

Seminested PCR for detection and identification of *Candida* species directly from blood culture bottles

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SUMMARY

We investigated the performance of a seminested PCR (snPCR) assay carried out directly from overnight incubated blood culture bottles of 50 newborn intensive care unit (NICU) patients with suspected candidemia and compared these, for sensitivity, specificity and reliability with results from blood cultures. All positive blood cultures (n=17) yielded positive results for snPCR, which detected the same *Candida* species, as did the yeast isolates of which 13 were *C. parapsilosis* and 4 were *C. albicans*. With both assays showing 32 negative samples and one sample positive with snPCR but negative with blood culture, sensitivity and specificity of snPCR were 100% and 97%, respectively. The patient with contradictory results exhibited a positive blood culture one week later yielding the same species as identified by snPCR. These are the first data demonstrating that snPCR from overnight blood culture bottles can be a potential tool for rapid detection and identification of *Candida* species, allowing follow-up of the "gold standard" blood culturing, as well.

KEY WORDS: Blood culture, Candidemia, Diagnosis, Seminested PCR (snPCR)

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INTRODUCTION

Candida species are the fourth most common cause of bloodstream infections (BSI) in hospitalized patients, and the most commonly isolated species include *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*. A recent study from Turkey (Kuzucu *et al.*, 2008) reported that *C. albicans* and *C. parapsilosis* were most common agents obtained from patients with invasive candidiasis in pediatric/neonatal intensive care units (PICU/NICU). However, diagnosis remains difficult and, more importantly, rapid identification of infecting *Candida* species is necessary for early effective and appropriate antifungal therapy (Foster *et al.*, 2007; Cuenca-Estrella *et al.*, 2005; Nguyen *et al.*, 1996; Pfaller *et al.*, 2000).

Over the last decades significant improvements have been made in culture media to increase the detection of *Candida* species in blood samples. One of these is the "BacT/ALERT" automated system, which is used in our routine laboratory and contains charcoal to adsorb inhibitors of microbial growth.

However, routine identification procedures from positive blood cultures require at least one additional day for the pure culture and germ tube test. An additional 2-4 days for chlamyospore formation and assimilation tests, especially for the germ tube negative isolates, which take up to 5 days or longer (Dooley *et al.*, 1994; Maaroufi *et al.*, 2004).

The current "gold standard" for detection of candidemia is blood culture. However, not only is it a time consuming method, but its sensitivity for early detection of infection has been reported to be as low as 50% (Rodriguez *et al.*, 1997; Ahmad *et al.*, 2002).

Molecular techniques are targeted to detect *Candida* species in a short period of time, with a high sensitivity and specificity.

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For this purpose several PCR methods have been developed, such as nested PCR, multiplex PCR, Taq-man PCR, Light-Cycler PCR and fluorescent PCR (Maaroufi *et al.*, 2004; Bougnoux *et al.*, 1993; Innings *et al.*, 2007; Metwalley *et al.*, 2007; Dunyach *et al.*, 2008; Shin *et al.*, 1999).

Seminested PCR (snPCR) has been standardized and established by Ahmad *et al.* (2002) using universal and species specific primers for the detection of 4 *Candida* species (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*) in sera which yielded results with high specificity and sensitivity. However, it required a separate serum sample in addition to a blood culture, which may not be obtainable in all instances. Therefore, we aimed to apply snPCR directly to the overnight blood cultures of critically ill patients to introduce a more rapid diagnosis of presumptive candidemia and to compare the results with those of the BacT/ALERT blood culture system in terms of consistency and reliability.

MATERIALS AND METHODS

Clinical specimens

In this retrospective study, fifty blood cultures were collected between January 2007-January 2008 from hospitalized patients with suspected candidemia due to the presence of risk factors such as use of broad spectrum antibiotics and clinical signs such as nutrition intolerance, lethargy, apnea, thrombocytopenia in the NICU at Marmara University Hospital.

A 1 ml sample was taken from each of the pediatric BacT/ALERT blood culture bottles after overnight incubation, and was stored at 4° C to be used in snPCR later; bottles were incubated for a complete follow-up of cultures for 7 days.

Conventional identification of the yeast isolates

Gram stained smears from bottles with a positive growth signal were examined microscopically to confirm the presence of yeasts and subcultured on Sabouraud dextrose agar (SDA).

