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Determination of candidal vulvovaginitis prevalence in patients referred to the gynecology clinic of Imam Khomeini Hospital, Noor City, Iran

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Original Article

Abstract

BACKGROUND: Candidal vulvovaginitis is an infection of the female genital tract that is caused by overgrowth of Candida, especially Candida albicans (C. albicans), and it may sometimes be recurrent. This research aimed to determine Candidal vulvovaginitis prevalence in women by phenotypic tests and polymerase chain reaction (PCR).

METHODS: This descriptive cross-sectional study was performed in Noor City, Iran, in 2017, on 36 patients with Candidal vulvovaginitis. Samples were isolated and identified by direct and phenotypic tests. Then, to confirm the isolates, molecular PCR and sequencing were used. Frequency determination was performed for them using SPSS software.

RESULTS: Out of 15 isolates, 10 (66.67%) belonged to the C. albicans species and 5 (33.33%) isolates belonged to Candida glabrata (C. glabrata). PCR-sequencing showed that out of 15 isolates, 10 isolates were positive for C. albicans, and all 5 isolates were C. glabrata.

CONCLUSION: C. albicans and C. glabrata species were the most common cause of Candidal vulvovaginitis. Although C. albicans is still the predominant species in patients with Candidal vulvovaginitis, the increase in non-albicans species is a fact that requires further research by researchers in this field.

KEYWORDS: Gynecology; Candida; Polymerase Chain Reaction

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Introduction

Candidal vulvovaginitis is a fungal disease characterized by symptoms such as itching and thick white discharge. The disease is caused by different species of Candida. 70%-75% of healthy adult women develop Candidal vulvovaginitis at least once during their reproductive years.[1] Genital candidiasis is most often caused by an increase in the number of Candida albicans (C. albicans), which is a natural flora in the vagina. Approximately, 5% of these patients develop this disease four or more times a year, which is called recurrent Candidal vulvovaginitis.[2] The most common cause of this infection (80%-90%) is C. albicans, but in patients with recurrent Candidal vulvovaginitis, 10% to 20% of cases are created by non-albicans species.[1] Some believe that the widespread use of antifungal drugs has shown a prevalence of non-albicans species because one- to three-day treatment regimens of imidazoles may stop the growth of C. albicans but cause an imbalance in the vaginal flora. This matter can facilitate the overgrowth of other species of Candida.[3] Most non-albicans Candida species are

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resistant to antifungal drugs; Candida glabrata (C. glabrata) and Candida krusei (C. krusei) are more resistant to fluconazole than other species.[4] Therefore, accurate identification of Candida species will be very important in the treatment and control of the disease. In previous years, species were diagnosed and even classified based on clinical manifestations, morphology of the organism in the culture medium, and some biochemical and physiological reactions. These classical methods are very laborious and time-consuming and take several days to identify isolates of a culture.[5] In contrast, molecular methods that have replaced the previous methods in recent years have a higher speed and accuracy. Various polymerase chain reaction (PCR) methods have been reported to differentiate some species of Candida. The species of Candida that cause Candidal vulvovaginitis are the normal flora of the lower genitalia in 20% to 50% of asymptomatic healthy women.[6] Sasani et al. reported that the overall prevalence of vulvovaginal candidiasis among Iranian women was 47% and C. albicans was the most prevalent microbe.[7] Mirhendi et al. isolated different C. albicans (66.5%) with the highest frequency, followed by Candida parapsilosis (C. parapsilosis) (8.6%), C. tropicalis (8.2%), C. glabrata (6.1%), C. krusei (4.6%), Candida kefyr (C. kefyr) (2.5%), Candida guilliermondii (C. guilliermondii) (0.7%), and Candida lusitaniae (C. lusitaniae) (0.35%) in different specimens of 280 patients.[5] Kord et al. reported that, out of 304 yeast colonies isolated from the vaginal specimen, 204 were C. albicans.[2] Jacob et al. reported that approximately 75% of 50279 women were diagnosed with vulvovaginal candidiasis.[1] Therefore, accurate identification of Candida species will be very important in the treatment and control of this disease. In previous years, species were identified based on clinical manifestations, morphology of the organism in the culture medium, and some biochemical and physiological reactions that were very time-consuming and costly. In contrast, molecular methods have become more rapid and accurate in recent years. Thus, determining the prevalence of Candidal vulvovaginitis based on the type of causative species by molecular method is necessary to adopt an appropriate treatment method. This research aimed to determine the prevalence of Candidal vulvovaginitis by PCR in women referred to a gynecology clinic in Noor City, Iran.

