Original Article

Identification of Candida Species Associated with Blood Infection by Multiplex PCR and Phenotypic Characteristics

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Abstract

Background and Aim: The correct identification of Candida species is one of the most critical procedures in prognostic and therapeutic significance, allowing an early and appropriate antifungal therapy. This study aimed to evaluate multiplex PCR as a rapid diagnostic method and traditional phenotypic tests in identifying *Candida* species isolated from candidemia cases.

Methods: In this study, 38 *Candida* spp were isolated from culturing of human blood obtained from patients suspected to candidemia. The isolated species were evaluated by phenotypic and molecular methods including carbohydrate assimilation test, colony colour on CHROMagar Candida, chlamydoconidia production, germ tube formation and Multiplex PCR. Multiplex PCR was performed using specific primers of 4 common species. The results of multiplex PCR were compared with those obtained from phenotypic tests.

Results: According to multiplex PCR findings, the isolated *Candida* species were identified as *C. albicans*, *C. parapsilosis, C. glabrata*, and *C. tropicalis*. Phenotypic tests identified that 23 (60.52%), 8 (21.05%), 5 (13.15%), and 2 (5.26%) isolates belonged to *C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*, respectively, that were confirmed by multiplex PCR results. *C. albicans* and *C. parapsilosis* had the same carbohydrate assimilation pattern but were differentiated based on their colonies color on CHROMagar and the ability of *C. albicans* to produce chlamydoconidia and germ tube. *C. glabrata* (100%) and *C. tropicalis* (100%) assimilated trehalose and cellobiose, respectively.

Conclusion: Our study showed that the both phenotypic and molecular techniques provide appropriate information for identification of *Candida* spp from blood samples.

Keywords: *Candida* Species; Candidemia; Phenotypic Tests; Multiplex PCR

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Introduction

The genus *Candida* contains more than 400 asexual yeasts, although a small number of them cause infections in humans. This genus includes some opportunistic species causing various infections including candidiasis of the skin, nails, and mucous tissues (vaginal and oral - pharyngeal) as well as life - threatening invasive diseases including candidemia which is a bloodstream infection. *Candida* spp. is considered the fourth and third most common causes of nosocomial and catheter - related blood infections, respectively. In the United States, the mortality rate due to *Candida* infections has been reported to be 20-40% *C. albicans* is considered the main leading cause of invasive infections (3). In recent years, the emergence of non-albicans species has increased rapidly (1-4). Unfortunately, these species are associated with higher mortality rates and drug resistance in patients compared to *C. albicans*. Differences in characteristics such as drug resistance and pathogenicity between different *Candida* species may be due to phylogenetic distances between them.

Treatment of candidiasis varies according to the tissue involved, underlying diseases, risk factors, patients' immune condition, and the kind of species involved. Identifying the species that cause *Candida* infections is essential because it is better to prescribe appropriate drugs according to the drug sensitivity pattern of each species (1). Unfortunately, in most cases in Iran, treatment is still administered without identifying the type of species. Nowadays, the identification of *Candida* species is done using different methods that differ in terms of performance, differential power, and cost. In general, conventional methods such as serological tests, colony appearance examination, and chemical and morphological tests were used to separate and identify *Candida* species. To identify yeasts in clinical laboratories, a germ-tube formation test is usually performed first. Then other tests are used, such as carbohydrate fermentation and assimilation, chlamydoconidia production, and colony characterization on CHROMagar Candida which is considered a chromogenic media for rapid identification of Candida spp. The color of *Candida* spp. colonies can differentiate the species from each other (5, 6). Carbohydrate assimilation assay evaluates the ability of each isolate to utilize a specific carbohydrate as the only carbon source (7). Molecular techniques have overcome the limitations of phenotypic tests in recent years. Polymerase chain reaction (PCR) is used in many countries of the world due to its cost-effectiveness and high reproducibility (8). Multiplex PCR is practical in distinguishing various yeast species according to distinctive banding patterns. In this technique, several specific primers are used in a PCR reaction, so in the presence of various yeast species, the identification is performed accurately, quickly, and simultaneously. Many multiplex PCR studies consider several bands for unique yeast but if there are multiple species of yeast in a sample, it becomes difficult to interpret the results of several bands (9, 10). Despite the using molecular techniques for the identification of *Candida* spp. from clinical samples, phenotypic properties provide appropriate information for the identification of *C. albicans* and non - albicans species.

