Rapid Detection of *Candida albicans* in Clinical Blood Samples by Using a TaqMan-Based PCR Assay

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We describe a rapid and reproducible PCR assay for quantitation of the *Candida albicans* ribosomal DNA (rDNA) in clinical blood samples based on the TaqMan principle (Applied Biosystems), in which a signal is generated by cleavage of a template-specific probe during amplification. We used two fluorogenic probes based on universal, fungus-specific primers, one for the detection of *C. albicans* species DNA and one for the detection of all *Candida* genus DNA. *C. albicans* blastoconidia mixed with whole blood in a titration experiment yielded a linear PCR signal over a range of 3 orders of magnitude. The TaqMan-based PCR assay for *C. albicans* exhibited a low limit of detection (5 CFU/ml of blood) and an excellent reproducibility (96 to 99%). While the *C. albicans* species-specific probe had 100% specificity for *C. albicans*, all *Candida* genus-specific probes cross-reacted with other organisms likely to coinfect patients with *C. albicans* infections. On the basis of these data, we determined the *C. albicans* loads with a species-specific probe from 122 blood samples from 61 hematology or oncology patients with clinically proven or suspected systemic *Candida* infections. Eleven positive samples exhibited a wide range of *C. albicans* loads, extending from 5 to 100,475 CFU/ml of blood. The sensitivity and specificity of the present assay were 100 and 97%, respectively, compared with the results of blood culture. These data indicate that the TaqMan-based PCR assay for quantitation of *C. albicans* with a species-specific probe provides an attractive alternative for the identification and quantitation of *C. albicans* rDNA in pure cultures and blood samples.

Invasive candidiasis, a common cause of nosocomial infection, is a leading cause of infections among patients receiving bone marrow transplantations or those with leukemia or other cancers and is also associated with a high rate of morbidity and mortality (10, 18, 22). Given the rapidly fatal course of candidiasis, there is a need for improved methods for early diagnosis and subsequent initiation of antifungal therapy in order to have a significant impact on the death rate (1, 12). One such improvement is the application of rapid and sensitive methods based on PCR that enable the amplification and quantitation of a broad range of fungal pathogens directly from specimens and pure cultures.

Two quantitative real-time PCR systems have been described for *Candida* detection. An assay based on the PCR LightCycler system (Roche Molecular Diagnostics Systems, Indianapolis, Ind.) was applied by Löeffler et al. (15) for quantification of *Candida albicans* DNA in blood to which known numbers of blastoconidia had been added. Those investigators also used the assay to determine the fungal burdens in a small number of blood samples taken from patients with hematological malignancies. More recently, Guiver et al. (7) and Bowman et al. (3) described the automated detection of fungal DNA by using the TaqMan assay (Applied Biosystems, Foster City, Calif.) with clinical isolates and murine tissues, respectively. This assay, which takes advantage of the 5′-3′ nucleolytic activity of *Taq* polymerase (9), has been evaluated to quantita-

tively estimate the *C. albicans* species burdens in various mouse tissues, to monitor disease progression, and to measure antifungal drug efficacy (3). However, to our knowledge this assay has not yet been adapted to determination of the fungal load in clinical blood samples.

Here we describe a rapid and reproducible method for determination of the *C. albicans* ribosomal DNA (rDNA) loads in blood samples by using the TaqMan technology combined with the GeneAmp 5700 real-time sequence detection system (Applied Biosystems) (6, 8). The PCR assay based on the TaqMan principle generates a signal by cleaving a template-specific hybridization probe during amplification (9, 13). We first evaluated the sensitivity of this assay by adding *C. albicans* blastoconidia to blood, extracting total DNA, and demonstrating a linear response over a range of 3 orders of magnitude with a low limit of detection. We also evaluated the specificities of two fluorogenic probes (one *C. albicans* species-specific probe and one all *Candida* genus-specific probe) and the reproducibility and linear range of the TaqMan-based PCR assay. Finally, this assay was tested with template DNA prepared directly from 122 blood samples from hematology or oncology patients with either disseminated candidiasis or suspected disseminated candidiasis, and the results were compared with those of standard blood culture and identification methods.

