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Multiplex PCR identification of eight clinically relevant *Candida* species

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Invasive fungal infections, specifically candidemia, constitute major public health problems with high mortality rates. Therefore, in the last few years, the development of novel diagnostic methods has been considered a critical issue. Herein we describe a multiplex PCR strategy allowing the identification of 8 clinically relevant yeasts of the *Candida* genus, namely *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*. This method is based on the amplification of two fragments from the ITS1 and ITS2 regions by the combination of 2 yeast-specific and 8 species-specific primers in a single PCR reaction. Results from the identification of 231 clinical isolates are presented pointing to the high specificity of this procedure. Furthermore, several *Candida* isolates were identified directly from clinical specimens which also attests to the method's direct laboratory application. The results from the multiplex reactions with other microorganisms that usually co-infect patients also confirmed its high specificity in the identification of *Candida* species. Moreover, this method is simple and presents a sensitivity of approximately 2 cells per ml within 5 hours. Furthermore, it allows discrimination of individual *Candida* species within polyfungal samples. This novel method may therefore provide a clinical diagnostic procedure with direct applicability.

Keywords *Candida*, candidemia, diagnostic, identification, PCR, multiplex

Introduction

Invasive fungal infections represent a public health problem of major importance [1,2]. In particular, candidemia has been reported within similar rates in different countries, ranging from 0.20–0.38 per 1,000 admissions and from 3.0–4.4 per 100,000 patient days [3]. This emergence is often associated with human immunodeficiency virus (HIV) or advanced medical and surgical interventions that compromise patient immunity, e.g., bone-marrow or solid-organ transplants, aggressive chemotherapy and broad application of antifungal agents [2]. In fact, nosocomial fungal bloodstream infections are an increasingly significant cause of morbidity, with an estimated mortality of 25–38% [4]. *Candida albicans* is the most common and clinically relevant pathogen of the genus. However, there has been a significant trend towards the emergence of species other than *C. albicans*, with a particular increase in *C. glabrata* frequency [3,5,6] and to a lesser extent,

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C. parapsilosis and *C. tropicalis* [7]. In addition, given that several non-*C. albicans Candida* species are intrinsically resistant to common antifungal agents, accurate identification methods are critical for the establishment of appropriate antifungal therapy [7].

The cornerstone of laboratory detection of bloodstream fungal infections, including candidemia, remains direct examination and conventional blood culture. However, these methods are of limited clinical value since there are negative outcomes in as high as 50% autopsy-confirmed cases of candidemia. In addition, cultures may only become positive late in the infection [8]. Furthermore, most phenotypic methods of identification used in clinical laboratories are often time-consuming and may lead to inconclusive results. For example, phenotypic tests such as VITEK and ID32C systems require several days before biochemical reactions can be interpreted [9]. On the other hand, molecular approaches have the potential to detect candidemia with increased sensitivity and specificity. Buchman *et al*. were the first to demonstrate that detection of *C. albicans* in clinical specimens was possible by PCR amplification of the lanosterol-alpha-demethylase (L1A1) gene [10]. Other PCR-based techniques have been developed using amplification of target DNA, providing alternative strategies for the diagnosis and identification of fungal pathogens [11–14]. Fungal ribosomal genes are common DNA targets in PCR-based procedures for the identification of fungi at the species level. The highly variable sequences of internal transcribed spacer regions ITS1 and ITS2 flanked by the relatively conserved coding regions of 18S, 5.8S and 28S nuclear rRNA genes have been used in various PCR-based formats for the identification of medically important yeasts [13,15–17]. Even though these molecular techniques are highly sensitive and specific, their limited applicability has been the need for expensive equipment not readily accessible to many diagnostic laboratories.

