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Validation of the PCR–dHPLC method for rapid identification of *Candida glabrata* phylogenetically related species in different biological matrices

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ABSTRACT

Since two new species phylogenetically related to *Candida glabrata* with slightly different phenotypes and antifungal susceptibility profiles have been described, it seems to be necessary from clinical point of view, to develop a rapid and accurate identification system in order to distinguish between these three fungal species. We studied the performance of denaturing high performance liquid chromatography (dHPLC) as a faster (less than 7 min) and alternative novel technique for simultaneous analysis of *Candida* species in different biological matrices. The analyses show the good low limit of detection (LLOD) in all biological matrices studied (5.16–9.56 ng μL^{-1}, 4.14–4.70 ng μL^{-1} and 3.99–4.66 ng μL^{-1} for *Candida bracarensis*, *Candida nivariensis* and *C. glabrata*, respectively). 180 *Candida* isolates were analyzed in order to demonstrate the method suitability for screening analysis to identify *C. glabrata* and its cryptic species (*C. bracarensis* and *C. nivariensis*) in clinical routine.

1. Introduction

The incidence of opportunistic fungal infection has increased significantly in the past decades being an important cause of morbidity and mortality above all in immunocompromised hosts [1–3].

Candida spp. represents one of the most commonly isolated organisms for local and systemic infection [3–5]. Although, *Candida albicans* is the most prevalent species, the proportion of infection caused by *non-albicans Candida* strains is increasing particularly in Europe [1,6–13].

Candida glabrata is the third species in frequency isolated from bloodstream fungal infection, as stated in a recent study in Spain [14], concerning high mortality rates and reduced susceptibility to azoles [4,15–28].

Recently, due to the development in nucleic acid techniques, two new *C. glabrata* cryptic species has been described [29]: *Candida bracarensis* [30] and *Candida nivariensis* [31]. Their similar phenotypic characteristics with *C. glabrata*, can give rise to identify incorrectly unless molecular techniques are used [29].

Although different protocols have been described to identify *Candida* species using PCR, the most popular target for all of them are the internal transcribed spacer regions ITS1 and ITS2 [32–40]. The similarity in the DNA melting profiles and DNA melting temperature values of the *C. glabrata* cryptic species prevent the accurate differentiation of them when intercalating agents, such as SYBR Green, are used to perform the real-time PCR. Therefore, it is necessary to combine PCR with other complementary techniques, such as fluorescent probes, sequencing, high resolution melting (HRM) or denaturing high performance liquid chromatography (dHPLC).

Taking into account all the complementary techniques cited above, dHPLC has emerged as a versatile technology for screening analysis of slight differences in PCR amplicons, and has been successfully implemented in genotyping and detection of mutations in PCR products [3,41–44].

Therefore, regarding the clinical relevance of non-*albicans Candida* species, especially those from the *C. glabrata* complex, for successful guiding of the antifungal therapy and hence dismissing the associated mortality due to an invasive candidiasis episode, we decided to develop an accurate, cheap and fast identification protocol applied to distinguish between *C. glabrata* and its close-related cryptic species using dHPLC and compare this new protocol with conventional capillary sequencing considering the latter as a gold standard.

2. Materials and methods

2.1. Yeast strains

Reference strains of *C. glabrata* (UPV 04.229) and *C. nivariensis* (UPV 04.228 and UPV 04.230) were obtained from the Mycology Laboratory Collection at the University of the Basque Country, *C. bracarensis* (NCYC-3133 and NCYC-3397) from National Collection

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of Yeast Cultures (NCYC, Norwich, UK) and *C. albicans* (ATCC 64548), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 20019) from the American Type Culture Collection (ATCC, Manassas, VA, USA). All these yeast strains were used as control. In addition, 87 clinical isolates of *C. glabrata*, 39 clinical isolates of *C. albicans*, 8 clinical isolates of *Candida guilliermondii*, 9 clinical isolates of *C. krusei*, 25 clinical isolates of *C. parapsilosis* and 15 clinical isolates of *Candida tropicalis* were included in order to study and demonstrate the suitability of the method. The mentioned clinical isolates were obtained from the collection of the Clinical Microbiology Department at Basurto Hospital.

All the strains were maintained on Sabouraud dextrose agar with chloramphenicol (Becton Dickinson GmbH, Heidelberg, Germany) medium and periodically subcultured onto ChromID™ Candida plates (bioMerieux, Marcy l'Etoile, France) at 37 °C for 24 h for analyzing colony morphology and ensuring the purity of the subculture. The identity of all clinical isolates was confirmed by using the API C-AUX, biochemical gallery (bioMerieux), according to the manufacturer's instructions.

2.2. Sample preparation for real-time PCR and dHPLC assay validation

Different inert or biological matrices (water, urine, blood and sputum) were spiked with a 0.5 McFarland units homogeneous colony suspension of each *Candida* species considered in this experiment in order to verify the behaviour of the system using different types of clinical specimens. The DNA from each spiked sample was extracted and eluted as mentioned in the following subheading.

