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Usefulness of a multiplex PCR for the rapid identification of *Candida glabrata* species complex in Mexican clinical isolates

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ABSTRACT

Candida glabrata complex includes three species identified through molecular biology methods: *C. glabrata sensu stricto*, *C. nivariensis* and *C. bracarensis*. In Mexico, the phenotypic methods are still used in the diagnosis; therefore, the presence of *C. nivariensis* and *C. bracarensis* among clinical isolates is still unknown. The aim of this study was to evaluate the utility of a multiplex PCR for the identification of the *C. glabrata* species complex. DNA samples from 92 clinical isolates that were previously identified through phenotypic characteristics as *C. glabrata* were amplified by four oligonucleotides (UNI-5.8S, GLA-f, BRA-f, and NIV-f) that generate amplicons of 397, 293 and 223-bp corresponding to *C. glabrata sensu stricto*, *C. nivariensis*, and *C. bracarensis*, respectively. The amplicon sequences were used to perform a phylogenetic analysis through the Maximum Likelihood method (MEGA6), including strains and reference sequences of species belonging to *C. glabrata* complex. In addition, recombination and linkage disequilibrium were estimated (DnaSP version 5.0) for *C. glabrata sensu stricto* isolates. Eighty-eight isolates generated a 397-bp fragment and only in one isolate a 223-bp amplicon was observed. In the phylogenetic tree, the sequences of 397-bp were grouped with *C. glabrata* reference sequences, and the sequence of 223-bp was grouped with *C. bracarensis* reference sequences, corroborating the PCR identification. The number of recombination events for the isolates of *C. glabrata sensu stricto* was zero, suggesting a clonal population structure. Three isolates that did not amplify any of the expected fragments were identified as *Saccharomyces cerevisiae* through the sequencing of the D1/D2 domain region within the 28S rDNA gene. The multiplex PCR is a fast, cost-effective and reliable tool that can be used in clinical laboratories to identify *C. glabrata* complex species.

KEYWORDS: *Candida glabrata* complex. *Candida bracarensis*. *Candida nivariensis*. Multiplex PCR. Candidiasis. Mexico.

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INTRODUCTION

Candidiasis is the most frequent opportunistic mycosis in the world. *Candida albicans* is the main agent of candidiasis, but *Candida glabrata* has emerged as the second most important agent of invasive candidiasis in Central and Northern Europe and in the United States; however, in some Asian, African and Latin American countries, it ranks third or fourth place[1]. In Mexico, the etiologic diversity of invasive candidiasis is similar to that reported in other countries, where *C. albicans* is followed by the *C. glabrata* complex, *C. krusei*, *C. tropicalis* and the *C. parapsilosis* complex causes more than 90% of cases, whereas

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C. guilliermondii, *C. lipolytica*, *C. famata, C. zeylanoides*, *C. utilis*, *C. rugosa* and *C. boidinii* are infrequent agents[2,3]. The clinical relevance of the emergence of the *C. glabrata* complex as a frequent pathogen lies in the fact that this complex includes three species: *C. glabrata sensu stricto*, *Candida bracarensis* and *Candida nivariensis*[4-6], which can cause similar clinical manifestations although they are genetically diverse and differ regarding virulence factors and susceptibility to antifungals[3,7-10]. Therefore, the correct identification of the species of *C. glabrata* complex can lead to the appropriate choice of antifungal therapy. The identification of these species is not easy using phenotypic methods; thus, molecular tests have been developed[11-18]. Among these tests, amplification and sequencing of the internal transcribed spacer (ITS) and the D1/D2 domain of the large subunit rRNA gene regions stand out[4,18-22], as well as the amplification of gene fragments with specific oligonucleotides that allow an easy differentiation of species based on the amplicon molecular weight[23,24]. However, in many clinical laboratories, particularly in Latin America, molecular tests are still not used due to their costs as well as their methodological complexities, which has led to the erroneous identification of *C. nivariensis* and *C. bracarensis* as *C. glabrata* among clinical isolates.

