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Using Single Multiplex PCR Reaction to Identify *Candida* Species in Vaginal isolates: Comparison between Phenotypic and Molecular Methods

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ABSTRACT

Backgrounds: In this research, an attempt was made to identify *Candida* isolates collected from women with suspected vulvovaginal candidiasis using single Multiplex PCR reaction as a swift and valid method. Beside, this method was compared with phenotypic methods.
Materials & Methods: In this study, 250 vaginal swabs were collected from patients referring to obstetrics and gynecology specialists. In addition to phenotypic methods, multiplex PCR designed by species-specific primers was performed to identify *Candida* isolates in a single reaction. Descriptive statistics were analyzed by t-test and Chi-square test in SPSS software (Ver. 22) ($p< .05$).
Findings: According to the results, 92 positive samples were diagnosed using the culture method. Four species were identified by culturing the specimens on CHROM agar. The most common *Candida* species isolated was *C. albicans* (54.3%), followed by *C. parapsilosis* (28.2%), *C. glabrata* (17.4%), and *C. krusei* (1.0%). The most common *Candida* spp. identified by Multiplex PCR method were *C. albicans* (50.0%), *C. glabrata* (33.7%), and *C. parapsilosis* (6.2%). Also, three mixed infections with *C. albicans* and *C. glabrata* as well as *C. albicans* and *C. parapsilosis* were identified
Conclusion: In comparison to phenotypic methods, considering the cost-effectiveness of PCR methods, the single multiplex PCR reaction was shown to be efficient in epidemiological studies on pathogenic species.

Keywords: *Candida* species, Vulvovaginal candidiasis, Multiplex PCR, CHROM agar, Molecular biology.

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Introduction

Vulvovaginal candidiasis (VVC) is one of the most common infections of the female genital tract, which is often caused by *Candida albicans* (*C. alnicans*) [1, 2]. About 75% of women of reproductive age are likely to get this infection. In addition, about 15% of the female population suffers from recurrent infections [3, 4]. The most common symptoms of VVC include itching, burning, cheese-like discharge, atypical hemorrhage, painful intercourse, and erythema of the vaginal area and vulva. Notably, none of these symptoms are certain for VVC [5–7]. Almost 10–20% of women have complicated VVC, requiring dedicated diagnosis and therapeutic considerations. On the other hand, VVC could concomitantly arise with sexually transmitted diseases (STDs) [8]. The majority of healthy women with no complications have no recognizable predisposing factors. Nowadays, due to frequent treatment failures and recurrences, this infection has challenged the life quality of infected women as well as physicians [9].

Although *C. albicans* is known as the most common cause of VVC, there is a significant trend towards the emergence of other species such as *C*. *glabrata*, *C*. *parapsilosis,* and *C*. *krusei,* which show more resistance to the first-line antifungal treatments [10–12]. Hence, laboratory diagnosis seems necessary to identify *Candida* species involved in VVC. In this regard, some conventional methods have been used to identify and differentiate *Candida* species, including time-consuming and invalid techniques such as conventional culture techniques, colony morphology, serotyping, and morphological and biochemical methods [13–15].

Nowadays, polymerase chain reaction (PCR) is considered as a standard policy in every clinical laboratory, even in developing countries, due to its affordability and reproducibility [13, 15]. Recently, a variety of molecular biology methods have been performed for the genetic identification of *Candida spp*. Accordingly, some of these methods include standard PCR [16], multiplex PCR [17], PCR-RFLP [13], randomly amplify polymorphic DNA (RAPD)–PCR [18], and DNA sequence analysis [19]. However, routine use of many of these techniques in diagnostic laboratories is not practical because of time and cost issues [20]. Multiplex PCR is a swift diagnostic assay, which combines many specific-species primers in a single PCR tube. Therefore, it could be used to detect more than one species in clinical samples simultaneously.

Objectives: The present study attempted to identify *Candida* spp. isolated from women with suspected VVC using Multiplex PCR method as a swift and valid method and compared the obtained results with those of the conventional methods such as CHROM agar and germ tube formation.

Materials and Methods

Participants: This study was performed on 250 women with suspected vulvovaginal candidiasis, who referred to obstetrics and gynecology specialists in Birjand city during December 2019 to March 2020. This study was approved by the Ethics Committee of Birjand University of Medical Sciences, Iran (ir. bums REC.1397.367).

