

Improved Detection of *Candida albicans* by PCR in Blood of Neutropenic Mice with Systemic Candidiasis

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A PCR using primers aimed at the multicopy gene coding for the small subunit rRNA and resulting in the synthesis of a 180-bp fragment was evaluated for its use in diagnosing invasive candidiasis in comparison with blood culture. With the use of a *C. albicans*-specific probe, ± 10 to 15 *C. albicans* cells are detected in 100 μ l of whole blood by Southern analysis. A DNase pretreatment was critical in the purification process of yeast DNA from whole blood. Omission of the DNase pretreatment decreased assay sensitivity 10-fold. PCR analysis of blood specimens collected from mice with invasive candidiasis is more sensitive than blood culture (100 versus 67%, respectively) at 72 h after intravenous (i.v.) inoculation with *C. albicans*. Furthermore, the intensity of the hybridization signals increased with the progression of infection. In contrast, multiple blood samples from gastrointestinally colonized mice were all negative by PCR, indicating that the PCR assay is also specific and may, therefore, make a positive contribution to the detection and follow-up of invasive candidiasis.

The opportunistic pathogen *Candida albicans* is able to cause disseminated infections in immunocompromised patients. To reduce mortality in patients with invasive candidiasis, early initiation of antifungal drug therapy is critical (1). The clinical diagnosis is complicated because *C. albicans* commonly colonizes or causes mild infection of the mucous membranes or skin. It is, therefore, often difficult to judge the clinical significance of the finding of *C. albicans* in secretions or on the body surfaces when systemic infection is suspected in the case of occult fever or for other reasons. Microbiological evidence for the diagnosis of invasive candidiasis requires the demonstration of *C. albicans* in sterile body fluids, biopsies, or multiple consecutive blood cultures (7). There are, however, many problems inherent in these tests (12). Berenguer et al. (2), for instance, recently demonstrated that even by application of the lysis-centrifugation technique, a sensitivity of only 43% was established in autopsy-proven cases of invasive candidiasis. They also demonstrated that the sensitivity of the lysis-centrifugation blood culture technique correlated with the number of infected tissue sites, and therefore, these investigators stressed the need for more sensitive methods for detection of invasive candidiasis.

Since PCR has proven to be a powerful tool in the early diagnosis of several infectious diseases, it might also be a more sensitive alternative assay in the diagnosis of invasive candidiasis. Several PCR methods for the detection of *Candida* spp. in patient materials have been published (3, 6, 8, 10). However, the diagnostic usefulness of PCR remains to be established because of the reported limitations of the technique when whole blood is used (6, 8), the lack of verification with a species-specific probe for the amplified product (3), and the inability to detect medically important *Candida* spp. other than *C. albicans* (11).

The efficacy of application of PCR in the early diagnosis of systemic candidiasis has to be ascertained in comparison with

that of the blood culture technique, and furthermore, PCR results must be negative in patients who are merely colonized with *C. albicans*. In the present study, the diagnostic usefulness of PCR was studied by detecting *C. albicans* in whole blood of systemically infected mice and in gastrointestinally colonized mice in order to determine the sensitivity and accuracy of the PCR assay for discriminating between colonization and invasive candidiasis.

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MATERIALS AND METHODS

Animals and *Candida* strain. Specific-pathogen-free, 11- to 13-week-old female BALB/c mice weighing ± 22 g were used (Iffa Credo, L'Arbresle, France). *C. albicans* (ATCC 44858) was used throughout all the experiments. The yeast was stored at -80°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 10% (vol/vol) glycerol. Stationary-growth-phase cultures were obtained after incubation of the yeast in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C . The yeasts were washed in phosphate-buffered saline (PBS), counted in a hemocytometer, and adjusted to 5×10^4 cells per ml in PBS.

