Dual candidemia detected by nested polymerase chain reaction in two critically ill children

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The use of improved microbiological procedures associated with molecular techniques has increased the identification of Candida bloodstream infections, even if the isolation of more than one species by culture methods remains uncommon. We report the cases of two children presenting with severe gastrointestinal disorders and other risk factors that contribute to Candida infections. In the first patient, C. albicans DNA was initially detected by a nested-amplification and C. tropicalis was found later during hospitalization, while blood cultures were persistently negative. In the second child, there was amplification of C. albicans and C. glabrata DNA in the same samples, but blood cultures yielded only C. albicans. Both patients received antifungal therapy but had unfavorable outcomes. These two cases illustrate that PCR was more successful than culture methods in detecting Candida in the bloodstream of high risk children, and was also able to detect the presence of more than one species in the same patient that might impact therapy when the fungi are resistant to azole compounds.

Keywords Candida spp., PCR, polyfungemia, pediatric candidiasis

Introduction

Candida species are the fourth most common etiological agents of bloodstream infections in hospitalized patients and such cases are associated with high mortality rates [1]. Although Candida albicans remains the leading member of the genus associated with this form of infection, a growing number of non-C. albicans Candida isolates, many of which are azole-resistant, have been recovered from at risk patients. The latter includes, among other predisposing conditions, those patients with underlying diseases, hospitalized for prolonged periods with central venous or arterial catheters, receiving broad spectrum antibiotics and parenteral nutrition, [2,3]. More than 90% of invasive infections due to Candida are attributed to five species, i.e., C. albicans, C. tropicalis, C. glabrata, C. parapsilosis and C. krusei [1].

Although polymicrobial bacteremia has been extensively described, multiple fungal species isolated from the same blood culture bottles have been reported in only 3.0–5.2% of episodes, accounting for around 10% of all bloodstream infections in hospitalized patients with multiple co-morbidities [4–6].

The advent of PCR-based techniques has significantly increased the detection of one or more Candida species in bloodstream infections. Early detection and identification of Candida species could be critical for the success of therapy, especially with respect to infections caused by C. glabrata and C. krusei which are more resistant to azole antifungals [1,7,8].

We report two cases of critically ill children admitted to an intensive care unit with underlying gastrointestinal diseases and other risk factors predisposing to Candida
infection, in whom nested-PCR, but not blood cultures detected dual etiologic agents.

**Materials and methods**

**Patients**

**Patient 1.** A female, 4 years old, referred to the Hepatology Unit of the Children’s Hospital (University of São Paulo, Brazil), presenting with progressive familial intrahepatic cholestasis and liver cirrhosis. The child was admitted to the pediatric intensive care unit (PICU) presenting with signs and symptoms of sepsis and was promptly administered two broad spectrum antibiotics. The patient’s blood examination showed leukocytosis, severe thrombocytopenia and consumptive coagulopathy. The severity of illness was initially evaluated in accord with the pediatric risk of mortality (PRISM) procedures [9] and found to have a score of 21 (scores ≥10 indicate a high risk of mortality). The child developed bilateral bronchopneumonia, pulmonary hypertension, respiratory failure and septic shock. The patient did not receive parenteral nutrition, corticosteroid or cytotoxic drugs prior to or during her hospital stay, but had two central venous catheters. On day 13, urine culture collected by bladder catheterization yielded *C. albicans* (>100,000 CFU/ml) and fluconazole was administered (6 mg/kg/d) until day 20. However, no *Candida* species were recovered from eight blood samples sent for culture on days 3, 4, 6, 9, 12, 15, 16 and 18. The patient progressed with multiple organ failure and died on day 20, 8 days after the initiation of antifungal therapy.

**Patient 2.** A male, 10 years old, was referred to the pediatric surgery unit of the same hospital due to a short bowel syndrome. At admittance, the child had already undergone four small intestine resections and presented with a deep skin infection covering the low abdominal and the lumbar regions associated with severe malnutrition, and a consumptive coagulopathy. The PRISM score was 13, and the child developed severe sepsis. One arterial catheter was removed on day 2, the patient did not receive any parenteral nutrition or corticosteroid, but was already under broad spectrum antibiotics and acyclovir treatment prior to hospitalization. Six blood samples were sent for culture on days 1, 5, 7, 21, 27 and 35, and *C. albicans* was isolated on days 5 to 35. A catheter tip removed on day 16 also showed the presence of *C. albicans*. Fluconazole was administered beginning on day 7, for 10 days, then was replaced by amphotericin B for 23 days. The patient progressed with multiple organ failure and died on day 40.

