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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.

Table 1
Melting temperature values obtained in PCR using ITS86-ITS4 primer pair.

Microorganism	Melting temperature (° C)			
	Water	Urine	Blood	Sputum
C. bracarensis (*n* = 2)[a]	82.96 ± 0.22	84.34 ± 0.18	83.87 ± 0.24	84.51 ± 0.17
C. tropicalis (*n* = 15)[b]	83.47 ± 0.14	Not done	Not done	Not done
C. guilliermondii(*n* = 8)[b]	83.48 ± 0.22	Not done	Not done	Not done
C. parapsilosis (*n* = 26)[c]	84.16 ± 0.29	Not done	Not done	Not done
C. glabrata (*n* = 89)[c]	84.58 ± 0.23	82.97 ± 0.10	82.90 ± 0.14	83.08 ± 0.03
C. nivariensis (*n* = 2)[a]	84.68 ± 0.43	84.59 ± 0.45	84.63 ± 0.34	84.80 ± 0.48
C. albicans (*n* = 40)[c]	85.45 ± 0.30	Not done	Not done	Not done
C. krusei (*n* = 10)[c]	89.76 ± 0.07	Not done	Not done	Not done

[a] Reference strains.
[b] Clinical isolates.
[c] Reference strain and clinical isolates.

2.8. Comparison with other traditional methods

As there are many different identification and genotyping methods based primarily on the size of the obtained PCR amplicon, such as agarose gel electrophoresis, fragment analysis and microfluidic methods; we performed an additional validation step comparing the combined method using real-time PCR and dHPLC described above, to these other discrimination techniques.

3. Results

3.1. Candida species identification using LightCycler system

The amplification using real-time PCR with SYBR Green in a LightCycler 480 thermocycler allowed the identification and differentiation of *C. krusei* from other species of *Candida* considering a target located in the ITS2 region. Despite this promising result the technique was not capable of making accurate identification of the other species of *Candida*. This situation could be explained because of the melting curve slapping and therefore the similar melting temperatures of the PCR products obtained using this methodology (see Table 1). Taking into account the results mentioned above another identification system is necessary for accurate discrimination of different *Candida* species.

3.2. Chromatographic separation

3.2.1. Non-denaturing condition

The main utility of analyzing the PCR products under non-denaturing conditions was to assure the amplicons quality. Besides, this method was able to separate *C. glabrata* and its cryptic species from four other *Candida* species using the amplicon size variation.

To estimate the discrimination power of this technique, 180 *Candida* pure isolates (7 reference strains and 173 clinical isolates) comprising 7 different species (*C. albicans, C. parapsilosis, C. guilliermondii, C. tropicalis, C. glabrata, C. bracarensis* and *C. nivariensis*) were studied comparing the peaks profile obtained with pUC18 *HaeIII* digest ladder. All PCR products showed a single chromatographic peak and the sizes obtained for *C. parapsilosis*, *C. tropicalis*, *C. albicans* and *C. guilliermondii* oscillated between 230 and 300 bp (see Table 2).

Considering the results in the analysis of *C. glabrata*, *C. bracarensis* and *C. nivariensis* case, the amplification lengths were comprised between 340 and 360 bp. Despite the analysis based on under non-denaturing conditions was useful to perform the separation between *C. glabrata* complex species and other different species of *Candida*, it did not probe its usefulness to discriminate among *C. glabrata* and its cryptic species. Denaturing condition analysis was carried out in order to separate adequately the *C. glabrata* yeast complex.

3.2.2. Partially denaturing condition analysis

A mixture of PCR products of *C. glabrata*, *C. bracarensis* and *C. nivariensis* were injected at under optimized partially denaturing condition for the accurate identification of these three species. Optimal chromatographic conditions were set up taking into account the methodology described under Section 2.6 and are shown in Table 3. This analysis was accurate enough to confirm the identification of the species considered as it is shown in Fig. 1.

Following this procedure, we confirmed that none of the 87 *C. glabrata* clinical isolates analyzed in this experiment were misidentified, so there were no *C. glabrata* cryptic species in our clinical isolates collection (see Table 2).

Table 2
Summary of the retention time obtained in 180 reference and clinical samples using dHPLC method for the identification of *C. albicans, C. guilliermondii, C. parapsilosis, C. tropicalis, C. glabrata*, *C. bracarensis* and *C. nivariensis*. Figures represent the mean ± standard deviation of the retention time. Besides, in the non-denaturing column an estimate of the amplicon size in bp is included in brackets.

