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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

Molecular identification of clinical Candida isolates by simple and randomly amplified polymorphic DNA-PCR

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Abstract Thirty yeast isolates were obtained from specimens taken from different patients (sputum, vaginal and oral swabs). The isolates were identified by phenotyping and molecular methods. Six of the isolates were *Candida albicans*, identified by germ tube and CHROMagar. Three isolates were identified by CHROMagar as *C. krusei*. The remaining (21) clinical yeast isolates included *C. sphaerica*, *C. guilliermondii C. kefyr*, *C. famata*, *C. glabrata*, *C. parapsilosis* and *C. norvegensis* identified by Vitek2 and further confirmed by PCR. RAPD-PCR was also used in an attempt to identify DNA "fingerprints" for specific *Candida* spp. The DNA fingerprints of all *Candida* spp. except for *C. sphaerica* were determined.

Keywords Clinical *Candida* spp. · Phenotyping identification · Molecular identification PCR · RAPD-PCR

1 Introduction

Candida is a genus of yeasts, which includes many different species, such as *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. parapsilosis, C. kefyr, C. guilliermondii, C. famata, C. sphaerica* and *C. norvegensis* that are implicated in human disease (candidiasis) [1]. *C. kefyr, C. famata* and *C. norvegensis* although rarely recovered are now their isolation rate which has increased between twofold and tenfold over the last 15 years [2]. There are several types of candidiasis including mucosal candidiasis, cutaneous candidiasis and systemic candidiasis, and the incidence of all of these has increased due to a variety of factors such as more people living with HIV, the excessive use of antibiotics, organ transplantation and the use of invasive devices (catheters and artificial joints) [3]. The correct identification of *Candida* spp. is of great significance if this rise in *Candida* infection rates is to be addressed through the use of appropriately targeted treatments to comprehend the several ways by which *Candida* spp. avoid antifungal therapy and the immune response to the host causing infection [4]. Several methods have been used to identify yeasts, including phenotypic tests such as culturing and microscopic examination of the culture characteristics based on their colony appearance; germ tube tests which identify *C. albicans* on the basis of its ability to form germ tubes in serum; CHROMagar Candida that can be used for the identification of*C. albicans* and *C. krusei* based on strongly contrasted colors; the biochemical reaction-based Vitek 2 ID-YST system; and newer molecular methods which allow accurate yielded results and fulfilled in several hours compared with conventional techniques [5]. PCR-based techniques have been adapted as tools for clinical diagnosis of candidiasis targeting the DNA topoisomerase II encoding gene which is suitable as a target gene for identification of pathogenic *Candida* spp. [6]. Consequently, the aim of the current study was the evaluation of molecular methods in comparison with phenotypic methods, especially with respect to their speed and reliability of the identification. In this regard, a particular focus was RAPD-PCR, which has been previously used to detect DNA polymorphism of

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different *Candida* spp. in order to search for the source of differences that could be used as a DNA marker specific for each *Candida* spp. when electrophoresed in agarose gel, which potentially allows the identification of pathogenic *Candida* spp. The RAPD-PCR reaction was repeated on a set of DNA samples with several different primers, under PCR optimum conditions of programming and reagent concentration so as to provide different RAPD patterns for each primer [7]. This assay has been increasingly used for rapid, easy identification, without prior knowledge of the target's sequence [8].

2 Materials and Methods

2.1 Sample Collection

Thirty specimens were collected from patients between 17 and 50 years old attending Al-Yarmuk General Teaching Hospital suspected to have candidiasis as clinically identified by a physician; these included 7 sputum samples along with 13 vaginal and 10 oral swabs. Each sample was placed in a sterile tube containing transport media (Amies transport media) till reaching the laboratory [9].

2.2 Culture of Samples

All samples were inoculated on suitable culture media Sabouraud dextrose agar SDA (Himedia/India) and incubated for 3 days at 37 °C [9].

2.3 Yeast Identification

Single colonies were isolated from primary positive cultures and identified according to the criteria of [9] in the following tests:

2.3.1 Cultural Characteristics

The shape, color and texture of colonies were examined on Sabouraud dextrose agar [10].

