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Recent Advances in the Detection of Neonatal Candidiasis

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Abstract Neonatal candidiasis is serious and often fatal. Blood culture, the standard for diagnosis, has a sensitivity of 50% or less, and isolate speciation and susceptibility takes several days. This review explores recent advances in *Candida* detection using various diagnostic strategies.

Keywords Diagnosis · Neonate · *Candida*

Introduction

Neonatal candidiasis is a common complication in extremely preterm infants surviving beyond the first 2 weeks of life [1]. Approximately 10% of extremely low birth weight (<1,000 g birth weight) neonates develop candidiasis [2–4], and candidemia accounts for 12–15% of late-onset sepsis episodes in the neonatal intensive care unit (NICU) [1, 5]. Neonates diagnosed with candidiasis are at significant risk for mortality (20%) [1, 2, 6, 7] and morbidity among survivors, including retinopathy of prematurity, chronic lung disease, periventricular leukomalacia, and poor long-term neurodevelopmental outcomes [1, 2, 7–11].

Risk factors for invasive candidiasis in neonates include prematurity, broad-spectrum antibiotics (e.g., third-generation cephalosporins) [2, 3], histamine-2 receptor blockers [4, 12], postnatal steroids [13–15], and presence of an indwelling catheter [4, 5]. In addition, the underdeveloped immune system of the premature neonate lacks basic immunologic functions such as chemotaxis, cytokine production, production of antibodies, and phagocytosis [16].

Although the devastating consequences of neonatal invasive candidiasis have been clearly identified, early detection of candidiasis remains a challenge. More rapid, accurate, and reliable methods are needed to diagnose neonatal candidiasis. This article reviews recent advances in various methods aimed at detecting invasive candidiasis, with a special focus on the application of these methods to the neonatal population.

Body Fluid Cultures

Blood culture is the standard test for diagnosis of candidemia; however, this test confirms 50% or fewer of autopsy-proven cases of invasive candidiasis in adults [17]. In addition, this low sensitivity was observed in adults from whom more than 10 mL of blood was routinely collected for culture. In neonates, only 0.5–1 mL of blood is routinely available for culture, likely decreasing the sensitivity of the test substantially. In addition, even when a pathogen is isolated from blood culture, speciation and susceptibility testing can take several days. In a retrospective study of hospitalized patients (1 month to 65 years of age), there were 115 episodes of *Candida* bloodstream infections [18]. The mean time to detection was 35 h and 80 h for *C. albicans* and *C. glabrata*, respectively ($P<0.0001$), and the mean time to final speciation was 86 h and 154 h, respectively ($P<0.0001$).

While the diagnosis of candidemia via blood culture is lengthy, techniques such as peptide nucleic acid fluorescence in situ hybridization used in conjunction with blood

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cultures may shorten the time to preliminary species identification [19]. Once a blood culture is positive for *Candida*, this test can rapidly (within 2 h) identify up to five different *Candida* species with the use of fluorescent-labeled peptide nucleic acid probe. Although this test is potentially useful clinically, it requires a positive blood culture, lacks specificity, and is not able to fully distinguish between all clinically relevant *Candida* species.

Hematologic Markers

Hematologic parameters are often used to predict the presence of infection in neonates. However, the white blood cell count is not a useful aid in the diagnosis of *Candida* infections; as many as 40% of neonates with candidiasis have normal white blood cell counts [20].

Thrombocytopenia is another commonly used hematologic marker for candidiasis, but it lacks sensitivity and specificity. A single-center study found that thrombocytopenia was present in 84% of very-low-birth-weight (VLBW) neonates (<1,500 g birth weight) with candidemia [21]. However, in a second study of VLBW neonates, thrombocytopenia was only 20% sensitive for the presence of candidemia [22] and 16% sensitive for the presence of bacteremia. The mean platelet nadir was 66,500/mm^3 for Gram-positive sepsis, 46,000/mm^3 for Gram-negative, and 38,000/mm^3 for fungal sepsis.