Samples culture and phenotypic identification of *Candida* isolates: Participants' vaginal samples were taken with a sterile swab and cultured on Sabouraud dextrose agar (SDA Merck, Germany) culture medium. After incubation at 37 ^{0}C for 24, 48, and 72 hrs, the growth of colonies was observed and evaluated. To identify *Candida* species, phenotypic methods were used, including CHROM agar (CHROMagar™*Candida*, France) with incubation at 30 ^{0}C for 48 hrs. Thereafter, *Candida* spp. were identified based on their morphology and the color of their colonies**.** Then *albicans* and non-*albicans* species

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were differentiated based on germ tube formation after 2 hrs of incubation in serum at 37 ^{0}C and chlamydospore formation on corn meal agar-Tween 80 (CTA; Difco, Detroit, MI, USA)(30 ^{0}C for 48 hrs) [21, 22].

Molecular identification

DNA extraction: DNA extraction was performed according to the previously described boiling method with some modifications [23]. In brief, a fresh colony was transferred to a microtube containing 400 µL of distilled water and boiled for 15 min. Next, 200 µL of phenol-chloroform (1:1) solution was added to the microtube and vigorously mixed for 10 min. The supernatant was then separated by centrifugation at 12000 rpm and transferred to a clean tube. Then 1 volume of 96% ethanol was added to the tube and kept at -20 °C for 60 min. After that, the sample was centrifuged at 12000 rpm for 10 min to precipitate DNA. Finally, the precipitated DNA was dissolved in 50 µL of distilled water and stored at -20 °C after washing twice with 70% ethanol [13, 24]. The extracted DNA was used as a template in PCR reaction with appropriate dilution.

Primer design: Target primers were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/) [25]. The criteria for primers were also evaluated by primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were checked in term of cross-reactivity with the other species, the amplicon sizes of target species compatible with the rest of the target species in the same Multiplex PCR, melting temperatures, and the most stable segment of the target loci. To prevent cross-reactivity with non-target species, the gaps and mismatches were positioned in the 3′ end of primers. The primers were synthesized and shipped by Sinacolon Company (Tehran, Iran) (Table 1).

PCR conditions: Multiplex PCR was performed as previously described with some modifications [26] in a final volume of 25 µL. The mixture was prepared with 10 µL of master mix (Taq DNA Polymerase Master Mix, DNA biotech, Iran), 0.25 µL of each individual primer, and 2.5 µL of each DNA template and then adjusted to a final volume of 25 µL using Diethyl pyrocarbonate (DEPC) water. PCR thermal cycling conditions were as follow: an initial denaturation step at 95 ^{o}C for 5 min, followed by 35 cycles of denaturation at 95 ^{o}C for 45 s, 58 ^{o}C for 60 s, and 72 ^{o}C for 60 s and a final extension step at 72 ^{o}C for 5 min. The PCR reaction was carried out in an Applied Teq STAR 2X thermal cycler (PEQLAB, Germany). After that, the amplicons were detected by electrophoresis on 1.2% agarose gel and checked visually by Gel Doc system (BioRad, USA). The species identification was performed by comparing amplicon sizes with the reference band profile (Table 1). Moreover, the *Act1* gene was used for quality control purposes. All experiments were carried out in duplicate.

Statistical analysis: Descriptive statistics were analyzed using SPSS software (Ver. 16.0). T-test, Chi-square test, and Spearman's correlation were used to analyze the data. For all statistical analyses, a p value of $< .05$ was considered as significant.

Findings

Among 250 vaginal samples, 92 (36.8%) cases were positive for *Candida* species. The mean age (± SD) of the study participants was 33.3 (±1.02) years old. The patients' demographic information is shown in Table 2. According of the results, there was a significant association between age and the prevalence of *Candida* spp (p= .01). The results indicated that the participants in the age range of 25-35 years were prone to be infected with non-*albicans* species. On the other hand, there was no significant correlation between the prevalence of *Candida* spp. and clinical symptoms and history of infection

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Table 1) Primers employed in multiplex PCR amplification

Primers	Sequences (5'- 3')	Amplicons	References
C.alb	F-5'AGATTATTGCCATGCCCTGAG3' R5'CCATGTCGAACGTAGCGTATGC3'	606bp	22
C.gal	F5'ACCGTGCTTGCCTCTACA3' R5'GACATCTGAGCCTCGTCTGA3'	212bp	22
C.tr	F5'AGAACAAGAAAACAGTGAAGCAA3' R5'CCATGTCGAACGTAGCGTATGC3	126bp	22
C.par	F5'TACACCAAGCGACTCAGC3' R5'ACCAGCTGCTTTGACTTG3'	490bp	22
C.kr	F5'GGCGTTGTCCATCCAATG3' R5'CAGGAGAATTGCTGTTCCC3'	1159bp	22
C.dub	F5'GTCGGACATATACCTCCAACTC3' R5'CCATGTCGAACGTAGCGTAT3'	718bp	22

Table 2) Comparison of patients' demographic characteristics with frequency of *Candida albicans* and non-*albicans* species