2.3.2 Microscopic Test

The shape of the cells was examined microscopically after staining with crystal violet [11].

2.3.3 Germ Tube Test

The inoculum of yeast cells obtained from an isolated colony was suspended in 0.5 ml of human serum in a small tube and then incubated at 37 °C for 2–3 h. A drop of serum was transferred to a slide, covered by coverslip, and then examined microscopically under an oil immersion lens for the presence of germ tubes [12].

2.3.4 CHROMagar Culture (Liofilchem/Italy)

The CHROMagar plates were inoculated by streaking the isolated colonies using a sterile loop. The inoculated plates were then incubated for 48 h at 37 °C. The growth and color of the colonies were observed [13].

2.3.5 Vitek2 Test (BioMerieux/France)

A sterile swab was used to transfer a sufficient number of colonies of a pure culture and then suspended in 3.0 ml of sterile saline (0.50% NaCl) in a clear plastic test tube. The turbidity was adjusted to 1.80–2.0 using DensiChek^{TM} [14].

Identification cards were inoculated with yeast suspensions using a vacuum apparatus. The test suspension tube was placed into the "cassette" while inserting the transfer tube into the suspension tube. The filled cassette was placed into a vacuum chamber station, and then, the yeast suspension was introduced into micro-channels to fill all the test wells. The results contained on the card were compared to an identification database allowing unknown organisms to be identified within 18 hr of incubation [15].

2.3.6 Molecular Identification

DNA extraction DNA was prepared and purified according to the genomic isolation kit provided by Geneaid Company/Taiwan (Cultured Cell Protocol Procedure Ver.11.21.13). Liquid nitrogen (−170 °C) was used with the commercial kit since the very low temperature helped to prevent DNase activation [16]. The Nanodrop system (BioDrop/ UK) was used for the measurement of the concentration and purity of the DNA according to [17] using 2 µl of each DNA sample. According to [18], adequate DNA concentration ranged between 50*and*200 ng/µl to ensure a good yield of the desired PCR products. Thus, DNA concentration was diluted to 50 ng/µl.

PCR protocol (Specific and RAPD Primers)

The specific primers and their sequences were chosen according to [19] Table 1, and RAPD primers were those commercially available, as given in Table 2.

The amplification reaction programs for specific PCR were [initial denaturation: 95 °C for 5 min, (denaturation: 95 °C for 1 min, annealing: 58 °C for 45 s, elongation: 72 °C for 1 min) 40 cycles, final elongation: 72 °C for 10 min], while RAPD-PCR was [initial denaturation: 94 °C for 5 min, (denaturation: 94 °C for 1 min, annealing: 36 °C for 2 min, elongation: 72 °C for 1 min) 40 cycles, final elongation: 72 °C for 10 min].

Table 1 Specific primers

Candida species	Primer's name	Sequence (5−3′)	Amplified fragment size (bp.)
C. albicans	CABF59	TTGAACATCTCCAGTTTCAAAGGT	515
	CABR110	GTTGGCGTTGGCAATAGCTCTG	
C. parapsilosis I	CPPIF41	TGACAATATGACAAAGGTTGGTA	228
	CPPIR61	ACTTTTAAAACTGTTAACCGA	
C. parapsilosis II	CPPIF41	GGACAACATGACAAAAGTCGGCA	310
	CPPIIR69	TTGTGGTGTAATTCTTGGGAG	
C. krusei	CKSF35	GAGCCACGGTAAAGAATACACA	227
	CKSR57	TTTAAAGTGACCCGGATACC	
C. kefyr	CKFF35	CTTCCAAAGGTCAGAAGTATGTCC	532
	CKFR85	CTTCAAACGGTCTGAAACCT	
C.guilliermondii	CGLF41	CCCAAAATCACAAAGCTCAAGT	205
	CGLR61	TACGACTTGAAGTTGCGAATTG	
C. glabrata	CGBF35	CCCAAAAATGGCCGTAAGTATG	674
	CGBR103	ATAGTCGCTACTAATATCACACC	