Fungal Antigens

The two fungal antigens used most frequently for diagnostic purposes are 1−>3 β-D glucan (BG) and mannan (Table 1). Mannan is a high-molecular-weight polysaccharide present in the cell wall of *Candida*, and BG is an important structural component of the fungal cell wall. Two commonly used assays for mannan antigen detection include the Platelia *Candida* Ag (BioRad, Manes, La Coquette, France) and the Serion ELISA antigen *Candida* assay (Institute Virion/Serion, Wurzburg, Germany), which rely on monoclonal anti-mannan antibodies for detection [23]. Normal result values for these are <0.25 ng/mL and >1.15 U/mL, respectively. The Fungitell test kit (Associates of Cape Cod, East Falmouth, MA) is an antigen assay used to detect BG concentrations in an unknown sample based on a known calibration curve. A negative result has a value <60 pg/mL [23].

A study of 70 neonates admitted to the NICU with at least three mannan samples collected showed that the Platelia *Candida* mannan assay had an overall sensitivity and specificity of 94%, and a culture-proven sensitivity and specificity of 92% and 84%, respectively [24•]. These neonates were divided into two groups: those with proven (n=12) or probable (n=6) candidiasis (mean birth weight= 1,622 g), and those without candidiasis (n=52; mean birth weight=2,262 g). The antigen test was considered positive if there were two or more samples ≥0.5 ng/mL. Twelve neonates had proven candidiasis by positive blood culture and clinical sepsis; 11 (92%) of these had positive mannan assays. Of the remaining 58 neonates with negative blood cultures, nine (16%) had a positive antigen assay; six (67%) were considered to have invasive candidiasis based on a clinical diagnosis and lack of response to antibacterial treatment, while the other three were considered false positives. The mannan test was positive at a median of 8 days (range, 4–18 days) before blood culture.

A study of 51 patients (42% with invasive candidiasis) aged 3–65 years admitted to a tertiary care hospital for chemotherapy treatment evaluated Platelia *Candida* antigen assay (mannan) and Platelia *Candida* Ab/Ac/Ak assay (anti-mannan) levels before clinical evidence of disease [25]. Both mannan antigen and anti-mannan antibodies were detected before clinical evidence of candidiasis was apparent. Anti-mannan antibodies were detected significantly more frequently in patients who had experienced more than 15 days of neutropenia, compared with those with less than 15 days (P<0.05). Compared with controls (patients receiving chemotherapy without candidiasis), mannan antigen was detected more frequently in patients with candidiasis in the first 15 neutropenic days (odds ratio=3.7; 95% CI=1.4–9.7, P<0.05).

A follow-up study compared the use of several assays to detect mannan, anti-mannan antibodies, or BG in 21 neutropenic patients receiving chemotherapy with culture-proven invasive candidiasis and 30 controls without candidiasis undergoing myeloablative chemotherapy [23]. When using the Serion assay, the *Candida* mannan antigen and *Candida* anti-mannan antibodies were detected in 15/21 (71%) and 9/21 (43%) patients with candidiasis, compared with 7/30 (23%) and 3/30 (10%) controls, respectively. BG was detected in 16/21 (76%) patients with candidiasis, compared with 12/30 (40%). Overall, these tests produced a high number of false-positive results, leading to specificities ranging from 60% to 90%. The sensitivities were slightly better (ranging from 70%–79%), with the exception of the anti-mannan antibody test (sensitivity of 43%). *Candida* antigens were detected at a median of 11 days prior to culture confirmation of infection, with a range of 176 days pre- to 195 days post-culture confirmation, and antibodies were detected at a median of 20 days prior to culture confirmation (range of 197 days pre- to 8 days post-culture confirmation). These studies demonstrate that, at least in neutropenic patients, the timing of an infection may determine the most reliable diagnostic test.