Systemic infection in neutropenic mice. Neutropenia was induced by intraperitoneal administration of cyclophosphamide (Sigma) at 100 mg/kg of body weight 4 days prior to inoculation with *C. albicans*, followed by additional doses of 75 mg/kg on the day of inoculation and at 3-day intervals during the course of infection (14). This treatment resulted in persistent granulocytopenia of <100 granulocytes per μ l of blood from the time of inoculation with *C. albicans* up to termination of the studies. In each experiment 21 neutropenic mice were infected by inoculation of 200 μ l of 5×10^4 CFU of *C. albicans* per ml in the tail vein.

At 1, 6, 24, 48, 72, 96, and 144 h after inoculation with *C. albicans*, the mice were sacrificed, using three mice for each time point. At the above-indicated time intervals, blood was collected in EDTA-coated tubes by cardiac puncture. Of each blood sample, 100 μ l was directly cultured on Sabouraud dextrose agar (SDA) plates (Oxoid, Basingstoke, England) and incubated for 5 days at 37°C . Another 100 μ l of blood was used for DNA extraction.

The kidneys, spleen, liver, and lungs were removed from each mouse and separately homogenized in 20 ml of PBS for 30 s at 10,000 rpm in a VirTis homogenizer (VirTis Co. Inc., Gardiner, N.Y.). Two hundred microliters of the serial twofold dilutions of each homogenate was plated on SDA plates. The remainder of the homogenate, together with an equal volume of double-strength SDA, was mixed and poured into petri dishes. All plates were incubated for 48 h at 37°C .

Gastrointestinal colonization in immunocompetent mice. Gastrointestinal colonization was established by pretreating mice for 5 days with cefradine (1 g/liter, Velosef 250; Bristol Myers-Squibb, Woerden, The Netherlands). Cefradine was

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added to their drinking water throughout the experiment. Inoculation with 1.10^7 CFU of *C. albicans* was performed by gastric intubation. Feces were collected and cultured, at 2-day intervals. The mice were sacrificed 8 days after inoculation with *C. albicans*. Blood was sampled by cardiac puncture and collected into EDTA-coated tubes. One hundred microliters of blood was directly subcultured on SDA plates, and 100 μ l of whole blood was used for DNA extraction. Kidneys, lungs, liver, and spleen as well as stomach and gastrointestinal tract were removed, homogenized, and cultured as described above.

PCR sample preparation. For *Candida* DNA extraction from blood we used a method modified from Buchman et al. (3). Briefly, 0.1 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 1% Triton X-100) was added to 0.1 ml of whole blood. After lysis, cell debris and *Candida* yeast cells were pelleted by centrifugation (Eppendorf; Merck, Amsterdam, The Netherlands) at $16,000 \times g$ for 5 min. The pellet was resuspended in 0.2 ml of lysis buffer. To remove free, non-yeast cell DNA, 7 μ l of DNase (10 mg/ml; Boehringer GmbH, Mannheim, Germany) was added and the samples were incubated at 37°C for 1 h. After centrifugation at $16,000 \times g$ for 5 min, the pellets were resuspended in 0.2 ml of TEG buffer (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA) containing 1.5 μ l of Lyticase (900 U/ml; Sigma Chemical Co., St. Louis, Mo.) and incubated for a further 1 h at 37°C. Subsequently, 3.0 μ l of pronase (15 mg/ml; Boehringer GmbH) and 10 μ l of 10% sodium dodecyl sulfate (SDS) were added and further incubated for 1 h at 37°C. DNA extraction and purification was realized by using Prep-A-gene DNA matrix according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). The matrix was eluted with 40 μ l of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Filter tips (Biozym, Landgraaf, The Netherlands) were used throughout the isolation procedure to avoid contamination.

