Serum Is More Suitable than Whole Blood for Diagnosis of Systemic Candidiasis by Nested PCR

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PCR assays for the diagnosis of systemic candidiasis can be performed either on serum or on whole blood, but results obtained with the two kinds of samples have never been formally compared. Thus we designed a nested PCR assay in which five specific inner pairs of primers were used to amplify specific targets on the rRNA genes of Candida albicans, C. tropicalis, C. parapsilosis, C. krusei, and C. glabrata. In vitro, the lower limit of detection of each nested PCR assay was 1 fg of purified DNA from the corresponding Candida species. In rabbits with candidemia of 120 minutes’ duration following intravenous (i.v.) injection of 106 CFU of C. albicans, the sensitivities of the PCR in serum and whole blood were not significantly different (93 versus 86%). In other rabbits, injected with only 105 CFU of C. albicans, detection of candidemia by culture was possible for only 1 min, whereas DNA could be detected by PCR in whole blood and in serum for 15 and 150 min, respectively. PCR was more often positive in serum than in whole blood in 40 culture-negative samples (27 versus 7%; P < 0.05%). Lastly, experiments with rabbits injected i.v. with 20 or 200 μg of purified C. albicans DNA showed that PCRs were positive in serum from 30 to at least 120 min after injection, suggesting that the clearance of free DNA is slow. These results suggest that serum is the sample of choice, which should be used preferentially over whole blood for the diagnosis of systemic candidiasis by PCR.

Systemic candidiasis is a major nosocomial infection in patients given immunosuppressive chemotherapy for cancer treatment or organ transplantation and in patients undergoing heart or abdominal surgery (18). Patients with candidemia have a poorer prognosis than those with nosocomial bacteremia (19, 25). Mortality rates among those with systemic candidiasis remain high, ranging from 50 to 80%, despite adequate treatment (11, 26). In the absence of pathognomonic signs or symptoms of systemic candidiasis, diagnosis is usually based on the isolation of Candida species from blood cultures or tissue biopsy specimens. However, since the sensitivity of blood cultures for diagnosis of systemic candidiasis is low at the early stage of the infection, and since it has been shown that the prognosis is better when treatment is started early, it is usually recommended that antifungal therapy be started as soon as a strong suspicion of systemic candidiasis exists (9, 16, 20). On the other hand, such empiric antifungal therapy may be unnecessarily toxic and costly, and it may increase the selective pressure towards more-resistant Candida species (29). Thus, efforts have been made to develop more-sensitive methods for the earliest possible diagnosis of systemic candidiasis. One of these involves the PCR method in which different targets of Candida DNA have been tested: either single-copy genes such as the actin (15), chitin synthase (14), HSP 90 (7), and lanosterol-14 α-demethylase-encoding genes (3, 4) or multicopy genes such as the gene coding for rRNA (12, 13, 22, 23). Hybridization (8, 12, 22, 23) and nested PCR (4, 6) experiments have been used to identify all the amplifiers at the Candida species level. The best of these assays are those which can identify all the species most commonly involved in candidemia: Candida albicans, C. tropicalis, C. krusei, C. parapsilosis, and C. glabrata (8, 12–14, 22). It has been demonstrated that PCR can be performed either on whole-blood samples (3, 4, 10) or on serum samples (5, 6, 15). However, the efficiencies of the same PCR assay applied simultaneously to serum or whole blood have never been formally compared. These might not be equivalent, since the DNAs present in the two types of samples are probably different in origin. Indeed, only free template DNA should be detectable in serum samples, since fungal cells are eliminated by centrifugation without having been lysed to release intracellular DNA (6). By contrast, when whole-blood samples are used, both free DNA and intracellular DNA could be present when the sample is drawn from the patient. However, because of the presence in blood of PCR inhibitors, such as hemoglobin, a decontamination step, including lysis of blood cells and washing, is performed first. These steps probably eliminate free Candida DNA, leaving intracellular Candida DNA as the sole possible target for the PCR assay (3, 4, 8, 23). Thus, depending on the sample used, the origin of the detected DNA probably varies. This may result in a difference in the sensitivity of the assay and in its clinical significance. To our knowledge these issues have not been fully investigated. This is why, in the present study, our efforts have been focused on that question. We have used a rabbit model of experimental candidemia specifically developed in this laboratory. We used DNA coding for the 5.8S rRNA and the adjacent internal transcribed spacer (ITS) as the target for amplification, and we have compared the positivities of the PCR on whole-blood and serum specimens, using blood cultures as the reference assay.

