IV: Clinical evaluation of a NASBA-based assay for detection of *Candida* spp. in blood and blood cultures

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SUMMARY

The number of life-threatening opportunistic fungal infections has shown a dramatic increase. However, the diagnosis of candidaemia remains difficult. Nucleic acid amplification assays may improve the detection rate and decrease the time needed for detection and identification of Candida spp. Whole blood samples of patients suspected of having candidaemia were analyzed using Nucleic Acid Sequence-Based Amplification (NASBA). Furthermore, aliquots of blood cultures of the patients after 2 days of culturing were tested. Eleven data sets from ten patients in two hospitals were generated. None of the whole blood samples was positive in the NASBA assay. Eight samples were positive in the NASBA assay after two days of culturing, whereas only two additional positive samples were found after longer incubation periods. Thus, a two-day culture step is sufficient to greatly improve the sensitivity of the NASBA assay. The NASBA assay detected Candida RNA in three patients. In one patient, the yeast was not detected by automated blood culturing, in another patient the NASBA assay detected the infection two days earlier than the blood culture system.

KEY WORDS

Candida spp., Nucleic Acid Sequence-Based Amplification (NASBA), blood culture

INTRODUCTION

The number of life-threatening opportunistic fungal infections in immunocompromised patients has shown a dramatic increase. Candida spp. account for the majority of these infections. The attributable mortality of Candida infections is approximately 38%\(^{19}\), and crude mortality rates exceed 50%\(^{7,12,18}\). The diagnosis of candidemia remains difficult. Automated blood culture systems are routinely used, but fail to detect yeasts in many cases\(^{2,11}\). Furthermore, prolonged incubation times or terminal subculturing of negative blood culture bottles may be necessary before yeast growth can be detected\(^{3,14,17}\).

Because of their sensitivity and speed, nucleic acid amplification assays may play an important role in improving the detection rate and decreasing the time needed for detection and identification of Candida spp. As little as 10 molecules of rRNA can be detected with a Nucleic Acid Sequence-Based Amplification (NASBA-) assay\(^{20}\). This assay makes use of primers directed against conserved regions of the 18S rRNA of medically important fungi and specific probes for the identification of different Candida spp. When using whole blood samples, 1-10 cfu of C. albicans could still be detected.

In a previous study, we showed that the NASBA assay could detect candidemia, whereas no Candida RNA was detected in blood culture medium alone, or in blood cultures from healthy volunteers, uninfected patients or patients colonized with Candida spp. When we used the NASBA assay to analyze culture-positive as well as negative blood cultures from patients with a proven candidemia after 7 days of culturing, we showed that compared with automated blood culturing, the number of positive blood cultures increased from 21% to 34%\(^{4}\).
Here, we present the results of a clinical evaluation of the NASBA assay. Whole blood samples of patients suspected of having candidemia were analyzed. However, since candidemia is characterized by a low number of yeast cells in the bloodstream, a short culture step may further increase the detection rate. Therefore, aliquots of blood cultures from the patients after 2 days of culturing were also tested. The results were compared with automated blood culturing as well as results from the NASBA assay on aliquots of blood cultures after 7 days of culturing.

MATERIALS AND METHODS

Clinical samples. Blood and blood culture samples were obtained from the University Medical Center (UMC), Utrecht, the Netherlands and the Catharina Hospital, Eindhoven, the Netherlands, between March 2000 and June 2001. The study design was approved by the Medical Ethics Committee of the UMC Utrecht (protocol no. 99/104). Patients in the intensive care unit (ICU) were included when there was a clinical suspicion of candidemia and a presence of two or more systemic inflammatory response syndrome (SIRS)-criteria: temperature < 36°C or > 38°C; tachycardia > 90 beats/min.; CO₂ < 32 mm Hg; respiratory rate > 24 breaths/min.; leukocytes < 4 x 10⁹/l or > 12 x 10⁹/l; > 10% immature (band) forms. Patients in the hematology ward were included when they had neutropenia (<100 granulocytes/µl) and did not respond to broad spectrum antibiotic treatment for 48-72 hours. In the Catharina Hospital Eindhoven, the inclusion criteria also included culturing of yeasts from 2 or more foci. Patients or their relatives were required to comprehend and sign an informed consent form. EDTA-blood was drawn from the patients on day 1 (before treatment with antymycotic agents was started), divided into 1 ml aliquots, and stored at -70°C. Two blood culture sets were drawn on day 1: one from a central venous catheter (if present) and one from a peripheral vein (if possible). Each blood culture set consisted of one aerobic and one anaerobic blood culture bottle (BacT/Alert, Organon Teknika, the Netherlands). In the UMC Utrecht, FAN aerobic bottles were used. On days 3 and 5 another blood culture set was drawn from a peripheral vein (if possible, otherwise from the central venous catheter). Blood cultures were cultured in the BacT/Alert monitoring system for 7 days. After 2 days of culturing, three 1 ml samples were taken from each bottle and stored at -70°C until use in the NASBA assay. This was repeated after 7 days of culturing, or when blood cultures were positive in the BacT/Alert monitoring system. The species was identified using CHROMagar plates (bioMérieux, Den Bosch, the Netherlands) and VITEK analysis (bioMérieux).