Table 2 RAPD primers

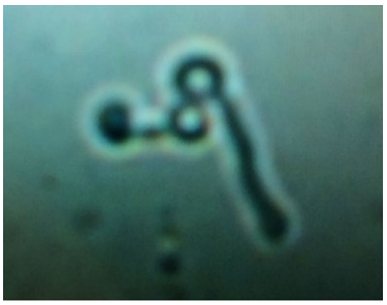
Primer name	Sequence (5−3′)
OPI_06	AAGGCGGCAG
OPL_05	ACGCAGGCAC
OPM_20	AGGTCTTGGG
OPE_16	GGTGACTGTT
OPQ_01	GGGACGATGG

PCR amplification was performed in a final volume of 20 µl. Each reaction consisted of 5 µl of the PCR ready mix (Bioneer/Korea), 11 µl of double distilled water (ddH2O), 2 µl of template DNA and 2 µl of each primer. Seven µl of PCR amplified products was electrophoresed on 1.2% agarose gel for Candida species PCR and 1.5% for RAPD-PCR (2 h 5 V/cm, 1X Tris-borate buffer). The DNA bands were visualized under UV light and photographed after staining the agarose gels with ethidium bromide (5 µg/ml).

3 Results and Discussion

3.1 Isolation and Conventional Identification of Yeast-Like Isolates

A total of 30 specimens of yeast-like isolates were obtained from patients between 17 and 50 years old. These specimens included sputum, vaginal and oral swabs. The colonies of the yeast isolate appeared on Sabouraud dextrose agar medium as raised, smooth, glabrous yeast and white to cream colored. Under microscopic examination with an oil immersion lens and crystal violet staining under the

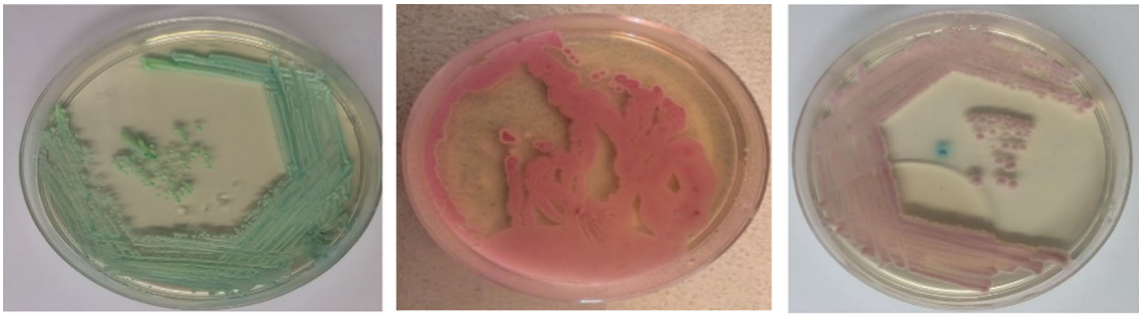


C. albicans

Fig. 1 Germ tube formation when incubated in serum at 37 °C for 2–3 h

blastoconidia of the yeast, isolates exhibited different morphological appearances, including subspherical, ellipsoidal, ovoid and elongated morphologies. These variable morphologies suggested the presence of many yeast cells, and it was therefore not possible to differentiate between yeast species. To distinguish *C. albicans* from other yeast isolates, the germ tube formation test using human serum was performed. Out of 30 isolates tested, only six isolates gave a positive germ tube result for *C. albicans* (a short hyphal projection arising laterally from a yeast cell with no constriction at the point of origin) (Fig. 1).

