

Monochrome LightCycler PCR assay for detection and quantification of five common species of *Candida* and *Aspergillus*

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Invasive fungal pathogens, especially in immunocompromised hosts, can result in life-threatening infections. Current laboratory/radiological methods for fungal identification are time-consuming and lack sensitivity and specificity. A monochrome, multiplex, real-time PCR assay for the identification and quantification of *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Aspergillus flavus* and *Aspergillus fumigatus* is described here. Detection of each of these fungi was specific and demonstrated 100 % concordance with biochemical/culture identification in all 60 isolates tested. Samples from 16 febrile neutropenic patients with haematological malignancies were also analysed and the utility of the assay in clinical samples was reconfirmed without false-negative results. The sensitivity of this assay was 0.1 pg fungal genomic DNA, corresponding to three cells, for *C. albicans*, *C. krusei*, *C. tropicalis* and *A. flavus*, and 0.01 pg fungal genomic DNA, i.e. less than one cell, for *A. fumigatus*. The analysis allows a low-cost, simple, rapid and sensitive alternative for clinical identification and quantification of these five common fungal species.

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INTRODUCTION

Invasive fungal infections complicate immunocompromised states that can occur secondary to cancer chemotherapy, organ transplantation and human immunodeficiency virus infections. *Candida* and *Aspergillus* infections are common opportunistic infections in this group of patients (Barnes *et al.*, 1996; Coleman *et al.*, 1998; Krcmery *et al.*, 1999; Dasbach *et al.*, 2000). Occasionally, resistance to commonly used antifungal agents has been encountered (Newman *et al.*, 1994; Rex *et al.*, 1995; Nguyen *et al.*, 1996; Pfaller *et al.*, 1998). In this scenario, early, simple, rapid and accurate identification and quantification of pathogenic fungi are critical for timely and appropriate management of affected patients.

Conventional diagnostic tests for fungal infections are based on radiological findings, cultures and histopathological examination of suspected tissues, which entail some delay and may be inaccurate. Serological tests lack specificity and sensitivity and are limited by the patient's inability to mount an antibody response, due to the underlying immunosuppression.

Several studies have discussed the usefulness of non-culture-based, non-invasive methods for fungal detection and identification (Mitchell *et al.*, 1994; Gottfredsson *et al.*,

1998; Reiss *et al.*, 1998; Walsh & Chanock, 1998). PCR-based methodologies have demonstrated high specificity and sensitivity for detection and quantification of *Candida* and *Aspergillus* species (Yeo & Wong, 2002; Atkins & Clark, 2004). Real-time PCR assays decrease the risk of carry-over contamination, as the tests are conducted in a closed system and no laborious post-PCR analyses are required. An enzymic method for the prevention of contamination, based on uracil-DNA glycosylase, further increases the reliability of the test and the turnaround time is less than 1 h (Bretagne *et al.*, 1995).

PCR methodologies that record fluorescence in real time when samples pass photodetection diodes within the instrument have been described. This can be achieved by using double-stranded DNA-specific dyes, such as SYBR green, or by sequence-specific fluorescence resonance-energy transfer (FRET) hybridization probes. The sensitivity of this technique is better than or similar to that of other PCR methods (Yeo & Wong, 2002). Identification by using this technique, however, needs a labelled probe for each species.

In our laboratory, we successfully developed an alternative assay that is species-specific, sensitive and rapid for identification and quantification of *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Aspergillus flavus* and *Aspergillus*

fumigatus. This assay consists of a panel of two duplex and one single monochrome LightCycler (Roche) PCRs. It utilizes species-specific primers and SYBR green, without the need for expensive, sequence-specific hybridization probes.