MATERIALS AND METHODS

Fungal culture. *C. albicans* strain ATCC 24433 was cultured on Sabouraud-glucose-agar for 72 h at 30°C. Serial dilutions of fungal cells were prepared with sterile saline suspensions that were adjusted to a 0.5 McFarland standard (which is approximately 10^6 CFU/ml).

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FIG. 1. Quantitation of *C. albicans* blastoconidia in seeded human blood specimens and comparative sensitivities of DNA detection by using the CA-FAM and All-CAN-TET probes. Serially diluted *C. albicans* blastoconidia were mixed with blood, and the extracted DNA was used in duplicate as the template in the TaqMan assay. (A) The relative fluorescence refers to the increase in the fluorescence emission of the reporter dye relative to that of the passive reference dye. The threshold fluorescence was determined by using the CA-FAM probe. No signal was obtained when double-distilled water was used as a negative control. (B) Log_{10} concentration of blastoconidia plotted against the mean C_T values generated from the amplification plots in panel A (CA-FAM probe) and those generated with the All-CAN-TET probe.

TABLE 1. Comparative specificity of DNA detection by using the *C. albicans* species-specific and all-*Candida* genus-specific fluorescent probes

Genomic DNA[a]	$C_T{}^b$	
	CA-FAM	All-CAN-TET
S. cerevisiae	−	16.91 ± 0.14
C. parapsilosis	−	20.22 ± 0.20
C. albicans	17.54 ± 0.11	20.23 ± 0.27
A. nidulans	−	20.31 ± 0.26
Penicillium spp.	−	20.92 ± 0.07
C. krusei	−	21.28 ± 0.10
C. guilliermondii	−	21.29 ± 0.18
C. glabrata	−	21.57 ± 0.03
C. tropicalis	−	21.64 ± 0.29
C. norvegensis	−	21.67 ± 0.21
A. flavus	−	21.84 ± 0.13
A. fumigatus	−	22.73 ± 0.24
Curvularia spp.	−	22.74 ± 0.01
Fusarium spp.	−	23.20 ± 0.19
Alternaria spp.	−	24.05 ± 0.07
H. werneckii	−	24.45 ± 0.16
P. verrucosa	−	24.54 ± 0.21
E. purpurescens	−	24.77 ± 0.16
A. niger	−	25.30 ± 0.14
Auxarthron spp.	−	26.20 ± 0.11
C. neoformans	−	26.36 ± 0.18
S. apiospermum	−	26.39 ± 0.22
Chaetomium spp.	−	32.19 ± 0.01
Klebsiella spp.	−	−
H. influenzae	−	−
M. morganii	−	−
S. maltophilia	−	−
P. mirabilis	−	−
P. aeruginosa	−	−
Human lymphocyte line	−	−
Cytomegalovirus	−	−

[a] One nanogram of purified DNA was analyzed.
[b] Values are expressed as means ± standard deviations. Data are from triplicate samples from two to three experiments. A negative value was assigned for all C_T values equal to 40.

34.04 ± 0.51; and for 0.05 pg, 35.62 ± 0.54. These are equivalent to standard errors of 0.17, 0.18, 0.20, 0.21, and 0.22, respectively. The same concentrations of *C. albicans* genomic DNA used as external standards (1,000 to 0.05 pg) were used for the quantitation of DNA in clinical samples.