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TABLE 1. Oligonucleotides used in nested PCR

<table>
<thead>
<tr>
<th>Species (accession no.) and primers</th>
<th>Sequence (5′→3′)</th>
<th>Nucleotide positiona</th>
<th>Fragment size (bp)</th>
<th>Tempb (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans (L47111)</td>
<td>Oligonucleotide 1: CAL1</td>
<td>AACTTGCTTTTGCGGCTGGGC</td>
<td>73</td>
<td>386</td>
</tr>
<tr>
<td>Oligonucleotide 2: CAL3</td>
<td>TGGACGTATACCGCGGCAGC</td>
<td>439</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. tropicalis (L47112)</td>
<td>Oligonucleotide 1: CTR1</td>
<td>ATTCTTTGTGCGCGGACGC</td>
<td>73</td>
<td>373</td>
</tr>
<tr>
<td>Oligonucleotide 2: CTR3</td>
<td>GGCCACTAGCAAAATAGCG</td>
<td>426</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. glabrata (L47110)</td>
<td>Oligonucleotide 1: CGL1</td>
<td>ATGCTATTTTCCTGCCTGTC</td>
<td>128</td>
<td>234</td>
</tr>
<tr>
<td>Oligonucleotide 2: CGL3</td>
<td>TGNATCCAGTGGGAAACTC</td>
<td>342</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis (L47109)</td>
<td>Oligonucleotide 1: CPA1</td>
<td>GCCGAGGATTANACTCAACC</td>
<td>123</td>
<td>336</td>
</tr>
<tr>
<td>Oligonucleotide 2: CPA3</td>
<td>GGAAGAAGTTTGGGAGTIG</td>
<td>439</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. krusei (L47113)</td>
<td>Oligonucleotide 1: CKR2</td>
<td>ACTACACGTGGTAGCGGAA</td>
<td>43</td>
<td>360</td>
</tr>
<tr>
<td>Oligonucleotide 2: CKR3</td>
<td>AAAAGCTAGTTCCGCTGG</td>
<td>383</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal control</td>
<td>Oligonucleotide 1: M1</td>
<td>AACTTGCTTTTGCGGCTGGGCCCTCGGTTTCTTCTCGTA</td>
<td>491</td>
<td>66</td>
</tr>
<tr>
<td>Oligonucleotide 2: M3</td>
<td>TGGAGCTTTACCGCGGAAATGCGGAAACTCCGACTTGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a In the ITS sequence.  
b Annealing temperature.
In vitro sensitivity and specificity of Candida nested PCR. As indicated in Fig. 1, the limits of purified Candida DNA detection by nested PCR ranged from 1 to 0.1 fg, depending on the Candida species. By inoculation of human and rabbit blood samples with either C. albicans or C. tropicalis cells, a PCR assay was able to detect the Candida DNA extracted from as few as 100 CFU/ml for C. albicans and 30 CFU/ml for C. tropicalis. There was no difference between the results of experiments performed with human and rabbit blood (data not shown).

The observed specificities of the species-specific Candida nested PCRs were all 100%. Indeed, the inner primers designed for a given Candida species never amplified DNA from any of the other four Candida species (Fig. 1 and Table 2) or from any of the other fungal, bacterial, or parasitic species tested. No cross-reactivity with human DNA was observed (Table 2).

Sensitivity of C. albicans nested PCR applied to whole blood and serum, compared to that of quantitative blood cultures in the rabbit model. In the five rabbits injected with 10^8 CFU of C. albicans ATCC 2091, results of quantitative blood cultures showed that 90% of the microorganisms present in the blood 1 min after injection were cleared within 4 min. Blood cultures then remained positive, with a low concentration of C. albicans (1 to 17 CFU/ml) during the remaining 115 min of the experiment. Nested PCRs performed on whole blood were positive in 26 of 30 (86%) samples tested, comprising all of the 11 samples in which the counts of C. albicans were greater than 10 CFU/ml, and 15 of the 19 samples in which the counts were equal to or lower than 10 CFU/ml (Fig. 2). When PCR was performed on serum, a total of 28 of 30 (93%) samples were positive. The two negative serum samples were from the same rabbit and were drawn at 1 and 5 min after injection, when cell concentrations of C. albicans were high (Fig. 2). Negative PCRs were not due to the presence of inhibitors in whole-blood or serum samples, since the internal positive PCR controls were always positive (Fig. 3).

When a smaller inoculum of 10^5 CFU of C. albicans ATCC 2091 was injected, a candidemia of brief duration was observed in five rabbits and no candidemia was observed in one rabbit at 1 min postinjection, and the PCRs performed on whole blood and on sera were positive in 4 of 6 and 3 of 6 of these rabbits, respectively (Table 3). The blood cultures remained negative.