Accurate and rapid identification of candidemia agents is important in terms of treatment and prevention and determines the correct type of treatment so that delay in diagnosis is life - threatening. This study aimed to evaluate combined multiplex PCR and phenotypic methods in identifying *Candida* species isolated from candidemia cases.

Methods

Sample preparation

Samples were obtained from 38 candidemia cases identified in the Imam Khomeini hospital complex (where blood sampling is a part of the standard diagnostic protocol for candidemia) during October 2019 to March 2020. This study was confirmed by the Ethics Committee of Tarbiat Modares University, Tehran, Iran (Code: IR.MODARES.REC.1398.103). Patients were in different wards of the hospital and of both sexes with an age range of 1-90. Blood samples were cultured on SDA (sabouraud dextrose agar, Merck, Germany) medium containing 50 mg/L chloramphenicol, and incubated at 37°C for 48 hrs. After the incubation period, all yeast colonies were harvested and stored in 20% glycerol for subsequent differential tests.

Chromogenic agar culture

0.5 ml of blood samples from suspected candidemia patients were cultured on CHROMagar Candida medium (CHROMagar Candida Company, Paris and France) under the recommendations of the manufacturer. The media were incubated at 37°C for 48 hrs. *Candida* spp was identified in accordance with the manufacturer's instructions based on the color and appearance of colonies (7, 11).

Germ - tube formation test

Each isolate was inoculated into a sterile tube containing fetal bovine serum (0.5 mL) (FBS). After incubation at 37°C for 2.5 hrs, the tubes were microscopically examined. The germ tube is a narrow tube in which there is no septum or compression at the junction between cells. The formation of germ tubes is characteristic of *C. albicans* and *C. dubliniensis* species. *C. albicans* 10231 and *C. glabrata* 90030 were used as positive and negative controls, respectively (7, 12, 13).

Chlamydoconidia production test

To perform chlamydoconidia production test, grown colonies on SDA were seeded in three parallel lines on a fragment of Tween 80 - enriched cornmeal agar medium located between two slides. After incubation at a temperature of 30°C for 72 hrs in a moist chamber, microscopic observation was performed. Double - walled round spores were considered chlamydoconidia. Chlamydoconidia production is characteristic of *C. albicans* and *C. dubliniensis* (14, 15).

Carbohydrate assimilation test

Yeast nitrogen base (YNB, Difco) medium was used to perform the carbohydrate assimilation test. From 24

to 48 - hour culture of each isolate, yeast suspension was made in 2 mL of YNB medium by inoculating a large amount of colony. The carbohydrate disks including galactose, xylose, sucrose, trehalose, and cellobiose were then placed at a suitable distance from each other on the plates containing the culture medium. After incubation at 37°C for 3 to 4 days, the presence or absence of growth around each carbohydrate indicated its absorption and assimilation (7, 16).

Fungal growth at 45 °C

The ability to grow at 45°C is a reliable test for differentiating the two species *C. albicans* and *C. dubliniensis* from each other. *C. albicans* strains can grow at 45°C unlike *C. dubliniensis* (17).

Molecular identification of *Candida* spp. by multiplex - PCR assay

The phenol - chloroform method previously proposed by Esfahani et al. to extract DNA from yeast cells (18). Briefly, 10 µl of fresh yeast colony was added to 100 µl of glass beads, 300µl of phenol chloroform/ isoamyl alcohol, and 300 µl of lysis buffer (0.5% sodium dodecyl sulfate, 25 mM EDTA, 250 mM NaCl, and 200 mM Tris-Hcl). After centrifugation for 5 min at 10000 ×g, the supernatant was moved to another tube containing sodium acetate (0.1ml), isopropanol, and chloroform (300 µl). The tube was then placed at -20°C for 10 minutes. The next step was