Microorganism	dHPLC retention time (min)	
	Non-denaturing (T° = 50° C)	Partially-denaturing (T° = 58 °C)
C. parapsilosis (*n* = 26)[a]	5.44 ± 0.04 (233 bp)	N.D.[d]
C. tropicalis (*n* = 15)[b]	5.53 ± 0.04 (248 bp)	N.D.[d]
C. albicans (*n* = 40)[a]	5.75 ± 0.03 (256 bp)	N.D.[d]
C. guilliermondii(*n* = 8)[b]	6.25 ± 0.12 (269 bp)	N.D.[d]
C. nivariensis(*n* = 2)[c]	6.75 ± 0.06 (335 bp)	2.14 ± 0.07
C. glabrata(*n* = 88)[a]	6.78 ± 0.04 (339 bp)	2.49 ± 0.05
C. bracarensis(*n* = 2)[c]	7.05 ± 0.05 (361 bp)	1.44 ± 0.04

[a] Reference strains and clinical isolates.
[b] Clinical isolates.
[c] Reference strains.
[d] Not done.

Table 3
Optimized conditions for the identification of *C. glabrata*, *C. bracarensis* and *C. nivariensis* using dHPLC method.

Temperature (58 °C)	Time (min)	Flow-rate (mL/min) (0.9)	
Gradient		% A	% B
Loading	0	48	52
Start gradient	0.5	43	57
Stop gradient	5	34	66
Start clean	5.1	48	52
Stop clean	5.6	48	52
Start equilibrate	5.7	48	52
Stop equilibrate	6.1	48	52

3.3. dHPLC assay validation

3.3.1. Comparison between non-denaturing dHLPC to other similar genotyping techniques

As mentioned in the methods subheading, we performed an additional validation step comparing the method combining real-time PCR and dHPLC to other discrimination techniques, such as agarose gel electrophoresis, capillary fragment analysis and microfluidic methods. All these techniques gave equivalent results and demonstrate their suitability to discriminate between *C. glabrata* complex and other clinically relevant species of *Candida*, but all of them failed in the discrimination between the three *Candida* close related species included in the *C. glabrata* complex. Only using the partially denaturing dHPLC we obtained a successful discrimination between them.

3.4. Selectivity

Selectivity is the ability of an analytical method to differentiate the analyte in the presence of other components in the sample. So, samples according to the ICH guidelines requirements were used to analyze the selectivity of the technique [48]. Following this methodology we did not obtain any interfering peaks as it can be shown in Fig. 2.

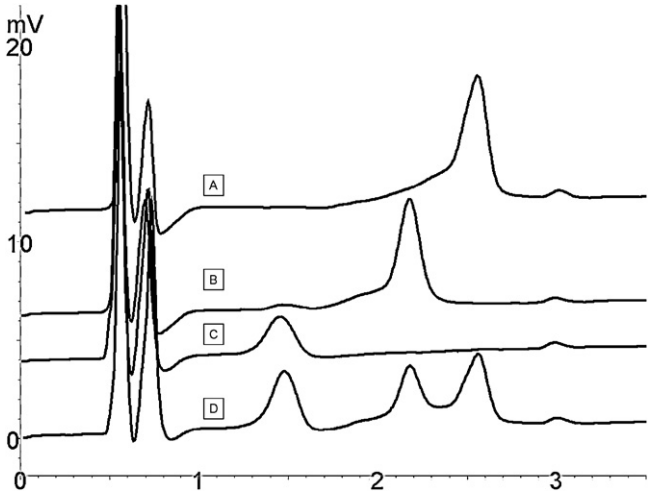

Fig. 1. dHPLC profile from a PCR product mixture of *C. glabrata* and close related reference strains in urine matrix and the individual reference strains used as marker for identification being the elution order *C. bracarensis* NCYC-3133, *C. nivariensis* 04.228 and *C. glabrata* 04.229, respectively. (A) *C. glabrata* 04.229 reference strain chromatographic peak, (B) *C. nivariensis* 04.228 reference strain chromatographic peak, (C) *C. bracarensis* NCYC-3133 reference strain chromatographic peak and (D) PCR product mixture of *C. bracarensis*, *C. nivariensis* and *C. glabrata* reference strains chromatographic peaks in elution order.

3.4.1. Linearity and lower limit of detection (LLOD)

We obtained from the calibration curves, determination and positive correlation coefficients values nearly to the unit value in each matrix considered. The lower limits of concentrations (LLOD) were calculated as mentioned on the assay validation subheading. The concentration values obtained for *C. glabrata*, *C. bracarensis* and *C. nivariensis* for each biological specimen are recorded in Table 4.