Methods

This was a cross-sectional study to isolate Candida. Human samples infected with the fungus in women with fungal infections were randomly collected from 36 women in the clinic (Imam Khomeini Hospital, Noor City, Mazandaran Province) during the first three months of spring 2017 and transferred to the laboratory under sterile conditions. For laboratory tests, the subject was placed in lithotomy for examination and a sample of cervical and vaginal secretions was removed for microscopic examination by placing a spaculum using a sterile cotton swab. After collecting the samples and transferring them to the laboratory, they were transferred to Sabouraud dextrose broth medium (Merck, Germany) and then incubated at 30 °C for 24 hours. After this period, 100 µl of turbidity-containing tubes were cultured in Sabouraud dextrose agar (SDA) (Merck, Germany) under sterile conditions next to the flame and under the biological hood to allow the fungi in the sample to grow in this nutrient medium. The plates were then incubated at 30 °C for 24 hours. All the collected samples cultured on SDA were cultured linearly on CHROMagar medium (Merck, Germany), and after gluing around the plates, they were incubated at 30 °C for 48-72 hours. After that, they were kept at room temperature. In the present study, corn meal agar (CMA) medium (Merck,

Germany) containing 80% Tween was used. After preparation of the medium, freshly grown yeast colonies were removed from SDA medium with a sterile ounce and cultured as shallow and parallel grooves in CMA medium, and then they were incubated at 30 °C for 24 to 48 hours. After incubation time, under a microscope, the presence of false and true mycelium, chlamydoconidia (chlamydospores), and blastoconidia was examined. After the time elapsed for the fungi to grow, the colonies, which differed in shape, color, size, opacity, and transparency, were cultured separately by observing CMA. Green, blue, and purple colonies were identified. Single colonies were obtained by replanting in CMA. After careful observation, classification, and coding, microscopic examination was performed. Smears were prepared from the colonies and dried and fixed on the slide. Then lactophenol blue staining was performed to observe the fungus. After drying, a drop of immersion oil was poured on the slide and microscopic examination with 100 lenses was performed. To purify and isolate the selected colonies of Candida, in a sterile and freshly prepared culture medium, under sterile conditions and a laminar hood, 4 regions were cultured and incubated at 30 °C for 24 hours. After identifying Candida, the culture medium of broth SDA with 25% sterile glycerol was poured into a sterile microtube and some sterile single fungal colonies were cut with a sterile loop and inoculated into the microtube. In the next step, it was incubated at 30 °C for 24 hours and then placed at -80 °C for long-term storage.

Molecular detection: For this purpose, the boiling method was used in such a way that the first 1 cc (1000 microliters) of each sample was added to each of the microtubes under completely sterile conditions, and the microtubes were centrifuged at 10000 revolutions per minute (rpm) with a microcentrifuge for 2 minutes. Then the supernatant was discarded and the microtubes containing the sediment were completely covered with latex and placed in boiling water for 15 minutes; finally, the deoxyribonucleic acid (DNA) of each sample was extracted. In this study, a pair of 18SrDNA primers (Pishgam Company, Iran) was used to amplify the genes (Table 1).

PCR kit (CinaClon, Iran) was used for PCR test. According to kit, PCR reaction material was prepared (Table 2) and then performed.

The microtubes were placed in a thermal cycler and PCR steps included: 5 minutes at 95 °C to separate 2 strands of DNA (initial denaturation), 34 cycles including 45 seconds at 94 °C for denaturation in the annealing step, 1 minute at 55 °C for binding primers, 1 minute at 72 °C for polymerization by Taq polymerase (annealing extension), and finally 7 minutes at 72 °C for final expansion (final extension) (Table 3).

1 µl of dye or loading buffer (CinaClon, Iran) was mixed with 5 µl PCR product and poured into the gel well (samples were placed in each well, respectively, one of the wells was PCR and ladder). Using a Gel Documentation System (GelDoc), bands were interpreted. The information was entered into the SPSS software (version 21, IBM Corporation, Armonk, NY, USA) and a frequency determination was performed for data.