### RESULTS

### Statistical analysis

The differences between PCR positivity rates on serum and on whole blood were tested by using the chi-square test with Yates' correction at the 5% level of significance.
thereafter, but the PCRs performed on whole blood were still positive in two rabbits and one rabbit at 5 and 15 min postinjection, respectively (Table 3). In addition, the PCRs performed on sera were positive for 11 of the 35 samples drawn from 5 to 150 min postinjection but were always negative for samples drawn 180 min after injection. Overall, among the 40 culture-negative samples drawn later than 1 min after injection, 3 (7%) were positive when PCR was performed on whole blood and 11 (27%) were positive when PCR was performed on serum (P < 0.05%).

**Sequence comparison.** The sequences of the 386-bp fragments amplified from C. albicans ATCC 2091 and from the sera of infected rabbits were strictly homologous. They differed from the corresponding GenBank C. albicans sequence (accession no. L47111) only by the insertion of a G base between positions 353 and 354 (99.7% homology).

**Purified Candida DNA clearance from rabbit blood using nested PCR.** When purified C. albicans DNA was directly injected into rabbits at a dose of 200 μg, the PCRs were positive in serum samples from 1 to at least 120 min. They were positive from 1 to at least 30 min when only 20 μg was injected (Table 4).

**DISCUSSION**

We designed a nested Candida PCR assay in which an amplified fragment of the Candida ITS repeated region was used as a template for five different inner primer pairs, which were chosen for specific amplification of the DNAs of the five species most frequently causing human candidiasis (2, 28). The in vitro specificity of our five PCRs was 100%. The sensitivity was similar to that published elsewhere for PCR targeting repeated genes and using specific probe hybridization assays for species differentiation (8, 12).

For in vivo evaluation, we used an experimental model of infection in rabbits in which candidemia was studied over a period of 120 min after injection of a 10⁸-CFU C. albicans bolus. This inoculum was similar to that used in a previously described model of experimental candidemia in rabbits (1). Because of the large volumes of the blood samples that could be drawn from rabbits, we could precisely compare the sensitivities of our nested PCR in whole blood and serum. Such a strict comparison had not and probably could not have been performed in studies of experimental candidiasis in smaller laboratory animals (6, 15, 17). Compared to quantitative blood cultures, the overall observed sensitivity of our PCR assay was 86% for whole blood and 93% for serum. The PCR assay was negative in four whole-blood and two serum samples which were positive in culture. The sampling times at which the PCR was negative in whole blood differed from those at which it was negative in serum, possibly due to the different origins of the template DNA. Considering, first, that template DNA in whole blood originated from DNA extracted ex vivo from Candida cells circulating in the blood and, second, that the four PCRs with false-negative results in whole blood were performed on samples with low Candida counts (5, 4, 2, and 2 CFU/ml), negativity may reflect difficulty in extracting DNA ex vivo by cell lysis, as reported elsewhere (23, 24). PCR was positive in 28 of 30 serum samples for which there was no such ex vivo cell

**FIG. 2.** Sensitivity of quantitative blood cultures compared to that of nested PCR performed on whole blood and on serum from five rabbits infected with 10⁸ CFU of C. albicans. Each rabbit is represented by a circle at each sampling time. ⓞ, positive nested PCR in both whole blood and serum; ⓜ, positive nested PCR in whole blood and negative nested PCR in serum; ⓜ, negative nested PCR in whole blood and positive nested PCR in serum.

**FIG. 3.** Coamplification of internal PCR control and rabbit blood samples. Lanes 1, 2, 3, and 4, whole-blood samples; lanes 5 and 6, serum samples which exhibited a negative C. albicans nested PCR; lanes 7 and 8, two whole-blood samples for which this PCR was positive. Amplification of the internal control is indicated by the presence of a 491-bp fragment, and amplification of C. albicans DNA in blood is indicated by the presence of a 386-bp fragment. M, molecular weight marker.
was used for the detection of Candida during the culture-positive candidemic periods. Both in whole-blood samples and in serum samples drawn human candidemia. However, we also showed that PCR was more sensitive than cultures in diagnosing candidon sera remained positive for a longer period, confirming that following injection, but PCRs performed on whole blood and , blood cultures were positive only during the 1st min, and from nested PCR performed on whole-blood and serum samples. A slow clearance of free DNA was also observed in the rabbits that we injected with purified C. albi-

In conclusion, our results showed that serum samples should be used preferentially over whole blood to diagnose candidemia by PCR. They also confirmed that Candida template DNA which can be detected by PCR during the candidemic episodes corresponds both to DNA from intact cultivable or noncultivatable Candida cells, and to free DNA released in vivo. Whether the same will be observed in neutropenic and post-surgery patients who are at high risk of Candida infection is now being investigated in a prospective clinical trial.

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