The results obtained for each species in each biological matrix were equivalent except for *C. bracarensis.* Assays of this species of *Candida* in water and urine matrices showed better sensibility. We found no reliable explanation to this phenomenon and further research is need in this field.

3.4.2. Precision and accuracy

Precision and accuracy were assessed spiking water, urine, blood and sputum with low (5 ng μL^{-1}), medium (15 ng μL^{-1}) and high (35 ng μL^{-1}) concentration of *Candida* species. The intra ($n = 6$) and inter-day ($n = 18$) precision and accuracy expressed as relative standard deviation (%RSD) and relative error (%RE), respectively, are summarized in Table 5. The values we obtained in our experiment were acceptable since the RSD and RE are lower than 20% and 15%, respectively which are the tolerable limits in accordance with FDA and ICH recommendations.

4. Discussion

The amplification using real-time PCR with SYBR Green considering a target located in the ITS2 region is not capable of making accurate identification of different species of *Candida*, except for *C. krusei* which has got a melting temperature higher than other *Candida* species. The simplest explanation to this, is the nearly overlapping melting curve profiles of the PCR products obtained using this methodology that prevented an accurate discrimination between the most common *Candida* species involved in the aetiology of candidemia and invasive candidiasis (see Table 1). As it was mentioned in other parts of this manuscript, the implementation of other identification methods is mandatory for accurate discrimination of different emerging species of *Candida*, especially when these could be close-related such as *C. bracarensis*, *C. glabrata* and *C. nivariensis*. Although there are many approaches trying to solve this problem such as HRM analysis, capillary fragment analysis, conventional PCR, etc. with similar results in discriminating the *C. glabrata* complex from other *Candida* species, not all of them are suitable for clinical purposes and adaptable to the daily overwork in the clinical laboratory.

Capillary electrophoresis and microfluidic analytical devices have already claimed their place in most molecular biology laboratories. In fact these techniques can be applied to speed up existing genotyping and mutation identification protocols using old techniques such as conventional gel electrophoresis, RFLP or others. Some of the advantages of these sorts of molecular biology techniques are the small sample volume requirement, fast processing, great accuracy, reliability and easy multiplexing, [49,50] but these techniques have two major disadvantages. First, they are not suitable for processing a small number of samples thus doing so, represent a waste of reagents and therefore increase the price of the technique. Second, even if they are suitable for accurate discrimination of *C. glabrata* complex from other common species of *Candida*; they fail in distinguishing using the same PCR amplicon, *C. glabrata* from its cryptic species *C. nivariensis* and *C. bracarensis*.

We used dHPLC of the PCR products obtained with real-time PCR technique as an easy, reliable and sensitive technique that showed to be a good analytical alternative not only for accurate identification of different *Candida* species (at non-denaturing conditions), but for accurate discrimination among cryptic species such as *C.*