Primers and PCR protocol. PCR was performed on 10 μ l of the DNA samples, which corresponded to 25 μ l of the original blood sample. The following primer set, generating a 180-bp fragment spanning the V4 region of the small subunit rRNA gene, was used: 532 (5'-TATTAAAGTTGTTGCAG-3') and 651 (5'-CTGCTTTGAACACTCTAATTT-3') (13). PCR was performed in 100 μ l of PCR solution containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM MgCl₂, 200 μ M (each) dNTP, 50 pmol of each primer, 0.1 mg of gelatin per ml, 1 U of *Taq* DNA polymerase (Promega, Leiden, The Netherlands), and 10 μ l of sample specimen. The reaction mixture was overlaid with 100 μ l of mineral oil (Sigma) to prevent evaporation and was preincubated for 5 min at 94°C for DNA denaturation. Forty cycles of amplification were performed with a PCR processor (Biomed GmbH, Theres, Germany). Each cycle consisted of a denaturation step at 94°C for 1 min, a primer-annealing step at 52°C for 1.5 min, and a chain-elongation step at 74°C for 1 min. After 40 cycles, a temperature-delay step of 5 min at 74°C completed elongation. Thirty microliters of the amplified PCR product was analyzed by agarose (1.5%, wt/vol) gel electrophoresis.

Southern blot analysis of PCR products. DNA was transferred from agarose to Hybond Plus nylon filters (Amersham International, plc, Amersham, United Kingdom) by overnight diffusion blotting in 0.4 N NaOH. The PCR products were analyzed with the *C. albicans*-specific probe (5'-GTAGCCATTTATGGC GAACC-3') (13). The membranes were preincubated at 37°C in a hybridization solution (5 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1 \times Denhardt's solution) for 1 h. Hybridization occurred overnight at 37°C with 20 pmol of T4 polynucleotide kinase (Life Technologies, Ltd., Renfrewshire, Scotland) [γ -³²P]ATP (3,000 Ci/mmol; Amersham)-labelled oligonucleotide probe. The filters were washed once for 15 min at 37°C and once for 15 min at 55°C with 2 \times SSC-0.1% (wt/vol) SDS. Autoradiography was performed with Kodak Royal X-Omat film and a 3.5-h exposure time.

RESULTS

PCR sensitivity. In order to develop a test with a better sensitivity than that of blood culture, special effort was put into determining the method of DNA extraction and into creating optimal storage conditions for the blood samples. As a guideline for the DNA extraction, the method described by Buchman et al. (3) was used. An essential step in the DNA extraction procedure proved to be the digestion of non-yeast DNA by DNase pretreatment. Figure 1 shows the results of making a 10-fold dilution series of *C. albicans* in whole sheep blood and performing the DNA extraction with and without DNase pretreatment. By application of DNase pretreatment we were able to detect 10 to 15 cells of *C. albicans* per 100 μ l of whole blood. Leaving out the DNase treatment, only 150 to 200 cells of *C. albicans* per 100 μ l could be detected, thus reducing the sensitivity of the PCR assay at least 10-fold.

Before starting the in vivo experiments, several conditions were studied with regard to the storage of blood samples for PCR analysis. Several blood samples were spiked with a 10-fold dilution series of *C. albicans* in whole sheep blood, and the

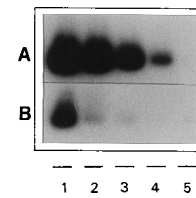


FIG. 1. Southern blot analysis of *C. albicans* PCR products in whole sheep blood with (A) and without (B) DNase pretreatment. Lanes: 1 through 5, *C. albicans* cells spiked at 2,300, 230, 115, 23, and 0 (negative control) per 100 μ l of whole blood, respectively.

following three procedures were tested: (i) direct extraction by the standard procedure, (ii) partial processing of the samples up to the pronase treatment step and subsequent completion of the rest of the standard protocol 1 week later after the samples had been kept at -20°C , and (iii) freezing of the samples at -20°C for 1 week and subsequent extraction of DNA by the standard protocol. We found that direct extraction or partial processing incurs no loss in reproducibility or sensitivity. However, freezing the blood samples at -20°C for 1 week prior to DNA processing was associated with some loss of reproducibility and sensitivity of the PCR assay (Fig. 2). We therefore decided to routinely process the blood samples obtained in the in vivo experiments with the two-stage extraction procedure, i.e., procedure (ii), so that all samples belonging to one in vivo experiment could be processed in a single PCR.

