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PCR Monitoring of Response to Liposomal Amphotericin B Treatment of Systemic Candidiasis in Neutropenic Mice

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When a diagnosis of invasive candidiasis has been made, treatment with toxic fungicidal agents is inevitable. The crucial decision of when to stop such treatment is difficult to make, because cultures are often negative despite ongoing invasive candidiasis and can therefore not be used as a reliable parameter of effective therapy. In the present study, the use of PCR in monitoring the therapeutic efficacy of antifungal treatment with liposomal amphotericin B was evaluated by using neutropenic mice with systemic candidiasis. Blood cultures of infected mice treated with different doses of liposomal amphotericin B were only positive at the early onset of the infection process and became sterile within 3 days; this was true even with mice treated with 1 mg of liposomal amphotericin B per kg of body weight that experienced a relapse of infection 14 days later. A significant correlation between presence of *Candida albicans* in the kidneys and PCR results obtained with blood was demonstrated. Thus, PCR results obtained with blood samples correlated well with the therapeutic efficacy of antifungal treatment.

Invasive candidiasis is an important cause of mortality and morbidity, particularly in immunocompromised patients. Unfortunately, efficient diagnosis is hampered by the lack of clinical parameters that can be used to recognize invasive candidiasis. A combination of microbiological, histological, and serological results may sometimes contribute to the diagnosis, but these tests are not 100% sensitive and specific (3). Recently, non-culture-dependent assays such as the PCR have gained increasing importance in the rapid diagnosis of infections in patients at risk (10). The contribution of PCR assays in the diagnosis of fungal infections has now been demonstrated with a wide variety of clinical specimens, including blood, serum, respiratory secretions, urine, peritoneal fluid, and cerebrospinal fluid (2, 4–7, 9, 12). It has been demonstrated that PCR has a better sensitivity than blood cultures in detecting *Candida albicans* infection in persistently neutropenic mice (14). Apart from helping to diagnose invasive candidiasis, an ideal assay should also help in monitoring the response to treatment and thus in determining the duration of therapy. Since toxic fungicidal agents are used, achieving accurate estimates of the time at which treatment can be stopped is clinically highly relevant. In the present study, we evaluated the use of PCR in monitoring the response to antifungal treatment with a liposomal formulation of amphotericin B (AmB) (AmBisome) in neutropenic mice with systemic candidiasis. PCR results were compared with blood culture results at several time points during the course of the infection.

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MATERIALS AND METHODS

Animals and *C. albicans* strain. Specific-pathogen-free, 11- to 13-week-old female BALB/c mice weighing 22 ± 1.3 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 *C. albicans* in Sabouraud maltose broth (Difco Laboratories) for 24 h at 37°C. Yeast cells were washed in phosphate-buffered saline (PBS), counted in a hemocytometer, and adjusted to 5×10^4 cells per ml in PBS.

Antifungal agent. AmB, encapsulated in small unilamellar vesicles consisting of hydrogenated soybean phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol in a molar ratio of 2:1:0.8, was kindly provided by Vestar Inc. (San Pimas, Calif.) as a lyophilized preparation. The powder was reconstituted with distilled water at 65°C according to the manufacturer's instructions to give a liposomal suspension containing 4 g of AmB per liter and 35 g of lipid per liter.

Systemic infection in neutropenic mice. A model of invasive candidiasis in persistently neutropenic mice, as previously described, was used (15). Briefly, neutropenia was induced by intraperitoneal administration of cyclophosphamide (Sigma Chemical Co., St. Louis, Mo.) 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 (15). This treatment resulted in persistent granulocytopenia of <100 granulocytes per μl of blood from the time of inoculation with *C. albicans* up to termination of the studies.

In each experiment, 19 neutropenic mice were infected by inoculation of 200 μl of 5×10^4 *C. albicans* CFU/ml in the tail vein. AmB was administered intravenously (i.v.) for 4 consecutive days at a dose of 1, 7, or 14 mg/kg of body weight. At 1 h or 3, 6, 14, 21, and 35 days after inoculation with *C. albicans*, groups of three to four mice were sacrificed and blood was collected in EDTA-coated tubes by cardiac puncture. Of each blood sample, 100 μl was directly cultured on Sabouraud dextrose agar (SDA) plates (Oxoid, Basingstoke, England) and incubated for 5 days at 37°C. Another 100 μl 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.). Residual AmB was eliminated from the homogenates by washing the sediment after centrifugation three times with PBS. Two hundred microliters of the serial 10-fold dilutions of each homogenate was plated on SDA plates. Two milliliters of the homogenate and the remainder of the homogenate, together with 18 ml and an equal volume of double-strength SDA, respectively, were mixed and poured into petri dishes. All plates were incubated for 48 h at 37°C.