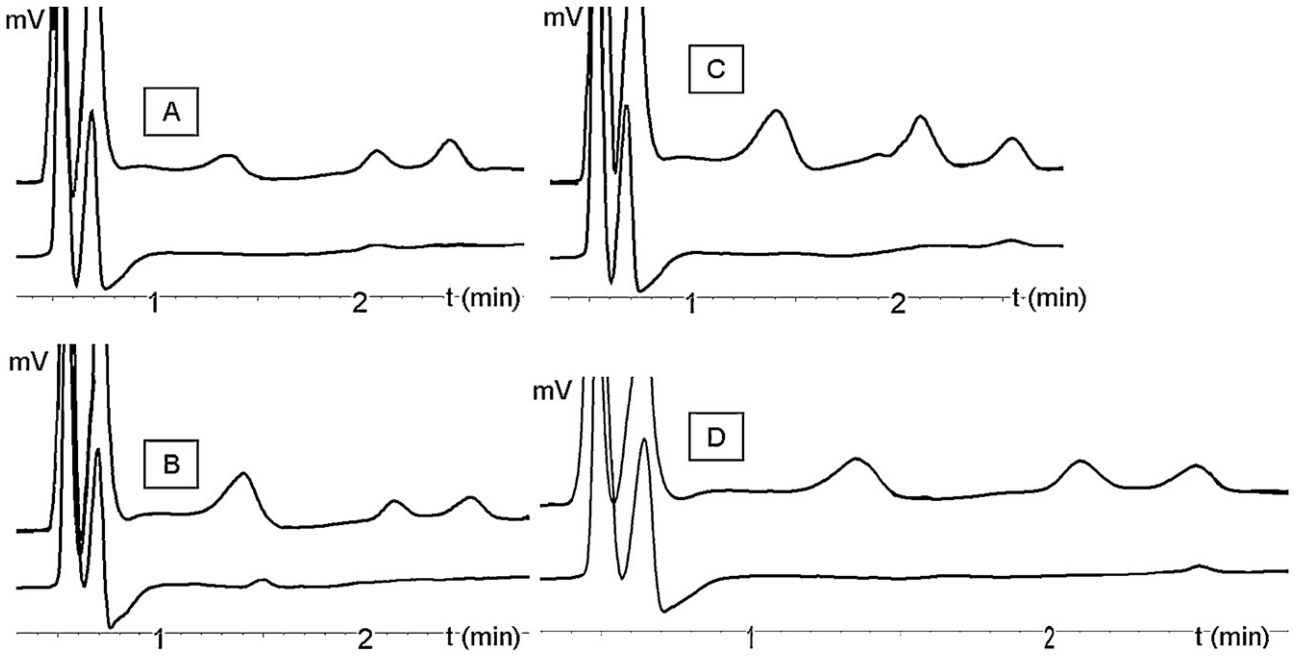

Fig. 2. Chromatograms obtained using different biological matrices. (A) Blood matrix, (B) urine matrix, (C) sputum matrix, (D) water matrix. Upper traces in each subfigure: chromatogram of spiked samples at LLOD with *C. bracarensis*, *C. nivariensis* and *C. glabrata*. Lower traces: chromatogram of blank samples. The peaks at the beginning of each trace represent the injection peak.

Table 4
Low limits of detection (LLOD) (ng μL^{-1}) obtained for the analytes studies in water, urine, blood and sputum.

Microorganism	Water	Urine	Blood	Sputum
	LLOD (ng μL^{-1})	LLOD (ng μL^{-1})	LLOD (ng μL^{-1})	LLOD (ng μL^{-1})
C. bracarensis	5.16 ± 0.58	5.56 ± 0.80	8.13 ± 0.90	9.56 ± 1.04
C. nivariensis	4.70 ± 0.49	4.14 ± 0.44	4.59 ± 0.78	4.52 ± 0.47
C. glabrata	4.02 ± 0.42	4.66 ± 0.49	3.99 ± 0.42	4.66 ± 0.50

Table 5
Intra-day ($n = 6$) (above) and inter-day ($n = 18$) (under) precision (RSD %) and accuracy (RE %) values at the LLOD, medium (15 ng μL^{-1}) and high (35 ng μL^{-1}) concentration levels.

	LLOD (ng μL^{-1}) Intra-day				Medium (ng μL^{-1}) Intra-day				High (ng μL^{-1}) Intra-day			
	Water	Urine	Blood	sputum	Water	Urine	Blood	sputum	Water	Urine	Blood	sputum
RSD %												
C. bracarensis	1.56	11.03	9.37	2.54	0.85	3.66	5.19	2.08	0.52	1.39	1.59	0.41
C. nivariensis	1.16	5.28	5.31	5.47	0.85	1.37	2.60	1.74	0.39	2.53	0.46	0.07
C. glabrata	1.37	1.21	5.08	1.93	1.08	0.99	2.02	1.83	0.94	1.38	0.97	0.63
RE %												
C. bracarensis	3.83	10.33	4.95	7.51	2.28	7.86	8.50	3.87	1.04	2.31	3.02	2.87
C. nivariensis	7.29	6.53	9.11	8.24	4.15	2.29	2.91	5.73	1.59	3.07	1.43	1.26
C. glabrata	6.19	9.02	8.95	9.45	4.25	2.14	5.46	1.24	1.18	1.65	1.92	1.26
	LLOD (ng μL^{-1}) Inter-day				Medium (ng μL^{-1}) Inter-day				High (ng μL^{-1}) Inter-day			
	Water	Urine	Blood	sputum	Water	Urine	Blood	sputum	Water	Urine	Blood	sputum
RSD %												
C. bracarensis	5.60	11.20	9.31	4.65	0.90	2.90	6.05	3.50	2.32	4.92	3.13	4.99
C. nivariensis	4.32	5.91	3.82	4.07	4.07	1.69	2.54	4.27	0.83	2.39	1.22	1.55
C. glabrata	7.32	6.88	5.49	6.26	3.50	1.61	4.56	1.21	2.53	1.08	0.99	0.70
RE %												
C. bracarensis	4.63	14.44	9.80	4.37	0.75	2.25	4.72	2.74	1.63	3.33	2.87	3.96
C. nivariensis	3.41	4.94	3.66	3.54	3.42	1.28	2.44	5.75	0.71	1.80	0.86	1.23
C. glabrata	6.82	4.82	5.84	7.63	3.83	1.13	4.30	0.89	1.99	1.11	1.28	0.68

