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Development and validation of a molecular method for the diagnosis of medically important fungal infections

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SUMMARY

The increasing incidence of severe fungal infections highlights the need for rapid and precise identification methods in clinical mycology. The aim of this study was to develop and validate a culture-indipendent molecular approach that could allow the detection of fungal pathogens in clinical samples, with particular attention to the identification of drug-resistant *Candida* and *Aspergillus* species. A real-time multiplex PCR assay was developed using TaqMan probes specific for highly discriminating ITS sequences. In its multiplex format the assay showed a high specificity, clearly discriminating among different species, as well as a high sensitivity (20 CFU/1 mL sample), making it a potentially useful starting point for the development of a more complete molecular diagnostic assay.

KEY WORDS: Real-time PCR, Invasive fungal infections, Drug-resistant fungal species

In the last decades the increasing number of at-risk patients has led to a higher incidence of opportunistic fungal infections. Moreover, the emergence of uncommon fungal pathogens constitutively resistant to some antimycotic drugs, makes a rapid and specific diagnosis crucial for guiding early effective therapy (Marr, Carter *et al.*, 2002; Martino, Subira *et al.,* 2002; Pfaller and Diekema 2004; Nucci and Marr 2005; Zaoutis, Argon *et al.*, 2005, Mancini *et al.*, 2005).

Current microbiological diagnostic methods in fungal infections rely on several parameters such as serological assays (fungal antigen detection) and, for definitive species identification, morphological and biochemical characteristics of cultured fungi (Kaufman 1992; Yoshida, Obayashi *et al.,* 1997; Merz and Roberts 2003; Alexander and Pfaller 2006). Even though these approaches represent the cornerstone of fungal microbiological diagnosis, they have some disadvantages: they are time-consuming and rely on experienced laboratory staff. Moreover, a conventional mycological identification approach may be elusive in some cases, especially when morphological characteristics are not easily differentiated. Therefore there is an increasing move towards molecular approaches to be used as a complement to the traditional approach.

The past decade has seen many advances in fungal molecular diagnostics, and the PCR assay is one of the most useful. In contrast to other methods, a PCR-based approach has the potential of being time-saving, highly specific and endowed with a good sensitivity (Atkins and Clark 2004). Common molecular targets for rapid detection and identification of fungal pathogens are sequences within the ribosomal DNA (rDNA)

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gene complex. This section of genome includes the variable DNA sequences of the internal transcribed spacer (ITS) regions. Although not translated, the ITS coding regions have a critical role in the development of functional rRNA (Musters, Boon *et al.,* 1990; Lalev and Nazar 1999; Iwen, Hinrichs *et al.,* 2002), and their variability among fungal species shows promise as "signature regions" for molecular species-specific identification (Iwen, Hinrichs *et al.,* 2002; Pryce, Palladino *et al.,* 2003; Leinberger, Schumacher *et al.,* 2005, Mancini *et al.*, 2005, Mancini *et al.*, 2006). Current molecular methods targeting the ITS regions for the identification of fungi include the use of genus- or species-specific primers and probes, restriction length polymorphism of amplified DNA or direct sequence analysis of amplified DNA. The breadth of application shows that ITS regions have great potential as targets in molecular-based assays for the identification of fungi at the species level.

Given this background, the aim of the study was the development of a real-time PCR assay as a rapid method for fungal detection and identification. Starting from sequences of reference strains in the *GeneBank* database (NCBI, National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov) and of clinical isolates present in the culture collection of our institute, we designed a pair of slightly degenerated universal fungal primers and a probe targeting conserved ribosomal 5.8S coding region (*Panfungal* Real-time PCR) and two different pairs of slightly degenerated primers and several species-specific probes recognizing highly discriminating ITS 2 regions of different *Candida* and *Aspergillus* species. Thermodynamic features of primers and probes were analysed using tools available on the following web sites: Integrated DNA Technologies (*www.idtdna.com/analyzer/Applications/OligoAnalyzer*); Primer3 (*frodo.wi.mit. edu/cgi-bin/primer3/primer3_ www.cgi*); Hyther (*ozone2.chem.wayne.edu/ Hyther/hytherm1main. html*). Primer and probes used are free of secondary structures, have appropriate melting temperature and do not cross-react with other species. Primers and probes are designed following rules described elsewhere (Dorak 2006). Different probes were tagged with different fluorophores to allow their use in a multiplex assay.

The assay was designed to promptly identify mycotic genera or species constitutively resistant to antifungal agents. In particular, for *Candida* spp. three different probes were designed able to distinguish resistant (*C. glabrata* and *C. krusei*) from other usually susceptible species (*C. albicans, C. tropicalis, C. parapsilosis*).