Isolated yeasts were tested for germ tube and chlamydospore production and identified to species level with the API ID 32C assimilation system (bioMérieux, France) (Larone, 2002).

Extraction of *Candida* DNA directly from blood culture bottles

Each sample from the overnight blood cultures was centrifuged at 720xg for 2 min to remove the charcoal (Microfuge, Hettich, Germany), supernatants were recentrifuged at 4400xg for 5 min and after treating with 200 U Lyticase (L4025, Sigma, Germany) the resultant supernatants were used for DNA extraction which was carried out by the QIAmp tissue protocol (QIAGEN, Hilden, Germany). To verify DNA extraction method, we performed spiked blood culture bottles inoculated with the ATCC reference strains (*C. albicans* 90028, *C. parapsilosis* 22019, *C. tropicalis* 750, *C. glabrata* 90030).

Sn PCR

In order to amplify the target DNA region, in the first amplification step we used a 22 bp forward primer CTSF (5'-TCGCATCGATGAAGAACGCA GC-3') and a 25 bp reverse primer CTSR (5'-TCTTTCCTCCGCTTATTGATATGC-3') (IDT Inc., Gralville, USA) capable of amplifying the 3' end of 5.8S rDNA and the 5' end of 28S rDNA, including the intervening spacer region. A second amplification step was carried out in four separate tubes for each *Candida* species using specific forward primers derived from the ITS 2 regions of *C. albicans* (CADET, 5'-ATTGCTTGCGGCG-GTAACGTCC-3'), *C. parapsilosis* (CPDET, 5'-ACAACTCCAACTTCTTCCA-3'), *C. tropicalis* (CTDET, 5'-AACGCTTATTTTGCTAGTGGCC-3') and *C. glabrata* (CGDET, 5'-TAGGTTTTAC-CAACTCGGTGTT-3'), in addition to the reverse primer CTSR.

In each amplification step, 0.5% bovine serum albumin (BSA) (Fraction V, Sigma, Germany) was added to the reaction mixture to bind possible PCR inhibitors in the sample (Maaroufi *et al.*, 2004).

Thermocycling conditions of snPCR steps 1 and 2 were performed under the same conditions except for cycling numbers and primers, as described previously (Ahmad *et al.*, 2002). Four ATCC reference strains (*C. albicans* 90028, *C. parapsilosis* 22019, *C. tropicalis* 750, *C. glabrata* 90030) were used as positive controls and deionized water was used as a negative control.

The final PCR products were detected under U.V.light and photographed (TFX 20M Vilber-Lourmat, Marne LaValle, France).

The detection limit of the technique was investigated by blood culture bottles spiked with serial concentrations of *C. parapsilosis*. Briefly, five pediatric BacT/ALERT blood culture bottles were separately inoculated with 1 ml of serially diluted yeast suspensions plus 1 ml of human blood obtained from volunteers giving final concentrations of $0.5-2.5 \times 10^3$, $0.5-2.5 \times 10^2$, $0.5-2.5 \times 10^1$, 0.5-2.5, 0.05-0.25 CFU/ml, respectively. In other words, simulated patient samples containing the above-mentioned number of microorganisms were inoculated into blood culture bottles. One additional blood culture bottle was used as negative growth control without being seeded. Following inoculation, all the bottles were immediately placed in the BacT/ALERT system. After 18-24 hours of incubation, 1 ml of sample from each bottle was aseptically collected and the culture bottles were reincubated until an alarm signal indicated positivity.

RESULTS

Candida growth in BacT/ALERT System was positive for 17 of the patients within 2-4 days of incubation. However, samples obtained from overnight blood culture bottles of those 50 hospitalized patients, 18 (36%) were found to be positive by snPCR with universal primers (Table 1);

of these 18 samples 14 were identified as *C. parapsilosis* and 4 as *C. albicans* by species-specific primers (Figure 1). No *C. tropicalis* or *C. glabrata* species were detected. All the isolates were identified to be the same species both by conventional methods and snPCR.

In one patient, whose snPCR was positive for *C. parapsilosis*, the blood culture remained negative despite 7 days of incubation; however, the following blood culture from the same patient, obtained one week after the initial one, was positive for *C. parapsilosis*.

Therefore the sensitivity of snPCR compared to blood culture results was found to be 100 %, while specificity was 97%.

The remaining 32 (64 %) samples were negative for candida with both methods.

