1999).

Extraction of Fungal DNA

There are several different approaches to the extraction of fungal DNA from blood. Most workers start by removing red cells and then extract the white cell pellet. However, fungal DNA can also be extracted from serum, plasma or environmental specimens (Costa *et al.*, 2001; Haugland *et al.*, 2002). We have used proteinase K and lyticase to effect lysis and QIAmp columns for DNA purification (Qiagen, Crawley, UK). The protocol is included later in this chapter.

Target Sequences, Probes and Primers for Invasive Fungal Infection

Although some workers have used single copy genes as targets for fungal pathogens most attention has focused on conserved multi-copy genes, such as ribosomal RNAs, where it is possible to select primers that amplify all the major fungal species associated with invasive disease. Sufficient variation between rRNA sequences is present such that the organism may be identified to the species level from the internal sequence of the PCR amplicon. Either of the two classes of rRNA present in fungal cells, the nuclear 18S or the mitochondrial 16S may be targeted. The mitochondrial gene is present in greater abundance (*i.e.* one per mitochondrion) but there may be specificity problems associated with using this gene.

The primers shown in Table 1 bind to regions of the 18S rRNA gene, which are highly conserved throughout the whole fungal kingdom and enable the detection of all medically relevant fungal pathogens. The amplicons produced are between 490 bp and 504 bp in size, depending on the fungal species and include a variable region. Hybridization probes that detect the clinically most relevant fungal pathogens, *A. fumigatus* and *C. albicans* have been described (Loeffler *et al.*, 2000). The *A. fumigatus* probe is not species-specific but the other fungal species that match perfectly, including several other *Aspergillus* species are not known human pathogens. The probes hybridise to the *A. fumigatus* and *C. albicans* amplicons at adjacent sites, separated

Table 1. Primers and probes for fungal real-time PCR.

Oligonucleotide	Specificity	Sequence (5' to 3')
Forward primer (18SF)	Fungal	ATTGGAGGGCAAGTCTGGTG
Reverse primer (18SR)	Fungal	CCGATCCCTAGTCGGCATAG
Hybridization probe acceptor	*A. fumigatus* *C. albicans*	**LC**-TGAGGTTCCCCAGAAGGAAAGGTCCAGC **LC**-TGGCGAACCAGGACTTTTACTTTGA
Hybridization probe donor	*A. fumigatus* *C. albicans*	GTTCCCCCCACAGCCAGTGAAGGC -**F** AGCCTTTCCTTCTGGGTAGCCATT –**F**

LightCycler Red 640 (**LC**) and fluorescein (**F**).

by two bases. On hybridisation the fluorescein and LightCycler Red 640 fluors are brought into close proximity and the efficiency of FRET increases.

Standards for Fungal Real-time PCR

A. fumigatus conidia (Figure 1) grown on Sabouraud Dextrose Agar (SDA) can be used as an external standard for real-time PCR. Conidia are counted and then spiked into uninfected blood to provide useful extraction and PCR efficiency controls.

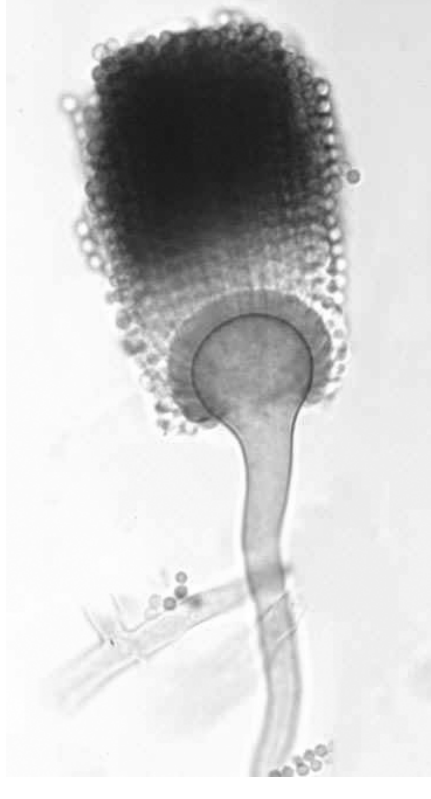

Figure 1. The micrograph shows an *Aspergillus fumigatus* conidial head. The vesicle is approximately 40 microns in diameter.

Protocols

Preparation of *A. fumigatus* Standards

Aspergillus fumigatus was cultured on SDA for 72 h at 30°C. Aspergillus colonies were quantified by hemocytometry and diluted to 10^1-10^6 CFU/ml and then spiked into blood samples from healthy volunteers for use as a quantified standard. Colonies grown in this way are also suitable for the preparation of a naked DNA positive control as described below.

Fungal Nucleic Acid Extraction from Blood

All solutions should be prepared using molecular biology grade reagents and plastics, before dividing into aliquots of suitable size ready for use. Proteinase K stock (Roche Diagnostics, Mannheim; 20 mg/ml) may be stored at –20°C. Defrosted protease K is stable for at least 2 months when stored at 2-8°C and should not be subjected to multiple freeze/thaw cycles All manipulations should be performed in class II cabinets to reduce the possibility of contamination.