centrifugation at $12000 \times g$ for 15 minutes. The pellet was washed using ethanol 70%, and 50 µl of distilled water was added. The sequence of primers for multiplex PCR is shown in Table 1 (8). The multiplex PCR mixture included 0.5 µM of each of the specific primers for *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis* complex, and *C. tropicalis*, 25 µl of Master mix (SinaClon BioScience Co., Karaj, Iran), and 2 µl genomic DNA template in a final volume of 50 µl. PCRs were carried out in a thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Foster City, CA, USA), and cycling conditions included a step at 95˚C for 5 min (beginning denaturation), followed by 35 cycles of 95˚C for 30s (denaturation) and 60˚C for 30s (annealing), 72 ˚C for 30s (extension) and 72 ˚C for 8 min (extension end). Products were separated by electrophoresis in a 2% agarose gel together whit Gel Red (BioTium Corporation, USA). After electrophoresis, the bands were observed and examined using a UV light. The species identification was possible by comparison with a 100-bp DNA ladder (Fermentas, USA). All tests were performed in triplicate. Positive controls in this study included: *C. albicans* 10231 (606 bp), *C. glabrata* 90030 (212 bp), *C. tropicalis* 13801 (126 bp), and *C. parapsilosis* 22019 (490 bp). A specific band was detected for each species.

Table 1. The primer sequences applied for multiplex PCR

Candida spp.	Primers	Sequence (5'to3')	Amplicon size (bp)	Ref
C. glabrata	IGS 1- Fcalb	5'-AGATTATTGCCATGCCCTGAG-3'	606	[8]
	IGS 1- Rcalb	5'-CCATGTCGAACGTAGCGTAT-3'		
C. glabrata	Hypothetical protein- Fcgla	5'- ACCGTGCTTGCCTCTACA-3'	212	[8]
	Hypothetical protein- Rcgla	5'-GACATCTGAGCCTCGTCTGA-3'		
C. parapsilosis	Plp 1- Fcpara	5'- TACACCAAGCGACTCAGC -3'	490	[8]
	Plp 1- Rcpara	5'-ACCAGCTGCTTTGACTTG-3'		
C. tropicalis	IGS 1- Fctro	5'-AGAACAAGAAAACAGTGAAGCAA-3'	126	[8]
	IGS 1- Rctro	5'-CCATGTCGAACGTAGCGTAT-3'		
C. dubliniensis	IGS 1- Fcdub	5'-GTCGGACATATACCTCCAACTC-3'	718	[8]
	IGS 1- Rcdub	5'-CCATGTCGAACGTAGCGTAT-3'		

R: reverse, F: forward, calb: *C. albicans*, cgla: *C. glabrata*, cpara: *C. parapsilosis*, ctro: *C. tropicalis*, cdub: *C. dubliniensis*

Results

In this study, 38 *Candida* isolates collected from candidemia cases were identified phenotypically based on their colonies morphology and color on CHROMagar Candida, chlamydoconidia production, germ-tube formation, carbohydrate assimilation, and

genotypic method by multiplex PCR. The colonies developed on CHROMagar Candida had completely different colors, and this difference was more visible after 48 hours of incubation (Figure 1). Colonies that appear as green on CHROMagar Candida medium belong to *C. albicans* or *C. dubliniensis;* however, in

this study, all of them were identified as *C. albicans* (100%) due to their ability to assimilate xylose and grow at 45°C. *C. parapsilosis* isolates (100%) produced pink colonies, *C. glabrata* isolates (100%) produced purple colonies, and *C. tropicalis* isolates (100%) produced blue colonies.
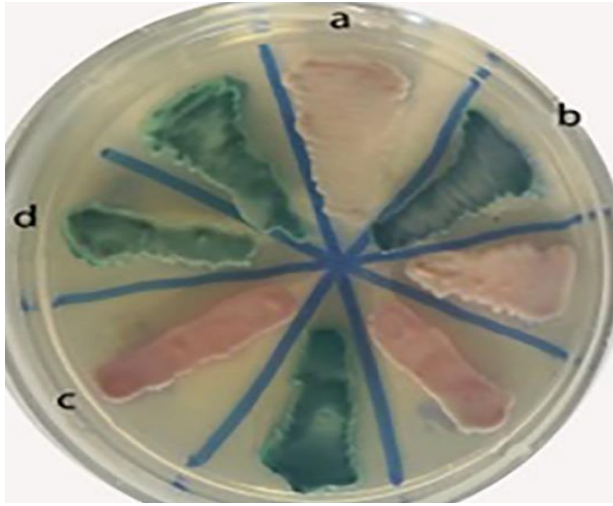

Figure 1. Identification of *Candida* species by CHROMagar Candida medium based on the color and appearance of colonies; (a) *C. parapsilosis*; pink, (b) *C. tropicalis*; metallic blue , (c) *C. glabrata*; mauve and (d) *C. albicans*; green.