Of the blood culture bottles inoculated by simu-

TABLE 1 - Comparison of blood culture and snPCR results for *Candida* species.

snPCR	Blood Culture		Total
	Positive	Negative	
Positive	17 (34%)	1 (2%)	18 (36%)
Negative	-	32 (64%)	32 (64%)
Total	17 (34%)	33 (66%)	50 (100%)

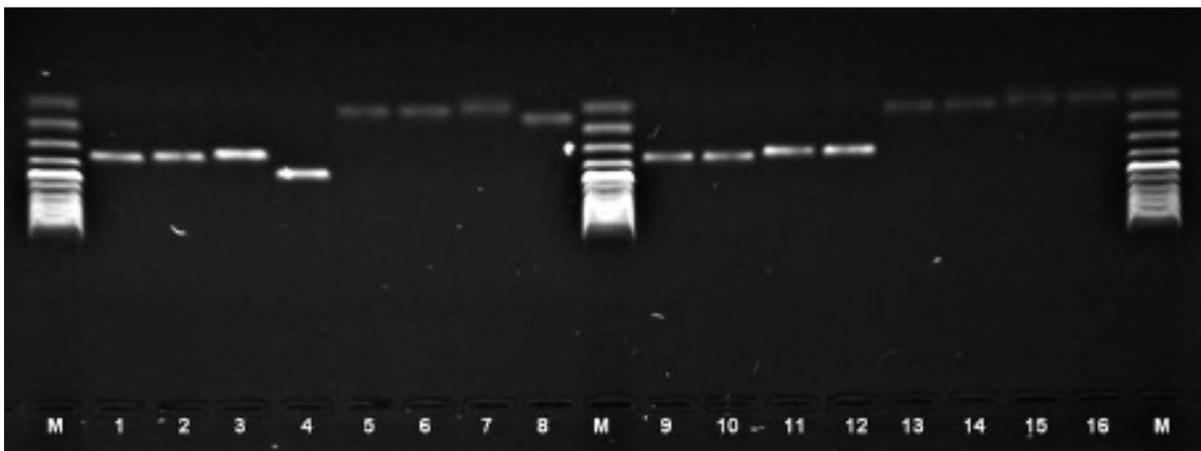


FIGURE 1- Semi-nested PCR results for *Candida* sp. colonies and blood culture bottles. M: 100 bp molecular marker. sn-PCR 1.step results from colonies, Lane1: *C. albicans*, Lane 2: *C. tropicalis*, Lane 3: *C. parapsilosis*, Lane 4: *C. glabrata*. sn PCR 2.step results from colonies: Lane 5: *C. albicans*, Lane 6: *C. tropicalis*, Lane 7: *C. parapsilosis*, Lane 8: *C. glabrata*. sn-PCR 1.step results for blood culture bottles: Lane 9: *Candida* sp., Lane 10: *Candida* sp., Lane 11: *Candida* sp., Lane 12: *Candida* sp. Sn-PCR 2.step results for blood culture bottles: Lane 13: *C. albicans*, Lane 14: *C. albicans*, Lane 15: *C. parapsilosis*, Lane 16: *C. parapsilosis*.

lated samples with $0.5-2.5 \times 10^3$, $0.5-2.5 \times 10^2$, $0.5-2.5 \times 10^1$, 0.5-2.5, 0.05-0.25 CFU/ml of test microorganism, the first three gave a positive growth signal within 48 hours of incubation while the fourth bottle became positive on the fourth day and the last and negative control blood culture bottles were negative on 7th day of incubation. The three samples yielding growth signal were also found to be positive on snPCR. Hence, snPCR seems to have a sensitivity limit of 5-25 CFU/ml microorganism detection in original blood samples.

DISCUSSION

Culturing blood specimens for detection of candidemia is the "gold standard" in clinical microbiology laboratories. However, its low positivity is a major drawback in diagnosing hematogenous candidiasis. Even in autopsy verified candidiasis, the positive outcome of blood cultures can range between 40-60% (Ahmad *et al.*, 2002). By using "BacT/ALERT", one of the continuously monitored automated blood culture systems, we could only detect 17 (34%) positive results for candida. On the other hand, blood cultures are time consuming and at least 7 days must pass for a reliable negative result (Innings *et al.*, 2007, Borst *et al.*, 2000).