PCR sample preparation. For *Candida* DNA extraction from blood we used a method modified from that of Buchman et al. (2, 14). Briefly, 0.1 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100) was added to 0.1 ml of whole blood. After lysis, cell debris and *Candida* cells were pelleted by centrifugation (Eppendorf centrifuge; Merck, Amsterdam, The Neth-

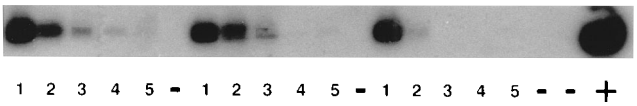

FIG. 1. Southern blot analysis of *C. albicans* PCR products obtained by amplification of different volumes of processed blood samples spiked with 2,000, 200, 20, 10, 2, and 0 (negative control [−]) *C. albicans* cells per 100 μl of blood. Lanes 1 to 5 represent the dilution series obtained by amplification of 5, 10, and 15 μl of processed blood (lane sets from left to right); +, positive control (whole blood spiked with *C. albicans* cells).

erlands) at 16,000 × *g* for 5 min. The pellet was resuspended in 0.2 ml of lysis buffer. To remove free, non-yeast-cell DNA, 7 μl of DNase (10 mg/ml; Boehringer Mannheim Gmbh, Mannheim, Germany) was added and the samples were incubated at 37°C for 1 h. After centrifugation at 16,000 × *g* for 5 min, 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 μl of Lyticase (900 U/ml; Sigma Chemical Co.) and incubated for a further 1 h at 37°C. Subsequently, 3.0 μl of pronase (15 mg/ml; Boehringer Mannheim) and 10 μl of 10% sodium dodecyl sulfate (SDS) were added and incubation was continued for 1 h at 37°C. DNA extraction and purification were 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 μl of TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA).

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

Primers and PCR protocol. PCR was performed in a 100-μl volume under standard conditions with 5 and 10 μl of the DNA samples. 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′-CCTGCTTTGAACACTCTAATTT-3′) (10). PCR was performed in 100 μl of PCR solution, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 50 pmol of each primer, 0.1 mg of gelatin per ml, 1 U of *Taq* DNA polymerase (Promega, Leiden, The Netherlands), and 5 or 10 μl of sample. The reaction mixture was overlaid with 100 μl 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. A 30-μl volume 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′) (10). Membranes were preincubated at 37°C in a hybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's solution) for 1 h. Hybridization occurred overnight at 37°C with 20 pmol of T_4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham International, plc)-labelled oligonucleotide probe. The filters were washed once for 15 min at 37°C and once for 15 min at 55°C with 2× SSC–0.1% (wt/vol) SDS. Autoradiography was performed with Kodak Royal X-Omat film and a 3.5-h exposure time.

RESULTS

Optimization of *Candida* PCR. To rule out an inhibitory effect of residual AmB on the PCR assay, blood of mice pretreated with AmB at 17 mg/kg for 4 days was spiked with a 10-fold dilution series of 2,000 to 2 cells of *C. albicans* and incubated for 30 min at 37°C and then tested in the PCR assay. No difference in the lower limit of detection was found between blood of treated and untreated mice (data not shown). In a previous study, we used 10 μl of DNA sample in the final PCR (10). In the present study, we tried to improve the sensitivity of the PCR assay by adding several volumes of extracted DNA obtained from blood samples spiked with different numbers of yeast cells. No significant differences were obtained by using either 5 or 10 μl of extracted DNA sample in this in vitro experiment. However, the sensitivity of the PCR decreased 100-fold if 15 μl of the same DNA sample was used (Fig. 1). We thus decided to use 5 μl as well as 10 μl of DNA samples in the current study.

PCR monitoring of invasive candidiasis. After challenge with *C. albicans*, AmB was administered for 4 consecutive days at a dose of 1, 7, or 14 mg/kg of body weight. In mice treated with 1 mg of AmB per kg, an initial drop in CFU per organ in lungs, livers, and spleens was observed and then there was an increasing number of CFU per organ from 14 to 21 days after inoculation (Fig. 2). The number of CFU in the kidneys, however, remained stable and increased from 10^4 at day 14 to 10^6 at day 35.