Extraction of RNA. Blood and blood culture samples were frozen at -70°C for at least 20 minutes. After thawing, 0.9 ml lysis buffer (0.32 M sucrose; 10 mM Tris-HCl (pH 7.5); 5 mM MgCl₂; 1% Triton X100) was added and the samples were centrifuged for 5 minutes at 13,000 g. The supernatant was removed, and this step was repeated once. The pellet was then resuspended in 100 µl enzyme buffer containing 2 mg/ml lyticase (Sigma-Aldrich, Steinheim, Germany), 4 mg/ml lysing enzymes (Sigma-Aldrich) and 0.17% β-mercaptoethanol in 50 mM Tris-HCl (pH 7.5)/10 mM EDTA, and incubated at 37°C for 10 minutes. When FAN aerobic blood cultures were used, the samples were centrifuged for 1 minute at 13,000 g, and the supernatant was transferred to a fresh tube. One ml RNAzol (Campro Scientific, Veenendaal,
the Netherlands) was added and RNA was extracted according to the manufacturer's instructions, with minor modifications: 600 µl isopropanol was added to the aqueous phase instead of 500 µl; after washing with ethanol the pellet was dried for 10 minutes at 56°C, and RNA was dissolved for 10 minutes at 56°C in 40 µl water that was treated with UV-light for 2 hours. Samples were stored at -70°C until further use.

**Primers and probes.** The oligonucleotides used in this study are shown in Table 1. The specificity of the probes and the sensitivity of the NASBA assay have been described previously.\(^4,20\)

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>The primers and probes used in this study</td>
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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Target</th>
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<tr>
<td>primer 1</td>
<td>AATTCTAATACGACTCATAATAGGAGAGA-CATGCGATTGCAGAAAAGTTA(^a)</td>
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</tr>
<tr>
<td>primer 2</td>
<td>GATGCAAGGTTCGGCATATGAG-ATGTCTAAGTATAAGCAATTTA(^b)</td>
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<th>Probe</th>
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<tr>
<td>1912</td>
<td>ATCTCGACCTCTTGGAAGAGATGT</td>
<td><em>C. glabrata</em></td>
</tr>
<tr>
<td>1913</td>
<td>ATCCCCGACTGTGGGAAGGGATGT</td>
<td><em>C. albicans</em>; <em>C. tropicalis</em>; <em>C. parapsilosis</em>; <em>C. viswanathii</em>; <em>C. guilliermondii</em></td>
</tr>
<tr>
<td>1914</td>
<td>AGCCCGACCTCTGGAAAGGGCTGTA</td>
<td><em>C. lusitaniae</em></td>
</tr>
<tr>
<td>2176</td>
<td>CAATGTCTTCTGGGACTCTCTGT</td>
<td><em>C. tropicalis</em></td>
</tr>
<tr>
<td>9566</td>
<td>CCCTCGGGCTTTGATG</td>
<td><em>C. krusei</em></td>
</tr>
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\(^a\) italics: T7 promotor sequence  
\(^b\) italics: generic sequence used for ECL-detection

**NASBA.** Five µl RNA samples were taken up in a pre-reaction mixture with a final volume of 15 µl, containing 53 mM Tris-HCl (pH 8.5), 16 mM MgCl\(_2\), 93 mM KCl, 6.7 mM DTT, 1.3 mM of each dNTP, 2.7 mM of each rNTP, 20% (v/v) dimethyl sulfoxide and 0.27 µM of each primer. The whole mixture was first incubated at 65°C for 5 minutes followed by 5 minutes at 41°C. Then 5 µl of an enzyme mixture containing 2.1 µg BSA, 6.4 U AMV-RT (Seigaku, Rockville, MD), 0.08 U RNase H and 32 U T7 RNA polymerase in 1.5 M sorbitol was added. The reaction mixture was incubated for 90 minutes at 41°C.