The aim of this work was to evaluate the usefulness of a multiplex PCR assay (targeting the ITS1 region and the 5.8S ribosomal RNA gene) for the identification of the species of *C. glabrata complex* and to confirm the multiplex PCR results through a phylogenetic analysis.

MATERIAL AND METHODS

Clinical isolates and culture conditions

Ninety-two clinical isolates of the *C. glabrata* complex (Cgl-1 to Cgl-92) were included in this study. The isolates were obtained from vaginal exudates, blood samples, biopsies, semen samples, abscesses, peritoneal dialysis fluid and catheter tips collected in two tertiary hospitals (1 and 2) located in Mexico City, during the period of January-June 2017. Sixty-five isolates (Cgl-1 to Cgl-65) were obtained from hospital 1, and twenty-seven (Cgl-66 to Cgl-92) from hospital 2. Each isolate was recovered from a single patient. All isolates were previously identified by the automated system VITEK® 2 Compact (bioMeriux, France) as *C. glabrata*, with 93-98% probability of identification. *C. glabrata* ATCC® 2001™, *C. bracarensis* Cb-1 and ExV75 were included as reference strains. The Cb-1 strain was identified by proteomics through the MALDI-TOF MS spectrometry (score value 2.1), and was kindely provided by *Dr. Rosa Areli Martínez Gamboa, Instituto Nacional de Ciencias Médicas y de la Nutrición "Salvador Zubirán"*, Mexico City. The ExV75 strain was identified by sequencing the ITS region and reported by Trevino-Rangel *et al.*[25].

Isolates were grown in Sabouraud agar containing chloramphenicol, at 28 °C. Yeasts were kept at room temperature in a saline solution (NaCl 0.8%).

Extraction of genomic DNA

Yeasts were cultured in YEPG medium (1% yeast extract, 2% peptone, 2% glucose), cells were harvested by centrifugation and washed three times with sterile distilled water. DNA from the yeasts was extracted and purified using the Yeast DNA Preparation kit (Jena Bioscience, GE), following the manufacturer's instructions. DNA samples were stored at 4 °C.

PCR

DNA from the yeasts was amplified through multiplex PCR, using the primers designed by Romeo *et al.*[23]. The reaction mixture (25 µL) contained: 1X PCR buffer, 100 pmol/µL of each oligonucleotide (UNI-5.8S 5'-ACCAGAGGGCGCAATGTG-3', GLA-f 5'-CGGTTG GTGGGTGTTCTGC-3', BRA-f 5'-GGGACGGTAAG TCTCCCG-3', NIV-f 5'-AGGGAGGAGTTTGTATCT TTCAAC-3') (Sigma-Aldrich, USA), 200 µM of each dNTP (Jena Bioscience), 2.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Jena Bioscience), and 10 ng of genomic DNA. As a negative control, sterile Milli-Q® water (Merck Millipore, USA) was included. Reactions were carried out in a thermocycler T100™ (Bio-Rad, Laboratories, Inc., USA).

The PCR cycles parameters were as follows: preheating at 95 ° C for 5 min; then 34 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 50 s, and a final extension at 72 °C for 10 min. Amplicons were analyzed by electrophoresis in 1.7% agarose gels (Pronadisa, ES) stained with 3X GelRed™ (Biotium, USA). The electrophoresis was performed in TBE (45 mM Tris-Base, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 100 V for approximately 45 min. A 100-bp DNA molecular size marker was used (Jena Bioscience). Amplified DNA fragments were visualized in a UV transilluminator and then documented (Gel DocTM EZ Documentation System, Bio-Rad Laboratories). The expected molecular weights of the amplification products were 397, 293 and 223-bp for *C. glabrata sensu stricto*, *C. nivariensis*, and *C. bracarensis*, respectively. All amplicons were sequenced in both directions at the Genomic Services Unit in LANGEBIO (CINVESTAV, Mexico).