METHODS

Fungal isolates. In total, 60 isolates of *C. albicans* (n = 11), *C. krusei* (n = 10), *C. tropicalis* (n = 11), *A. flavus* (n = 10), *A. fumigatus* (n = 11), *Candida glabrata* (n = 1), *Candida parapsilosis* (n = 1), *Candida famata* (n = 1), *Cryptococcus albidus* (n = 1), *Trichosporon capitatum* (n = 1), *Alternaria* sp. (n = 1) and *Aspergillus ferreus* (n = 1), including seven reference strains, were used in this study (Table 1). These isolates were kindly provided by the Laboratory of Mycology, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia.

Fungal DNA isolation. DNA was isolated by using the Puregene DNA purification system (Gentra). Two loops of fungal biomass from 3-day-old cultures were mixed in cell-suspension solution and the manufacturer's recommendations for DNA isolation were followed.

To assess clinical applicability, the sensitivity of the assay was further determined in starting materials that mimicked clinical samples. To achieve this, two loops of fungal biomass from 3-day-old cultures were mixed into 2 ml peripheral blood from healthy donors. Fungal genomic DNA was isolated by using the Puregene DNA purification system (Gentra) with some modifications. After lysing red blood cells with red blood cell lysis solution, white cells were lysed with 2 ml cell-lysis solution and centrifuged at 12 000 g for 5 min. The pellet, containing fungal cells, was resuspended in 300 µl cell-suspension solution and

treated with 6 U lytic enzyme, following the manufacturer's recommendations. DNA concentrations were measured with a spectrophotometer and DNA was stored at -80 °C.

Clinical samples from 16 febrile and neutropenic paediatric patients with haematological malignancies were obtained after Institutional Review Board approval. These samples were leftovers from diagnostic samples and were processed as for the spiked blood samples.

Primer design. The primers targeted the rRNA gene, which is distinct for each fungal species. In total, 64 specific primers were tested: 58 primers were designed based on the rRNA gene sequence (GenBank accession numbers are given in Table 2) and six primers were described by Luo & Mitchell (2002). Specificity of each pair of primers, with no cross-reaction between species, was tested by standard PCR and confirmed by real-time PCR.

Conventional PCR and preparation of DNA standard. HotStarTaq polymerase (Qiagen) with individual primer pairs was used to perform block PCR to amplify specific products, yielding a distinct-sized amplicon for each species (Table 2). Each 25 µl reaction contained 10 ng DNA, 3 mM MgCl₂, 200 µM each dNTP, 0.5 U enzyme and 12.5 pmol each primer. PCR conditions were 10 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1.5 min and a final extension step of 72 °C for 7 min. Amplified products were visualized in 3 % agarose gels. Each specific amplicon was cloned into the PCR 2.1 vector (TOPO TA cloning system; Invitrogen). Plasmid DNA containing the fungal fragment was purified by using a QIAprep Spin Miniprep kit (Qiagen) as described by the manufacturer. Serial tenfold dilutions of plasmid DNA were prepared, containing from 1 pg to 0.01 fg, representing about 200 000–2 copies of the corresponding gene.

LightCycler-based PCR assay. Five species were tested in two duplex and one single real-time assay. Duplex PCR panel 1 contained two pairs

Table 1. Fungal strains used in this study

All clinical isolates were available from the Mycology Laboratory, King Faisal Specialist Hospital and Research Center.

Organism	n	Real-time PCR	Cross-reaction
<i>Candida albicans</i>			
ATCC 14053	1	<i>C. albicans</i> +	—
Clinical isolates	10	<i>C. albicans</i> +	—
<i>Candida tropicalis</i>			
ATCC 13803	1	<i>C. tropicalis</i> +	—
Clinical isolates	10	<i>C. tropicalis</i> +	—
<i>Candida krusei</i>			
ATCC 14243	1	<i>C. krusei</i> +	—
Clinical isolates	9	<i>C. krusei</i> +	—
<i>Aspergillus fumigatus</i>			
ATCC 14110	1	<i>A. fumigatus</i> +	—
Clinical isolates	10	<i>A. fumigatus</i> +	—
<i>Aspergillus flavus</i> clinical isolates	10	<i>A. flavus</i> +	—
<i>Candida glabrata</i> ATCC 90030	1	—	—
<i>Candida parapsilosis</i> ATCC 90018	1	—	—
<i>Candida famata</i> clinical isolate	1	—	—
<i>Cryptococcus albidus</i> ATCC 10666	1	—	—
<i>Trichosporon capitatum</i> clinical isolate	1	—	—
<i>Alternaria</i> sp. clinical isolate	1	—	—
<i>Aspergillus ferreus</i> clinical isolate	1	—	—