A study of four antigen tests (mannan [Platelia *Candida* antigen], anti-mannan antibodies [Platelia *Candida* anti-

Table 1 Overview of diagnostic tools available for the diagnosis of candidemia

	Study population	Patients, *n*	Sample volume	Sensitivity	Specificity
Blood culture [17]	Adults	37		50%[a]	100%
Fungal antigens					
Mannan [24•]	Neonates	70	300 μL	92%	84%
Mannan [23]	Chemotherapy	51		71%	77%
[26]	Children and adults	92	300 μL	41%	100%
1−>3 β-D glucan [23]	Chemotherapy	51		76%	60%
1−>3 β-D glucan [26]	Children and adults	92	500 μL	47%	100%
Fungal antibodies					
Anti-mannan [23]	Chemotherapy	51		43%	90%
Anti-mannan [26]	Children and adults	92		47%	100%
PCR					
Semi-nested [26]	Children and adults	92		88%	100%
Nested [28]	Hospitalized patients	110 (24 neonates)	200 μL	86%	54%
Real-time [28]	Hospitalized patients	110 (24 neonates)	200 μL	81%	96%
Real-time [31]	Inpatients	23		93%	66%
Real-time [31]	Inpatients	23		77%	100%
Real-time [32]	Immunocompromised	384	2,500 μL	88%	94%
NASBA [37•]	Samples from positive blood cultures	10		100%	100%

NASBA nucleic acid sequence–based amplification; *PCR* polymerase chain reaction

[a] <50% sensitivity and 100% specificity in the clinical setting

body], BG [Fungitell], and semi-nested polymerase chain reaction [snPCR]) examined 109 blood samples from 92 children and adults: 27 hospitalized patients (aged 6 months to 88 years) with culture-proven candidemia (32 samples); 39 hospitalized patients with suspected candidemia (51 samples); 10 samples from outpatient women with *C. albicans* vaginitis; and 16 samples from healthy controls [26]. The sensitivity and specificity for each test were as follows: mannan assay 41% and 100%, anti-mannan and BG 47% and 100%, and snPCR 88% and 100%, respectively. Testing for both mannan and anti-mannan antibodies increased the sensitivity to 75%. The snPCR test, which allows for greater specificity compared with standard PCR by introducing a second primer within the amplified region from the first PCR reactions, identified five patients whose infection was due to more than one *Candida* species. Although fungal antigen tests are encouraging, they are expensive and not yet ready for use in standard practice (particularly in neonates, for whom there is little information on their performance).

DNA PCR

PCR is a process capable of identifying a specific target DNA sequence (primers) for a given pathogen using sequence amplification techniques. Nested PCR uses two sets of primers in order to minimize sample contamination. Real-time PCR uses fluorescent dyes to detect and quantify amplified DNA sequences as they are made during the PCR process, rather than afterward, which allows earlier DNA identification and target quantification.

Over the past few decades, PCR has been extensively used as a viral diagnostic test and is becoming a focus to discover faster and more accurate diagnostic methods for candidemia (Table 1). In addition to providing a more accurate diagnosis, PCR has the potential to identify the specific *Candida* species in a shorter amount of time and the ability to decrease the lower level of detection to less than ten colony-forming units per mL [27]. This level of detection is critical in the setting of low organism burden in the blood that is commonly seen with invasive candidiasis.

Two different PCR techniques, nested and real-time PCR, were compared in a study of 200 blood samples from 110 hospitalized patients (24 neonates) [28]. All samples were tested by blood culture, nested, and real-time PCR [28]. There were 36 positive blood cultures for yeast: 30 single *Candida* species, three mixed *Candida*, and three non-speciated yeasts. The sensitivity and specificity were 86% and 54% for nested PCR, and 81% and 96% for real-time PCR, respectively.

Real-time PCR was used to compare the LightCycler SeptiFast PCR test (Roche Diagnostics, Penzberg, Germany) with the Bactec 9,240 blood culture system (Becton Dickinson, Heidelberg, Germany) [29]. The SeptiFast PCR test has the ability to identify 25 different bacterial or fungal pathogens (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *Aspergillus fumigatus*) [30]. The study examined 101 samples undergoing both diagnostic tests from 77 hospitalized adult patients (median age=59 years for those with positive PCR, 52 years for negative PCR) [29]. Of the eight patients with invasive fungal disease, two were both blood culture and PCR positive, two were blood culture positive and PCR negative, and four were blood culture negative and PCR positive. The sensitivity of the blood culture was only 50%, compared with 75% for PCR.