Sequences analysis

All DNA sequences from the isolates were edited with the program BioEdit ver. 7.2.5.[26]. Subsequently, they were analyzed with the BLAST (Basic Local Alignment Search Tool) program[27] available at www.blast.ncbi.nlm.nih.gov/blast.cgito verify the identity, similarity and "e-value" between sequences.

Phylogenetic analysis

All sequences were aligned using the Muscle algorithm within MEGA6 with default options[28]. The statistical method of Maximum Likelihood was used, a phylogeny test that includes the bootstrap method with 1,000 pseudo replicates; then, replicates were analyzed with the MEGA6 program[28]. As a reference, sequences of *C. glabrata* complex deposited in GenBank (*C. bracarensis* AY589573.2 and MF187327.1, *C. nivariensis* AY620957.1, *C. glabrata* AY198398.1)[23] were included for comparisons. A sequence of *C. albicans* (JN882343.1) was used as an outgroup.

Recombination detection and linkage disequilibrium

The recombination and linkage disequilibrium was calculated (DnaSP version 5.0)[29]. The ZZ statistic[30] was used to verify the effect of intragenic recombination on nucleotide variation by analyzing the level of linkage disequilibrium.

RESULTS

PCR

From the total of 92 isolates, 88 amplified a 397-bp fragment, compatible with *C. glabrata sensu stricto*; whereas only one (Cgl-60), showed a 223-bp amplicon, suggestive of *C. bracarensis.* The reference strains (ATCC® 2001™, Cb-1 and ExV75) amplified the expected fragments (Figure 1). None of the isolates amplified a 293-bp fragment, corresponding to *C. nivariensis*.

Sequence analysis

The 397-bp sequences (accession no: MK583352 – MK583407 and MK583409 – MK583440) analyzed by BLAST revealed an identity and similarity of 100% ("e-value" equal to zero) with partial sequences of the ITS1-5.8S-ITS2 region from *C. glabrata* (gb|MK026347.1, gb|MH699024.1, gb|LC389240.1, gb|LS398123.1, gb|MG599239.1, gb|MH016268.1, gb|MF033154.1, gb|LC317501.1).

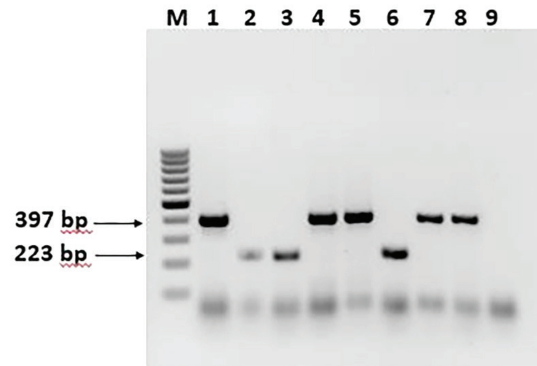

Figure 1 - Electrophoresis of the multiplex-PCR for the identification of clinical isolates of the *Candida glabrata* complex. M: molecular weight marker 100-bp; lane 1: *Candida glabrata* ATCC® 2001™ strain; lane 2: *Candida bracarensis* Cb-1 strain; lane 3: *Candida bracarensis* ExV75 strain; lane 4: isolate Cgl-59; lane 5: isolate Cgl-61; lane 6: isolate Cgl-63; lane 7: isolate Cgl-90; lane 8: isolate Cgl-92; lane 9: negative control (sterile water instead of DNA).

The 223-pb sequence analyzed by BLAST (from the isolate Cgl-60, accession no: MK583408) showed 100% identity and similarity ("e-value" equal to $2e^{-111}$) with partial sequences of the ITS1-5.8S-ITS2 region from *C. bracarensis* (gb|JN882340.1, gb|GU199439.1, gb|GU199438.1, gb|NR136973.1, gb|MF084287.1, gb|MF187327.1, gb|KP674715.1, gb|KP131680.1).