Table 2. Primer pairs designed to amplify DNA specifically from the listed species of pathogenic fungi

	Primer sequence (5'→3')	Description*	Amplicon size (bp)	Melting temp. (°C) (mean ± SD)	GenBank accession no.
<i>C. albicans</i>					
Forward	TTTATCAACTTGTCACACCAGA	ITS1	273	85.7 ± 0.7	L47111
Reverse	ATCCCGCCTTACCACTACCG	ITS2			L28817
<i>C. tropicalis</i>					
Forward	CAATCCTACCGCCAGAGGTTAT	ITS1	357	83.5 ± 0.9	AF287910
Reverse	TGGCCACTAGCAAAATAAGCGT	ITS2			AF268095
<i>C. krusei</i>					
Forward	GATAGAGGCCTACCATGGTTTG	SSU rRNA	429	87.1 ± 0.8	M60305
Reverse	AAGGCCTGCTTTGAACACTC	SSU rRNA			M55528
<i>A. fumigatus</i>					
Forward	TATGCAGTCTGAGTTGATTATCG	ITS1	359	92.7 ± 0.8	AF176662
Reverse	ACCTTAGAAAAATAAAGTTGGGTG	ITS2			AF138288
<i>A. flavus</i>					
Forward	CTCCACCCGTGTTTACTGT	ITS1	199	92.8 ± 0.8	AF138287
Reverse	GCGTTCTTCATCGATGCCT	5.8S rRNA			AF027863

*ITS, Internal transcribed spacer; SSU, small subunit.

of primers that identify *C. albicans* and *A. flavus*, whilst duplex PCR panel 2 included primers that are specific for *C. krusei* and *C. tropicalis*. The *A. fumigatus* pair of primers was used singly.

A LightCycler FastStart DNA master SYBR green kit (Roche) was used in a 10 µl reaction containing 3.5 mM MgCl₂, 2.5 pmol each primer and 1 µl DNA. Samples were run in triplicate. The program consisted of a denaturation step (95 °C for 10 min), followed by 45 cycles of touch-down PCR (10 s at 95 °C, 5 s at 64–61 °C and 10 s at 72 °C) and a melting-curve step (50–98 °C). The experiment was completed in approximately 55 min. The PCR process was monitored by fluorescence quantification of the DNA-binding dye SYBR green for detection of double-stranded amplified DNA in real time and the melting analysis allowed identification of the species by a characteristic melting profile.

RESULTS AND DISCUSSION

The increasing frequency of fungal infections and high mortality associated with disseminated fungal disease has underlined the importance of rapid detection of pathogenic fungi. Early identification and quantification of fungal load should improve the outcome of invasive fungal disease, as well as guide physicians to rationalize the use of antifungal agents. Conventional identification methods, based on phenotypic features, are often time-consuming, not very sensitive and subjective. In contrast, molecular approaches based on PCR are usually quicker, highly sensitive and more objective. Indeed, several reports have demonstrated the potential use of these new methods in identification and/or quantification of infectious agents (reviewed by Atkins & Clark, 2004).