A study of 58 serum samples from 23 patients used the LightCycler PCR system and two different fungal-specific primers, L18 and internal transcribed spacer (ITS) [31]. The control group included ten healthy volunteers. Thirteen of the 23 (56.5%) patients had culture-proven candidemia. The L18 primer detected the presence of *Candida* DNA in 92% of positive cultures (12/13) but was not able to identify individual species. Use of the ITS primers allowed for the detection of *Candida* DNA in 77% of positive cultures (10/13), and the ITS primers allowed for rapid species differentiation between *C. albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. parapsilosis*. The sensitivity and specificity of these two primers when used separately were 93% and 66% for L18 and 77% and 100% for the ITS primers, respectively. Although this assay may hold promise, it is not yet commercially available.

A three-center study compared blood cultures with real-time PCR using the MolYsis Complete5 kit (Molzym, Bremen, Germany) in 384 immunocompromised patients (including 55 children) with 468 infectious episodes [32]. The MolYsis Complete5 kit is used prior to PCR sample analysis to lyse white blood cells prior to bacterial or fungal cell wall destruction to maximize recovery of pathogen DNA. Seven of eight (88%) episodes of culture-proven candidemia were PCR positive, and 28/460 (6%) negative blood cultures had a positive PCR. The sensitivity of the PCR test combined with the use of the MolYsis kit was 88%, with a specificity of 94%. Positive PCR samples allowed for detection of fungal disease 3 days earlier than blood culture results.

PCR testing shows promise as a tool for the diagnosis of invasive candidiasis. The current limitations of this test include lack of probes that can detect multiple *Candida* species simultaneously, lack of specificity, potential for sample contamination leading to false-positive results, and difficult sample preparation. Additionally, these tests are understudied in the neonatal population.

Nucleic Acid Sequence–Based Amplification

Real-time nucleic acid sequence–based amplification (NASBA) is a technology used to amplify RNA from either RNA or DNA, as opposed to PCR, which amplifies only DNA. These tests are able to detect a target RNA without any background DNA contamination, decreasing the number of false-positive results. NASBA is a very sensitive diagnostic test with a lower level of detection of one colony-forming unit per mL [27], less than that required for PCR. NASBA has often been used to diagnose RNA viruses such as enterovirus [33, 34]. There are a few reported cases of it being used to diagnose fungal infections [35, 36], and no reports of its use in the diagnosis of bloodstream infections (Table 1).

Molecular beacons are hairpin probes used in conjunction with NASBA composed of nucleic acids that fluoresce once they are bound to their target [37•]. Investigators developed five molecular beacons and the corresponding primers to detect Gram-negative, Gram-positive, *Candida*, and *Aspergillus* organisms. The *Candida* primer was able to detect eight different species and the *Aspergillus* primer four species. Although there were few fungal blood samples (n=10), the probes were found to be reliable with a sensitivity, specificity, positive predictive value, and negative predictive value of 100%. However, due to the small number of samples, difficulty of use, and sparse amount of data, this test is not currently standard practice.

Conclusions

The accurate and timely diagnosis of invasive *Candida* disease in the neonatal population is challenging. However, recent focus has been aimed at investigating and improving alternative methods of diagnosis. Identification of a single colony of *Candida* in the blood is clinically significant. In spite of its poor sensitivity, blood culture continues to be the standard diagnostic test. While blood culture remains the current gold standard, other tests, such as fungal antigen tests, may currently be realistic adjunctive tests in specific populations. Real-time PCR and NASBA technologies show promise as diagnostic tools for patients in the NICU; however, they are currently understudied in this population. Accurate, rapid, and sensitive diagnosis of invasive *Candida* disease is needed for the neonatal population, in whom the burden of disease is high and outcomes are poor.

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