Three isolates (Cgl-2, Cgl-28, and Cgl-32) showed no amplification even after the testing of different DNA concentrations. Therefore, a region of the D1/D2 domain of the 28S rDNA gene was amplified and sequenced with the universal oligonucleotides NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3')[31]. A 588-bp amplicon was obtained from the three isolates. The BLAST analysis of these sequences showed 100% identity and similarity with the sequence of *Saccharomyces cerevisiae* gb|LC334458.1, corresponding to the 28S ribosomal RNA region.

Phylogenetic analysis

The phylogenetic tree showed three groups supported by a 99% bootstrap: group I included all isolates that amplified the 397-bp fragment and reference sequences of *C. glabrata* distributed in three subgroups. The subgroup Ia included the majority of isolates from hospital 1; the subgroup Ib included most isolates from hospital 2and the subgroup Ic included three isolates from hospital 1. The group II included the reference sequence (AY620957.1) of *C. nivariensis* and group III included that single isolate (Cgl-60), as well as the reference sequences (MF187327.1, AY589573.2, Cb-1 and ExV75) corresponding to *C. bracariensis* (Figure 2).

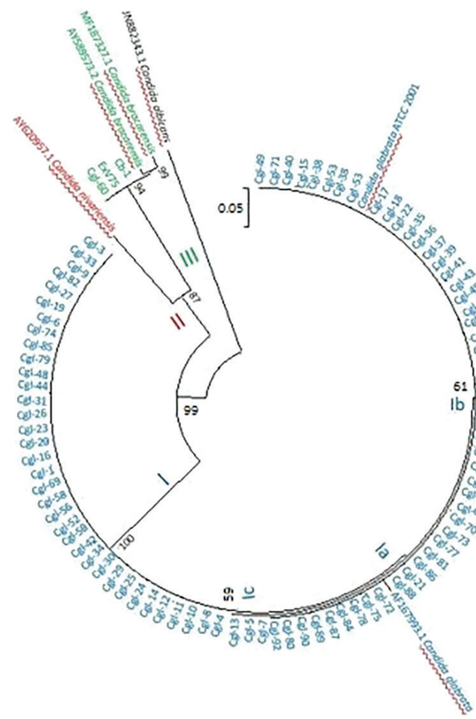

Figure 2 - Maximum Likelihood (ML) tree obtained from the ITS1 region-5.8S ribosomal RNA gene sequences of the *Candida glabrata* clinical isolates. The branch support was inferred by a non-parametric bootstrap for ML analysis. As the outgroup, a sequence of *C. albicans* (JN882343.1) was included.

Recombination detection and linkage disequilibrium

The ZZ value for *C. glabrata sensu stricto* isolates from hospital 1 was 0.0308 (P<0.001) and 0.0000 for the ones coming from hospital 2, considered statistically significant after the Bonferroni procedure; meanwhile, the minimum number of recombination events Rm was 0 for both hospitals. These results suggest that the isolates have a clonal mode of reproduction.

DISCUSSION

Although *C. albicans* remains the most isolated species, *C. glabrata* has emerged as a common agent of superficial and invasive candidiasis[3]. From the report of *C. bracarensis* and *C. nivariensis* as closely related species to *C. glabrata*, there have been several molecular studies in the United States, Europe and Asia, in which the existence of *C. bracarensis* and *C. nivariensis* has been reported. In addition, these species are potentially resistant to fluconazole, and have been wrongly identified as *C. glabrata* through conventional methods[7,24,32-36]. In Latin America, the presence of *C. bracarensis* and *C. nivariensis* is little known[10,37] due to the fact that the identification of *Candida* spp. is carried out on a routine basis associated with phenotypic methods, such as API ID32C, API20C AUX V5.0 (bioMerieux), or VITEK® 2 Compact[24,25,36]. However, these methods fail to differentiate among species belonging to *C. glabrata*[33] complex, thus the use of molecular methods is required. Although many molecular markers have been reported for the identification of species within *C. glabrata* complex, they have not yet been implemented in clinical laboratories due to their complexity, high cost of dedicated equipment, or because they have not been validated. We report in this study the usefulness of a multiplex PCR assay, which allowed us to distinguish *C. glabrata, C. bracarensis* and *C. nivariensis* using four oligonucleotides directed to the ITS1-5.8S ribosomal RNA region[23].