**Controls.** One positive and at least two negative controls for the amplification were used for each NASBA reaction. As a positive control for the amplification, 0.70 fg *C. albicans* RNA was used. In the negative controls, no template was added. Negative controls were positioned at the beginning and end of the series of samples that was tested. As a positive control for the whole procedure of isolation and amplification, two blood samples of each patient were spiked with 50 µl of physiological salt solution containing 10\(^2\) and 10\(^4\) colony forming units (cfu) of *C. albicans* (CBS 562, Centraal Bureau voor Schimmelcultures, Utrecht, the Netherlands).

**ECL-detection.** For electrochemiluminescence (ECL)-detection, the NucliSens Basic Kit Detection Reagents in combination with the NucliSens Reader (Organon Teknika, Boxtel, the Netherlands) were used according to the manufacturer's instructions. Amplification products were simultaneously hybridized to the specific capture probes (Table 1) as well as to the
generic ECL probe provided in the Basic Kit. This probe hybridizes to the generic sequence, incorporated by primer 2 during amplification. Amplification products were diluted 1:20 before hybridization with capture probes 1912, 1913, 1914 or 9566, and a 1:200 dilution was used when amplicons were hybridized with probe 2176. The ECL procedure involves standard use of 5 ml tubes (Falcon, Becton Dickinson, le Pont de Claix, France) for the relatively small sample volumes, which greatly reduces the risk of carry-over contaminations. Furthermore, fresh filtertips were used for each pipetting step and all contaminated waste was disposed in closed plastic bags. Hybridization took place at 41° C for 30 minutes. ECL-signals were considered positive when \( \geq 50\% \) of the positive control (0.70 fg \( C. albicans \) RNA).

RESULTS

Ten patients were included in the trial: 4 patients from the UMC Utrecht and 6 patients from the Catharina Hospital Eindhoven. One patient was suspected of candidemia on two different occasions. From this patient, two data sets were obtained.

None of the whole blood samples was positive in the NASBA assay. Blood cultures as well as the NASBA assay were negative for patients 1, 2, 3, 4 (two data sets), and 9. Bacteria, but not yeasts, were cultured in blood cultures of patients 6, 8 and 10 (patient 6: \textit{Enterococcus faecalis} and \textit{Escherichia coli}; patient 8: \textit{Staphylococcus epidermidis}; patient 10: \textit{Staphylococcus epidermidis} and \textit{Staphylococcus haemolyticus}). NASBA assays performed on samples from patients 6 and 8 were all negative for yeast. However, several blood culture samples of patient 10 hybridized with probe 1913 or 2176 in the NASBA assay (Table 2).

Blood cultures from two patients were positive for \textit{C. albicans} (patients 5 and 7, Table 2). All \textit{C. albicans} positive cultures were taken on day 1: three out of four blood cultures (two anaerobic bottles and one aerobic bottle) of patient 5, and one (aerobic) blood culture of patient 7. Blood cultures taken on days 3 and 5 of patient 5 showed bacterial growth (\textit{Enterococcus faecalis} and \textit{Staphylococcus aureus}, Table 2). Several samples of patients 5 and 7 hybridized with probes 1913 and/or 2176 in the NASBA assay (Table 2).

DISCUSSION

Eleven data sets from ten patients in two hospitals were obtained over a period of 16 months. This low number is due in part to the fact that during the study the therapeutic regimen in the University Medical Center Utrecht was changed: all patients at the surgical ICU received fluconazole-prophylaxis during the second half of the study period, and therefore did not meet the inclusion criteria (according to which the first samples should be obtained before antimycotic treatment was commenced). However, the number of patients that were included during the first half of the study period and from the second hospital was still low.