Systemically infected mice. To test the sensitivity and in vivo accuracy of the *Candida* PCR assay, we used an induced systemic *C. albicans* infection in mice with cyclophosphamide-induced neutropenia. Since cyclophosphamide is metabolized in the liver into metabolites which actively cross-link DNA chains, control experiments were performed to check whether blood containing active cyclophosphamide metabolites would interfere with the PCR. Therefore, purified *C. albicans* DNA was added to blood, collected from mice 1 and 6 h after they had received intraperitoneal cyclophosphamide, and incubated at 37°C. At 15, 30, and 60 min of incubation, samples were taken and the DNA was extracted and assayed by PCR. From these experiments it was concluded that active cyclophosphamide metabolites did not interfere with the *Candida* PCR assay of whole blood (data not shown).

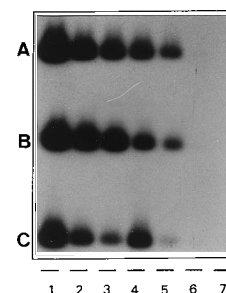


FIG. 2. Sensitivity and reproducibility results of the *C. albicans* PCR assay conducted with whole sheep blood processed by three different procedures: direct testing after DNA extraction by the standard procedure (A), partial processing of the samples up to the pronase treatment step and subsequent completion of the rest of the standard protocol 1 week later after the samples had been kept at -20°C (B), and freezing of the samples at -20°C for 1 week and subsequent extraction and testing according to the standard protocol (C). Lanes: 1 through 5, *C. albicans* DNA from 3,000, 300, 150, 30, and 10 cells per 100 μ l of whole blood, respectively; lanes 6 and 7 are negative controls. Lane 6, 100 μ l of processed blood sample; lane 7, 100 μ l of sterile PBS (both lanes without spiked *Candida* cells).

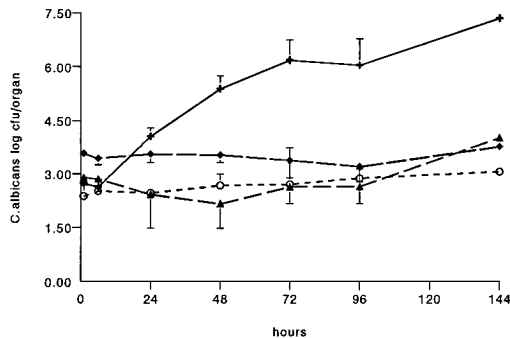


FIG. 3. Progression of systemic infection in neutropenic mice, expressed as the mean number of CFU of *C. albicans* in the kidney (+), spleen (O), lung (▲), and liver (◆), obtained from three separate in vivo experiments. In each experiment, 21 mice were inoculated with 10⁴ CFU of *C. albicans* and three mice were sacrificed at 1, 6, 24, 48, 72, 96, and 144 h after i.v. inoculation. Each symbol represents the geometric mean, ± the standard deviation, for three experiments using three mice each (n = 9).

After the mice were i.v. challenged with 10⁴ CFU of *C. albicans*, the mean number of CFU per organ increased (kidney) or remained more or less constant (lung, liver, and spleen) over the course of the experiment (Fig. 3). The infection model is reproducible since the number of CFU per organ remained in the same order of magnitude (Fig. 3). The results of the blood cultures and PCR are given in Table 1. Blood culture results varied considerably and showed CFU counts ranging from 0 to 45 CFU per 100 µl of blood. Early after the onset of the infection as well as at 72 h into the infection, blood cultures tended to yield higher numbers of CFU. At 24 and 48 h, the CFU counts were often lower than at 1 and 72 h. In Fig. 4, PCR results of the blood samples from each mouse, from experiments 1 and 3, indicate that with the progression of the infection more DNA targets became available for amplification. However, this increase in PCR signal did not correlate with the number of CFU of *C. albicans* cultured from the same blood sample (Table 1). This difference in the sensitivities of the PCR assay and blood culture was scored at each sampling moment (Table 1). PCR was more often positive at 48 and 72 h and has, therefore, a better sensitivity than blood culture. In only one blood sample, *C. albicans* was cultured (1 CFU per 100 µl) but no PCR signal could be obtained (experiment 2; 24 h).