Overall, out of the 38 isolates examined, 23 (60.52%) strains were positive for germ-tube production (Figure 2a). Moreover, these 23 isolates were able to produce chlamydoconidia on cornmeal agar (Figure 2b) and green colonies on CHROMagar Candida, indicating the presence of *C. albicans*.
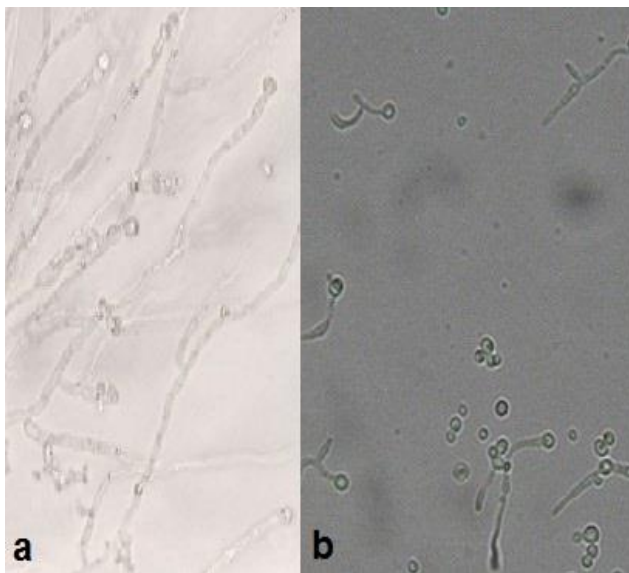

Figure 2. Chlamydoconidia (a) and germ tube (b) formation assay as a differential method for identification of *C. albicans*

In this study, 31 isolates had the same pattern in the carbohydrate assimilation test, among them eight isolates produced pink colonies on CHROMagar Candida but were unable to produce germ tubes and chlamydoconidia, which were identified as *C. parapsilosis.* Among 31 isolates with a similar assimilation pattern, 23 isolates produced green colonies on CHROMagar Candida medium, which were then identified as *C. albicans* due to xylose assimilation and growth at 45 °C (Figure 3, Table 2). Also, two (5.26%, 5 of 38) isolates produced blue colonies on CHROMagar Candida but were unable to produce germ tubes and chlamydoconidia, which were identified as *C. tropicalis.* The carbohydrate assimilation pattern also confirmed these two isolates as *C. tropicalis* species. *C. tropicalis* isolates had the same carbohydrate assimilation pattern. Moreover, 13.16% (5 of 38) of the studied strains produced purple colonies on CHROMagar Candida but were only able to assimilate trehalose, which was confirmed as *C. glabrata*. The carbohydrate assimilation pattern was the same in 100% of *C. glabrata* strains.
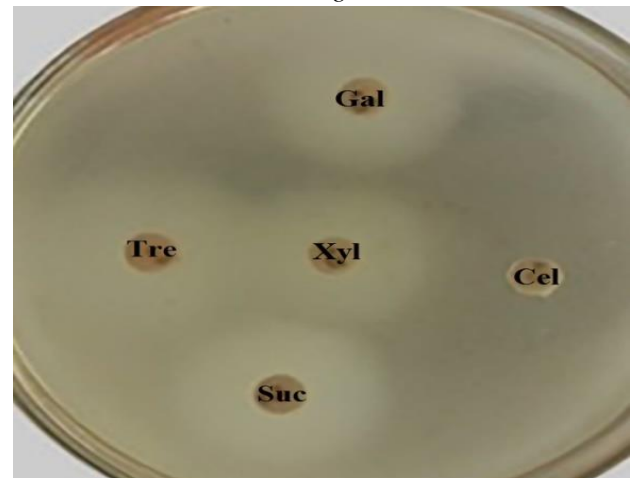

Figure 3. Carbohydrate assimilation assay in *C. albicans*; sucrose (Suc), cellobiose (Cel), trehalose (Tre), xylose (Xyl), and galactose (Gal).