Table 1. Pair primers used in this study

Primer sequence	Sequence	Product size
Forward	5´TCCGTAGGTGAACCTGCGG3´	C. albicans
	3 TCCTCCGCTTATTGATATGC5´	535 bp
Reverse	3CGATTGTGGACCCCTCCTG5´	C. glabrata
	3´GCTAACACCTGGGGAGGAC5´	871 bp

C. albicans: Candida albicans; C. glabrata: Candida glabrata

Table 2. Materials for polymerase chain reaction (PCR)

Sample	Values (µl)
Water	16.05
Buffer	2.50
MgCl2	0.75
dNTP	0.50
Taq DNA polymerase	0.20
Primer forward	1.00
Primer reverse	1.00
DNA template	3.00
Total	25.00

dNTP: Deoxynucleotide triphosphate; DNA: Deoxyribonucleic acid; MgCl2: Magnesium dichloride

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Results

According to the results, 10 isolates (66.67%) out of 15 isolates belonged to the C. albicans species and 5 (33.33%) isolates belonged to C. glabrata. Based on the results of PCR test, out of 15 isolates, 10 isolates were positive for C. albicans, and all 5 isolates were C. glabrata. The sequencing for identifying the samples was also performed. Sequencing results also showed that the samples isolated from patients' vaginal secretions belonged to C. albicans and C. glabrata (Table 4) (Figure 1).

Table 3. Thermal cycle of polymerase chain reaction (PCR)

Step	Phase	Temperature (°C)	Time
1	Initial denaturing	95	5 minutes
2	Denature in annealing	94	45 seconds
3	Annealing	55	1 minute
4	Extension	72	1 minute
5	Step 2 (34x)	94	45 seconds
6	Final extension	72	7 minutes
7	Final storage	4	∞

Discussion

In this study, different phenotypic, biochemical, and molecular tests were used to determine Candida species in patients with recurrent Candidal vulvovaginitis. Results of this study showed that C. albicans and C. glabrata were isolated from patients suffering from candidal vulvovaginitis. Vaginitis is a common disease of the genital area that affects about ten million medical clients each year.[8] According to statistical data in the United Kingdom (UK) in the last decade, there has been a sharp increase in the incidence of Candidal vulvovaginitis.[9]

Table 4. Sequencing results to identify the studied samples

Sample	Organism
SH1-ITS1	Candida albicans Eukaryota, Fungi, Dikarya, Ascomycota, Saccharomycotina, Saccharomycetes, Saccharomycetales, Debaryomycetaceae, Candida/Lodderomyces clade, Candida
SH2-ITS4	Candida albicans Eukaryota, Fungi, Dikarya, Ascomycota, Saccharomycotina, Saccharomycetes, Saccharomycetales, Debaryomycetaceae, Candida/Lodderomyces clade, Candida
SH3-ITS1	Candida glabrata Eukaryota, Fungi, Dikarya, Ascomycota, Saccharomycotina, Saccharomycetes, Saccharomycetales, Saccharomycetaceae, Nakaseomyces, Nakaseomyces/Candida clade
SH4-ITS4	Candida glabrata Eukaryota, Fungi, Dikarya, Ascomycota, Saccharomycotina, Saccharomycetes, Saccharomycetales, Saccharomycetaceae, Nakaseomyces, Nakaseomyces/Candida clade

SH-ITS: Shaygan-Internal transcribed spacer

>**SH1-ITS1-**

TGCTTTGGATTTGATTATTGCCCCATGTGTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAGCCTGCCGCCAGAGGTCTA
AACTTACAACCAATTTTTTATCAACTTGTCACACCAGATTATTACTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATC
GATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCATATATTCGTGAATCATCCAATCTTTGAACGCACATTGCGCCCTCT
GGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGC
TTGAAAGACGGTAGTGGTAAGGCGGGATCGCTTTGACAATGGCTTAGGTCTAACCAAAAACATTGCTTGCGGCGGTAACGTCTAC
TACGTATATCTTCAAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAACATATCAATAAGCGGAGGAATTGATTTATTT
CGTTTTCCCTAATAAAATGCGCTACAGGGCTTTCGGGCGGTGTCATCCGTCTGATTTGATTACTCCAGCCCAATCCAGCTACCACA
TTGTATAGGTATCAGCTTCAGTGTCCAGGAGAAGAGTGGAACTCACACTGTAGACGTGCAATTCGTACATACGTCGAGGACACCA
GGGGCAAGGGCCACTCTCTCCTCTGTACTTACTCTTATAAGCACATCCTCGCGACCATCTTGACCCTGTACACTGCTATTCACCAT
ATAAATATAATCCTCTTGA

