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Research Article

Identification of *Candida* spp. isolated from vaginal swab by phenotypic methods and multiplex PCR in Duhok, Iraq

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ABSTRACT

Background: *Candida* species are the second most common cause of vulvovaginitis worldwide. The purpose of this study was to identify the species of vaginal *Candida* isolates by using phenotypic and Multiplex PCR techniques.
Methods: 91 isolates from patients admitted to Azadi hospital and Maternity hospital in Duhok city were collected. The vaginal swab specimens were inoculated on Sabouraud dextrose agar. Colonies were then sub cultured on Chromogenic Candida agar. Genomic DNA extraction was performed using a Genomic DNA Extraction kit. For rapid identification of *Candida* spp., specific primers based on the genomic sequence of DNA topoisomerase 11 of *C. albicans*, *C. parapsilosis I*, *C. parapsilosis II*, *C. guilliermondi*, *C. dubliniensis*, *C. krusei*, *C. kefyr and C. glabrata*, *C. tropicalis I*, *C. tropicalis II*, *C. lusitaniae* were used. The multiplex PCR products were separated by electrophoresis in 1.5% agarose gel, visualized by staining with ethidium bromide, and photographed.
Results: 4 *Candida* species, namely C. *albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* were distinguished by Chromogenic Candida agar on the basis of colony colour and morphology. PCR with the primer mixes yielded 7 different sized of PCR products corresponding to C. *albicans*, *C. guilliermondii*, *C. dubliniensis*, *C. glabrata*, *C. kefyr*, *C. krusei* and *C. tropicalis II*. The analysis revealed *C. glabrata* and *C. albicans* were the most common species isolated with the percentage 40% and 30% respectively.
Conclusions: This study concluded that phenotypic characteristics on selective agar medium such as chromogenic candida agar are useful for presumptive identification of *Candiada* spp. with the support of molecular method such as multiplex PCR.

Keywords: *Candida* species, Multiplex PCR, Vulvovaginitis, Chromogenic candida agar

INTRODUCTION

The genus *Candida* includes many pathogenic species of yeasts that cause a variety of clinical syndromes in humans, ranging from superficial infection to invasive disease in immunocompromised patients.[1] *Candida* species are the second most common cause of vulvovaginitis worldwide.[2] The prevalence of vulvovaginal candidiasis is increasing due to extensive utilization of broad-spectrum antibiotics as well as increased cases of immunocompromised patients.[3,4] Nearly 75% of women over 25 years of age, reported to have at least one episode of physician approved vulvovaginal candidiasis (VVC) during their lifetime and 5% experienced recurrent type; which is defined by getting infected for at least 4 times in a one-year period.[5] However, 20-50% of women have *Candida* species in their vaginal flora without showing any clinical symptoms.[5,6] *C. albicans* is the most common and

clinically relevant species that account for 85-90% of vulvovaginal candidiasis.[5] However, there has been a significant trend towards the emergence of other species such as *C. glabrata*, *C. krusei and C. parapsilosis* which show more resistance to the first antifungal treatments.[7] Hence, the differentiation of diverse species of *Candida* in the laboratories seems necessary. Traditionally, the identification and classification of *Candida* species were done by time consuming and unreliable methods such as serotyping,[8] colony morphotyping,[9] conventional culture techniques, and morphological and biochemical analysis.[10] Nonetheless, the improvement in molecular assay technology for identifying Candida species, such as Randomly Amplified Polymorphic DNA analysis (RAPD), has overcome these limitations during the last couple of years. However, methods such as single and direct PCR or Multiplex PCR have not been used extensively despite being highly sensitive and specific with a shorter turn-around time.[11-14] Multiplex PCR is a rapid diagnostic assay which combines many specific species primers in one PCR tube. Hence, it could be used to identify more than one species in a specimen simultaneously.[15] The majority of studies on *Candida* infection in Iraq were focused on vaginal candidiasis. These studies included isolation, identification of etiological agents, study of some factors that predispose to the disease and antifungal susceptibility tests for some *Candida* isolates.[16-19] Molecular diagnosis of *Candida* species has been carried out in few studies such as Epidemiological and molecular study of *Candida* species in vagina was carried out on patient women attending outpatient consultation clinics for gynecology in Baghdad and Najaf governorates.[20] In Duhok province there is no any study dealing with the diagnosis of *Candida* spp. using multiplex PCR technique, therefore this study is aimed to isolate and identify different species of *Candida* species associated with vulvovaginal candidiasis in patient's population in Duhok, Kurdistan, Iraq by using morphological and molecular methods. Multiplex PCR method was also evaluated as a rapid and reliable method to identify *Candida* species by comparing the results with the traditional methods such as colour and morphology of the colonies on chromogenic candida agar medium.