In order to overcome the limitations of blood cultures, various PCR-based methods to detect the presence of candida in blood have been developed. However, blood-containing samples, such as blood cultures, whole blood or serum, may include PCR inhibitors such as SPS (sodium polyanethol sulfonate), EDTA, heparin, hemoglobin, lactoferrin and lipids which may need to be removed or inactivated (Alam *et al.*, 2007; Fredricks *et al.*, 1998; Kreader *et al.*, 1996). BacT/ALERT blood culture bottles contain carbon particles as potent PCR inhibitors. To remove those particles as far as possible, we centrifuged the aliquots at low speeds. To do so, we modified the speeds used in the study of Shresta *et al.*, (2002) and allowed the charcoal to settle, but not the yeasts or fungal DNA. The best results for a sufficient number of yeast in the initial supernatant were obtained at 720xg for 2 min as indicated by observing yeast colonies on subcultures on SDA plates.

In a recent study, where serum samples of candidemic patients were used, the sensitivity of snPCR was found to be 88% (Alam *et al.*, 2007). In our study by using samples withdrawn directly from overnight blood culture bottles, we could detect candida by snPCR in all 17 blood culture positive patients (17/17) and all the *Candida* isolates were identified as the same species detected by snPCR (Figure 1, Table 1). We found the sensitivity and specificity of snPCR to be 100% and 97%, respectively, due to an additional snPCR positive but culture negative patient. There are at least two reasons for the remarkable difference in sensitivity between our study and the earlier one. Firstly, samples taken directly from blood culture bottles in our study may contain higher amounts of DNAs due to a possible fungal growth provided by overnight incubation. Secondly, we added 0.5% BSA to the mixture which optimized the amplifying procedure by binding PCR inhibitors such as lipids, and hence increased the sensitivity of the PCR (Kreader *et al.*, 1996; Maaroufi *et al.*, 2004).

The patient who was positive for *C. parapsilosis* with snPCR but negative on blood culture became positive for the same species on repeat blood cultures collected 7 days after the first set. Retrospective analysis of the patient's records demonstrated that, as part of a surveillance study, this patient had rectal and axillary colonization with *C. parapsilosis* by one week of life and this persisted for several weeks.

This indicates that snPCR can detect very low numbers of candida or its DNA, which may result in a "false negative" blood culture. Although the detection limit of snPCR was declared to be as low as 1 cell per ml of serum in the study of Suhail *et al.* (2002), it was 5-25 fungal cells in our study. The difference in detectable cell numbers between the two studies may be due to the difference in study conditions. That is, the sera in the former study were obtained from blood samples in plain tubes, whereas we tried to extract fungal DNA from the blood culture bottles containing PCR inhibitors such as charcoal.

Combining these data with previous findings, it can be stated that snPCR is in full agreement with the gold standard.

The disadvantage of snPCR is the need to perform 4 separate PCR sets in the second amplification step. However, the advantages of this

method are many when applied under our study conditions:

- 1) in general, for logical reasons sampling for both culture and PCR could not always be performed on the same day. By using samples obtained directly from overnight blood culture bottles we did not need a second whole blood or serum;
- 2) as discussed earlier, overnight incubation allowed a period of time for presumptive fungal growth providing higher amounts of DNA for PCR;
- 3) the laboratory can produce results in 24-48 hours, even for dual infections caused by more than one *Candida* species;
- 4) the method has potential to provide earlier alerts for candida present in the blood for patients who are candidemic but negative on blood cultures for various reasons.

This is the first study showing a rapid, reliable, highly sensitive and specific method for detection and identification of the clinically relevant *Candida* species directly from blood culture bottles which can give a positive impulse to clinicians for appropriate treatment without having to wait for the growth and additional laboratory procedures for identification at species level. In recent years, three genetically distinct groups (groups I, II, and III) of *C. parapsilosis* have been renamed as distinct species: *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*. Even though by using snPCR we cannot differentiate these distinct species from each other, we suggest that it is not necessary to perform an additional test for the novel species for they are already detected at very low frequencies and do not exhibit problemw in terms of in vitro antifungal susceptibility and therapeutic approach (Silva AP *et al.*, 2009, Lockhart SR *et al.*, 2008).

In conclusion, snPCR has the potential to diagnose candidemia in critically ill patients rapidly and accurately, and samples obtained directly from overnight blood culture bottles are to be preferred over whole blood or serum since these provide an opportunity for a simultaneous follow-up of blood cultures.

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