

Identification of *Candida* species causing Vaginal Candidiasis Using Multiplex PCR

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ABSTRACT

Aims: To determine the sensitivity and specificity of a multiplex PCR assay for the identification of major species of candida involved in vaginal candidiasis, and evaluation of this method in comparison with routine phenotypic culture identification.

Methods: 100 isolates of candida from vaginal swabs were collected. Research and identification of *Candida* spp, with routine phenotypic culture identification (germ-tube test in serum at 37°C for 3 hours), were performed. Each sample was analyzed with multiplex PCR. Samples given discrepant results between routine phenotypic and PCR identification methods were resubcultured on CHROMagar *Candida* plates. The fungus-specific primers ITS1, ITS2, CA3, and CA4 were used. For the identification of other species (*C kefyr*, *C famata* and *C dubliniensis*), ITS1F, ITS1K, and ITS2D primers were used.

Results: Multiplex PCR correctly identified all samples, including those with single species, or with mixed species.

Conclusion: This multiplex PCR assay provides a good method to confirm the conventional culture based technique for the identification and speciation of the most frequently isolated *Candida* species.

Key words: *Candida Albicans*; multiplex PCR; Vaginal Candidiasis

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INTRODUCTION & RATIONALE

Candida albicans is both a ubiquitous commensal and an opportunistic human pathogen capable of causing mucosal and systemic disease in immune compromised individuals. *Candida albicans* is a commensal organism which is a common inhabitant of the mucosal membranes of humans⁽¹⁾. However, when the balance of the normal flora is altered, during antibiotic or hormonal therapy, *C. albicans* can cause uncomfortable mucosal infections such as, vaginitis, oral thrush, diaper rash, conjunctivitis and nail bed infections. In immune compromised individuals, such as HIV patients, cancer chemotherapy patients, transplant recipients etc., *C.albicans* can become a serious problem causing life threatening disseminated infections⁽²⁾.

Oropharyngeal and vaginal candidiasis are most commonly observed in immune competent people with predisposing factors such as antibiotic, glucocorticosteroid, and hormone therapies, whereas life threatening systemic infections is limited to severely immune compromised patients⁽³⁾.

The conventional identification of pathogenic fungi in the clinical microbiology laboratory involves the examination of colony and microscopic morphologies and the assessment of various biochemical reactions^(4,5). It often requires three or more days, and may be inaccurate. Moreover, the presence of more than one *Candida* species in the vagina of the same host is not infrequent.

In recent years, numerous DNA-based methods such as DNA–DNA reassociation⁽⁶⁾, DNA fingerprinting⁽⁷⁾, and Southern hybridization with appropriate DNA probes^(8,9) have been reported to recognize *Candida* species in culture or in clinical materials. However, these genotypic methods have the disadvantage of being laborious and time-consuming, and also require specialized equipment.

The aims of the present study were to determine the sensitivity and specificity of a multiplex PCR method applied to vaginal *candida* isolates obtained in our laboratory, for the identification of *Candida* species that are frequently isolated from the vagina swabs and to evaluate this method in comparison with routine phenotypic culture identification. The method is based on the size variability of the ITS1 region in different species and on the amplification of a specific DNA fragment of the ITS2 region of *C albicans*. The test has allowed us to identify *Candida* species using seven species-specific oligonucleotides in a single PCR tube.

Drawing from this strategy we studied the structure of other species of *Candida* and generated in the same region other primers that were able to amplify specific fragments. Indeed for the identification of other three species, *C kefyri*, *C famata* and *C. dubliniensis* we designed the following primers ITS1F (5-CCAGCG CTT AAT TGC G-3), ITS1K (5-ATC GTC TGA ACA AGGCCT GC-3), ITS2D (5-GAG AAC CAA GAG ATC CGT TGTTG-3).

MATERIALS AND METHODS

A total of 100 isolates of *Candida* were collected from vaginal swabs of females suffering from vaginal candidiasis.

All positive cultures, *Candida* genus yeasts were identified presumptively (Germ-tube test in serum at 37°C for 3 hours)⁽¹⁰⁾ and definitively with API 20C AUX (bioMérieux, France) sugar fermentation test^(11,12). Each isolate was analyzed with multiplex PCR⁽¹³⁾. Samples giving discrepant results between routine phenotypic and PCR identification methods were resubcultured on CHROMagar *Candida* (Hi Media Laboratories, India) plates to identify possible mixed yeast culture^(14,15).

The fungus-specific primers ITS1 (5-TCC GTA GGT GAA CCTGCG G-3) and ITS2 (5-GCT GCG TTC TTC ATC GAT CG-39)⁽¹⁶⁾ were used to amplify a small conserved portion of the 18S rDNA region, the adjacent ITS1, and a small portion of the 28S rDNA region, generating PCR products for *C. glabrata*, *C. guilliermondii*, *C. lusitanae*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. In addition, *C. albicans*-specific primers CA3 (59-GGT TTG GAAAGA CGG TAG-39) and CA4 (59-AGT TTG AAG ATA TAC GTGGTA G-39)⁽¹⁷⁾ were also included in the PCR mixture to amplify a portion of the ITS2 region of *C. albicans*.