Cultures from lungs, livers, and spleens of mice treated with 7 and 14 mg of AmB per kg became sterile within 3 days of

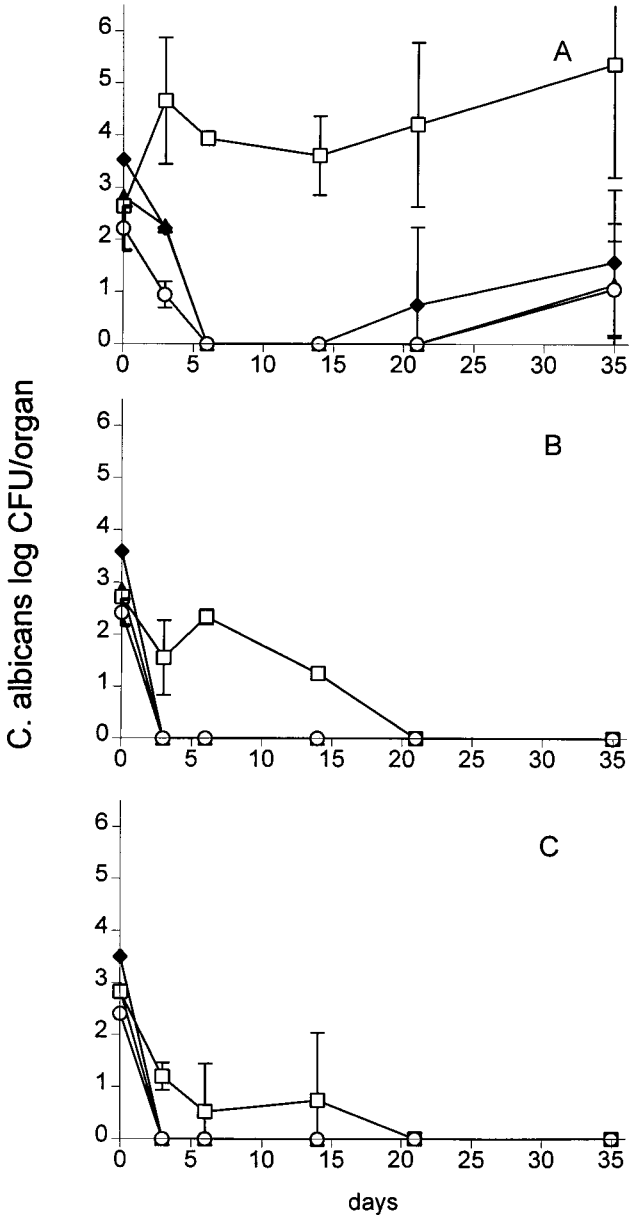

FIG. 2. Progression of systemic infection in neutropenic mice treated with 4 daily doses of 1 mg of AmB per kg (A), 7 mg of AmB per kg (B), 14 mg of AmB per kg of body weight (C). Data are expressed as the mean number ± standard error (error bars) of CFU of *C. albicans* in kidneys (□), spleens (○), lungs (▲), and livers (◆). Data are based on three to four mice each group.

TABLE 1. Blood culture and PCR hybridization results for neutropenic mice after i.v. inoculation with *C. albicans*

Time	Result with AmB regimen[a]:								
	1 mg/kg			7 mg/kg			14 mg/kg		
	No. of CFU[b]	PCR^{5c}	PCR^{10d}	No. of CFU	PCR^{5}	PCR^{10}	No. of CFU	PCR^{5}	PCR^{10}
1 h	0, 6, 4	+, +, +	+, +, +	1, 1, 7	−, +, +	+, +, +	8, 6, 6	+, +, +	−, −, −
3 days	0, 93, 0	−, +, −	+, +, +	0, 0, 0	−, −, −	−, −, −	0, 0, 0	−, −, −	−, −, −
6 days	0, 0, 0	−, +, +	+, +, +	0, 0, 0	−, +, −	+, +, +	0, 0, 0	−, −, −	−, −, −
14 days	0, 0, 0	−, −, −	+, +, +	0, 0, 0	+, −, −	+, +, −	0, 0, 0	+, +, −	−, −, −
21 days	0, 0, 0, 0	−, +, +, +	+, −, −, +	0, 0, 0, 0	+, +, −, −	+, +, +, +	0, 0, 0	+, +, +	−, −, −
35 days	0, 0, 0	+, +, +	+, +, +	0, 0, 0	+, +, −	+, +, −	0, 0, 0	−, −, −	−, −, −