2.3. DNA extraction

The DNA was extracted using a MagNAPure 96 system (Roche Applied Science, Mannheim, Germany) according with the manufacturer's instruction. After extraction, the DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer at 260 nm (A_{260} nm) (Thermo Fisher Scientific, Wilmington, USA).

2.4. Real-time PCR amplification

The primers pair used for universal yeast amplification were ITS86-F [5′-GTG AAT CAT CGA ATC TTT GAA C-3′] and ITS4-R [5′-TCC TCC GCT TAT TGA C-3′] (TibMolbiol, Berlin, Germany) targeting ITS2 region. The amplicons length oscillated between 198 and 372 bp [45].

Each 20 μL reaction mixture contained 10 μL DNA Master SYBR Green, 0.3 μM of ITS86 primer, 0.5 μM of ITS4 primer, 3.4 μL of PCR-grade water and 5 μL of fungal DNA [45].

The PCR protocol was performed in a LightCycler 480 thermocycler (Roche Applied Science) and the cycling conditions were as follows: Initial denaturalization (10 min at 95 °C), 30 cycles of denaturalization, annealing and extension (10 s at 95 °C, 15 s at 58 °C and 15 s at 72 °C, respectively) and lastly this step was followed by a melting-curve analysis from 58 °C to 95 °C and, afterwards, cooling to 40 °C.

The obtained PCR products were purified using a High Pure 96 UF Cleanup kit (Roche diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

2.5. Sequencing

Amplicons were re-amplified in both directions (forward and reverse) with the same primers as those used in real-time PCR. Sequencing reactions were performed in a 20 μL volume with 4 μL of BigDye™ terminator cycle sequencing ready reaction mix (v3.1; Applied Biosystems, Foster City, CA, USA), 0.4 μL primer, 25 ng of PCR template and PCR-grade water to obtain a final volume of 20 μL. Excess of chromophore was removed with Illustra™ AutoSeq™ G-50 columns (GE Healthcare, Little Chalfont, UK) according to manufacturer's instructions.

ITS2 sequences were determined with the 3130 Genetic Analyzer (Applied Biosystems) and compared to the GeneBank public database using the BLAST software provided by the National Centre for Biotechnology Information.

2.6. dHPLC analysis

dHPLC analysis was performed by WAVE MD System 4000 plus (Transgenomic, Omaha, NE). Basically, 5 μL of each PCR product were loaded onto the autosampler of the system and separated on a DNASepMD cartridge, which contains an electrostatically neutral hydrophobic polystyrene–divinylbenzene particle matrix, which binds DNA in the presence of the ion-pair reagent 0.1 M triethylammonium acetate at pH7. Bound DNA was eluted with 0.1 M triethylammonium acetate in 25% acetonitrile. Reactives were purchased from Transgenomic. Finally, the elution product was monitored spectrophotometrically by UV absorption at 260 nm and analyzed using Navigator software version 1.5.4.

Prior to identification analysis, it is necessary to ensure the PCR products size yield and quality. So, all the amplicons were injected in the Wave system at non-denaturing conditions, that is, at 50 °C [46] and thereafter, the amplicons size was determined using pUC18 *HaeIII* digest ladder (Transgenomic Omaha, NE) in parallel analysis.

In order to perform the strain identification, the analysis was carried out under partially denaturing condition. The temperature was optimized taking into account, the peaks resolution determined by titration analysis for each analyte, 1–3 °C above and below the mean temperature predicted by the software.

2.7. Assay validation

The following parameters were tested in this study: (1) selectivity, (2) linearity, (3) lower limit of detection (LLOD), (4) precision and (5) accuracy in different biological samples in order to ensure the suitability of the analytical method in the same way that is proposed by the FDA and the ICH guidelines for bioanalytical method validation [47,48].

Selectivity was tested to ensure that the possible peaks from blanks do not interfere on the separation. So, water, human blood culture, urine and sputum from different source were used as blank under optimized chromatographic conditions. The obtained chromatograms of the different blanks were compared with chromatograms spiked at concentration near the LLOD.

In order to calculate the method linearity, calibration curves were built between 1 and 35 ng μL^{-1} range at nominal concentrations of 1, 5, 15, 25 and 35 ng μL^{-1} to each analyte ($n = 3$) prepared in water, human blood culture, urine and sputum in three different days. Each calibration was evaluated by its correlation coefficient, slope and intercept.

The LLOD was calculated following two different ways depending where some chromatographic peaks at the analytes retention time have been seen or not. In the former case (when peaks are seen) the LLOD was calculated multiplying 3 times the signal-to-noise ratio, in the later case (when no peaks are seen) the LLOD was equal to 3 times the standard deviation of the blank signal.

Intra and inter-day precision and accuracy were evaluated at low, medium and high concentration levels ($n = 6$) that are expressed as relative standard deviation (%RSD) and relative error (RE), respectively.