>**SH4-ITS4-**

CGATTGTGGACCCCTCCTGATTTGAGGTCAACTTAAGACGTCTGTCTGCCCAGGGGGGTTAAAACACTCACTTATCCCTCCCTAG
GGTGACACCGAGTTGGTAAAACCTAATCCCCTATTAACCCCCGCCGCTCATGCAAACGAATTCCAGATTAACACAGAAGCTTGCG
CTCGTGTCCTCCATACTGATATGGCCTATGTGTTCTTGTTAACTCCCAAACGAGAGTATCACTCACTACCAAGCCCATTGTGTTTGA
GAAGGAAATGACGCTCAAACGGGCATGCCCACCGGAATACCTCACGGCCCTCTGTGCGTTCGTAGATTCGATGATCCACGGAATT
CCGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCACCAGATCCATTGTTGAAAGTTTTGAAGTTGT
TTTCTACTAAAAGAAATCTTGTGTTGACTGAATTAGTTTAAAAAAATATATGTTTGTGTTTGCATCCACTGGGAGAACTCCCCCCCCT
TGAGAAAAATTCCCCCCACGCACCAAGGAATTTGTGGGAAGCCAACCTCCCCGTTTGTGTTTTATTTTAAAGAGGCCAATCCCCGG
GATTAAAAGGCTTCCTTGGAAGTTTAAAGATCAAAGGGCCGCCCCAGCCTTTGGTTGCTCCCTTCCCCCACTGGCGAAGAAACAA
CAAGGGTAGAATTAAGCGCCGCCTGAACACATGGCTTCCAAGCAGAAAAAATATACAGGAAACTCCCGAACCGCTGCTATGGTCC
CACACCAAGAAAGGGCTATCTCCCCGACTCAAGAAAATCCACTACACTTCCTCTTATGGAACCCTTCCCC

>**SH5-ITS1-**

GGTGCGCATTTAACTCATTTGTCTGAGCTCGGATAGAGAATCCCTGGGGACGACCAGTGTTACACTCCTGAGGTTCCTAAAAAATT
TTCTCTGCTGTGAATGCAATTTCTCCTGCCGGGGCATGCGGGCCCGGTTGGTGGGCGTTTCCCTCTGGGGGGAGGGAACTTACA
AAGAGCTGTGAGAGTGGGTGGATCTCTTGTGGCATTGGGAGGTGTTTTATCACTTCAGGCCCTTTTTCTAATCCCTTGACACAGGG
GAGTTTCCTTTGCTACAATGCTTTTGATCATTGGGGGACCTCCCCCTTTCCGGGGGGGGAGTCCCCCCCAGGACCTTACACTGCC
CAACTGTATTTTGAAAACTATCATCAAGGGGTGGTTTCGTTTTTTTACTTAACCCAACATCACTTTATACGAGCGGACCACTGCCAT
GCACCACCTGTAACTCTGCCCCCGAAGGGGACTTCCTATCTCTAGTATTGTAATACCATGACCCCACCTGGTTTTGATCTTCCTTTC
CCCCCCCGGTAATCCGGGGGGCCCGCCTGTTTGGGCGGCCCCTCCCTTCTCATTTAAGTTTTGTTTGGGCTACCTGAACCCCCC
CGTTTTTTGATTAACTGCGTTATTGTCGCCAATAAAGGACGGAAACACCCCTGAACACTTAGTTTTCTTATTTTACGGCGGGAACTA
GCAGGATGGGGGAGGCGTGTTCGCTCCCTACTAGTTTCTCACCTCCCGTGATTAACGACGGAAGAGACAGCATCCGTCTCTTGTG
CTTCCTCCACATCACAAACTATTCACGCTAGGACGTAGATTCCGCTCGTCTTCTCCTGTTACACTCTACCCCTCTCTCAGCCGGAA
GCAACGGCGGTGGAGTCGGATGGTATTGAAATTCTTATGAAACCAGACGTCCGGC

>**SH6-ITS4-**

AGAAACTTGGGAATCCTACTGATTTGAGGTCAAGTTTGAAGATATACGTAGTAGACGTTACCGCCGCAAGCAATGTTTTTGGTTAG
ACCTAAGCCATTGTCAAAGCGATCCCGCCTTACCACTACCGTCTTTCAAGCAAACCCAAGTCGTATTGCTCAACACCAAACCCAGC
GGTTTGAGGGAGAAACGACGCTCAAACAGGCATGCCCTCCGGAATACCAGAGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACGAATATCTGCAATTCATATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTT

Figure 1. Sequence determined for the studied samples to identify the Candida species