[a] Mice were treated with 1, 7, or 14 mg of AmB per kg for 4 days and sacrificed at the indicated intervals; data are from individual animals in each of three separate experiments.
[b] Data are per 100 μl of blood.
[c] PCR^{5}, hybridization signals obtained by using 5 μl of processed blood for PCR. +, positive signal; −, negative signal.
[d] PCR^{10}, hybridization signals obtained by using 10 μl of processed blood for PCR. +, positive signal; −, negative signal.

treatment (Fig. 2). In contrast, the number of CFU in the kidneys of mice treated with 7 and 14 mg of AmB per kg decreased only slowly and cultures became sterile between days 14 and 21 (Fig. 2). Eight of nine blood samples taken 1 h after the challenge grew *C. albicans* (Table 1). Of the mice treated with 1 mg of AmB per kg, only one had a *C. albicans*-positive blood culture for the sample taken 3 days after the challenge. All other blood samples taken later in the infection process in all three treatment regimens failed to grow *C. albicans.*

The PCR results obtained by amplification of 5 μl of target DNA (Fig. 3) of blood samples of mice treated with 1 mg of AmB per kg show an initial decrease in PCR hybridization signals, which were absent on day 14. However, at day 21 PCR gave positive results again (Table 1). PCR with 10 μl of target DNA (Fig. 4) actually demonstrated that a constant level of target DNA was present in the blood; however, the intensity of the hybridization signals of samples taken on day 35 was higher.

Mice treated with 7 mg of AmB per kg showed positive PCR results only at 1 h and 35 days if 5 μl of target DNA was used. PCR with 10 μl showed a similar profile. However, more often a positive PCR result was found between 1 h and 35 days (Fig. 3 and 4; Table 1). Samples from mice treated with 14 mg of AmB per kg gave several positive PCR signals over time if 5 μl was used; however, 5-μl target DNA samples were all PCR negative at day 35. If 10 μl was used, only negative PCR signals were obtained (data not shown).

A positive correlation between the PCR results obtained by using 10 μl of processed sample for all three treatment regimens and the positive kidney cultures of the mice was demonstrated ($P = 0.04$; Fisher extract test). No such correlation could be demonstrated with the data from PCR with 5 μl of processed blood sample.

DISCUSSION

Generally, it takes 2 to 7 days for *Candida* spp. to grow from patients' specimens. Therefore, microbiological culture cannot be regarded as an early diagnostic tool. Furthermore, even the best blood culture technique often gives negative results despite evidence of systemic infection (1). A breakthrough in diagnostic microbiology has been the introduction of the PCR technique for the detection of *Candida* spp. in several clinical specimens (8, 11). In a previous study, we demonstrated that PCR was more sensitive than culture in detecting *C. albicans* in blood from neutropenic mice with invasive candidiasis (14). Once the diagnosis of invasive candidiasis has been made, treatment with toxic fungicidal agents is inevitable. The decision of when to stop treatment is even more difficult to make, since negative cultures are not always indicative of a therapeutic response. Therefore, the aim of the present study was to examine the usefulness of PCR as an assay to monitor the response to AmB in neutropenic mice with invasive candidiasis.

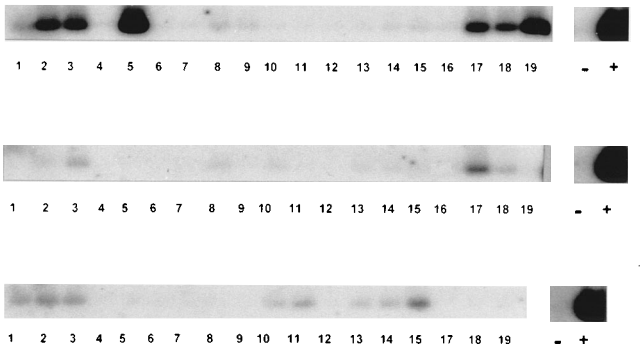

FIG. 3. Southern blot analysis of 5 μl of *C. albicans* PCR products obtained from blood samples from mice treated with 1 (top), 7 (middle), and 14 (bottom) mg of AmB per kg. Lanes 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 16, and 17 to 19 represent mice sacrificed at 3 h and 3, 6, 14, 21, and 35 days after i.v. inoculation, respectively. +, positive controls (whole blood spiked with *C. albicans* cells); −, negative controls (whole blood of uninfected control mice).