The formation of a germ tube is associated with an increase in protein and ribonucleic acid synthesis due to the presence of a stimulating substrate in the germ tube solution, i.e., human serum or some other nourishing media [15]. A differential culture medium (CHROMagar) was also used to distinguish *C. albicans* and *C. krusei* according to their contrasted colony color. The same six isolates that had the ability to produce a germ tube gave a green color in CHROMagar, again indicating *C. albicans*; of the remaining 24 yeast isolates, three showed a pink color, indicating *C. kru-*



(a) *C. albicans* (b) *C. krusei* (c) other yeast

Fig. 2 Growth of *C. albicans, C. krusei* and other yeast-like cultures on CHROMagar medium at 37 °C for 48 h. **a** *C. albicans*, **b** *C. krusei*, **c** other yeast

Table 3 Results of Vitek2 YST card system, the identification results for the yeast isolates are assigned to six confidence levels

Excellent identification	Very good identification	Good identification	Acceptable identification	Low discrimination	Unidentified organism
C. sphaerica	*C. sphaerica*	*C. sphaerica*	*C. famata*	*C. kefyr*	1
				C. sphaerica	
C. sphaerica		*C. sphaerica*	*Cryptococcus laurentii*	*C. famata*	1
				C. guilliermondii	
C. albicans		*C. albicans*		*C. famata*	1
				C. albicans	
C. albicans		*C. albicans*			1
C. albicans		*Cryptococcus laurentii*			1
C. norvegensis					1
C. parapsilosis					1
C. krusei					
C. krusei					
C. krusei					
C.guilliermondii					
C. glabrata					

Excellent identification with 96–99% probability, very good identification with 93–95% probability, good identification with probability 89–92%, acceptable identification with 85 to 88% probability, low discrimination and unidentified organism

sei and the remaining 21 a white to pink color indicating other yeast isolates (Fig. 2) [20]. The specificity and sensitivity of CHROMagar for a presumptive diagnosis of *C. krusei* and *C. albicans* has been evaluated as exceeding 99% in each case [21].

The presence of these colors is thought to be due to a reaction between the chromogenic substrate cleaved by species-specific enzymes, thereby creating the differences in colors for *C. krusei* and *C. albicans* [21]. According to [22], 6-chloro-3-indolyl β-D-glucopyranoside was a substrate used as a chromogenic medium to identify the presence of *Candida* spp. when reacted with β-glucosidase and converted to insoluble, colored products that precipitate onto the membrane. The resulting colored spot requires no special equipment for visualization. To confirm the *Candida* spp. that were identified previously based on phenotypic methods (germ tube and CHROMagar) and to identify the remaining 21 yeast isolates at the species level, a Vitek2 yeast card automated system based on biochemical tests was used. The identification results for the yeast isolates obtained from this system are assigned to six confidence levels: (i) excellent identification with 96–99% probability, (ii) very good identification with 93–95% probability, (iii) good identification with probability 89–92%, (iv) 85–88% with acceptable identification, (v) low discrimination and unidentified organism as shown in Table (3).

Of the 30 yeast-like isolates, three had low discrimination between *C. guelliermondii* and *C. famata*, *C. sphaerica* and *C. kefyr* likewise *C. famata* and *C. albicans*, 20/30 clinical isolates could be identified with high confidence (excellent, very good, good and acceptable confidence levels), and 7/30 isolates were unidentified. The difficulty of differentiating

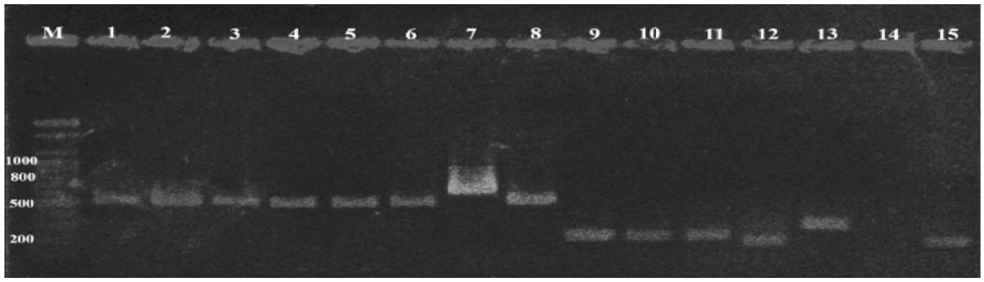


Fig. 3 Electrophoregram of amplified PCR products for Candida isolates. *M* indicates the lane containing the 100-bp molecular size DNA marker. *Lanes 1–6* for *C. albicans*, *lane 7* for *C. glabrata*, *lane 8* for *C. kefyr*, *lanes 9–11* for *C. krusei*, *lanes 12* and *15* for *C. guilliermondii*, *lane 13* for *C. parapsilosis* II and *lane 14* for *C. parapsilosis*