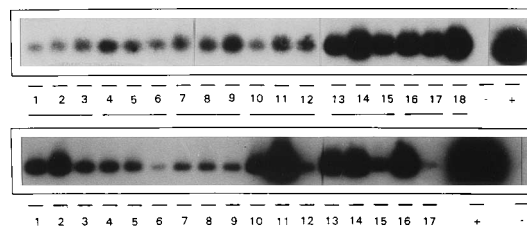


FIG. 4. Southern blot analysis of *Candida* PCR amplificates from the blood of each mouse with systemic candidiasis sacrificed at the indicated time intervals. Lanes: 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 15, 16 and 17, and 18 represent mice sacrificed at 1, 6, 24, 48, 72, 96, and 144 h after i.v. inoculation, respectively. +, positive controls; -, negative controls (processed blood of uninfected mice). The top gel corresponds to experiment 1, and the bottom gel corresponds to experiment 3 (Table 1).

Gastrointestinal colonization of mice. From the previous results we concluded that the PCR assay may be more sensitive than blood culture for the detection of systemic *C. albicans* infection. To gain insight into the ability of the PCR assay to accurately discriminate between colonization and invasive candidiasis, a model for gastrointestinal-tract *C. albicans* colonization in mice was established.

After inoculation of six mice with 10⁷ CFU of *C. albicans*, feces were cultured from the animals at days 2, 4, and 6. Feces cultures grew *C. albicans* in a yield of 14 to 1,500 CFU per mg of feces. In contrast, feces samples of cefradine-treated mice before inoculation were all negative for *C. albicans*.

Eight days after inoculation with *C. albicans*, the mice were sacrificed and several organs were cultured. The stomach and gastrointestinal tract grew *C. albicans* in each case. In contrast, kidneys, liver, lungs, and spleen were always sterile. Cultures of blood samples obtained at day 8 via cardiac puncture were all negative, as were the results of PCR with the same blood samples (data not shown).

DISCUSSION

Several previous studies have described the development of specific PCR techniques for the detection of *C. albicans* and assessed their sensitivity in vitro and in vivo. Buchman et al. (3), Miyakawa et al. (10), and Holmes et al. (5) were able to detect ±10 *Candida* cells per 100 µl of 1 ml of blood. Buchman et al. (3), Kahn (8), and Crampin and Matthews (4) were able

TABLE 1. Individual blood culture and PCR results obtained from neutropenic mice sacrificed at the indicated times after i.v. inoculation with *C. albicans*, in each of three separate experiments

Time (h)	Results ^a for expt:						Sensitivity (%) ^b	
	1		2		3		Culture	PCR
	No. of CFU ^c	PCR ^d	No. of CFU	PCR	No. of CFU	PCR		
1	39, 7, 6	+, +, +	9, 12, 2	+, +, +	6, 3, 5	+, +, +	100	100
6	6, 1, 3	+, +, +	3, 3, 4	+, +, +	0, 3, 0	+, +, +	78	100
24	1, 0, 0	+, +, +	3, 1, 1	+, -, +	8, 4, 0	+, +, +	67	89
48	2, 0, 0	+, +, +	0, 0, 0	+, +, -	20, 7, 1	+, +, +	44	89
72	0, 0, 0	+, +, +	27, 15, 13	+, +, +	34, 45, 14	+, +, +	67	100
96	0, 0, - ^e	+, +, -	-, -, -	-, -, -	5, 1, -	+, +, -	50	100
144	1, -, -	+, -, -	-, -, -	-, -, -	-, -, -	-, -, -	100	100

^a Each result is for a single mouse (three mice per experiment).
^b Sensitivities were determined presuming all samples should be culture and PCR positive.
^c Per 100 µl of blood collected in EDTA-coated tubes.
^d Using 25 µl of pretreated blood.
^e Mouse died prematurely.

to detect *C. albicans* not only in blood samples but also in other clinical specimens including sputum and urine. However, since these specimens were usually positive by conventional culture as well, PCR did not contribute much to the actual diagnostic sensitivity. Furthermore, most assays described were too specific, in the sense that they were designed to detect *C. albicans* only.

