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One-Hour Detection of *Candida albicans* and *Candida dubliniensis* in Blood Samples Using the Smart Cycler[®]

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ABSTRACT

Background: *Candida albicans* is the most important cause of invasive human mycoses. Recently, a very closely related species, *C. dubliniensis*, has been identified as an opportunistic pathogen of immunosuppressed patients. As these two species are difficult to distinguish by classic biochemical methods, there is a need for a rapid diagnostic test that will ensure proper patient management.

Methods: Primers were selected for their ability to specifically amplify a *tef1* (elongation factor 1 alpha) gene fragment from both *Candida* species. Specificity of the amplification was verified using purified genomic DNA from a panel of fungal species. Within the *tef1* amplicon, a region differentiating *C. albicans* and *C. dubliniensis* was selected and used to design two molecular beacon probes.

Results: The sensitivity of the assay was approximately 10 genome copies for both *C. albicans* and *C. dubliniensis*. The assay was specific since species-specific fluorescent signal of molecular beacon probes was only detected in the presence of the cognate genomic DNA target. Then, to simulate a fungal infection, serial dilutions of exponentially growing cultures of either *Candida* species were used to spike blood samples. These spiked blood samples were then quickly prepared for PCR amplification by using a simple 10 minutes protocol. The two *Candida* species could easily be differentiated with detection levels of approximately 20 CFU/mL of blood for both species.

Conclusion: This differential *C. albicans-C. dubliniensis* molecular diagnostic test is therefore a sensitive and specific assay for which results can be obtained within one hour of blood sample reception in the lab. This assay will enter in pre-clinical research for validation on patient blood samples.

INTRODUCTION

The incidence of invasive fungal infections due to *Candida* species has risen considerably in recent years. In the USA, candidiasis cases almost doubled from 1980 to 1990 in intensive care units, and *Candida* is now the fourth leading cause of bloodstream infections. Despite available anti-fungal therapy, mortality associated with candidiasis is over 30 %. Although *Candida albicans* is the main pathogen implicated, there is a shift towards infections by non-*albicans* species. In particular, the new species *C. dubliniensis* has been identified as an important opportunistic pathogen of immunocompromised patients.

Traditional diagnosis of *Candida* infections is slow and complicated. Only a minority of deep-seated infections give positive blood cultures, and positive results from other sites may represent colonization rather than invasive disease. The ability to diagnose and identify candidiasis may be enhanced by the use of molecular techniques, such as PCR. In particular, the discrimination of *Candida albicans* from *Candida dubliniensis* is difficult to establish by classic biochemical methods, as these two species have almost identical phenotypes; yet, both species can be differentiated by their genetic profiles. There is a need for a rapid diagnostic

test that will ensure proper patient management, as *C. dubliniensis* is capable of developing stable fluconazole resistance *in vitro* at a high frequency.

We have designed a differential *C. albicans-C. dubliniensis* molecular diagnostic test that is sensitive and specific, and for which results can be obtained within one hour of blood sample reception in the laboratory.

METHODS

Organisms and Growth Conditions

Fungal and yeast isolates were cultured on Sabouraud medium for 48 hours at 30 °C. Broth cultures were performed in YPD medium. Bacterial strains were cultured on sheep blood agar or in brain heart infusion broth at 37 °C for 24 hours.

DNA Extraction from Reference Strains and Sequencing

Genomic DNA was extracted using the G-NOME Yeast DNA extraction kit from QBiogene (Carlsbad, CA). Universal sequencing primers were designed for all fungi based on the alignment of the elongation factor 1 alpha (*tef1*) sequences available in databases. A 1100-bp fragment of the *tef1* gene was amplified and sequenced from genomic DNA of the following species: *Aspergillus fumigatus*, *Candida albicans* (5 strains), *C. dubliniensis* (2 strains), *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*, *Cryptococcus neoformans*, *Pichia anomala* and *Saccharomyces cerevisiae*.

PCR Assay

I: Selection of amplification primers and molecular beacon probes. Based on a multiple sequence alignment of the fungal and yeast *tef1* sequences, primers were selected for their ability to specifically amplify a 149 bp fragment from both *Candida albicans* and *C. dubliniensis* species. Within this amplicon, a region differentiating both species by two nucleotides was selected and used to design two molecular beacon probes labeled with different fluorophores (FAM and TET).

II: Strains tested. Specificity of the *C. albicans-C. dubliniensis* primers was verified using DNA from a panel of fungal and yeast species, including several other *Candida* species. These experiments were performed using standard thermocyclers and agarose gel electrophoresis. Ubiquity and sensitivity of the real-time PCR assay was determined by using serial two-fold dilutions of purified genomic DNA from 6 reference strains of *Candida albicans*, and from 6 reference and clinical strains of *Candida dubliniensis*.