Real-time PCR has been shown to be a highly sensitive and specific diagnostic tool for the detection of fungi, which also allows quantification, albeit expensive (Hsu *et al.*, 2003; Pryce *et al.*, 2003; Selvarangan *et al.*, 2003; White *et al.*, 2003). To minimize costs and expand the application of real-time

PCR, we developed a novel, probe-free, real-time PCR assay consisting of a tandem of two duplex reactions and one single reaction. This multiplex test is based on species-specific primers and SYBR green dye, without the need for dual-labelled fluorogenic oligonucleotides. The LightCycler system allows analysis of the melting temperature of amplicons, which depends on the G+C content, sequence length and compositional variation in nucleotide bases, and facilitates identification.

We initially used 10 isolates (including the reference strains listed in Table 1), two each of *C. albicans*, *C. krusei*, *C. tropicalis*, *A. flavus* and *A. fumigatus*, to standardize conventional PCR conditions and specificity of the primers to avoid any cross-amplification. Sixty-four specific primers were tested to select the best primer pairs, considering both specificity and sensitivity. Primers were designed to yield amplicons of different sizes, each one characteristic of one species (Table 2). Primer pairs were then selected based on specificity, demonstrating no cross-amplification between species. These primers were finally tested by real-time PCR to determine their distinct melting profile. Thus, primers that yielded optimal results for each fungal species and provided discriminatory melting profiles to allow multiplex reactions (Fig. 1) without compromising the sensitivity for each species were selected and are listed in Table 2. The melting temperatures for *C. albicans*, *C. krusei*, *C. tropicalis*, *A. flavus* and *A. fumigatus* were 85.7 ± 0.7, 87.1 ± 0.8, 83.5 ± 0.9, 92.8 ± 0.8 and 92.7 ± 0.8 °C, respectively (Table 2), when assayed individually.

Based on their melting profiles, we designed the real-time PCR assay as two duplex reactions, one for identification of *C. albicans* and *A. flavus* (easily discriminated by a difference of 7 °C) and the second for *C. krusei* and *C. tropicalis* (with a

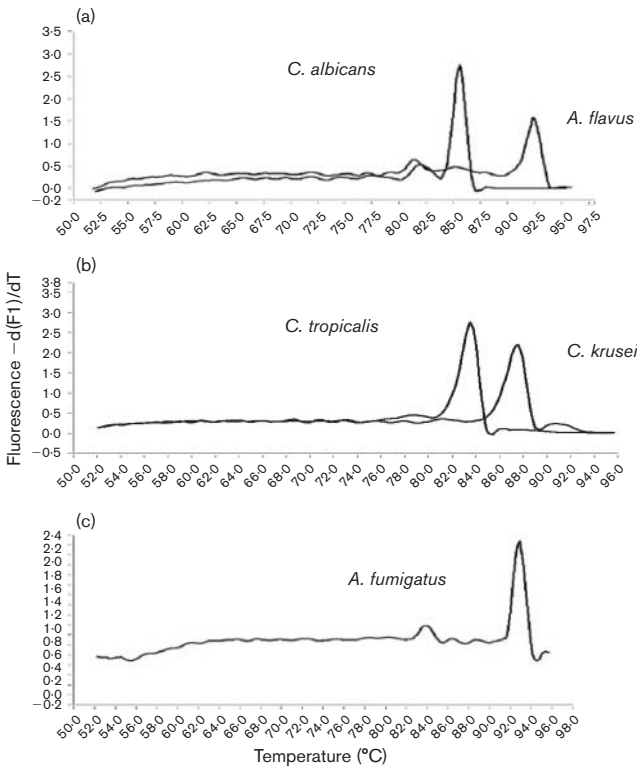


Fig. 1. Characteristic melting peaks discriminate *C. albicans* and *A. flavus* in duplex LightCycler PCR panel 1 (a), and *C. tropicalis* and *C. krusei* in duplex LightCycler PCR panel 2 (b). The melting peak of the single LightCycler assay for *A. fumigatus* is shown in (c).

difference of 3·6 °C). *A. fumigatus* was identified in an independent, single reaction. These different temperatures in each of the duplex reactions ensure reliable identification of each fungal species, as shown in Fig. 1.