bracarensis and *C. nivariensis* which belong to the *C. glabrata* cryptic species complex (at partially denaturing conditions), maintaining the same advantages of capillary electrophoresis and microfluidic techniques, but also suitable for processing a small number of samples at a reasonable price.

This approaching scheme has the advantage that is very simple, cheap, and relatively easy to perform (once it is optimized). Other approaches described in the literature by Romeo et al. using multiplex PCR [29] is less sensitive as it uses conventional PCR or more complex ones such as the Septifast® commercial assay (Roche Diagnostics Limited, Burgess Hill, U.K.) using real-time PCR with HybProbes® are very expensive, quite time consuming and have not been validated for other biological samples except for whole blood.

We obtained no interfering peaks when testing for the system selectivity. This indicates the high selectivity of the method, and could be of interest in complex samples which could be contaminated with other spurious flora.

Revising the existing literature, there are few clinical oriented articles in this field. Goldenberg et al. used dHPLC for unequivocal identification of seven *Candida* species that are frequently isolated from blood cultures [3]. Their work revealed that dHPLC allowed to distinguish between many different *Candida* species analysing the peak profile of mixed fungal PCR amplified ITS2 DNA fragments with no overlapping of them. This state of art finding enhances our results and makes improbable to obtain interfering peaks even under denaturing conditions where size dependency is lost. Before this last step in the processing of the sample, the specificity and accuracy of the real-time PCR and non-denaturing dHPLC steps in the identification of the *C. glabrata* complex assures that misidentification at the denaturing step is improbable. Besides, recent candidemia surveillance studies conducted in Europe show that *C. albicans, C. parapsilosis* complex, *C. glabrata* complex, *C. tropicalis* and *C. krusei* represent more than 95% of the isolates involved in a bloodstream *Candida* infection. As we mentioned before, accurate differentiation of *C. glabrata* complex from these other species can be done even using old techniques such as conventional PCR using gel electrophoresis, thus misidentification using dHPLC at non-denaturing and denaturing steps is unreliable.

The correlation coefficients and determination coefficient values ($R^2 \geq 0.99$) estimated from the calibration curves, demonstrate the good linearity and strong correlation between the chromatographic peak area of interest vs. the analyte concentration for all the different matrices and analytes used.

The LLOD concentrations obtained for each matrix analyzed was nearly the same regarding the matrix and the *Candida* species used for the experiment, except for *C. bracarensis* where we obtained better sensibility when using the water and urine matrices compared to more complex matrices such as blood and sputum. Though there is no fulfil answer to these results after revising the literature, one plausible hypothesis to explain this question could be the relative amount of target and non-target DNA in each matrix as in the complex ones (blood and sputum) we have nearly doubled the LLOD compared to the simple ones (urine and water).

Although our results obtained for *C. bracarensis* confirm the hypothesis mentioned above, the ones obtained for *C. glabrata* and *C. nivariensis* did not. So something else besides the complexity of the biological matrix of the sample is interfering in the LLOD of the technique. Our preliminary results show that the *Candida* species could probably have a role in the explanation of this phenomenon and further research on this field is needed. We could assume that maybe high related species such as *C. glabrata* and *C. nivariensis* would have similar LLOD and more distanced ones such as *C. bracarensis* would have a different behaviour.

Summarising, this is the first experiment to our knowledge that validates the use of dHPLC for accurate identification of the cryptic species included in the *C. glabrata* complex and estimates different accuracy and precision parameters. Besides it points the necessity of further research on the field to explain some different behaviour of the proposed technique when using different *Candida* species.

5. Conclusion

This work shows that PCR amplification of the ITS2 region followed by dHPLC have been successfully applied using different biological matrices for simultaneous identification analysis of *Candida* species using non-denaturing condition, and the rapid and accuracy discrimination of *C. glabrata* from its cryptic species, *C. bracarensis* and *C. nivariensis*, using denaturing conditions. Therefore the combination of the techniques described above seems to be promising in the routine identification analysis of phylogenetically close-related *Candida* species.

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