Although some patients who fitted the inclusion criteria may have been missed, these numbers are indicative of the low number of patients suspected of candidemia in the Netherlands. It is possible that this truly reflects a minimal number of systemic \textit{Candida} infections. It is known that in Europe, \textit{Candida} spp. account for less bloodstream infections
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<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sample¹</th>
<th>BacT/Alert²</th>
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<th>NASBA² day 7</th>
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<td>-</td>
<td>1913</td>
<td>n.d.</td>
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<tr>
<td></td>
<td>1b</td>
<td>Candida albicans</td>
<td>1913</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>1c</td>
<td>Candida albicans</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>1d</td>
<td>Candida albicans</td>
<td>-</td>
<td>n.d.</td>
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<tr>
<td></td>
<td>3a</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<tr>
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<tr>
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<td>3a</td>
<td>(Staphylococcus epidermidis)</td>
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<td>(Staphylococcus epidermidis)</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>5a</td>
<td>(Staphylococcus epidermidis; S. haemolyticus)</td>
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<tr>
<td></td>
<td>5b</td>
<td>(Staphylococcus epidermidis; S. haemolyticus)</td>
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<td>-</td>
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1. ¹a = aerobic blood culture, day 1, central venous catheter
   1b = anaerobic blood culture, day 1, central venous catheter
   1c = aerobic blood culture, day 1, peripheral blood
   1d = anaerobic blood culture, day 1, peripheral blood
   3a = aerobic blood culture, day 3
   3b = anaerobic blood culture, day 3
   5a = aerobic blood culture, day 5
   5b = anaerobic blood culture, day 5

2. ²n.d. = no data (cultures were positive for yeast after 2-4 days of culturing)
   day 2 = NASBA on blood culture samples after 2 days of culturing
   day 7 = NASBA on blood culture samples after 7 days of culturing
   1913 = NASBA product hybridized with probe 1913
   2176 = NASBA product hybridized with probe 2176

3. ³samples were taken from blood cultures on day 3 instead of day 7
than, e.g., in North America, where *Candida* spp. are the fourth most common bloodstream pathogens. In Europe, *Candida* spp. rank 8th. However, it is also believed that the number of systemic *Candida* infections is underestimated. Tan et al. reported in 1992 that the number of systemic mycoses in the Netherlands may be as high as hundreds per year, even though only a few cases were reported in their survey. Although these data are quite old, the fact that only four patients from the University Medical Center Utrecht were included in our study (and therefore suspected of having candidemia) over a period of 16 months, whereas around 40 blood cultures positive for *Candida* spp. are seen annually in this hospital, supports this latter hypothesis. Furthermore, taking into account that in up to 65% of the cases automated blood culture systems fail to detect yeasts, the true number of systemic *Candida* infections may be much higher than expected.

We have previously established a detection limit for *C. albicans* of 1-10 cfu/ml blood. In the present study, none of the whole blood samples was positive in the NASBA assay. *Candida* spp. were cultured from the blood of two of the patients, and *Candida* RNA was detected in blood cultures of three. It seems likely that the other patients were not infected. The number of cells in the blood of the three patients with *Candida* RNA-positive blood culture samples may have been too low to detect without a culture step. The number of *Candida* cells in candidemia has been quantitated by using colony counts obtained directly on blood agar plates by the lysis centrifugation technique. It was shown that 26.5% of all samples had < 1 cfu/ml blood, and that another 27.9% had between 1 and 10 cfu/ml. Eight samples were positive in the NASBA assay after two days of culturing. Only two additional positive samples were found: one after three days, and one after seven days of culturing. This indicates that a two-day culture step is sufficient to greatly improve the sensitivity of the NASBA assay.

One sample each of patients 3 and 9 hybridized with probe 2176 in the NASBA assay (results not shown). When NASBA products hybridize with probe 2176 as well as probe 1913, the species is identified as *C. tropicalis*. However, in this case the products hybridized with probe 2176 only. Probe 2176 cross-hybridizes with *Saccharomyces cerevisiae*, which is one of the main causes of contaminations in fungal amplification assays. Therefore, it is likely that these NASBA-products were derived from a *S. cerevisiae* contamination.