We then validated this tandem multiplex assay by using 50 additional independent isolates, blinded to the operator, and spiked into peripheral blood from healthy donors. Fungal DNA was extracted as described in Methods. Results obtained by this novel molecular assay were concordant with the biochemical/culture identification in 100 % of cases (Table 1), suggesting that it is a highly efficient tool for fungal species determination in a clinical setting.

These encouraging data prompted us to further analyse samples from paediatric patients with haematological malignancies. We obtained leftover samples (14 peripheral bloods, one skin biopsy and one nasal scraping) from 16 febrile and neutropenic patients at the time that microbiology cultures and radiological studies were requested. Results are listed in Table 3 and reconfirm the utility of the tandem, multiplex, real-time PCR assay for identification of fungal infection. No false-negative results were obtained. Four samples that tested positive by our molecular assay and overall showed low fungal loads failed to grow in culture tests. Further clinical studies, including follow-up of patients, are ongoing to evaluate the significance of these observations.

Targets for detection of fungal pathogens in previous studies include the rRNA gene (Yamakami *et al.*, 1998; Skladny *et al.*, 1999), mitochondrial gene (Bretagne *et al.*, 1998; Jones *et al.*, 1998; Yamada *et al.*, 2002; Bretagne, 2003), the internal transcribed spacer regions (Spreadbury *et al.*, 1993; Burnie *et al.*, 1997) and other genes (Tang *et al.*, 1993; Kanbe *et al.*,

Table 3. Correlation of molecular and laboratory/radiological tests in febrile neutropenic paediatric oncology patients

Patient	Real-time PCR	No. cells (ml blood) ⁻¹	Culture/chest CT scan
1 (skin)	<i>A. flavus</i>	129*	<i>A. flavus</i>
2	<i>C. albicans</i>	398	<i>C. albicans</i>
3	<i>C. albicans</i>	44	—/+
4	<i>C. albicans</i>	685	—/suspicious
5	<i>C. albicans</i>	86	—
6 (nasal)	<i>A. flavus</i>	100*	—
7	<i>C. albicans</i>	146	—
8	<i>C. albicans</i>	190	—
9	—	0	—/+
10	—	0	—
11	—	0	—
12	—	0	—
13	—	0	—
14	—	0	—
15	—	0	—
16	—	0	—

*Total no. cells quantified in the sample

2003). However, we based our assay on the rRNA gene because of its advantages over other molecular targets, i.e. greatly increased sensitivity due to the existence of multiple gene copies per genome (80–160 copies) (White *et al.*, 1990).

As this novel multiplex PCR assay is based on real-time PCR, it has the advantage of quantification. We therefore developed standards to generate calibration curves for each of the five fungal species (Table 4). Specific amplicons, generated by conventional PCR, were TA-cloned and the resulting plasmids were diluted serially from 1 pg to 0.01 fg, corresponding to about 200 000–2 copies. We subsequently determined the sensitivity of the real-time PCR assay. The minimal amount that we could detect was 1 fg, corresponding to 218 copies of the *C. albicans* amplicon, 210 copies of the *C. krusei* amplicon, 214 copies of the *C. tropicalis* amplicon and 222 copies of the *A. flavus* amplicon. The limit of detection for *A. fumigatus* was 0.1 fg (approx. 21 copies). The linear range of the assay was at least from 1 pg to 1 fg plasmid DNA. Table 4 lists the standard-curve values and Fig. 2 plots the results for *C. albicans* as an example.