Variables	*Candida* spp.		
	C. albicans N=52 N(%)	Non-*albicans* Species N=40 N(%)	*P*-Value
Age group <25 25-35 36-45 >45	14(70.0) 16(47.1) 10(43.5) 12(10.0)	6(30.0) 18(48.4) 13(52.4) 0	.001
History of infection Yes No	33(55.9) 19(63.3)	26(44.1) 11(36.7)	.97
Clinical symptoms Yes No	20(58.7) 32(57.1)	14(41.2) 24(42.9)	.29

(p= .97 and p= .29, respectively) (Table 2). In the present study, the differentiation of *C. albicans* from non-*albicens* species was carried out based on the germ tube test and chlamydospore production. The results revealed that chlamydospore production and germ tube formation were detected in 55.9 and 38.4% of the isolates, respectively (Fig. A.1). Then species identification was done on chromogenic medium based on color production as follows: light green colonies were identified as *C. albicans*, blue-violet colonies as *C. tropicalis*, pink colonies with rough borders as *C. krusei*, pale pink colonies with smooth borders as *C. parapsilosis,* and glossy lavender/dark pink colonies as *C. glabrata* [27, 28]. The results of phenotypic methods are shown in Figure B.1.

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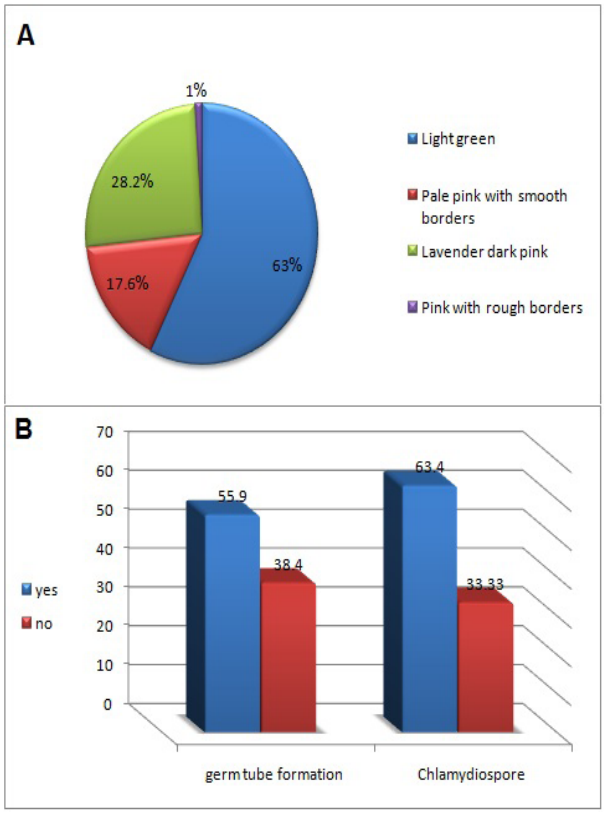

Figure 1) Frequency of *Candida* species identified by phenotypic methods; A: Morphology examination, B: CHORM agar culture

The most common *Candida* species identified using CHROM agar medium was *C. albicans* (54.3%, 50 of 92), followed by non-*albicans* species (46.7%, 42 of 92) including *C. galabrata* (28.2%, 26 cases), *C. parapsilosis* (17.4%, 15 cases), and *C. krusei* (1.0%, 1 case) (Fig. 2). In single multiplex PCR (mPCR), species identification was performed by species-specific primers. The results of the mPCR reaction are shown in Figure 3. Among the 92 vaginal samples, 96.7% were identified with one *Candida* species, and 3.3% were identified with mixed infections (more than one *Candida* species). Accordingly, the frequency of *Candida* spp. was as follows: *C. albicans* was identified in 52 cases (50.0%), C. *glabrata* in 34 cases (33.7%), C. *parapsilosis* in three cases (6.2%), *C. glabrata* and *C. albicans* in two cases (2.1%), and *C. parapsilosis* and *C. albicans* in one case (1.0%), respectively.

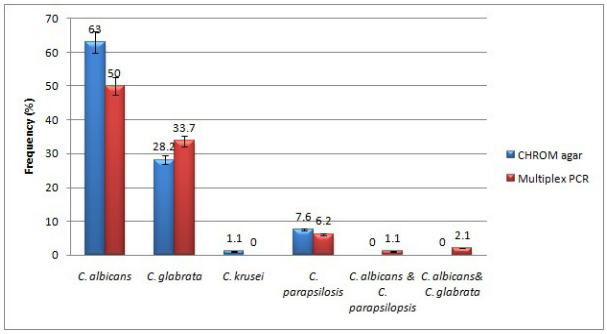

Figure 2) Frequency distribution of *Candida* species by phenotypic and multiplex PCR methods

Discussion

Vulvovaginal candidiasis is the most common infection caused by *Candida* spp. in the female genital tract, which has been known as a challenge for gynecologists and infected patients for decades. Although *C. albicans* is known as the most common cause of VVC infection, other *Candida* species with high drug resistance, such as *C. glabrata*, have recently been replaced [7, 12, 29, 30]. Studies have suggested that the diagnosis of vulvovaginitis caused by other *Candida* species may be very effective in selecting a successful treatment protocol [26, 31]. In this regard, many studies have been conducted, each of which has introduced some molecular methods and techniques to identify *Candida* species isolated from clinical specimens [13–15].