We describe a rapid and simple multiplex PCR-based method able to specifically identify 8 clinically relevant *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*) based on the amplification of particular DNA fragments of the ITS1 and ITS2 regions. The method combines two yeast-specific primers and eight *Candida* species-specific primers in a single PCR reaction yielding two amplicons of different sizes for each species. In addition, this method provides the following advantages over currently available techniques: (1) it can be used to test several clinical samples, including blood culture bottles and urine samples, (2) whole yeast cells may be employed directly in the PCR mixture, (3) it is highly specific and sensitive, with a detection limit of 2.15 ± 0.25 cells/ml, (4) it has the potential to discriminate individual *Candida* species in polyfungal infections to a maximum ratio of 1:10, and (5) it presents good reproducibility among different PCR thermal cyclers and within different laboratories. Altogether, the features of this method point to a novel and highly advantageous application in the identification of *Candida* species in both clinical diagnosis and epidemiological studies.

Materials and methods

Yeast and bacterial strains

A total of 231 yeast isolates (90 *Candida albicans*, 61 *C. parapsilosis*, 25 *C. tropicalis*, 19 *C. krusei*, 18 *C. glabrata*, 13 *C. guilliermondii* and 5 *C. lusitaniae*) were recovered from clinical specimens in two Portuguese medical institutions, one in the northern region (Hospital de São João, Porto) and the other in the southern area of the country (Hospital de Santa Maria, Lisboa). The following type Candida strains; *C. albicans* ATCC 18804, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. lusitaniae* ATCC 34449, *C. guilliermondii* ATCC 6260 and *C. dubliniensis* ATCC MYA-646 were used in the present studies. The identification of yeast isolates was carried out by conventional biochemical techniques in both medical institutions using the VITEK (bioMérieux Vitek, Inc., MO, USA) or ID32C (bioMérieux Vitek) yeast identification systems. The isolates were stored on yeast extract peptone dextrose (YEPD) agar plates. In addition, *Aspergillus fumigatus* MUM 98.02, *A. niger* MUM 03.01, *A. flavus* MUM 00.29, *A. terreus* MUM 94.09, *A. nidulans* MUM 98.32, *Cryptococcus neoformans* var. *neoformans* ATCC 28957,*Saccharomyces cerevisiae* Y10000, as well as *Mycobacterium tuberculosis* H37Rv, *M. avium* 3509, *Escherichia coli* HB101, *Staphylococcus aureus* 0400, *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6051 were used for PCR cross-reactivity testing.

Furthermore, 27 BACTEC blood culture bottles (Becton Dickinson Microbiology Systems, MD, USA) which were identified as positive for bacteria and/or yeasts through the use of a blood culture instrument and Gram staining and 19 urine samples found to be positive for yeasts and/or bacteria were also directly analysed by PCR. Identification was simultaneously carried out using the VITEK yeast biochemical card. Twenty blood culture bottles and 14 urine samples were noted to contain yeasts, while negative controls

included blood culture bottles and urine samples positive for different bacterial species. While no DNA isolation procedure was required for urine samples, *Candida* DNA was isolated from aliquots of blood culture bottles, in order to eliminate the presence of PCR inhibitory factors (see below).

Primer design

Yeast-specific universal primers UNI1 (5′-GTCAAAC TTGGTCATTTA-3′) and UNI2 (5′-TTCTTTTCCTC CGCTTATTGA-3′) were used to amplify the internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), including the 5.8S rRNA of the most relevant yeast pathogens associated with human disease [18]. In addition, the species-specific primers Calb, Cgla, Ckru, Cpar, Ctro, Clus, Cgui and Cdub were designed based on the sequence data for the ITS1 and ITS2 regions of the reference strains and of all clinical isolates from the *Candida* genus available in the EMBL/GenBank databases. The sequences were compared using the DNAMAN for Windows software (Lynnon Corporation, Quebec, Canada) in order to design primers to specifically amplify DNA from *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. guilliermondii* and *C. dubliniensis*, respectively (Table 1). In the multiplex PCR, the species-specific primers form a pair with the universal primer UNI2, with the exception of Clus, which pairs with UNI1 (Fig. 1).