**Blood samples**

One to 3 ml of the blood samples were inoculated into Bactec Peds Plus/F bottles and monitored on the Bactec 9240 system (Becton Dickinson, Franklin Lakes, NJ, USA). To avoid excessive blood collections of these two critically ill children, remnant volumes of EDTA tubes obtained to perform the routine blood count were used for PCR assays. Samples submitted for DNA extractions and amplifications were selected among those collected on the same days in which blood cultures were performed.

**DNA extraction**

DNA was extracted from 250 μl of EDTA-whole blood as described by Löeffler et al. [10] using 250 U/ml of recombinant lyticase (L-4276, Sigma-Aldrich, St. Louis, MO, USA) and the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. *Candida* reference strains were provided by the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. *Candida albicans* CBS 562, *C. glabrata* CBS 138, *C. parapsilosis* CBS 604, *C. tropicalis* CBS 94, and *C. krusei* CBS 573 were extracted as previously described [11], and used as positive DNA controls.

**PCR assay, RFLP technique and DNA sequencing**

The molecular biology laboratory in which the amplifications were carried out adopted procedures to minimize PCR carry-over according to a previously described protocol [12]. Briefly, reactions were set up in three separate rooms dedicated to master mix preparation, DNA extraction and amplification, respectively. A separate set, including equipment, supplies and pipetting devices was used in each room. The researchers handled reagents and samples with disposable gloves, DNA/RNase-free microtubes and pipetting tips, and stringent laboratory procedures were carried out in safety cabinets equipped with UV lights. Nested-amplifications were targeted to the *Candida* ITS sequence and were performed in 50 μl of total volume containing 1× amplification buffer, 2 mmol/l MgCl₂, 200 μmol/l of each deoxynucleoside triphosphate, and 2.5 U of Taq DNA polymerase (Labtrade, São Paulo, Brazil). For the first round, 0.4 μmol/l of ITS1/ITS4 primers [13], and 20 μl of DNA samples were added to the solution and amplifications were performed in a thermocycler (Biometra, Göttingen, Germany). A first denaturation step of 95°C for 5 min was followed by 40 cycles of 1 min at 95°C, 1 min at 47°C and 1 min at 72°C; and a final extension at 72°C for 5 min. For the second round, inner species-specific primers corresponding to *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* [13,14], were added to a new master mix containing 5 μl of the first PCR product. Amplifications were performed with the same reagent concentrations and cycling conditions, except for the annealing temperatures, which were 55°C for *C. albicans*.
and 52°C for the other species. DNA samples from patients were tested together with negative (sterile water instead of DNA) and positive (DNA from the five Candida reference strains) controls. The assay detection limit corresponded to 1–5 genome equivalents/ml of blood for all amplification systems (data not shown). The nested-PCR products were fractionated by electrophoresis and visualized in 2% ethidium bromide-stained agarose gels (Sigma-Aldrich).

To confirm the specificities of PCR results, as they could not be established through culture, amplification products were submitted to RFLP. Briefly, 100 ng of amplification products obtained from patients’ samples and from Candida reference strains were digested with 5 U of Nla III and Ava I (New England Biolabs, Ipswich, MA, USA) at 37°C for 3 h [15].

The entire PCR products were sequenced in both forward and reverse directions, in the ABI PRISM 3100 analyzer (Applied Biosystems, Carlsbad, CA, USA). Comparison of sequences was performed with the reference strains available in GenBank by means of the BLAST Assembled Genomes (http://blast.ncbi.nlm.nih.gov).