Mix EDTA treated blood (1 ml) with 5 ml RCLB (red cell lysis buffer containing 10 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$ and10 mM NaCl) and incubate on ice for 10-15 minutes. Centrifuge the lysate (3000g for 10 minutes) in a sealed rotor bench centrifuge, discard the supernatant and then add fresh RCLB to the pellet, incubate and recentrifuge to collect the cell pellet. Resuspend the pellet in 1 ml WCLB (white cell lysis buffer is RCLB with proteinase K stock solution added to a final concentration of 200 μg/ml) in a microfuge tube and incubate at 65°C for 45 minutes. Centrifuge at 3000g for 10 minutes and then discard the supernatant. Resuspend the pellet in 50 mM NaOH (200 μl) and then cover with a drop of mineral oil and incubate at 95°C for 10 minutes. Fungal material is recovered by centrifugation (3000g, 10 minutes), resuspended in lyticase solution (0.5 ml containing 300 μg/ml lyticase [Sigma, L-2524], 50 mM Tris, pH 7.5, 10 mM EDTA, and 28mM β-mercaptoethanol) and incubated at 37°C for 30 minutes to produce spheroplasts. The spheroplasts are harvested by centrifugation (10,000g

in a sealed rotor microcentrifuge for 10 minutes). Cell lysis in ATL buffer plus proteinase K (180 µl ATL and 20 µl stock proteinase K) and DNA extraction is done using the QIAamp DNA mini kit (Qiagen, Crawley, UK) reagents and tissue protocol. The eluted nucleic acid is stored at -70°C.

Real-Time PCR

Two examples of PCR protocols are given below:

SYBR Green I Method

The reaction components for the SYBR Green I assay for the LightCycler™ is shown in Table 2. Nine µl aliquots of this mix are transferred into separate tubes or wells and 1 µl of sample DNA is added. Many alternative formulations will give equivalent results. For example, the BioGene master mix and SYBR Green I can be replaced by the Roche LightCycler™ DNA Master SYBR Green I mix with appropriate volume adjustments.

The total volume (*i.e.* 10 µl) is carefully pipetted into a LightCycler capillary and the lid is applied loosely. The reaction mixture is spun to the bottom of the capillary by gentle centrifugation (2000 rpm for

Table 2. Mix for 10 µl LightCycler™ reactions using the SYBR Green I method.

Reagent	Volume (µl)
Master mix (BioGene, Kimbolton)*	5.0
H_2O (Sigma, Poole; PCR grade)	2.5
Primer mix (5 µM)	1.0
SYBR Green I (BioGene, Kimbolton; 1:1000)	0.5
Total volume	9

* These mixes contain buffer salts, stabilizer and polymerase. Versions are available containing different concentrations of magnesium ions. For the fungal 18S rRNA assay the final magnesium concentration was 3mM.

5 second) and then the lids are sealed. The capillaries are loaded into the LightCycler and run under the following conditions:

- Initial denaturation for 1 min at 95°C.
- Forty amplification cycles comprised of the following steps: denaturation at 95°C for 0s, annealing at 60°C for 5s and extension at 72°C for 10s with fluorescence acquisition in the F1 channel (530 nm). Maximum ramp rates are used throughout.
- Melting curve analysis follows amplification directly and is comprised of a maximum rate temperature transition to 45°C held for 5s and then a transition to 95°C at a rate of 0.2°C/s with continuous monitoring of fluorescence in the F1 channel.
- Finally the samples are cooled to 40°C prior to removal from the instrument.

The melt temperatures for the fungal 18S rRNA PCR products should be in the range 84-86°C. If required each reaction can be made in a total volume of 20µl providing the concentrations of reagents are maintained.

Hybridisation Probe Method

The master mix for detection of the fungal 18S gene using hybridization probes is shown in Table 3. Equal volumes of master mix and template are added to each capillary as described above. The amplification

Table 3. The master mix for detection of the fungal 18S gene using hybridization probes.

Reagent	Volume (µl)	Final
Roche LightCycler FastStart DNA Master Hybridization Probes	2	1X
$MgCl_2$ (25 mM)	1.6	3 mM
Primers (12 µM each)	1	0.6 µM each
Probes (30 µM each)	2	3 µM each
H_2O (PCR grade)	3.4	
Total volume	10	

program was as follows:

- Denaturation for 9 min at 95°C. This step is necessary to activate the hot start *Taq* polymerase.
- Forty-five amplification cycles comprised of the following steps: denaturation at 95°C for 1s, annealing at 54°C for 15s with fluorescence acquisition in the F2 channel (640 nm) and finally extension at 72°C for 25s. Maximum ramp rates are used throughout.
- Finally the samples are cooled to 40°C prior to removal from the instrument.

Separate reactions are run for detection of either *A. fumigatus* and *C. albicans* containing the respective probes. It is not necessary to determine the melting temperature of the probe/amplicon complex to confirm that the expected product has been formed due to the specificity of the probe reactions.

Analysis for Quantification

For each run in which quantification is required extracts of the *A. fumigatus* standards should be included run in parallel reaction tubes. A standard curve of crossing threshold cycle number against log CFU is plotted and used to in the calculation of the number of copies of the target sequence in the samples.

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