DNA isolation

For DNA extraction of yeasts in culture, cells were grown overnight in YEPD medium at 26°C with aeration on a mechanical shaker (150 rpm) [19]. Cells were harvested by centrifugation and the pellet suspended in 200 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). For cell disruption, 200 µl of 0.5 mm-diameter glass beads and 200 µl of phenol/chloroform (1:1) were added and the tubes were shaken for three 60-second intervals interspersed with periods of cooling on ice. After disrupted cell debris was removed by a 5 min centrifugation at 3,000 *g*, the supernatant was collected and 1 ml of cold isopropanol was added before mixing by inversion. The tubes were centrifuged for 3 min at 3,000 *g* and the sediment suspended in 400 µl of TE buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.0). A 5-min treatment with RNase A (1 mg/ml) at 37°C was then carried out before adding 10 µl of 3 M sodium acetate. DNA was further precipitated by addition of 1 ml of isopropanol, mixing by inversion and further centrifugation. Finally, the sediment was air-dried and the DNA suspended in 50 µl of ultra-filtered water. DNA content and purity were determined by spectral photometry at 260 and 280 nm and diluted to a 100 ng/µl final concentration. The DNA isolation from whole blood and blood culture bottles was adapted from elsewhere [20] with minor alterations. Four hundred µl of 10 × TXTE buffer (10 mM Tris-HCl, 1mM EDTA, 1% Triton X-100, pH 8.0) were added to 3.6 ml of blood sample and the mixture incubated for 20 min at room temperature to lyse blood cells. Yeast cells were collected by centrifugation at 3,000 *g* for 10 min and washed with 1 × TXTE buffer and with 20 mM Tris-HCl (pH 8.3). The erythrocyte-free pellet was then resuspended in 180 µl of ATL lysis buffer and 20 µl of proteinase K (1.7 mg/ml). The mixture was incubated at 65°C for 1 h, 200 µl of AL buffer were added and the sample was heated at 70°C for 10 min. After these steps, 200 µl of ethanol were added to each sample and the mixtures were applied to

Table 1 Universal and species-specific primers used in *Candida* species amplification and size of fragments visualized under agarose gel electrophoresis

Species	Primer name	Sequence (5′–3′)	Amplicon size (bp)*
Clinically relevant yeasts	UNI1 UNI2	GTCAAACTTGGTCATTTA TTCTTTTCCTCCGCTTATTG	Trost *et al.*, 2004 [18]
C. albicans	Calb	AGCTGCCGCCAGAGGTCTAA	583/446
C. glabrata	Cgla	TTGTCTGAGCTCGGAGAGAG	929/839
C. krusei	Ckru	CTGGCCGAGCGAACTAGACT	590/169
C. tropicalis	Ctro	GATTTGCTTAATTGCCCCAC	583/507
C. parapsilosis	Cpar	GTCAACCGATTATTTAATAG	570/370
C. guilliermondii	Cgui	TTGGCCTAGAGATAGGTTGG	668/512
C. lusitaniae	Clus	TTCGGAGCAACGCCTAACCG	433/329
C. dubliniensis	Cdub	CTCAAACCCCTAGGGTTTGG	591/217

*Amplicon sizes result from multiplex PCR amplification using yeast-specific primers (UNI1 and UNI2) and corresponding species-specific primer.

Fig. 1 Multiplex PCR strategy. Organization of fungal ribosomal genes with universal and species-specific primer targets indicated. Arrows indicate the direction of PCR amplification.

QIAamp mini spin columns with 2 ml collection tubes, centrifuged at 5,000 *g* for 1 min and washed twice with 500 µl of AW buffer. The columns were then washed twice with 50 mM EDTA and twice with AW buffer in order to chelate PCR inhibitory factors. DNA was eluted with 100 µl of previously heated AL buffer and kept at −20°C until PCR. ATL lysis buffer, AL buffer, AW buffer, proteinase K and the spin columns were purchased from Qiagen, Hilden, Germany.