Linear range. The linear range of the TaqMan system for the quantitation of *C. albicans* was determined by analysis of two identical dilution series of *C. albicans* genomic DNA that had been amplified by using either the CA-FAM or the All-CAN-TET probe. Both probes yielded different regression lines: the slopes were 3.794 and 3.787, respectively; the intercepts were 36.043 and 39.051, respectively; and the correlation coefficients (R^2 values) of the curves were 0.997 and 0.999, respectively (Fig. 2). Because the slopes were, on average, equivalent and the difference between the two intercepts was approximately 3, detection of the DNA standard with the All-CAN-TET probe within the linear range was overestimated by a factor of approximately 7 ($0.84 \log_{10}$). This result indicates that species-specific probes provide the basis for the very accurate quantitation of *C. albicans* DNA by the TaqMan assay.

Quantitation of DNA in clinical samples. The ability of the TaqMan assay to measure the *C. albicans* rDNA loads in 122 blood samples from 61 patients was assessed. Eleven samples tested displayed a signal by TaqMan analysis (Table 2). The *C. albicans* rDNA load ranged between 5 and 100,475 CFU/ml of blood when the results were compared to those for a DNA standard by using the CA-FAM probe. Six positive samples had weak to moderate *C. albicans* loads (<30 CFU/ml of blood), three had elevated *C. albicans* loads (>100 CFU/ml of blood), and two had highly elevated *C. albicans* loads (>1,000 CFU/ml of blood). Finally, 36 control blood samples from patients without clinical evidence of an invasive fungal infection tested negative for *C. albicans* by the TaqMan assay with
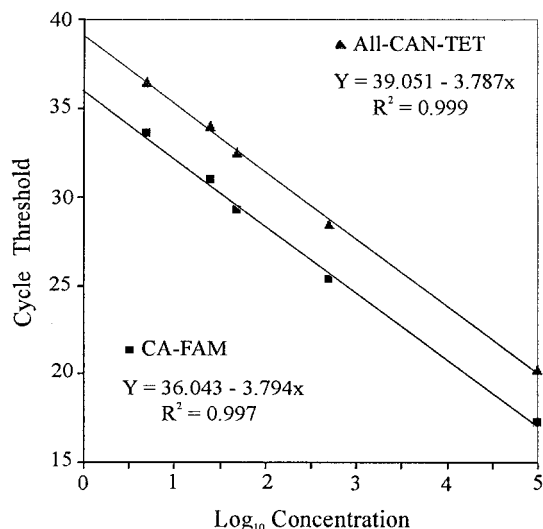
FIG. 2. Linear range for the TaqMan assay for *C. albicans* by using the CA-FAM and All-CAN-TET probes. Two identical dilution series of *C. albicans* DNA (1,000 to 0.05 pg DNA, corresponding to 10^5 to 5 CFU) were used as external standards. Each dilution was tested in triplicate. Linear regression analysis was performed on the mean C_T values for each dilution series plotted against the $\log_{10}$ concentration of standard DNA.

the CA-FAM probe. Thirteen culture bottles with blood from these patients showed that the patients had bacteremia (caused by *Lactobacillus* spp. [$n = 1$], *Enterococcus* spp. [$n = 2$], *Fusobacterium* spp. [$n = 3$], and *Staphylococcus epidermidis* [$n = 7$]).

Three blood specimens, which were culture negative, obtained from a single patient were identified as containing *C. albicans* by the TaqMan assay (5, 27, and 120 CFU/ml of blood; Table 2). This patient had received an allogeneic bone marrow transplant, had been severely neutropenic for several weeks, and had received prophylactic fluconazole, which may suppress *Candida* growth but not completely eradicate the organism. Of the patients who exhibited very high *C. albicans* loads (3,760 and 100,475 CFU/ml of blood) and in whom candidemia was documented by culture, one had a documented catheter infec-

TABLE 2. Efficiency of TaqMan-based detection with CA-FAM probe of *C. albicans* DNA from patient blood samples