Multiplex PCR was performed in duplicate with PCR reaction was performed in a 50 µl volume containing 20 µl of vaginal swab broth soln, 5X PCR buffer (Promega, USA), 2.5mM MgCl₂ (Promega, USA), 630 µM PCR nucleotide Mix - 10 mM each (Promega, USA), 2µl from each 7 primers, 1µl Go Tag DNA Polymerase (Promega, USA). The volume for each PCR reaction was completed to 50µl with nuclease free water (Promega, USA). PCR was carried out with a thermal Cycler (MyCycler, BIO-RAD) under the following condition: initial denaturation, 92°C, 2 min; 35 cycles of denaturation (95°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 1 min); and final extension, 72°C, 10 min⁽¹³⁾. A negative control run was performed with each test run by replacing the samples with sterilized water in the PCR mixture. A positive culture broth containing *C. albicans* was run in parallel with unknown samples, and this culture broth was used as a positive control. Gel electrophoresis was conducted in TBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA, pH 8.4) at 100 V for 1–2 h using gel composed of 2% agarose gel (Bioshop, Canada) containing ethidium bromide (Sigma, USA), and UV visualization were performed according to the protocols provided (G: box, SYNGENE, Cambridge, England). The length of the bands was measured by UVIssoft software.

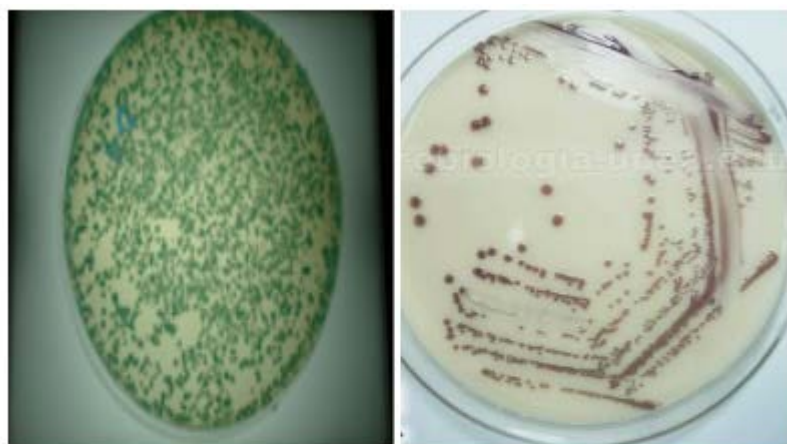
Table 1 lists these primers and the generated PCR products

Organism	ITS1	ITS1F	ITS1K	ITS2	ITS2D	CA3	CA4	PCR product (bp)
<i>C glabrata</i>	+	-	-	+	+	-	-	482–483/462–463
<i>C guilliermondii</i>	+	-	-	+	+	-	-	248/228
<i>C famata</i>	-	+	-	+	+	-	-	234/214
<i>C kefyri</i>	-	-	+	+	+	-	-	249/229
<i>C parapsilosis</i>	+	-	-	+	+	-	-	229/209
<i>C tropicalis</i>	+	-	-	+	+	-	-	218/199
<i>C albicans</i>	+	-	-	+	+	+	+	218–219/198–199/110
<i>C krusei</i>	+	-	-	+	+	-	-	182/166
<i>C lusitanae</i>	+	-	-	+	+	-	-	148/128
<i>C dubliniensis</i>	+	-	-	-	+	-	-	198

RESULTS

A total of 100 positive vaginal swabs were analyzed by routine phenotypic methods for species identification. The most frequently isolated species was *C albicans* (93 isolates, 93%). In CHROMagar *Candida* 98 isolates (98%) give green pigmentation and identified as *C albicans*, in the other hand 2 isolates (2%) give pink colonies and identified as *C glabrata* (figure 1). API identified as 98 isolates (98%) were *C albicans* and 2 isolates (2%) were *C glabrata*.

Figure 1 *Candida* sp on CHROMagar.



Candida albicans
Green color colonies on CHROMagar

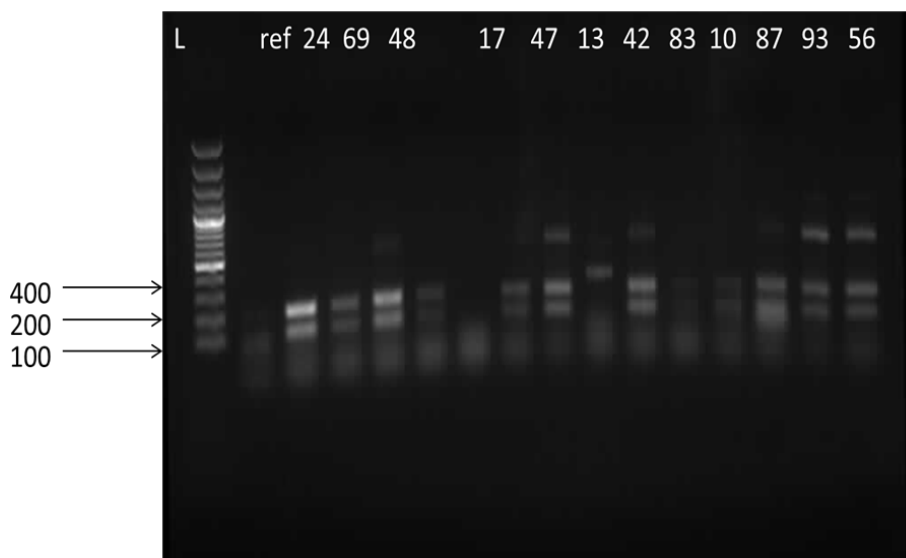
Candida glabrata
violet color colonies on CHROM agar