METHODS

Women with signs and symptoms of VVC were studied. About 99 Vaginal sampling of the participants which admitted to Azadi hospital and Maternity hospital (Duhok city, Kurdistan Region, Iraq) during a period from October 2013 to March 2014. Vaginal sampling of the participants (The age of the patients were between 15-45) performed by using a sterile swab by the principle researcher and was cultured simultaneously onto Sabouraud dextrose agar medium. Direct smears were prepared for Gram's staining from Isolates and diagnosed based on morphology of the culture medium and details of microscopic examination from positive cultures on Sabouraud dextrose agar.[21] Positive isolates were sub cultured on chromogenic candida agar (Rapid Labs Ltd, Essex) which differentiates *Candida* species on the basis of a color change.[22]

Genomic DNA extraction and purification were performed using a Genomic DNA Extraction kit (provided by Jena Bioscience GmbH/ Germany) based on the guidelines.

Table 1: Primers for PCR amplification used in this study and their sequences.

Set No.	No.	Target species	Forward primer	Reverse primer	Sizes of PCR product (bp)
Set S1	1	*C. albicans*	CABF59 (5-TTGAACATCTCCAGTTTCAAAGGT-3)	CADBR125 (5-AGCTAAATTCATAGCAGAAAGC-3)	665
	2	*C. parapsilosis I*	CPPIF41 (5-TGACAATATGACAAAGGTTGGTA-3)	CPPIR122 (5-TGTCAAGATCAACGTACATTTAGT-3)	837
	3	*C. parapsilosis II*	CPPIIF41 (5-GGACAACATGACAAAAGTCGGCA-3)	CPPIIR69 (5-TTGTGGTGTAATTCTTGGGAG-3)	310
	4	*C. guilliermondii*	CGLF41 (5-CCCAAAATCACAAAGCTCAAGT-3)	CGLR61 (5-TACGACTTGAAGTTGCGAATTG-3)	205
Set S11	1	*C. dubliniensis*	CDBF28 (5-AAATGGGTTTGGTGCCAAATTA-3)	CDBR110 (5-GTTGGCATTGGCAATAGCTCTA-3)	816
	2	*C. glabrata*	CGBF35 (5-CCCAAAAATGGCCGTAAGTATG-3)	CGBR103 (5-ATAGTCGCTACTAATATCACACC-3)	674
	3	*C. kefyr*	CKFF35 (5-CTTCCAAAGGTCAGAAGTATGTCC-3)	CKFR85 (5-CTTCAAACGGTCTGAAACCT-3)	532
	4	*C. krusei*	CKSF35 (5-GAGCCACGGTAAAGAATACACA-3)	CKSR57 (5-TTTAAAGTGACCCGGATACC-3)	227
Set 111	1	*C. tropicalis I*	CTPIF36 (5-GTTGTACAAGCAGACATGGACTG-3)	CTPIR68 (5-CAAGGTGCCGTCTTCGGCTAAT-3)	318
	2	*C. tropicalis II*	CTPIIF36 (5-CTGGGAAATTATATAAGCAAGTT-3)	CTPIIR121 (5-TCAATGTACAATTATGACCGAGTT-3)	860
	3	*C. lusitaniae*	CLTF39 (5-CATGTCGAAATGCAACCCCCCG-3)	CLTR119 (5-GCGTACACTTGTGGCCATCTTTA-3)	799