Table 4 The complete identification results obtained with Vitek2 and confirmed by PCR using the topoisomerase II encoding gene for pathogenic Candida spp

Isolates	Origin	Vitek2 system	PCR technique
1–2–3	Thrush	*C. albicans*	*C. albicans*
4	Vaginitis	*C. albicans*	*C. albicans*
5	Upper respiratory tract	*C. albicans*	*C. albicans*
6	Upper respiratory tract	Low discrimination *C. famata*/*C. albicans*	*C. albicans*
7–11	Thrush	*C. sphaerica*	–
8–9–10	Vaginitis	*C. sphaerica*	–
12	Vaginitis	Low discrimination *C. kefyr*/*C. sphaerica*	*C. kefyr*
13–14–15	Thrush	*C. krusei*	*C. krusei*
16	Vaginitis	*C. famata*	–
17–18	Vaginitis	Low discrimination *C. guilliermondii*/*C. famata*	*C. guilliermondii*
19	Vaginitis	*C. parapsilosis*	*C. parapsilosis*
20	Upper respiratory tract	*C. glabrata*	*C. glabrata*
21	Upper respiratory tract	*C. norvegensis*	–

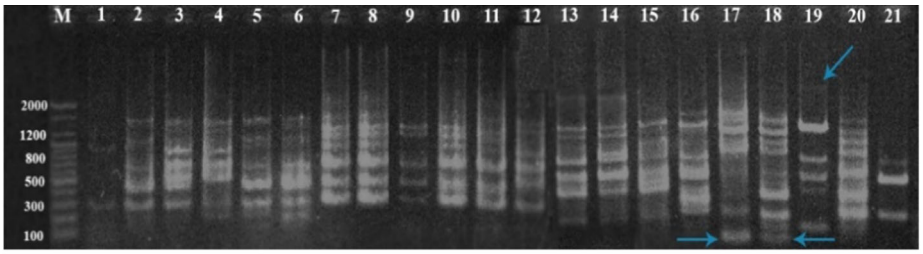
between species in the low discrimination category might be due to them having the same pattern of biochemical details. And the very typical pattern that does not correspond to any taxon in the database is often the reason for unidentified organisms [14].

3.2 Identification of *Candida* spp. at the molecular level

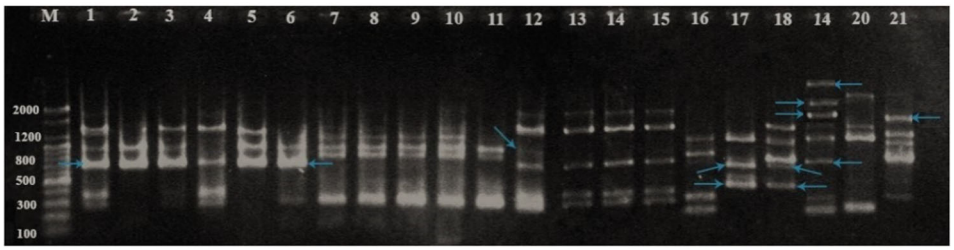
The genomic DNA was extracted efficiently from *Candida* isolates using a genomic DNA extraction kit to yield intact DNA with a good quality and high purity for use in PCR techniques. The concentration of the extracted DNA ranged between 903 and 2202 ng/µl, with a purity of 1.6–2 was obtained. Specific amplification of topoisomerase II encoding gene of different *Candida* spp. was performed in the present study to confirm the Vitek2 results, especially with respect to the low discrimination organism category. The DNA topoisomerase II encoding gene was used as a target gene since it is present in all eukaryotes and its sequence is well conserved and composed of species-specific regions and highly homologous regions [19]; species-specific primers worked specifically to amplify their own DNA topoisomerase II encoding gene and as expected. The nucleotide sequences of the topoisomerase II gene of *C. sphaerica, C. famata* and *C. norvegensis* have not been determined [23]. Moreover, it has been demonstrated that *C. parapsilosis* has two genotypes in the DNA topoisomerase II encoding gene [23], and to distinguish between these genotypes, two specific primer sequences, one for each of genotypes I and II, were used. As shown in Fig. 3, no PCR product was observed in lane No. 14 which means *C. parapsilosis* had the genotype group II.