Having obtained these high sensitivities with cloned DNA, we assessed the sensitivity in a more realistic clinical setting. We mixed two loops of fungal biomass from a 3-day-old culture of each species into 2 ml peripheral blood from healthy donors. Fungal genomic DNA was isolated as described in Methods and serial tenfold dilutions containing from 100 to 0.001 pg genomic DNA were prepared. Real-time tandem PCR analyses demonstrated that we could detect as little as 0.1 pg fungal DNA, representing about three cells of *C. albicans*, *C. krusei*, *C. tropicalis* and *A. flavus*. The reaction for *A. fumigatus* was even more sensitive; we were able to detect 0.01 pg DNA (less than one cell). These calculations are based on an estimate of 0.03 pg DNA per fungal cell, which is the mean yield of the kit used.

Considering that one cell has an average of 120 copies of the rRNA gene (White *et al.*, 1990), detection of less than one (0.3) cell for *A. fumigatus* and three cells for the other species will be equivalent to 36 and 360 copies of the rRNA gene, respectively. Therefore, the limits of detection when fungal genomic DNA and cloned amplicons were used as templates were very similar. Based on these results, when 2 ml blood from a healthy donor was spiked with a given fungal culture, we could detect as few as 33 cells (ml blood)⁻¹ (*C. albicans*, *C. krusei*, *C. tropicalis* and *A. flavus*) or three cells (ml blood)⁻¹

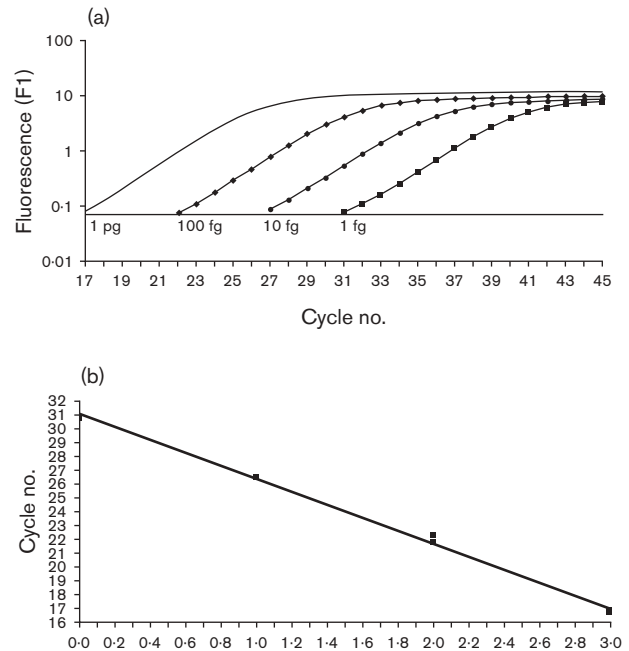


Fig. 2. Quantification of serially diluted plasmid DNA containing the amplified fragment from *C. albicans* (1 pg to 1 fg) using duplex LightCycler panel 1 primers (a). Parameters of the linear curve (b) are given in Table 4.

for *A. fumigatus*. These results are comparable with studies in which hybridization probes were used for sequence-specific, real-time detection (Loeffler *et al.*, 2000; Spiess *et al.*, 2003).

In addition, the real-time PCRs were performed successfully in half of the volume that is used conventionally in the LightCycler, i.e. 10 µl, allowing twice the number of reactions with each SYBR green kit.

In summary, a low-cost, highly specific and sensitive, LightCycler-based, real-time PCR assay was developed for rapid detection of *C. albicans*, *C. krusei*, *C. tropicalis*, *A. flavus* and *A. fumigatus*. The high sensitivity achieved allows quantification of fungal load, even in early stages of infection. It is hoped that this methodology will enable streamlining of the flow of diagnostic mycology laboratory work.

Table 4. Standard-curve values of the LightCycler PCR assay

Species	Slope	Intercept	Error	r
<i>C. albicans</i>	-4.671	36.87	0.078	-1
<i>C. tropicalis</i>	-4.332	35.47	0.0446	-1
<i>C. krusei</i>	-3.783	35.91	0.0611	-1
<i>A. fumigatus</i>	-3.581	30.66	0.101	-1
<i>A. flavus</i>	-4.030	34.75	0.176	-1

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