PCR amplification

Multiplex PCR amplification was performed in a 20 µl volume consisting of 0.8 × PCR buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8)], 3.5 mM $MgCl_2$, dNTP mixture (200 µM each), primer mixture (UNI1 and UNI2, 0.55 µM each; Cgui, 0.05 µM; Calb and Ckru, 0.15 µM each; Cgla, Ctro and Clus, 0.2 µM each; Cpar, 0.3 µM; Cdub, 0.4 µM), 1 U *Taq* DNA polymerase and 50 ng genomic DNA template, with the remaining volume consisting of sterilized water. The amount of template DNA used was quantified by spectrophotometry. A 2 µl volume was used when the template was urine or isolated *Candida* DNA, either from whole blood samples or aliquots from blood culture bottles. For colony-PCR, part of a single colony was suspended directly in the PCR mixture with a sterile toothpick. PCR was routinely carried out in a Biometra Tpersonal (Whatman Biometra, Goettingen, Germany) thermal cycler under the following cycling conditions: 40 cycles of 15 s at 94°C, 30 s at 55°C, and 45 s at 65°C, after a 10-min initial period of DNA denaturation and enzyme activation at 94°C. Additionally, multiplex PCR reactions were performed in four different thermal cyclers: iCycler and MyCycler (Bio-Rad, CA, USA), Primus 96 (MWG Biotech AG, Ebersberg, Germany) and GeneAmp PCR System 9600 (Perkin-Elmer, MA, USA) in order to confirm PCR reaction reproducibility. Negative control reactions were performed simultaneously with each test run by replacing the template DNA with sterilized water in the PCR mixture. Ten µl aliquots of each amplification product were separated by electrophoresis in a 2% agarose gel. Ethidium bromide staining (0.5 µg/ml) allowed the visualization of DNA fragments with a digital imaging system (Alpha Innotech Corporation, CA, USA) and species identification was possible by comparison with a 100-bp DNA ladder (Fermentas International Inc., Ontario, Canada). *Mycobacterium avium* subsp. *paratuberculosis* strain k10, *Mycobacterium tuberculosis* CDC1551, and *Nocardia farcinica* IFM10152 were tested using *in silico* PCR (http://www.in-silico.com/multiplex_PCR/).

Detection limit of Candida yeasts in whole blood

In order to determine the detection limit of *Candida* cells in whole blood by multiplex PCR, fresh human blood obtained from healthy volunteers was seeded with cells from each *Candida* species to a concentration of 5×10^2 CFU/ml. Yeast-cell number was estimated by haemocytometer counting and confirmed by plating serial dilutions of seeded blood onto Sabouraud agar plates and colony counting after 2 days of incubation at 30°C. The seeded blood was then serially diluted with whole blood to obtain yeast concentrations ranging from 2.5×10^2 to 1.25 cells/ml and 3.6 ml of the diluted samples were used for isolation of *Candida* DNA. Multiplex PCR amplification was then carried out using 2 µl of isolated DNA as template (see above).

Flow cytometry analysis

In order to eliminate the interference of DNA from dead cells, cell viability was determined for quantification of DNA in samples. Plasma membrane integrity was determined by examining cellular permeability to propidium iodide (Sigma-Aldrich Corporation, MO, USA) as described elsewhere [21]. Fluorescence analysis was carried out by flow cytometry with an EPICS XL-MCL (Beckman-Coulter Inc., CA, USA) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW.

Results

Sequence analysis and multiplex PCR strategy

Previously designed fungus-specific universal primers UNI1 and UNI2 were used to amplify the internal transcribed spacer regions 1 and 2, including the 5.8S rRNA region [18]. As shown in Fig. 1 and reported by Trost and colleagues, predicted PCR products obtained by amplification using these universal primers were found to vary among *Candida* species, ranging in length from 433 bp (*C. lusitaniae*) to 929 bp (*C. glabrata*) [18]. In addition, DNA from clinically

relevant yeasts other than *Candida*, including species from the genus *Cryptococcus*, *Saccharomyces* and *Trichosporon* would also be amplified into a single PCR fragment using the universal primers [18]. For most *Candida* species, the sizes of the obtained fragments are not sufficient to promote direct identification. Thus, species-specific variations within ITS1 and ITS2 sequences were used to design primers for amplification of an internal fragment with a lower length (Fig. 1), that could allow direct identification by only a PCR reaction avoiding the restriction analysis described by Trost and collaborators [18]. ITS1 and ITS2 sequences from the reference strains and from all clinical *Candida* isolates displayed in EMBL/GenBank database were analysed by intraspecies alignments using DNAMAN for Windows software (Lynnon Corporation, Quebec, Canada) to find blocks of conserved regions among the different strains. These sequences were compared inter-species in order to find variable regions that allowed the design of specific primers to each species. Interferences from both *C. orthopsilosis* and *C. metapsilosis* in *C. parapsilosis* identification and from *C. famata* and other species of the *C. guilliermondii* clade (*C. fermentati* and *C. carpophila*) in *C. guilliermondii* identification [22,23] were ruled out by sequence analysis and multiplex PCR. This *in silico* study was concluded with the design of primers in variable regions among species but at the same time conserved between strains (Table 1), thus excluding possible intraspecies variability.