In this study, species-specific primers were used to design a single Multiplex PCR reaction in order to determine *Candida* spp. isolated from vaginal specimens. Besides the Multiplex PCR method, conventional methods were also utilized to identify the species, and the results of both methods were finally compared with each other. By comparing the results of PCR and phenotypic methods, some differences were observed in the frequency of the identified species (Fig.1). Mixed infections were identified using PCR in cases where the results of CHROM agar, which is based on the colony color on the medium, were not distinguishable. Correspondingly, these defects were identified

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using Multiplex PCR technique and the standard strain of each species based on the size of the amplified species-specific primers identified by gel electrophoresis.

Carvalho et al. (2007) used a multiplex PCR technique to determine *Candida* spp. isolated from patients with suspected candidiasis. In their study, ITS1 and ITS2 primers were used to distinguish between two yeast-specific and eight species-specific primer pairs. In addition, they used pure colonies grown on culture medium to extract DNA [32]. Also, Taria et al. (2014) used multiplex PCR technique to identify *Candida* spp. isolated from infant's blood samples. Besides, they used ITS1 and ITS4 primers as external primers and species-specific primers as internal primers to identify *Candida* spp., including *C. albicans, C. glabrata, C, parapsilopsis*, *C. tropicalis,* and *C. krusei*, respectively [33]. In a study conducted by Mahmoudi Rad et al. (2011), a multiplex PCR technique was used to determine *Candida* spp. isolated from vaginitis specimens [14]. In their study, ITS1 and ITS2 primers were used to amplify the 18SrDNA and 28 SrDNA regions (first tube), and then CA3 and CA4 primers were used to identify *Candida* species (second tube), respectively. In their study, the single multiplex PCR reaction and species-specific primers were also used to identify *Candida* spp. Arastehfar et al. in 2019 invented the YEAST PANEL multiplex PCR assay, which identifies the most clinically important yeast species such as *Candida, Cryptococcus* spp., *Trichosporon*, *Rhodotorula,* and *Geotricum* spp [25]. The results obtained from the YEAST PANEL multiplex PCR assay were confirmed by those of MALDI-TOF MS and DNA sequencing. They acknowledged that due to the high accuracy and specificity of multiplex PCR assay, this method could be used for identification in routine laboratories and epidemiological studies.

Due to the increasing prevalence of non-*albicans Candida* species and their association with treatment difficulties due to the emergence of antifungal-resistant species worldwide, identification of these strains at the species level is of great importance [7, 34, 35]. Furthermore, in developing countries because of limited pecuniary support, the use of expensive and costly methods such as DNA sequencing, RFLP-PCR, RAPT-PCR, and MALDI-TOF MS is limited. This could lead to deficiencies in infection detection and mortality control in these regions [36–38]. Therefore, the PCR method could be employed as a useful identification tool due to its affordability, even in developing countries.

Thus, it was found that the single multiplex PCR reaction could be both economical and time-saving. In the present study, the boiling method was used instead of glass beads to extract the yeast genome; therefore, this method could be very cost effective and time-saving.

One of the limitations of the present study was that it was not possible to diagnose infections caused by other yeast species such as *Saccharomyces cerevisiae*, *Trichosporon*, *Rhodotorula,* and *Geotricum*spp with only a single PCR reaction well. Therefore, a second tube was needed to identify other non-*Candida* species.

Conclusion

This study showed that the high prevalence of *Candida* infection in VVC cases in eastern Iran is due to several species, mostly *C. albicans* and *C. glabrata***.** Due to the high cost of advanced methods, the identification of infectious agents in developing countries is mostly performed by conventional methods such as phenotypic assays, which waste time and are prone to errors. Considering the increasing prevalence of non-*albicans* species and mixed infections among VVC patients, precise determination of causative *Candida* species with more reliable methods such

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as molecular techniques could prevent patients from spending exorbitant costs for treatment. In addition, it is recommended to directly use the multiplex PCR method to identify samples in order to save detection time.

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Authors' contributions: FN designed and supervised the study. FGH carried out the statistical analysis and edited the final version of the manuscript. MZ and NGH helped in sample collection, and FN helped in conducting the study. All authors critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.
Consent to Participate: All participants signed a written consent form and was kept confidential.

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