The anaerobic blood culture taken from the central venous catheter of patient 5 on day 1 was positive for *C. albicans* after 4 days of growth (Table 2). Samples from both bottles of this blood culture set hybridized with probe 1913 in the NASBA assay. This probe hybridizes with *C. albicans*, *C. tropicalis*, *C. viswanathii*, *C. parapsilosis* and *C. guilliermondii*. Because *C. albicans* was grown from this blood culture, it is most likely RNA of this species which was detected. Since the samples for the NASBA assay were taken after two days of growth, the NASBA assay clearly led to earlier detection. Both the aerobic as well as the anaerobic blood culture taken from a peripheral vein instead of the central venous catheter of the same patient (patient 5) on the same day (day 1) showed growth of *C. albicans*. However, samples taken from these bottles did not hybridize with any of the *Candida* probes used. It is known that nucleic acid amplification assays can be inhibited when large amounts of target DNA are present. Therefore, the extracted RNA from these samples was diluted 5 and 10x, and another NASBA was performed (results not shown). Again, the samples did not hybridize with any of the probes. Since yeast growth was detected in these blood cultures very rapidly (in the aerobic bottle after 2 days, and in the anaerobic bottle after 3 days of culturing), it is possible that the...
number of yeast cells in the samples was extremely high. In that case, a greater than 10x
dilution may have been necessary in order to detect target RNA. This was not further
examined, due to lack of material.

Blood cultures from the same patient (patient 5) taken on days 3 and 5 showed bacterial,
but not yeast growth. However, a sample taken after two days of culturing from a blood culture
of day 5 hybridized with probe 1913 (Table 2). A sample taken from the same blood culture
bottle after seven days of culturing did not show any results in the NASBA assay. It is possible
that the yeast cells were degraded by antifungal agents present in the blood of the patient.
Patient 5 had been treated with fluconazole for 5 days at the time of blood sampling for this
culture. The fact that yeast RNA was still detectable in a sample that was drawn when the
patient was receiving antifungal treatment may indicate treatment failure. This may have
remained undetected by the automated blood culture system because of bacterial overgrowth.

One blood culture from patient 7 showed growth of *C. albicans* after three days. A sample
from this bottle taken after three days of culturing was also positive in the NASBA assay (1a,
Table 2). A sample from another blood culture bottle from day 1 hybridized with probe 2176
(1c, Table 2). Another sample, taken after two days of culturing of a blood culture from day 5,
hybridized with probe 2176 as well as 1913 (indicative for *C. tropicalis*; 5b, Table 2). Since
only *C. albicans* was cultured and no *C. tropicalis* was ever found in any culture from this
patient, the most likely explanation for these results is that sample 1c is a *S. cerevisiae*
contaminant, as described above, and sample 5b contains a mixture of *C. albicans* and *S.
cerevisiae* RNA.

Several samples from patient 10 hybridized with either probe 2176 or 1913 (Table 2). Since all negative controls were free of contaminants and because of the stringent measures for
prevention of contaminations that are taken in our laboratory, it is not likely that all samples
which hybridized only with probe 2176 are the result of *S. cerevisiae* contaminations of the
NASBA assay. In rare cases, *S. cerevisiae* causes bloodstream infections (Taylor et al. reported
one case in their hospital over a seven year period16). A combined infection with *S. cerevisiae*
and *C. albicans* is therefore possible. It is, however, striking that all three samples which
hybridized with probe 2176 were taken from one blood culture set. *S. cerevisiae*, also known
as 'baker's yeast', is used widely in industrial baking and brewing, and is commonly present in
the environment. Since the three samples were taken from blood cultures which were drawn at
the same time from a central venous catheter, it is very well possible that the blood cultures
were contaminated with yeast cells from the skin of either the patient or the health care worker,
for instance. The fact that blood cultures in many cases fail to detect yeasts might explain why
they were not detected by the automated blood culture system.

Three samples from patient 10 hybridized with probe 1913, indicating that this patient
possibly suffered from candidemia. No *Candida* spp. were cultured from the blood of this
patient. However, we have previously shown, that the NASBA assay performed on aliquots of
blood cultures is more sensitive than the BacT/Alert monitoring system4.

Our NASBA assay uses separate amplification- and detection procedures. Real-time
detection of *C. albicans* with the Light Cycler system has been used successfully10. It is
possible to combine NASBA amplification with the use of molecular beacons. This results in
an isothermal real-time detection assay, without the need of expensive thermal cyclers.
Quantification of the NASBA products is possible with ECL- as well as molecular beacon
detection.

In conclusion: in three out of ten patients the NASBA assay detected Candida RNA. In one patient the yeast was not detected by automated blood culturing, in another patient the NASBA assay detected the infection two days earlier than the blood culture system. This is encouraging for the use of NASBA for the detection of systemic Candida infections. However, this study should be repeated with a larger number of patients before sound conclusions can be made.

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