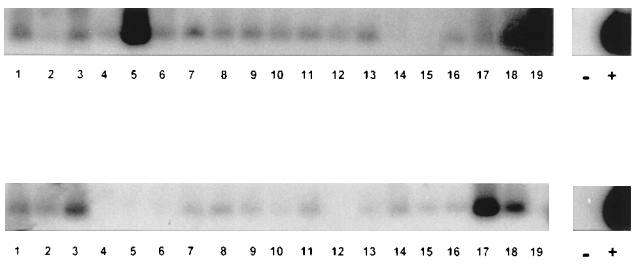

FIG. 4. Southern blot analysis of 10 μl of *C. albicans* PCR products obtained from blood samples of mice treated with 1 (top) and 7 (bottom) mg of AmB per kg. Lanes 1 to 3, 4 to 6, 7 to 9, 10 to 12, 13 to 16, and 17 to 19 represent mice sacrificed at 3 h and 3, 6, 14, 21, and 35 days after i.v. inoculation, respectively. +, positive controls (whole blood spiked with *C. albicans* cells); −, negative controls (whole blood of uninfected control mice).

Berenguer et al. (1) demonstrated in a clinical setting the existence of a correlation between the number of organs infected by *Candida* spp. and the number of positive blood cultures. Our results showed that blood cultures of infected mice treated with three different doses of AmB were positive only at the beginning of the treatment and became sterile within 3 days, even for mice treated with 1 mg of AmB per kg that experienced a relapse of infection after 14 days. On the other hand, a significant correlation between positive kidney cultures and the PCR results was demonstrated. Likewise, hybridization signals of the PCR increased with increasing numbers of *C. albicans* CFU in the kidneys of untreated mice (14). This correlation between positive kidney cultures and positivity of the PCR can only be explained by the fact that intact *Candida* cells are still present and detectable by PCR in the peripheral circulation. Since in the DNA extraction procedure a DNase treatment step is incorporated before the actual lysis of the *Candida* cells, the possibility that we are detecting circulating *C. albicans* DNA only is ruled out. Blood culture results for the same samples were negative, however, probably because of the low level or complete absence of metabolic activity of the *Candida* cells or the inhibitory presence of residual AmB.

Strong hybridization signals were observed at day 35 with mice that had been treated with a regimen of 7 mg of AmB per kg for 4 days, suggesting a relapse of the infection despite negative cultures from the kidneys, livers, lungs, and spleens at that moment. This hypothesis could not be further analyzed since we did not evaluate the progression of infection beyond day 35. Samples obtained with regimen of 14 mg of AmB per kg showed negative PCR results at day 35. This regimen might thus be more effective than 7 mg of AmB per kg, implicating that the mice treated with 7 mg of AmB per kg had not completely cleared the *Candida* cells. On the other hand, false-positive signals are not a problem with this PCR since multiple blood samples from gastrointestinally colonized mice (14) and normal control mice were all negative by PCR.

PCR with 5 μl of processed target DNA was less sensitive than PCR with 10 μl of target DNA of blood samples in the in vivo experiment for mice treated with 1 or 7 mg of AmB per kg, indicating that more target DNA increases the sensitivity of the PCR assay. Application of 15 μl of processed target DNA in the PCR, however, caused false-negative results, probably due to inhibitory factors present in the blood samples. For the same reason, no hybridization signals were obtained by using 10 μl of target DNA of blood samples of mice treated with 14 mg of AmB per kg, whereas the same samples were PCR positive if 5 μl was used. This indicates the narrow window between negative results due to inhibitory factors and the detection limit of the assay. Therefore, to exclude false-negative PCR results it may be necessary to routinely use several concentrations of target DNA in the PCR assay. This limitation in sensitivity of the PCR might be overcome in the near future if rRNA instead of DNA is used, since there is a high copy number of rRNA present in each cell. Another advantage of rRNA is that it can be amplified in a commercially available nucleic acid sequence-based amplification system, which is faster than PCR (13) and whose enzymes are less sensitive to inhibitory factors. The advantage of commercial systems is that they are easy to perform and detection formats are less laborious, which makes the introduction of these techniques into all microbiology laboratories possible.

We conclude that in this neutropenic murine model of invasive candidiasis, PCR results correlated well with the response to the therapy administered. PCR is much better in this respect than routine blood cultures. However, before this assay can be used as a universal monitoring tool, PCR needs to be further evaluated in this or other animal models and in a clinical setting as well.

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