38 candida isolates were correctly and accurately identified using multiplex PCR compared to phenotypic techniques. This method identified 23 isolates as *C. albicans*, so using special primers designed for this species, the product band length was 606bp. Multiplex PCR identified 8, 5, and 2 isolates as *C. parapsilosis* (490 bp), *C. glabrata* (212 bp), and *C. tropicalis* (126 bp), respectively (Figure 4, Table 3). Due to the observation of the specific band of *C. albicans* following multiplex PCR and the results of phenotypic tests, among the 38 isolates studied, there were no isolates of *C. dubliniensis*.

Table 2. Identification of *Candida* species using selective CHROMagar Candida, carbohydrate assimilation, chlamydoconidia production, and germ-tube formation methods

Number	CHROMagar Candida	GT	CH	CA					Identified Candida spp.
				Gal	Xly	Suc	Tre	Cel	
1	Blue	-	-	+	+	+	+	+	*C. tropicalis*
2	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
3	Purple	-	-	-	-	-	+	-	*C. glabrata*
4	Green	+	+	+	+	+	+	-	*C. albicans*
5	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
6	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
7	Purple	-	-	-	-	-	+	-	*C. glabrata*
8	Purple	-	-	-	-	-	+	-	*C. glabrata*
9	Green	+	+	+	+	+	+	-	*C. albicans*
10	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
11	Green	+	+	+	+	+	+	-	*C. albicans*
12	Blue	-	-	+	+	+	+	+	*C. tropicalis*
13	Green	+	+	+	+	+	+	-	*C. albicans*
14	Green	+	+	+	+	+	+	-	*C. albicans*
15	Green	+	+	+	+	+	+	-	*C. albicans*
16	Green	+	+	+	+	+	+	-	*C. albicans*
17	Green	+	+	+	+	+	+	-	*C. albicans*
18	Purple	-	-	-	-	-	+	-	*C. glabrata*
19	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
20	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
21	Green	+	+	+	+	+	+	-	*C. albicans*
22	Green	+	+	+	+	+	+	-	*C. albicans*
23	Green	+	+	+	+	+	+	-	*C. albicans*
24	Green	+	+	+	+	+	+	-	*C. albicans*
25	Green	+	+	+	+	+	+	-	*C. albicans*
26	Green	+	+	+	+	+	+	-	*C. albicans*
27	Green	+	+	+	+	+	+	-	*C. albicans*
28	Pink	-	-	+	+	+	+	-	*C. parapsilosis*
29	Green	+	+	+	+	+	+	-	*C. albicans*
30	Green	+	+	+	+	+	+	-	*C. albicans*
31	Green	+	+	+	+	+	+	-	*C. albicans*
32	Green	+	+	+	+	+	+	-	*C. albicans*
33	Green	+	+	+	+	+	+	-	*C. albicans*
34	Green	+	+	+	+	+	+	-	*C. albicans*
35	Green	+	+	+	+	+	+	-	*C. albicans*
36	Green	+	+	+	+	+	+	-	*C. albicans*
37	Purple	-	-	-	-	-	+	-	*C. glabrata*
38	Pink	-	-	+	+	+	+	-	*C. parapsilosis*

Carbohydrate assimilation (CA), Chlamydoconidia production (CH), and Germ-tube formation (GT), Carbohydrates abbreviation: sucrose: Suc, cellobiose: Cel, trehalose: Tre, xylose: Xyl, and galactose: Gal

Table 3. Identification of *Candida* species from blood samples based on phenotypic characteristics and Multiplex-PCR