III: Real-time PCR assay. The real-time multiplex PCR assay using species-specific molecular beacon probes was carried out using a Smart Cycler® thermal cycler (Cepheid, Sunnyvale, CA). Detection of the PCR products was made in real-time by measuring the fluorescent signal emitted by the molecular beacons when they hybridize to their respective target at the beginning of each annealing step.

Preparation of Spiked Blood Samples

To simulate a fungal infection, serial dilutions of exponentially growing cultures of either *Candida* species were used to spike blood samples. These spiked blood samples were then quickly prepared for PCR amplification by using a simple 10-minute protocol using a rapid extraction procedure. Blood from 3 different donors were tested to demonstrate the versatility of the cell lysis and DNA extraction method.

RESULTS

PCR Assay

Specificity of the primers was demonstrated by testing genomic DNA from a panel of microbial species, including 67 *Candida* strains representing 24 species. Amplification was observed only with DNA from either *C. albicans* or *C. dubliniensis*. The analytical sensitivity of the real-time PCR assay was approximately 10 genome copies for all *C. albicans* (n=6) and *C. dubliniensis* (n=6) strains tested (Figure 1). The assay was specific since species-specific fluorescent signals of molecular beacon probes was only detected in the presence of the cognate genomic DNA target.

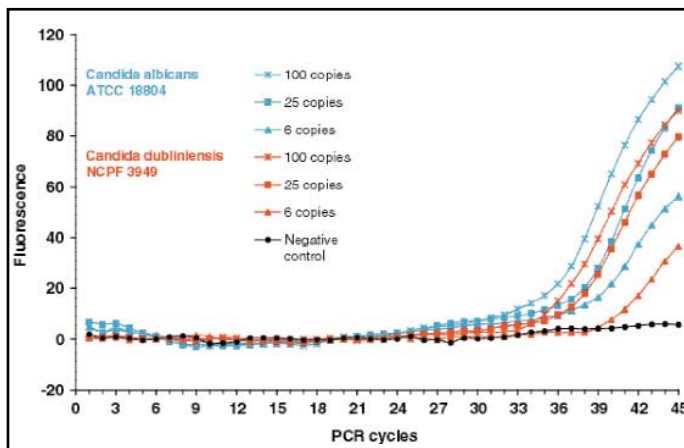


Figure 1: Detection of *Candida albicans* and *Candida dubliniensis* in blood by the real-time PCR assay. Results with blood samples spiked with either species are shown. The detection limit was approximately 20 CFU/mL of blood for both species.

Detection of *Candida albicans* and *C. dubliniensis* in Blood

The two *Candida* species could easily be differentiated with detection levels of approximately 20 CFU/mL of blood for both species (Figure 2). The analytical sensitivity of the real-time PCR assay was the same in blood samples from all 3 donors tested, demonstrating the potential of the DNA extraction method for future clinical studies. An amplification control revealed that there was no significant PCR inhibition.

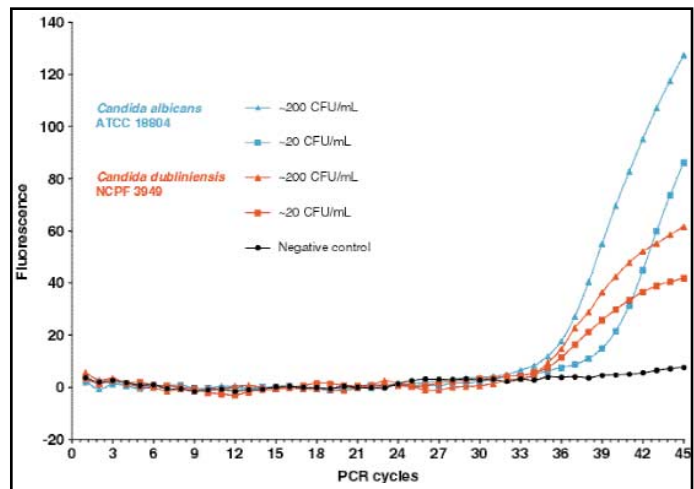


Figure 2: Analytical sensitivity of the real-time PCR assay using purified genomic DNA. The detection limit was less than 10 genome copies for both *C. albicans* and *C. dubliniensis*.

CONCLUSIONS

- ◆ We have developed a specific and ubiquitous assay capable of detecting and identifying down to 20 CFUs of *C. albicans* and *C. dubliniensis* per mL of blood within one hour.
- ◆ For the speciation of *C. albicans* and *C. dubliniensis*, this molecular test is superior to phenotype-based methods.
- ◆ Further validation of this assay will include pre-clinical research on real patient blood samples.

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