Diagnosis[a]	TaqMan result (CFU/ml)	Blood culture result	Blood origin[b]
AML	5	Negative[c]	PAC
AML	5	Positive	PER
SCLC	6	Positive	PER
Gastric tumor	7	Positive	PER
AML and breast tumor	17	Negative	PER
AML	27	Negative[c]	PER
AML	120	Negative[c]	PAC
Colon tumor	339	Positive	Unknown
Colon tumor	525	Positive	PAC
Pancreas tumor	3,760	Positive	CVC
Metastatic breast tumor	100,475	Positive	PAC

[a] AML, acute myeloid leukemia; SCLC, small-cell lung cancer.
[b] PAC, Port-a-cath; PER, peripheral blood; CVC, central venous catheter.
[c] The three blood culture samples were from the same patient.

tion with a large number of positive blood cultures and a larger number of colonies from catheter samples compared to the colony counts from peripheral blood (Isolator quantitative culture), and the second had extremely high *C. albicans* loads and a documented catheter infection, but only one of four blood cultures was positive.

Fifteen (12%) of the 122 blood cultures were positive for *Candida* spp.: 6 for *C. albicans*, 3 for *C. glabrata*, 2 for *C. tropicalis*, 1 for *C. guilliermondii*, 1 for *Candida lipolytica*, 1 for *C. lusitaniae*, and 1 for a mixture of *C. albicans* and *C. glabrata*. Eleven (9%) EDTA-anticoagulated blood specimens were positive by the TaqMan assay with the CA-FAM probe; six of these were culture positive for *C. albicans*, one was culture positive for *C. albicans* and *C. glabrata*, and four remained negative by blood culture. Among the 44 (36%) blood specimens that were TaqMan positive with the All-CAN-TET probe, 29 were culture negative for *Candida* spp. and 15 were culture positive for *Candida* spp. Compared with the results of blood culture, the sensitivity and specificity of the TaqMan PCR assay with the CA-FAM probe observed with blood samples were 100 and 97%, respectively; those for the TaqMan PCR assay with the All-CAN-TET probe were 100 and 72%, respectively. Finally, for both probes, the TaqMan assay had a very high negative predictive value of 100%.

DISCUSSION

Most commercial or in-house fungal DNA quantitative methods are based on DNA amplification with conventional thermocyclers, followed by hybridization with biotin- or digoxigenin-labeled probes. These methods use end-point measurement to quantify the amount of amplified product. At any given cycle within the exponential phase of PCR, but not during the plateau, the amount of product is proportional to the initial amount of template. In contrast, the real-time TaqMan PCR assay for the quantitation of *C. albicans* rDNA enables accurate calculation of the amount of PCR product at a point in the early exponential phase of the reaction (C_T) and eliminates the concern that a reaction plateau will be reached at different cycles. This allows determination of the initial fungal rDNA load from the amount of PCR product by reference to an external standard.

By using the TaqMan assay, the smallest amount of *C. albicans* DNA tested and reliably quantified is 5 CFU/ml of blood with either the species-specific or the genus-specific probe. In practice, however, a relatively high fluorogenic signal was displayed by unspiked whole blood from healthy volunteers. This drawback was associated solely with the all *Candida* genus-specific probe and can result in false-positive reactions. To circumvent this, the All-CAN-TET probe was assigned a higher baseline value, which, following PCR, enables differentiation of PCR-specific and nonspecific products. This result was in accordance with those reported by Shin et al. (20), who applied a 5′ exonuclease assay to quantify medically significant species of *Candida* from blood culture bottles using fluorescent DNA probes and luminescence spectrometric detection. The investigators used a positive cutoff value for the All-CAN-TET probe approximately five times higher than the cutoff value for the CA-FAM probe.

The TaqMan assay for *C. albicans* quantitation exhibited a

good reproducibility of 96% when the C_T values for dilution series of six different extracts of purified *C. albicans* DNA in the range of 1,000 to 0.05 pg were analyzed. Moreover, triplicate amplifications with each dilution performed in each assay allow us to calculate a high reproducibility of 99%. A similar reproducibility was exhibited by another recently described quantitative test for *Candida* with the LightCycler instrument (15).