Multiplex PCR amplification

In order to develop a protocol that could allow the specific identification of each *Candida* species studied in a single reaction, a simple step-by-step protocol of multiplex PCR optimization was developed [24]. The results of multiplex PCR using isolated DNA from each species allowed the identification of up to eight clinically relevant yeasts in a single PCR reaction by the use of eight species-specific primers together with the previously described universal primers [18]. Multiplex PCR directly from living/intact yeast whole cells (commonly referred as colony-PCR [25]) was also assessed. PCR conditions were maintained and the DNA was replaced by whole yeast cells, where the initial 10 min at 94°C in the PCR protocol was sufficient to disrupt cell integrity allowing DNA release. Multiplex PCR reactions performed with yeast cells yielded both universal and species-specific amplicons (Fig. 2B).

Identification of clinical yeast isolates

To validate the methodology described, a wide scan of 231 clinical isolates, previously characterized by conventional phenotypic systems, was carried out using the multiplex PCR-based approach. Comparison of the results of the two identification approaches (Table 2) showed discrepancies in species identification for 18 isolates representing 7.8% of the total. The multiplex PCR identification of the discrepant isolates was further confirmed using PCR fingerprinting (Fig. 2E) [26], which demonstrated the accuracy of the multiplex PCR identifications. No isolates of *C. dubliniensis* were found in the clinical specimens provided by the health care institutions. For this reason, results for *C. dubliniensis* are not included in the agarose gel presented in Fig. 2B. The most frequent misidentifications of the VITEK/ID32C analysis were of *C. parapsilosis* (7/18 of all discrepant identifications), followed by *C. albicans* (5/18), *C. tropicalis* (4/18), *C. krusei* (1/18) and *C. guilliermondii* (1/18). In addition, the most common misidentification was *C. tropicalis* for *C. parapsilosis*. Taking into account the misidentification percentage for each species regarding the number of isolates wrongly identified by the VITEK or ID32C systems, *C. tropicalis* was the most often misidentified species (16% of the *C. tropicalis* isolates), followed by *C. parapsilosis* (11.5%), *C. guilliermondii* (7.7%), *C. albicans* (5.6%) and *C. krusei* (5.3%).

Identification of Candida species in polymicrobial mixtures

Theoretically, the strategy here described would be able to simultaneously identify in a single multiplex PCR reaction coexisting yeast species in mixed samples. In order to experimentally assess this hypothesis, samples with two or three mixed *Candida* species were tested. The results showed that the multiplex method discriminates the different species present in polyfungal samples (Fig. 2C) by the amplification of the fragments corresponding to each *Candida* species present. In fact, multiple species can be identified to a maximum cell ratio of 1:10, where for lower ratios the less abundant yeast species could not be detected (data not shown). In addition, in the case of polymicrobial samples (mixed fungal and bacterial cells) no detectable PCR products were obtained using as template *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* bacterial DNA, although *Candida* species could be identified without interference (data not shown). This confirms the specificity of the proposed methodology. In addition, common microorganisms which usually cause infection in immunocompromised populations with candidemia, such