For multiplex PCR using primer mixes each one of the primer pairs were designed for a species of *Candida* was grouped, based on the following criteria: (a) no primers in a group form dimers and/or interrupt PCR amplification ; (b) the number of the group should be as small as possible; (c) each primer pairs yields one major PCR product, and each species of *Candida* should clearly be distinguished by the size (bp) of the PCR products, these primers were described by Kanbe and his colleagues (2002) for sequencing of the *Candida* DNA topoisomerase II genes.[23] In this study, three sets of primer groups were prepared. These groups were designated S1, S11 and S111, and referred to as a 'primer mix 'in this study. S1 was composed of four specific primer pairs for identification of *C. albicans, C. parapsilosis I, C. parapsilosis II and C. guilliermondii*; S11 was for *C. dubliniensis, C. krusei, C. kefyr and C. glabrata*; and S111 was for *C. tropicalis I, C. tropicalis II, C. lusitaniae*. For the set S1 and S11 primers the PCR was performed in 25 µl reaction mixture consisting of approximately 5 µl of template DNA, 10 µl of Taq DNA Polymerase 2X ReadyMix, 1 µl of forward and reverse primers (1 µl) for each one and 2 µl of PCR-grade water all of these kept in a single tube. While for set S111 primers the amount of water was increased to the 4 µl in the reaction mixture. The species-specific primer pairs used in each mix and calculated sizes of PCR products generated by each set of the mixes are listed in Table 1.

The PCR cycle parameters were as follows; one cycle of initial denaturation at 95°C for 5 min; then 35 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72˚Cfor 1 min; and followed by one cycle of final extension two at 72°C for 2 min. PCR products were analysed by agarose gel electrophoresis in 1X TBE buffer at 100 V for 120 min in gel composed of 1.5% agarose. 100bp ladder DNA Marker was run with PCR products for sizing of the bands. Gels were stained with ethidium bromide solution (concentration of 0.5 µg/ml) for 30 min, then visualized with a UV transilluminater and photographed.

RESULTS

Phenotypic examination showed that *Candida* colonization was detected in 40 patients. *Candida* species growing on Sabouraud dextrose agar (Figure 1) were characterized by producing white colonies to cream coloured, smooth and waxy as stated by Ellis and his colleagues (2007);[24] Babic and Hukic (2010).[22] Microscopic appearance of *Candida* were spherical to subspherical and showing budding (Figure 2).

Out of 40 samples of vaginal swabs which showed positive result on SDA, 34 isolates were identified on chromogenic candida agar (Table 2). The colony colour and morphology of 34 isolates were examined and *Candida* species were suspected *Candida albicans* (Emerald, with metallic shine), *C. glabrata* (White, shiny), *C. krusei* (Light pink) and *C. tropicalis* (Red purple, some strains with the small white outer ring) (Figure 3).

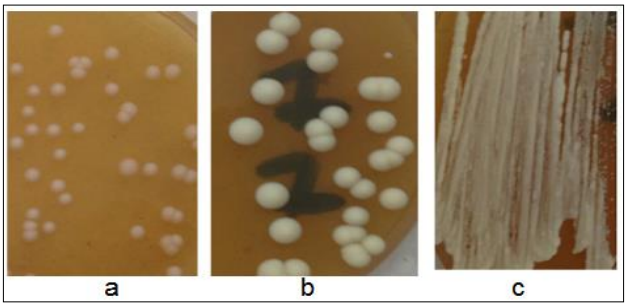

Figure 1 (a, b c): Growth of *Candida* species on Sabouraud dextrose agar.

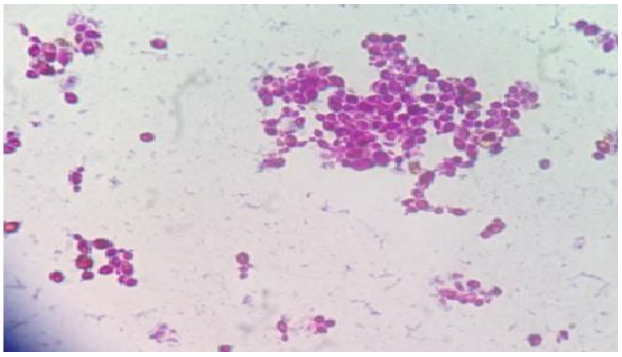

Figure 2: Microscopic appearance of *Candida* (Gram's stain x100).