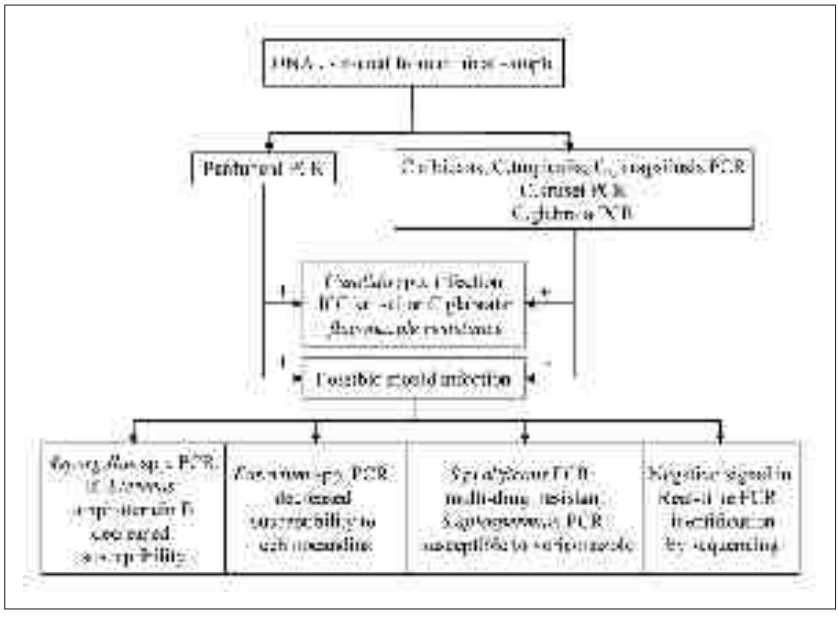

FIGURE 1 - *Possible clinically-oriented flow-chart in the molecular diagnosis of invasive fungal infection using the described molecular approach.*