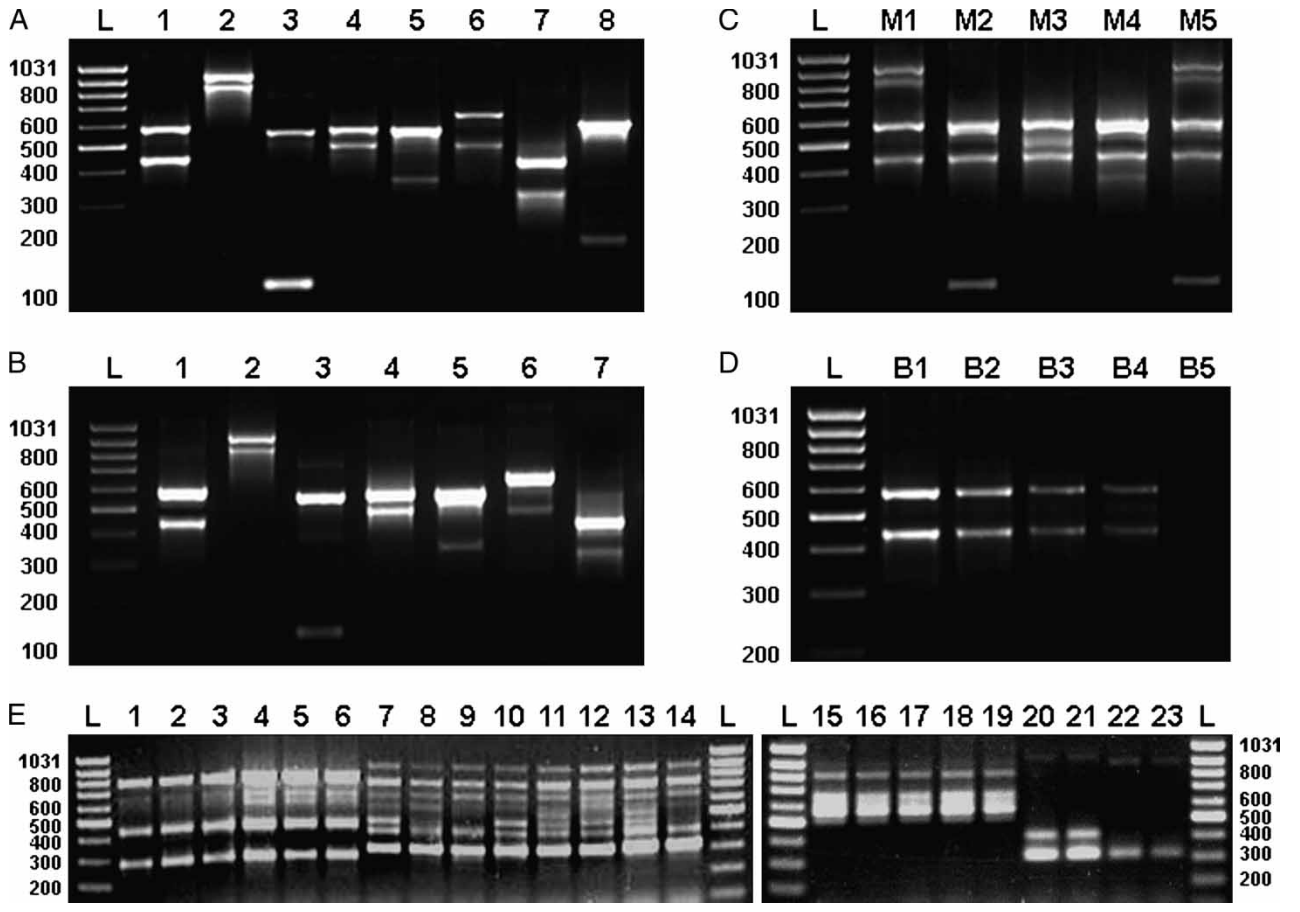

Fig. 2 Agarose gel showing the results obtained for multiplex PCR of isolated yeast genomic DNA (A) or yeast whole cells (B) as template. Lanes: L, 100-bp DNA ladder; 1, *C. albicans*; 2, *C. glabrata*; 3, *C. krusei*; 4, *C. tropicalis*; 5, *C. parapsilosis*; 6, *C. guilliermondii*, 7, *C. lusitaniae*; and 8, *C. dubliniensis*. (C) Multiplex PCR of *Candida* mixtures in 1:1 ratios. Lanes: L – 100-bp DNA ladder; M1-*C. albicans* + *C. glabrata*, M2-*C. albicans* + *C. krusei*, M3-*C. albicans* + *C. tropicalis*, M4-*C. albicans* + *C. parapsilosis* and M5-*C. albicans* + *C. glabrata* + *C. krusei*. (D) Sensitivity of multiplex PCR. DNA was isolated from whole blood with different levels of artificial infection with *Candida albicans*. Lanes: L, 100-bp DNA ladder; B1, 2.5×10^2; B2, 2.5×10^1; B3, 5; B4, 2.5; and B5, 1.25 cells/ml. (E) PCR fingerprinting using primer T3B of the *Candida* isolates with different identification using multiplex PCR and VITEK/ID32C. Lanes: L, 100-bp DNA ladder; 1, *C. albicans* type strain; 2–6, *C. albicans* (as identified by multiplex PCR); 7, *C. parapsilosis* type strain; 8–14, *C. parapsilosis* (as identified by multiplex PCR); 15, *C. tropicalis* type strain; 16–19, *C. tropicalis* (as identified by multiplex PCR); 20, *C. krusei* type strain; 21, *C. krusei* (as identified by multiplex PCR); 22, *C. guilliermondii* type strain; and 23, *C. guilliermondii* (as identified by multiplex PCR).

as *Mycobacterium tuberculosis*, *M. avium* and *Nocardia farcinica* did not present cross-reactivity with *Candida* species (data not shown).

Identification of yeasts directly from urine and blood culture bottles

In order to directly apply this methodology to clinical samples, direct detection and identification of yeast cells in blood culture bottles ($n = 27$) and urine samples ($n = 19$) was assessed. To avoid cellular growth, the PCR reactions were performed in the samples from blood culture bottles immediately after detection of growth and in the case of urine samples, frozen aliquots were tested after positive indicators of development was obtained. The results for the *Candida* species present in the analysed samples were in accordance with the presumptive identification provided by the health care institutions that carried out simultaneous phenotypic identification (Table 3). No cross-reactivity was detected in control samples positive for bacteria (7 samples) and at the same time, no interference in the identification of *Candida* species was observed in polymicrobial samples (2 samples constituted *C. albicans* with either *S. aureus* or *P. aeruginosa*).