The specificities of the probe sets used in real-time PCR assays for *C. albicans* detection are often established by using closely related species and DNA of human origin (7, 15). In our study, the CA-FAM probe had 100% specificity for *C. albicans*. In contrast, the All-CAN-TET probe cross-hybridized with DNA purified from other species such as *S. cerevisiae, A. fumigatus, A. flavus,* and *Fusarium* spp. We recently reported similar cross-reactivities between this digoxigenin-labeled all *Candida* genus-specific probe (by PCR-enzyme-linked immunosorbent assay) and DNA from *Fusarium* spp. using colorimetric detection of amplified products from clinical blood samples (19). In addition, in a 5′ exonuclease assay for *Candida,* the All-CAN-TET probe was shown to hydridize with products amplified from *S. cerevisiae, A. fumigatus,* and *A. flavus* (20). The investigators recommended further analysis with *S. cerevisiae-* and *Aspergillus*-specific probes to differentiate between these species and *Candida* species. Hence, a positive PCR result with the all *Candida* genus-specific probe does not exclude the presence of species other than the common *Candida* species, and the concomitant use of species-specific probes is necessary.

Among the most important factors that limit the direct application of the all *Candida* genus-specific probe to blood samples is its design from the highly conserved 5.8S region of rDNA (16, 20). Recently, human DNA-derived sequences were detected in blood specimens from healthy individuals by using real-time PCR with a probe targeting conserved regions of bacterial 16S rDNA (17). In our study with blood samples from individuals without clinical signs or symptoms suggesting infection or other disease, the threshold was generally exceeded during later cycles of amplification with the all *Candida* genus-specific probe. A preliminary search of GenBank showed a region of similarity between the sequence of this probe and that of 5.8S human rDNA (data not shown). We are in the process of creating clone libraries from PCR products amplified from blood. When the sequences of these clones match human DNA-derived sequences, redesign of a new all *Candida*-specific probe permitting sole detection of the major clinically important species of *Candida* can be achieved by use of the sequence from the less conserved internal transcribed spacer 2 region, between the 5.8S rRNA gene and the 28S rRNA gene. This region is distinctive and has great potential for use as a taxonomic tool (4, 21). The redesign of all *Candida* genus-specific probes can also be performed by use of the recently commercialized TaqMan Minor Groove Binder (Applied Biosystems), which enhances the melting temperature of the oligonucleotide, resulting in shorter probe sequences.

The greatest impact of the real-time PCR assay on routine diagnostic assays may be the saving of time from the time of sample collection to the time of a final diagnosis. As the fungal DNA extraction step is still a limiting factor, requiring more than half of a working day, this PCR assay reduces the time to achieve a final result by several hours, allowing a report to be made on the same day that the test is performed. The detection of fungemia by conventional culture methods can be difficult. It is generally attributed to very small fungal loads in clinical samples (2, 14). Recently, in a preliminary study that used the LightCycler PCR assay, quantitation of fungal DNA showed that two of nine PCR-positive patients with hematological malignancies had fungal loads of more than 100 CFU/ml of blood (15). In our study, the species-specific probe correctly determined *C. albicans* loads of more than 100 CFU/ml of blood in 5 of 11 PCR-positive hematology or oncology patients. It is interesting that the two samples exhibiting the highest *C. albicans* loads (>1,000 CFU/ml of blood) were both from patients with documented catheter infections, one of the most common conditions associated with fungemia. Moreover, the positive "gold standard" for *C. albicans* detection is difficult to define, because most disseminated infections are not detected until death and cultures might be falsely negative, for instance, because of the use of antifungal agents. In the present study, all four samples with positive PCR results and negative culture results were obtained from patients while they were receiving antifungal treatment. Given its high sensitivity, a prospective clinical study of the TaqMan-based PCR assay for the identification of patients at risk for invasive fungal infections is under way.

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