**Results**

From the total of eight blood samples collected from patient 1, those obtained on days 3, 4 and 6 after PICU admission amplified C. albicans DNA, while three samples collected on days 15, 16 and 18, after initiation of fluconazole therapy, detected C. tropicalis (Figs 1 & 2). For patient 2, three samples corresponding to those obtained on days 1, 5 and 7 after PICU admission amplified C. albicans DNA, and those obtained on days 21, 27 and 35 detected C. albicans and C. glabrata, simultaneously (Fig. 3).

Patient 1 had six samples that were amplified but which were negative in culture. RFLP was performed in these samples.
amplification products by means of the Nla III enzyme, which generated two fragments of 178 bp and 94 bp for the C. albicans reference strain and the amplicon of patient 1 (272 bp), while the Ava I enzyme produced two fragments of 251 bp and 106 bp for the C. tropicalis reference strain and the amplification product from the patient (357 bp). The same Nla III enzyme generated two fragments of 322 bp and 101 bp for the C. glabrata reference strain and the amplicon of patient 2 (423 bp) (Fig. 4).

DNA sequences of the amplicons were compared with GenBank C. albicans (accession number AB437012.1), C. tropicalis (AB437076.1) and C. glabrata (AB 355314.1) reference strains, and 98% homology was found between the patient 1 sequence and C. albicans, 98% homology between the patient 1 sequence and C. tropicalis, and 97% of homology between the sequence of patient 2 and C. glabrata.

Discussion

The presence of severe gastrointestinal disease has already been reported as a predisposing condition of candidemia [4] and both children presented in this case report had such infections, in addition to high risk of mortality and other factors which could contribute to Candida infections.

It is important to emphasize that in patient 1, C. albicans was detected in only one urine culture on day 11, while blood cultures were persistently negative. PCR indicated the involvement of C. albicans and C. tropicalis, although simultaneous amplification of both species was not observed. Nevertheless, detection of Candida in the bloodstream on day 3 instead of in the urine on day 13 would have anticipated antifungal therapy. In case 2, C. albicans was detected in blood cultures initiated on days 5 to 35 (fluconazole was introduced on day 7, for 10 days), but in none of the samples was another Candida species isolated.

In contrast, PCR amplified C. albicans and C. glabrata simultaneously beginning on day 21 until day 35. In this child, fluconazole was replaced by amphotericin B due to the deterioration of clinical and laboratory parameters. PCR could possibly have helped in earlier detection of the two Candida species in the bloodstream.

Recently, Garey et al. [16] demonstrated that a delay in fluconazole therapy in hospitalized patients with candidemia significantly impacts the length of ICU stay and mortality. These authors have claimed that new methods to avoid delays in appropriate antifungal therapy, such as rapid diagnostic tests or identification of unique risk factors, are needed.

Moreover, when a group of patients with candidemia was compared with a matched one without candidemia, the crude mortality among those in the first group was 61% compared with 12% in the other one, for an attributable mortality of 49% [17].

Routine diagnostic microbiological methods are neither sensitive nor timely enough to permit the early diagnosis of candidemia [1,18]. Even though culture isolation methods are still the standard reference to identify bloodstream infections, PCR should be simultaneously performed in an attempt to increase the number of Candida species detected, especially in patients at risk of developing mixed yeast infections [6].

A few reports have pointed out the usefulness of PCR-based techniques in detecting polyfungal candidemia, thus corroborating our results. Ahmad et al. [7] used a seminested-PCR (sn-PCR) with serum samples of 12 culture-proven and 16 clinically suspected candidemia patients. In the latter group, while blood cultures remained negative in all cases, samples from nine patients yielded positive results by PCR, and dual Candida infection was identified in two of them. In the group with proven candidemia, the sn-PCR detected Candida infection in all 12 patients and the presence of two Candida species in five patients, while blood cultures yielded dual infection in three cases. More recently, Alam et al. [8] reported dual Candida infection detected by sn-PCR in 14.8% of culture-proven patients, and in one case, the presence of three Candida species in the bloodstream, while only one Candida species was isolated by blood cultures.

The systematic use of PCR-based techniques coupled to microbiological identification will probably contribute to the detection of an increased number of candidemia episodes and polycandidemia in high risk patients, and potentially shorten the patients’ hospital stay, improving their outcome and reducing hospital charges.

Declaration of interest: The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
References


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