Consequently, the most common *Candida* spp. was *C. albicans* (28.57%) followed by *C. sphaerica* (23.8%), *C. krusei* (14.28%), *C. guilliermondii* (9.52%), *C. kefyr* (4.76%), *C. famata* (4.76%), *C. norvegensis* (4.76%), *C. glabrata* (4.76%) and *C. parapsilosis* (4.76%).

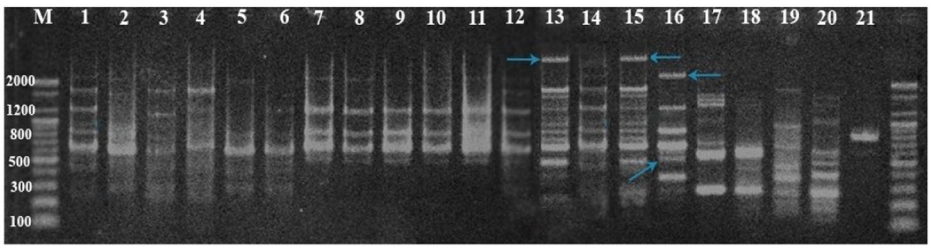
Table 4 shows the complete identification results obtained with Vitek2 and PCR (as a reference method) for pathogenic *Candida* spp.



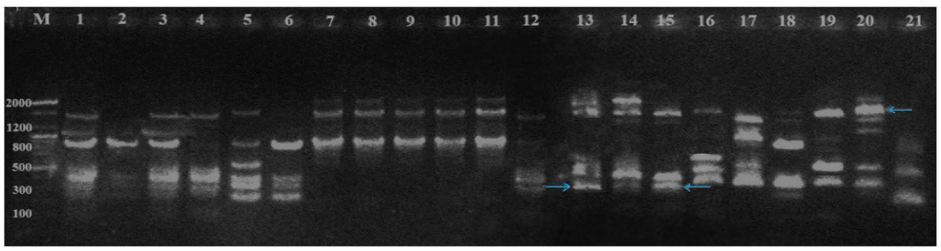
OPI_06



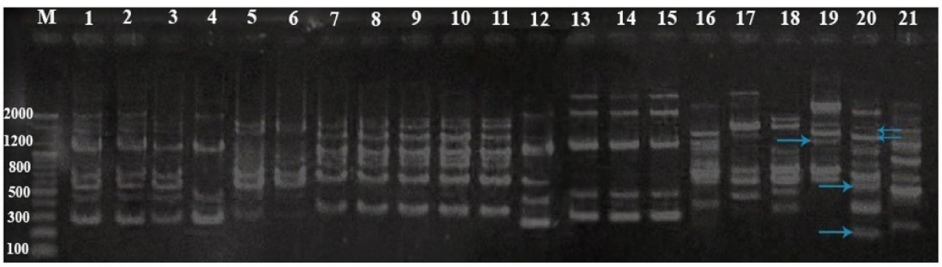
OPM_20



OPL_05



OPQ_01



OPE_16

Fig. 4 Electrophoregram of RAPD-PCR analysis of different *Candida* spp. *Lanes 1–6* for *C. albicans*. *Lanes 7–11* for *C. sphaerica. Lane 12* for *C. kefyr. Lanes 13–15* for *C. krusei*. *Lane 16* for *C. famata. Lanes 17* and *18* for *C. guelliermondii. Lane 19* for *C. parapsilosis*. *Lane 20* for *C. glabrata. Lane 21* for *C. norvegensis.* The *blue arrows*: a *single arrow* indicates unique bands arrows pointing toward each other indicated the monomorphic bands