A step forward in the application of the multiplex PCR described would be the detection of cases of candidemia directly from blood samples. In this sense

Table 2 Comparative results between identification in hospital centers (based on VITEK or ID32C) and identification by multiplex PCR of clinical isolates of *Candida* species. Multiplex PCR identification of the analysed yeast species by-pass the errors associated with VITEK/ID32C identification

VITEK/ID32C identification		Multiplex PCR identification*						VITEK/ID32C misidentifications
Species	No. of isolates	*C. albicans*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*C. glabrata*	*C. guilliermondii*	*C. lusitaniae*	(%)
C. albicans	90	85	3	0	0	1	0	1	5.6 (5/90)
C. parapsilosis	61	0	54	4	0	0	1	2	11.5 (7/61)
C. tropicalis	25	0	2	21	0	2	0	0	16.0 (*4/25*)
C. krusei	19	0	1	0	18	0	0	0	5.3 (1/19)
C. glabrata	18	0	0	0	0	18	0	0	0 (0/18)
C. guilliermondii	13	0	1	0	0	0	12	0	7.7 (1/13)
C. lusitaniae	5	0	0	0	0	0	0	5	0 (0/5)
Total	231	85	61	25	18	21	13	8	7.8

*PCR fingerprinting (Correia *et al.*, 2004) [26] confirmed the results obtained by multiplex PCR.

and to further characterize the sensibility of the methodology, the limit of detection in seeded whole blood was determined. Thus, cells of different *Candida* species were artificially inoculated in whole blood from healthy volunteers in concentrations ranging from 2.5×10^2 to 1.25 cells/ml and DNA was isolated from a 3.6 ml sample. Cells in the exponential phase of growth were used and their viability (assessed as plasma membrane integrity) was confirmed by flow cytometry in order to exclude from the presence of dead *Candida* cells that could contribute to the release of DNA, masking the sensitivity of the method (data not shown). Using the proposed multiplex protocol, the PCR products presented the characteristic mobility pattern, correctly identifying the species seeded in whole blood (Fig. 2D exemplifies the case of *C. albicans*). Additionally, the sensitivity of the multiplex PCR was found to be 2.15 ± 0.25 cells per ml of blood. On the other hand, in the particular case of simulated polyfungal infections, both species could be correctly identified to a minimum limit of approximately 20 cells/ml, although one of them was always detected to the minimum limit achieved for individual species (data not shown).