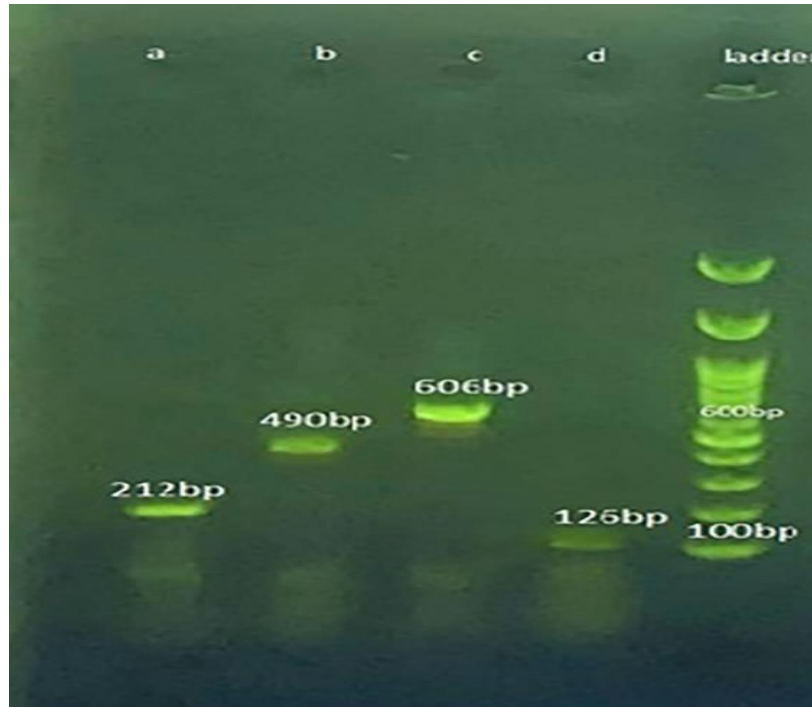| *Candida* species (N)	CHROMagar Candida (%)	Carbohydrate assimilation (%)	Germ - tube formation (%)	Chlamydoconidia production (%)	Multiplex - PCR
C. albicans (23)	23 (60.52)	23 (60.52)	23 (60.52)	23 (60.52)	23 (60.52)
C. parapsilosis (8)	8 (21.05)	8 (21.05)	0	0	8 (21.05)
C. glabrata (5)	5 (13.15)	5 (13.15)	0	0	5 (13.15)
C. tropicalis (2)	2 (5.26)	2 (5.26)	0	0	2 (5.26)

Figure 4. Identification of *Candida* species using Multiplex - PCR; a) *C. glabrata* (212 bp), b) *C. parapsilosis* (490 bp), c) *C. albicans* (600 bp) and d) *C. tropicalis* (126 bp).

Discussion

Candidemia caused by *Candida* spp was considered the fourth and sixth causes of nosocomial bloodstream infections in the world. The disease is associated with increased morbidity and mortality in patients with predisposing factors, such as cancer, corticosteroid and chemotherapy, transplant recipients, and patients with human immunodeficiency virus (HIV) (1). *C. albicans* accounts for more than 80% of all *Candida* strains isolated from blood, but the emergence of non-*C. albicans* species is worrying due to the inherent resistance of many of them to antimicrobial drugs. Therefore, identifying the species

causing candidiasis plays an important role in selecting an appropriate drug (9, 12).

The phenotypic techniques used to differentiate and identify *Candida* species are simple and inexpensive, although they have some limitations. *C. dubliniensis* strains have been identified as *C. albicans*, because both species share similar phenotypic characteristics such as formation of germ-tubes, production of chlamydoconidia in rice agar, biochemical pattern and same color of colonies on differential media CHROMagarTM Candida (7). The use of germ tube tests to identify *C. albicans* from other species is simple and cost-effective. However, differentiating pseudohyphae from germ tubes requires experience and high accuracy. In addition, identifying *C. albicans*

species with the help of germ tube requires large amounts of material. Some studies have reported the sensitivity of this test between 93 and 98.8% and its specificity between 73.3 and 100% (19, 20).

Marinho et ail. reported that 10% of *C. albicans* strains are unable to produce chlamydospores (7). Sullivan et al., showed that some atypical *C. albicans* strains are unable to produce chlamydoconidia (21). In this study, chlamydoconidia production was observed in all *C. albicans* isolates (60.52%), which produced green colonies on CHROMagar Candida. Although chlamydoconidia are not commonly produced by *C. dubliniensis*, if produced, they are abundant and usually in the form of triplets or adjacent pairs. The same result could be observed in *C. albicans* (21, 22).

Chromogenic media are cost - effective compared to other methods in terms of rapid detection of *Candida* species, easy preparation, and cost. CHROMagar Candida sensitivity and specificity have been reported to be 100 and 98.3% for *C. parapsilosis*, 96.9 and 97.9% for *C. albicans*, 98.5% for *C. tropicalis*, and 100% *C. krusei* and *C. glabrata* respectively (5). Nadeem et al. showed that this medium was able to identify most *Candida* species with high specificity and sensitivity, including *C. albicans*, *C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* (5).