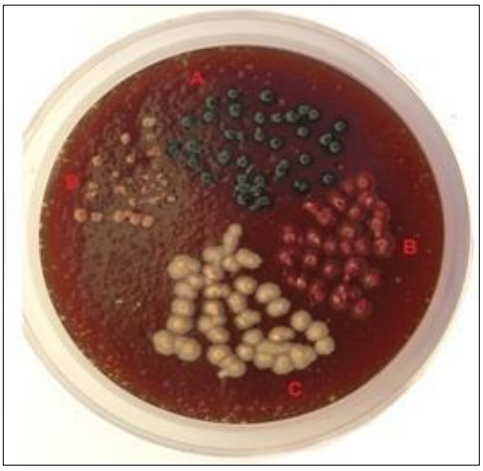

Figure 3: Chromogenic Candida agar plate showing chromogenic color change for A: *Candida albicans* (Emerald, with metallic shine), B: *Candida tropicalis* (Red purple). C: *Candida glabrata* (White, shiny), and *D: Candida krusei* (Light pink).

Twelve isolates were considered to be *C. albicans*. Eighteen isolates were considered to be *C. glabrata*. Two colonies were determined as *C. krusei*. Two small colonies with red purple were determined as *C. tropicalis* (Table 2).

Table 2: Identification of *Candida* species using chromogenic candida agar.

Candida species	Culture on chromogenic agar
	Vaginal swab
C. albicans	(12/40) 30%
C. glabrata	(18/40) 45%
C. krusei	(2/40) 5%
C. tropicalis	(2/40) 5%
Not detected on chromogenic agar	(6/40) 15%
***Candida* spp. identification**	(34/40) 85%

After determination of colony colour, PCR was done by using primers sets as shown in Table 1. However, Multiplex PCR was able to generate specific bands for 38 samples (95%) of total 40 different samples obtained from vaginal swabs (Table 3).

Seven of primer pairs amplified species-specific DNA fragments from genomic DNA template, the size of PCR products was from 205 to 860 bp and they were of a specific size corresponding to each species of *Candida* (Figure 4 and 5).

The DNA of *Candida albicans*, *C. guilliermondii*, *C. dubliniensis*, *C. glabrata*, *C. kefyr*, *C. krusei* and *C. tropicalis II*, was amplified by PCR and generated DNA fragments of 665, 205, 816, 674, 532, 227 and 860 bp, respectively. These were the exact sizes corresponding to each species (Table 1).

Molecular assessment of vaginal swab samples demonstrated that 40% of the patients showed *C. glabrata* followed by *C. albicans* (30%), *C. kefyr* (10%), *C. krusei* (5%), *C. tropicalis II* (5%), *C. dubliniensis* (2.5%) and *C. guilliermondii* (2.5%) (Table 3).

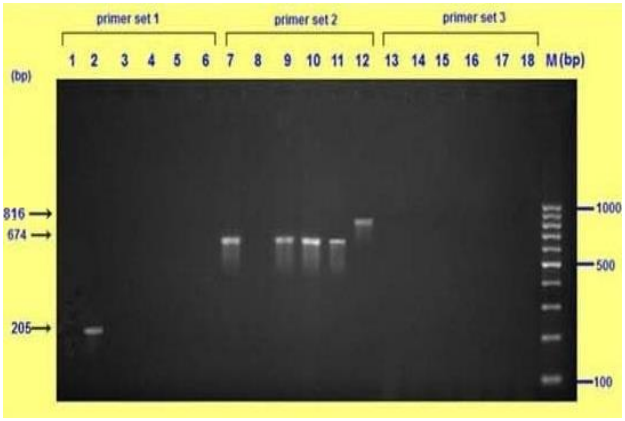

Figure 4: Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (M) indicates 100-bp DNA marker.

Lane (2): *C. guilliermondii* (205 bp). Lanes (7, 9, 10, and 11): *C. glabrata* (674 bp). Lane (12): *C. dubliniensis* (816 bp).

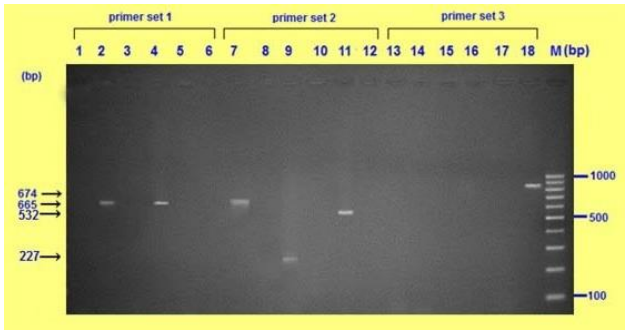

Figure 5: Agarose gel (1.5%) of PCR products obtained with species-specific primers. Lane (M) indicates 100-bp DNA marker.