Discussion

The development of a simple approach to the identification of *Candida* species based on the combined use of universal yeast primers and *Candida* species-specific primers was undertaken. The universal primers were first reported by Trost and colleagues, who further developed a method based on the enzyme restriction of PCR fragments in order to identify a set of fungal pathogens [18]. While universal primers provide a broad detection capability for yeast pathogens [18], in the present work, the presence of species-specific primers allow differentiation of *Candida* at the species level in a single PCR reaction. The multiplex PCR strategy herein described takes advantage of the high-copy number of rRNA genes, the differences in the length of ITS regions and the high variability of these regions among *Candida* species. This strategy renders the method with a test matrix able to identify eight of the most clinically relevant yeast species, including the newly emerging *C. dubliniensis*, as shown with the type strain (Fig. 2A). Together, both the universal and species-specific fragments result in characteristic band patterns, enabling easy identification of the *Candida* species in question. Similarly to other genotypic-based techniques, this method allows for the identification of species that have non-standard morphologic, cultural and biochemical characteristics. Additionally to the

Table 3 Comparative results between VITEK and multiplex PCR identification of *Candida* species present in blood culture bottles and urine samples

Clinical samples	Total	*C. albicans*		*C. glabrata*		*C. parapsilosis*		*C. tropicalis*		yeast-negative identification*
		VITEK	Multiplex PCR	VITEK	Multiplex PCR	VITEK	Multiplex PCR	VITEK	Multiplex PCR	
Blood culture bottles	27	13	13	4	4	2	2	1	1	7
Urine	19	12	12	2	2	0	0	0	0	5
Total	46	25	25	6	6	2	2	1	1	12

*Yeast-negative identification was proven by both VITEK and multiplex PCR.

amplification of isolated DNA, the identification of living/intact whole cells in the PCR mixture was also found to be possible (Fig. 2B), thus bypassing the time-consuming DNA isolation steps and reducing the time required for identification. Another advantage of this methodology resides in the rapid identification in the same multiplex PCR reaction of the etiologic agents involved in polyfungal infections (Fig. 2C). Such cases involving more than one yeast have been reported to occur with a frequency of approximately 5% among all candidemias [27]. Furthermore, yeast species was not hampered by the presence of bacteria frequently co-infecting candidemia patients.

The identification of *Candida* species was also carried out directly from clinical samples such as blood culture bottles and urine samples. The isolation of *Candida* DNA from aliquots of blood culture bottles required 2 h to lyse blood cells and recover DNA, 2 h for multiplex PCR amplification of isolated DNA and 1 h to perform agarose gel electrophoresis, including the time required to prepare the PCR mixture and agarose gel. Therefore, species can be identified within as little as 5 h, in contrast to phenotypic methods which can take several days [28]. Furthermore, this methodology is highly sensitive since the number of yeast cells detectable in artificially inoculated whole blood reached a minimum limit of 2.15 ± 0.25 cells/ml (Fig. 2D). This capacity may enable the method to detect low candidemias occurring in the early stages of infection, given that there are often less than 10 *Candida* cells circulating per ml of blood in these situations [29]. Moreover, this multiplex strategy is adaptable to diverse standard equipment currently in use in most clinical laboratories and if not, likely to be easily implemented. The technique is cost-effective since it only requires essentially PCR components and DNA isolation reagents. Furthermore, no DNA probes or expensive restriction enzymes are needed. Finally, the possibility of detection of candidemia cases directly from patients' whole blood is currently under investigation and efforts are being carried out to validate the applicability of the method in clinical settings.

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