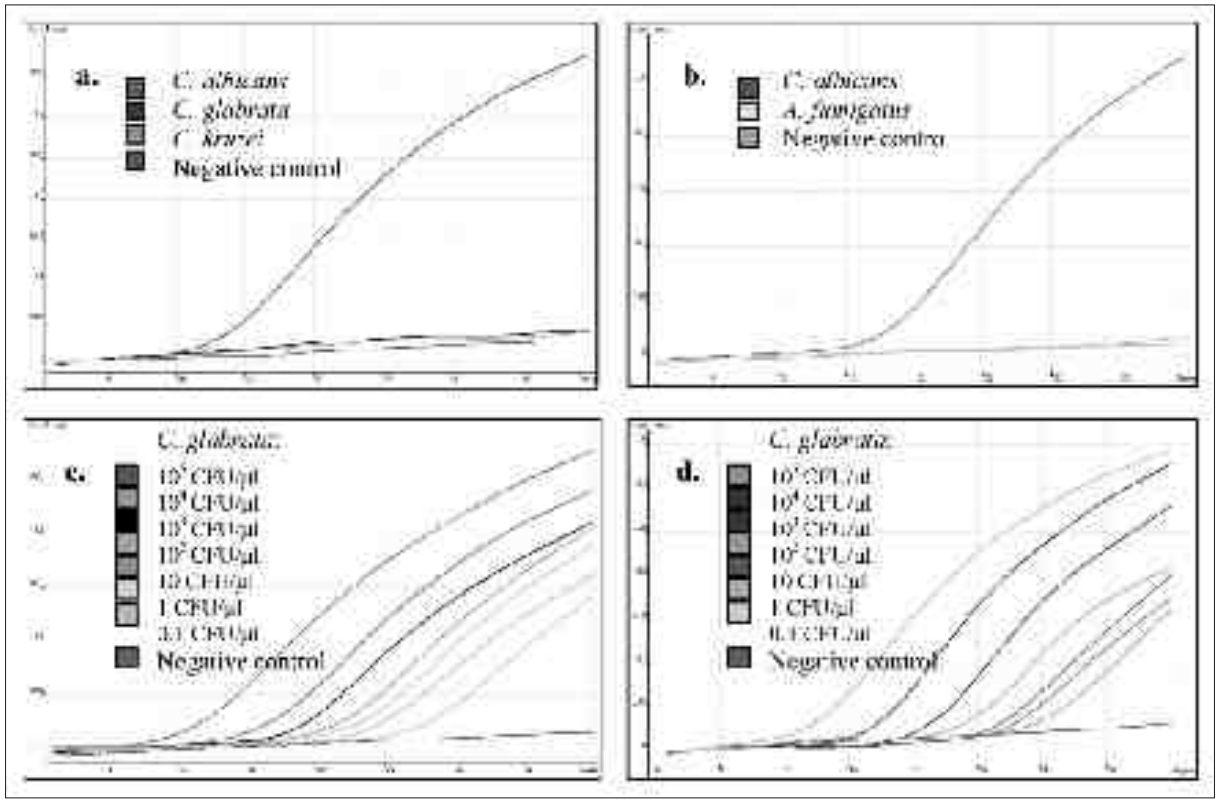

FIGURE 2 - Candida *spp. Real-time PCR.* (a-b) CaATP probe specificity. *CaATP probe is specific for* C. albicans, C. tropicalis, C. parapsilosis*. Its specificity was analyzed in Real-time PCR using DNA from* C. albicans, C. glabrata, C. kruzei ***(a)*** *and* A. fumigatus ***(b)***. *Data obtained show that CaATP probe specifically recognized its own target, that is* C. albicans *(red line in a.; dark green line in b.) and did not cross-react either with DNA from other species of* Candida *or with DNA from mould.* (c) Candida spp. Real-time PCR sensitvity. Candida *spp. Real-time PCR sensitivity was tested on serial dilutions of* C. albicans, C. tropicalis, C. parapsilosis, C. kruzei, C. glabrata*. Data in figure 2c show the assay sensitivity for* C. glabrata*: the assay detects fungal DNA with a sensitivity of 20 CFU/sample. Similar results were obtained for the other species of* Candida*.* (d) Candida spp. multiplex Real-time PCR sensitivity. Candida *spp. specific probes can be used together in a multiplex Real-time PCR without affecting assay sensitivity. Data in figure 2d show the assay sensitivity for* C. glabrata*; the reaction detects 20 CFU/sample. Similar results were obtained performing the assay on* C. albicans *and* C. kruzei*.*

As far as moulds are concerned, among *Aspergillus* spp. two different probes were designed to differentiate *A. terreus* from other species. These probes were designed to be used, in their final format, according to a highly flexible clinically-oriented flow-chart, as represented in Figure 1.

For pan-fungal PCR we used degenerated universal primers and a TaqMan probe hybridizing on a conserved regions of the 5.8S sequence. The assay was validated on extracted genomic DNA (QIAamp® DNA Mini Kit- QIAGEN) obtained from reference and clinical isolates of *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata, C. kruzei, C. guillermondi, C. lusitaniae, A. fumigatus, A. terreus, S. prolificans, S. apiospermum, F. solani, F. oxysporium*. For species-specific PCR assay we used universal degenerated primers hybridizing on conserved regions of fungal rDNA flanking the ITS2 region, and species-specific TaqMan probes hybridizing on specific sequences of ITS2. The assay was validated using genomic DNA from *C. albicans*, *C. tropicalis*, *C. parapsilosis, C. kruzei, C. glabrata*; *A. fumigatus* and *A. terreus*.

Each reaction was performed in 25 µl solution containing 2.5 µl of DNA template, 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen; Carlsbad,CA) (2X: 60 U/ml

Platinum® *Taq* DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, 40 U/ml UDG and stabilizers), 400 nM primers and 800 nM probe. All reactions were optimized to be performed under the same conditions: hold at 50°C for 2 min, hold at 95°C for 8 min, followed by 40 cycles with a denaturation step at 95°C for 30 s, an annealing step at 56°C for 1.5 min. Fluorescence was acquired during the annealing step. All the amplification reactions were performed using the Rotor-Gene 6 (Corbett Research, DiaTech).

In order to investigate the specificity of the species-specific probes, all of them were tested with DNA from *C. albicans*, *C. kruzei, C. glabrata, A. fumigatus* and *A. terreus* (Figure 2). We found that each probe specifically recognized its target and did not cross-react either with DNA from other *Candida* spp. or with DNA from mould species. Similarly, *Aspergillus* spp. probe did not react with DNA from other moulds and yeasts.

In order to investigate the sensitivity of the panfungal and the species-specific assays, DNA was extracted from 10-fold serial dilutions of cultured *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. kruzei*, *C. glabrata*, *A. fumigatus* and *A. terreus* containing a known amount of CFU (*colony forming units*). In particular, the sensitivity of the panfungal assay was evaluated on *C. albicans* and *A. fumigatus*. The sensitivity of the species-specific assays was determined by using the fungal species recognized by each probe. *Candida* spp. real-time PCR sensitivity was evaluated on 10-fold serial dilutions of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. kruzei*, *C. glabrata*. *Aspergillus* spp. real-time PCR was determined on 10-fold serial dilutions of *A. fumigatus*. The data obtained show that the panfungal assay, the *Candida* spp. assay and the *Aspergillus* spp. assay all share a high sensitivity allowing the detection of as few as 20 CFU in a 1 mL sample (Figure 2).

The probes specific for the different species belonging to *Candida* genus were also tested in a multiplex assay without affecting the overall sensitivity of the assay. This will detect and identify the five *Candida* species in a single reaction. The approach described is endowed with high sensitivity, specificity and reproducibility, and its high-throughput design could allow its use as a useful complement in the rapid identification of fungi at the species level. Indeed, the molecular specificity shown by the approach could also allow its use directly on clinical samples even if contaminated by resident flora. Finally, the sequences used in this assay and its multiplex approach, could also prove very useful for the design of future microarray-based assays which could allow the rapid identification of a broader panel of medically important fungi.

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