Multiplex PCR identification results matched phenotypic identification results for 100 positive samples, 98 samples containing *C albicans* (98%), and 2 samples containing *C glabrata* (2%).

The time from the first vaginal swabs rinse culture to species identification by routine phenotypic methods (subculture, germ tube and API 20CAUX) was 4 days (96 h). More slowly growing, individual isolates took as long as 6 days for growth and species identification by routine phenotypic methods (144 h, *C guilliermondii* isolate). For the multiplex PCR, on the other hand, no time was required to isolate and extract DNA from vaginal swabs rinse, 3 h was required for PCR amplification and 1.5 h was required for agarose gel electrophoresis analysis. Therefore, species could be identified in as little as 5 h, including the time required to prepare PCR reagent mixture, in contrast to routine phenotypic methods which took several days. The mycotic and bacterial colony count obtained from the vaginal rinse technique used for a quantitative study ranged from 10 CFU/ml to 5.466106 CFU/ml for the mycotic count and from 9.86105 CFU/ml to 15.36107 CFU/ml for the bacterial colony count. The limit of detection of the multiplex PCR was approximately 10 CFU/ml and was very close to that reported by Jaeger et al. (18).

The high quantity of bacterial colony count did not interfere with the detection and identification of yeasts with multiplex PCR. In fact, coexisting bacteria in vaginal swab rinse solution specimens did not produce any detectable PCR products. Therefore, the multiplex PCR method could also detect mixed yeast cultures missed by routine subculturing methods, even when the vaginal rinse solution contained bacteria (figure 2).

Figure 2: multiplex PCR.



L= LADDER 100 bp / ref= refrance

Candida albicans show bands at (218-219),(198_199),(110)

Candida glabrata show bands at (482-483),(462-463)

DISCUSSION

In order to improve the quality of our diagnostic research, we have set up a multiplex PCR assay for the identification of major species of *candida* involved in vaginal candidiasis to avoid any mistake in treatment and time consuming.

To the best of our knowledge this is the first application of a PCR assay on vaginal swab broth solutions obtained directly from the samples without DNA extraction.

The advantages of this method are as follows: it does not require use of expensive or toxic chemical substances such as proteinase K or phenol-chloroform, the total time from species identification is 5 h, compared to a mean of 5 days by routine phenotypic culture identification methods; identification of more than one species of yeast is possible in mixed cultures, with no cross-reaction or interference with bacteria and viruses likely to be present in vaginal rinse solutions; and no modifications or changes in routine practice were necessary for the clinicians or the laboratory technicians, who were able to use the same samples used for conventional morphological and metabolic examination for the PCR assay.

Finally, considering the continuous decrease of the cost of purchasing a thermal cycler and the reagents necessary to use it, the estimated cost for this assay is similar to that of routine phenotypic culture identification.

In our experience, at least 2 species of *Candida* are frequently isolated from vaginal swabs rinse solutions in laboratories: *C. glabrata*. In order to be able to detect the most common *Candida* species, we have found in the literature primers to detect *C albicans*, *C glabrata*, *C guilliermondii*, *Clusitaniae*, *C parapsilosis*, *C tropicalis* and *C krusei*⁽¹⁹⁾. Moreover, in order to increase the number of primers to include all the clinically important species of *Candida*, drawing from data in the literature, we have used other primers in the region between the conserved portion of the 18S rDNA region, the adjacent ITS1, and a small portion of the 28S rDNA region in order to detect *C kefyr*, *C famata* and *C dubliniensis*.

In our study, we conclude that the traditional method for detection of *candida* species as germ tube test or manual biochemical reactions may result in mistake results, in the other hand all the new methods for identification as API, CHROMagar, and PCR are best method for confirmation this identification.

The multiplex PCR is particularly suitable for use in a routine clinical microbiology laboratory because it can easily be automated; moreover it could be utilized for the rapid identification of *Candida* isolates from high-risk patients or patients with vaginal candidiasis, enabling prompt and appropriate treatment and thus reducing hospitalization time.

CONCLUSION AND RECOMMENDATIONS

This multiplex PCR assay provides a good method to confirm the conventional culture based technique for the identification and speciation of the most frequently isolated *Candida* species.

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