Lanes (2 and 4): *C. albicans* (665 bp). Lane (7): *C. glabrata* (674 bp). Lane (9): *C. krusei* (227 bp). Lane (11): *C. kefyr* (532 bp). Lane (18): *C. tropicalis II* (860 bp).

Table 3: Distribution and identification of *Candida* species using multiplex PCR in specimens of vaginal swabs.

Candida species	PCR-based identification
	Vaginal swab
C. albicans	(12/40) 30%
C. dubliniensis	(1/40) 2.5%
C. glabrata	(16/40) 40%
C. krusei	(2/40) 5%
C. tropicalis II	(2/40) 5%
C. kefyr	(4/40) 10%
C. guilliermondii	(1/40) 2.5%
Not identified	(2/40) 5 %
PCR-based for identification *Candida* species	(38/40) 95%

DISCUSSION

Vaginal yeast infection, alternately referred to as vaginal candidiasis or vulvovaginal candidiasis (VVC), is caused by a broad spectrum of Candida species in vagina with special symptoms including inflammation, irritation, itching and vaginal discharge. Vulvovaginal candidiasis (VVC) is routinely diagnosed without laboratory tests, which results in misdiagnosing. Although VVC is treatable, without appropriate treatment there is a probable risk for gaining of complications including pelvic inflammatory disease, infertility and ectopic pregnancy.[25] Hence, the reliable and rapid identification method of *Candida* species is a fundamental goal of microbiology laboratory. In this study we used a rapid, simple & reliable PCR system for definitive identification of 11 pathogenic *Candida* species, including those with poor susceptibility to antifungal drugs.

The multiplex PCR method is a highly sensitive and specific technique based on the results of previous studies.[26] Despite their demonstrated reliability,

molecular methods have not been routinely used to identify Candida species. Liguori and his colleagues compared different chromogenic and biological methods to PCR for *C. albicans* identification. They pointed out high incubation time, lack of experienced personnel, low sensitivity and specificity, and lower discrimination power as disadvantage of other methods and suggested using them for screening and preliminary assays, while introduced the PCR as a precise and simple to implement method with no requirement of toxic and expensive chemical reagent.[27] However, the differences in molecular weights of Multiplex PCR products ranged from 22 to 655 bp and there is no risk for misidentification when species were determined only by species-specific amplification method. In a study by Isogai and his colleagues (2010), they pointed out that molecular weights of PCR products were close to each other among *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis* and *C. krusei* when *Candida* spp. were identified using RFLP.[28] The results of this study showed that *Candida glabrata* was the more common in vaginal swap with prevalence of40% followed by *Candida albicans* (30%). This finding is in agreement with previous studies.[29-32] and could be due to the development of antifungal resistance, immune response, or hormonal changes among women.[32] Similarly Abu-Elteen (2001) stated that the most common species of *Candida* in vaginal swab was *C. glabrata*.[33] In this study, Chromogenic Candida agar allowed the presumptive identification of four *Candida* spp., namely *C. albicans*, *C. galbrata*, *C. krusei* and *C. tropicalis*. The results of the present study indicate that three major species of *Candida* were isolated from vaginal samples assigned to the species *C. kefyr*, *C. dubliniensis* and *C. guilliermondii* were not identified and distinguished from other species using phenotypic method. This finding is in agreement with previous study.[34,35] Therefore, the phenotypic characteristics on the selective agar medium such as chromogenic candida agar are useful to determine *Candida* species with support of molecular diagnosis. Hence, the Identification of *Candida* species with multiplex PCR is a practical and reliable method, and it is useful for the identification of some of clinically isolated *Candida* species, such as *Candida kefyr*, *C. dubliniensis* and *C. guilliermondii* which could not be identified by chromogenic candida agar.

CONCLUSION

The results of this study showed that *C. glabrata* is the most common *Candida* species in VVC among women with prevalence 40% followed by *Candida albicans* (30%). Chromogenic Candida agar allowed the presumptive identification of *C. albicans*, *C. galbrata*, *C. krusei* and *C. tropicalis.* A multiplex PCR which seemed to be a reliable and rapid technique was able to identify seven *Candida* spp., namely, *C. glabrata*, *C. albicans*, *C. kefyr*, *C. krusei*, *C. tropicalis II*, *C. dubliniensis* and *C. guilliermondii*. The latter two species have been reported for the first time in Kurdistan region, Iraq.

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