In contrast to findings from Hospenthal's study, CHROMagar Candida was able to distinguish between *C. glabrata* species with purple colonies and other *Candida* species forming pink colonies. However, in line with the results of other studies, the green colonies of C. albicans species were indistinguishable from those of C. dubliniensis (7, 23).

All strains that formed a green colony on CHROMagar Candida grew well at both 37 and 45°C. Accordingly, there was no *C. dubliniensis* among the isolates. Sullivan et al. also confirmed the lack of growth of *C. dubliniensis* species on SDA at 45°C, unlike *C. albicans* (24).

Nevertheless, Gales et al. showed that some *C. albicans* strains could not grow at 45°C. A possible reason can be attributed to false-negative observations in identifying *C. dubliniensis* despite the advantages of this test, such as simplicity, cost - effectiveness, and reproducibility (25).

The carbohydrate assimilation test is a reliable technique for identifying yeasts isolated from clinical specimens, especially in laboratories that lack equipment and materials (26, 27). It should be noted that this test is not very useful in differentiating *C. albicans* from *C. dubliniensis* since the carbohydrate

assimilation pattern in both species is similar, however, the absorption pattern of xylose in *C. dubliniensis* is different (28). Other studies reported the disability of *C. dubliniensis* to absorb xylose compared to *C. albicans* (29, 30).

Here, we found that five (13.15%) isolates were only able to absorb trehalose. According to the carbohydrate assimilation pattern in previous studies and their colonies' purple color on CHROMagar Candida these isolates were finally identified as *C. glabrata* species. The carbohydrate assimilation pattern identified that *C. tropicalis* isolates can absorb cellobiose in addition to sucrose, trehalose, xylose, and galactose. In another study, 100% of *C. tropicalis* isolates absorbed cellobiose (27).

In our study, traditional phenotypic tests revealed acceptable specificity and sensitivity. And all of these tests can be practical in differentiating *Candida* species, although there are limitations to the ability of some of them to differentiate species. However, the differential power of available tests can be increased by using several phenotypic methods simultaneously for the identification of *C. albicans* and non - albicans species.

In the present study, we used multiplex PCR for the recognition of 4 main species of *Candida* genus causing candidemia. Using multiplex PCR and specific primers of each of the four *Candida* species, all 38 isolates were identified. According to previous studies, more than one yeast species has been isolated in 5% of candidemia cases. Fortunately, the multiplex PCR technique can identify all species in a single sample at the same time (31).

Because the use of carbohydrate assimilation, colony formation on CHROMagar Candida, germ tube, and chlamydospore production cannot differentiate *C. albicans* from C. *dubliniensis* and the only phenotypic differential test between these two species based on conventional methods was the lack of growth of C. *dubliniensis* at 45°C. The multiplex technique helped confirm the absence of this species in our study.

Previous studies revealed that the multiplex PCR technique is simple, fast, and accurate and does not require expensive and poisonous materials. In recent years, molecular techniques have been widely used to identify the species causing candidiasis, but these methods have not yet become routine due to the need for expensive equipment and professional personnel.

However, sometimes the use of molecular techniques is necessary for accurate identification, such as epidemiological investigation, the distinction between

C. albicans and *C. dubliniensis* species, and repeated candidiasis (32).

Conclusion

In general, identification and differentiation of *Candida* spp. is possible using a set of phenotypic and genotypic techniques. Today, traditional techniques still have a particular place in identifying *Candida* species, especially in the absence of the necessary equipment for molecular methods. Also, they are time - consuming compared to molecular techniques. Multiplex PCR technique in identifying *Candida* species seems to be cost - effective, accurate, and reliable. The concomitant use of molecular techniques with phenotypic characteristics can provide appropriate information for the identification of *Candida* spp.

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Not declared.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethics

This study was approved by the Ethics Committee of Tarbiat Modares University, Tehran, Iran, with the Ethics Code: IR.MODARES.REC.1398.103.

Authors' contribution

M. Shams-Ghahfarokhi conceived, designed, and coordinated the study, B. Fallah carried out the experiments and writing the first version of the manuscript. M. R. Salehi contributed to the preparation of the clinical samples. All authors approved the article final version for publication.

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