Candida is the second most common cause of vaginal infections in the United States (US); 75% of women have had Candidal vulvovaginitis at least once, and approximately 40% to 50% have had a second experience of the infection during their reproductive years. A small number of this group (about 5%) also had recurrences of Candidal vulvovaginitis, and it is estimated that 10% to 20% of them have acute vulvovaginitis with symptoms and complications.[1,10]

66.67% of isolates in our study belonged to the C. albicans species and 33.33% belonged to C. glabrata. The most common yeasts isolated from the vagina are primarily C. albicans and then C. glabrata.[11,12] More than 200 strains of C. albicans have been identified, all of which are capable of colonization and can cause vaginitis. 25% to 40% of women have a positive Candida culture from a vaginal specimen.[13,14] Fortunately, molecular techniques make it possible to properly differentiate C. albicans from other species; therefore, accurate identification of species is essential for effective antifungal treatment given knowledge of resistance to antifungal drugs.[13] PCR in our study showed that out of

15 isolates, 10 isolates were positive for C. albicans, and all 5 isolates were C. glabrata (same phenotypic test). Different methods have been used to identify C. albicans and differentiate them from other species based on phenotypic and genotypic characteristics and differences in the sequence of specific pieces of DNA.[15] Several DNA-based methods such as karyotyping, specific sequence detection using probes, fingerprinting, restriction fragment polymorphism analysis, and replication of specific regions of DNA by PCR have been used to identify Candida species in clinical specimens.[16] Gupta et al. using morphological methods, chlamydoconidia formation on CMA, and colony examination in Candida chromium agar medium concluded that C. albicans was the main cause of leukoplakia and oral squamous cell carcinoma (OSCC).[17] Hedayati et al. found that, out of 234 patients with vulvovaginitis, 66 (28.2%) patients showed Candidal vulvovaginitis. Of these patients, 16 (24.2%) had recurrent Candidal vulvovaginitis. The age group of 20-29 years old had the highest frequency of Candidal vulvovaginitis (48.5%).[10] Falahati et al., in the study of drug resistance patterns in Candida species isolated from patients with vaginitis, reported that out of 150 tested samples, a total of 80 cases of Candidal vulvovaginitis were caused by C. albicans.[18] Mahmoudi Rad et al., by examining the prevalence of Candida species in patients with Candidal vulvovaginitis, concluded that the most common cause of recurrent and non-recurrent Candidal vulvovaginitis was C. albicans and then C. glabrata.[19] Gharaghani et al., by identifying Candida species in patients with Candidal vulvovaginitis using PCR-restriction fragment length polymorphism (RFLP) method, showed that cultures were positive for 160 (51.6%) vaginal samples. C. albicans (86.8%) was the most common species among the isolates, followed by C. glabrata (3.77%) and C. krusei (3%). Multispecies with two Candida were identified in nine patients.[20] Phenotypic methods are easy and inexpensive, but the results are general, and in some cases, unreliable. On the other hand, the use of these methods is usually time-consuming, and obtaining false positive or negative results in them is inevitable, because, in this type of study, it is possible to create a switching phenotype in Candida species. Therefore, newer methods of molecular techniques have been considered. Genotypic methods have always been more reliable.[21] Although the use of these techniques requires more cost than phenotypic methods, their accuracy, precision, and speed are undeniable. Based on the results of this study that are consistent with previous research, it can be concluded that one of the important reasons for recurrent Candidal vulvovaginitis is the incomplete removal of Candida from the vagina after antifungal treatment. It remains low in the vagina and leads to the patient treated. When the normal hormonal and physiological conditions of the host change, the colonization of the organism increases and causes new clinical symptoms in the individual.[22] Therefore, it is recommended that the use of antibiotics be avoided separately for treatment and reduction of the disease. Differences in the frequency of species between two different groups of the population can be due to the low effect of drugs or their inappropriate use. Therefore, further studies in patients with recurrent infection to investigate the possible drug resistance of isolated species in other parts of the country and evaluation of drugs used in these patients can determine the specific points of the epidemiology of this disease.

Conclusion

In this study, two types of fungi, C. albicans and C. glabrata, were isolated from samples of women with Candidal vulvovaginitis. The frequency of C. albicans was more than C. glabrata; therefore, C. albicans has a greater

role in creating Candidal vulvovaginitis. Molecular methods are also more accurate than direct and phenotypic methods, but the results of both methods can confirm this disease.

Conflict of Interests

Authors have no conflict of interests.

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