The PCR method described in this paper uses primers aimed at the multicopy gene coding for the small subunit rRNA and results in a 180-bp fragment. The advantage is that with the use of a species-specific probe both yeast genus- and species-specific information can be obtained by a single PCR (13). To study the merits of this PCR method, its sensitivity and specificity were tested in neutropenic mice with an induced systemic infection and in immunocompetent mice with gastrointestinal colonization only. The sensitivity of this PCR method is on the order of 10 to 15 *C. albicans* cells per 100 μ l of spiked blood, which is comparable to the results of other methods (3, 5, 10). We also demonstrated that the digestion of non-yeast DNA is essential for obtaining such levels of sensitivity. Omitting the DNase pretreatment procedure reduces the sensitivity by at least a factor of 10 and may also result in specific amplicates, as was shown by Crampin and Matthews (4), although they used another primer set aimed at the HSP 90 protein. Other parameters for increasing the sensitivity, like concentrations of MgCl₂, dNTPs, and primers, have been optimized as described previously (13). Furthermore, it has been demonstrated that the storage conditions of blood samples influence the sensitivity and reproducibility of the PCR assay. We did not succeed in clarifying the negative effect of freezing, since by microscopy *Candida* cells looked intact and were still viable when checked by culture. Possibly, the *Candida* cells become fragile during freezing and thawing such that the DNase, during the pretreatment step, can reach some of the essential *Candida* target DNA, thereby reducing the sensitivity and reproducibility of the PCR assay.

For the neutropenic mice with a systemic *Candida* infection, a higher percentage of positive test results was obtained by PCR than was obtained by conventional blood culture. Kahn (8) also demonstrated positive PCR results with a candidemic murine model. However, in that study, blood samples obtained from five mice were pooled and were always positive by culture. In contrast, we demonstrated positive PCR results from blood samples of individual mice, some of which had sterile blood cultures at the same time. Positive PCR results may be due to the amplification of circulating nonviable *Candida* cells, e.g., those within phagocytic cells. It is this aspect of the PCR technique which makes the method more sensitive than blood culture. Makimura et al. (9) also obtained positive PCR results from culture-positive as well as culture-negative blood samples of mice infected with *C. albicans*. However, for DNA extraction they used a volume of blood which was twice that used for culture. Since the sensitivity of an assay is influenced by the volume tested, their results should be interpreted with great care. In our study we actually applied only 25 μ l of blood for PCR compared with 100 μ l for blood culture. Thus, the sensitivity of our PCR method may be further increased by using more target DNA in the final reaction. Despite the sensitivity of the PCR, PCR results obtained from blood samples of gastrointestinal colonized mice were always negative. This particular aspect makes this PCR method useful since it is able to discriminate between invasive candidiasis and colonization. The negative findings with the colonized model also underline

the target specificity of this PCR method, in that no false-positive results were obtained because of existing normal mouse bacterial flora.

Although the PCR assay of blood samples of mice with systemic candidiasis was not designed to give quantitative information, the intensity of the hybridization signal clearly increased with the progression of the infection. These findings suggest a correlation between the PCR results and the degree of dissemination of infection and make the technique possibly valuable as a clinical monitoring assay.

In conclusion, PCR with primers directed against the small subunit rRNA gene in combination with the *C. albicans*-specific probe provided a sensitive and specific assay for detecting *Candida* DNA in whole blood. This PCR method promises to be more sensitive than blood culture and may be an adequate technique in the early detection and follow-up of invasive candidiasis. Besides being sensitive, this PCR is specific, since PCR results obtained from blood samples of gastrointestinal colonized mice were negative; therefore, this PCR is diagnostically useful. Another positive diagnostic contribution is the relative speed of the PCR, since results are achieved within 2 days, whereas conventional blood cultures take 1 to 7 days or even longer. The true clinical relevance and utility of this test has to be proven in a prospective clinical study in which conventional blood cultures are evaluated in relation to PCR.

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