From the 92 studied isolates that were identified as *C. glabrata* through the VITEK® 2 Compact system, 95.6% confirmed the identification as *C. glabrata sensu stricto*, based on the amplicon size, the high percentage of identity and similarity revealed in the BLAST analysis, as well as their grouping in the phylogenetic tree, where they were clearly related to a reference sequence of *C. glabrata,* with a suitable bootstrap value. Likewise, the 99% identity and similarity of the 223-bp fragment and its grouping to the reference sequences of *C. bracarensis,* showed that the isolated Cgl-60 corresponds to *C. bracarensis*. This isolate was obtained from a vaginal exudate of a symptomatic woman, coinciding with the site of isolation in other reports[2,35], although it is important to mention that *C. bracarensis* has also been isolated from stool samples, sputum, blood and urine samples[32,37,38]. In the same way, other studies failed to find *C. nivariensis* probably because this is the less frequent species of the *C. glabrata* complex[7,24,34,39].

The BLAST analysis of the sequences of the D1/D2 region of the 28S rDNA gene obtained from the Cgl-2, Cgl-28 and Cgl-32 isolates that were not amplified by the multiplex PCR demonstrated that they do not correspond to the *C. glabrata* complex but to *S. cerevisiae*. These results highlight two important aspects: 1) the specificity of the multiplex PCR, as it managed to differentiate between two phylogenetically related yeasts (*C. glabrata* complex and *S. cerevisiae*); 2) although the vaginal infections caused by *S. cerevisiae* are rare, they must be taken into account in the differential diagnosis, particularly in women with risk factors for infection (previous treatment with corticosteroids or broad-spectrum antibiotics, and high-level exposure to an environmental source of *S. cerevisiae*)[40]. The isolates Cgl-2, Cgl-28, and Cgl-32 were obtained from women subjected to prolonged treatment with broad-spectrum antibiotics.

The findings of this study showed that four isolates (Cgl-2, Cgl-28, Cgl-32, and Cgl-60) had been misidentified as *C. glabrata* with a high level of probability (98-100%)

because the VITEK system® 2 Compact is not the most suitable method for the identification of *C. glabrata*[36,41]. The phylogeny constructed with the sequences from the isolates confirmed the identification by PCR; the clustering corroborated that 88 isolates corresponded to *C. glabrata sensu stricto*, and one to *C. bracarensis*. Furthermore, the data obtained from the linkage disequilibrium analysis and the recombination of *C. glabrata* isolates from hospitals 1 and 2 suggested the existence of a clonal population structure as shown by Lott *et al.*[42]. These results indicate the permanence and dissemination of one clone, which could represent an advantage since some researchers have speculated that the predominance of one genotype may have selective ecological benefits such as the decrease of virulent strains and of resistance to antifungal agents[43].

Based on the results obtained in this study, we recommend the multiplex PCR proposed by Romeo *et al*.[23] as a cost-effective, simple, fast and reliable tool for the identification of clinical isolates of the *C. glabrata* species complex. Although it is important to note that the cultivation of yeasts is still necessary to determine the susceptibility to antifungals, the combination of multiplex PCR and culture will contribute to a specific diagnosis, as well as to the understanding of the epidemiology and pathogenic relevance of these yeasts.

CONCLUSION

The multiplex PCR with oligonucleotides UNI-5.8S, GLA-f, BRA-f and NIV-f is an accessible, reliable and fast tool that can be used routinely in clinical laboratories to identify the species of the *C. glabrata* complex.

CONFLICT OF INTERESTS

The authors have declared that no competing interests exist.

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