As demonstrated in the table above, the isolates with low discrimination in Vitek2 between *C. albicans/ C. famata*, *C. guilliermondii/ C. famata* and *C. kefyr/ C. sphaerica* were confirmed as *C. albicans*, *C. guelliermondii* and *C. kefyr*, respectively. Vitek2 successfully identified the remaining *Candida* spp. when compared with PCR.

3.2.1 Interpretation RADP-PCR analysis

In the present study, the RAPD-PCR technique was also used to detect DNA polymorphism of different *Candida* spp. in order to search for the source of differences that could be used as a DNA marker specific for each *Candida* spp. The RAPD-PCR reaction was repeated on a set of DNA samples with several different primers, under PCR optimum conditions of programming and reagent concentration so as to provide different RAPD patterns for each primer [7]. Genomic DNA of the different *Candida* spp. was amplified by using the primers (OPI_06, OPL_05, OPM_20, OPE_16, and OPQ_01), and different DNA banding profiles were achieved with each primer (Fig. 4).

The primer OPE_16 generated five unique bands. *C. glabrata* was distinguished by having four unique bands with a molecular weight of about 1600, 1340, 524 and 192 bp, respectively. Finally, the unique band with the molecular weight of 1200 bp allowed the differentiation of *C. parapsilosis* from other *Candida* spp. The primer OPI_06 produced a single faint unique band, which was the biggest band with a molecular weight of approximately 2700 bp of *C. parapsilosis* and one monomorphic band of 100 bp, which differentiated *C. guilliermondii* from other Candida.

The primer OPM_20 generated six unique bands. Four of this distinguished *C. parapsilosis* from other *Candida* spp., theses (4) bands having molecular weight about (3000, 2150, 1770 and 700) bp, respectively. Also, *C. kefyr* and *C. norvegensis* were distinguished from other species by having one unique band with a molecular weight of 950, 1750 bp, respectively. With regard to the monomorphic band, *C. guelliermondii* was differentiated by having two monomorphic bands with a molecular weight of 650 and 450 bp. Also, another monomorphic band was identified as being present in *C. albicans*, with a molecular weight of 750 bp. The arbitrary primer OPL_01 primer produced only one unique band which distinguished *C. glabrata* from the other *Candida* spp., having a molecular weight of approximately 1700 bp. Another band was as a monomorphic band of *C. krusei*, with a molecular weight of 320 bp. The last OPL_05 primer generated two unique bands, the first of which was faint, and the second indicating *C. famata*, with molecular weights of 2300 and 520 bp, respectively. A single faint monomorphic band was also observed, and this was the biggest band produced by *C. krusei* with a molecular weight of 2900 bp. [24] found that species-specific monomorphic bands for pathogenic *Candida* spp. were used as DNA markers. The highest percentage of polymorphism, 96%, was obtained with the primer OPI_06, whereas the OPM_20 arbitrary primer showed the lowest polymorphism percentage (69%). The presence or absence of an amplified RAPD band (DNA polymorphism) resulted from rearrangements or mutations either at or between the priming sites. Several studies have supported the concept that genotypic differences among *C. albicans* isolates might be correlated with their invasive environments or different body sites [25]. Lastly, DNA markers were obtained for all studied *Candida* species except for *C. sphaerica*. These markers could be sequenced and used to prepare primers to specifically identify the isolates. Through using RAPD-PCR assay, it has been noted that the reason behind the misidentification of *C. albicans* and *C. famata*, that occurred in strain No. 6, between *C. sphaerica* and *C. kefyr* in strain No. 12 and *C. guilliermondii* and *C. famata* in strain No. 18 might be the genotypic similarity between them. This could be seen from the electropherogram images, in addition to the biochemical reasons which were mentioned previously.

4 Conclusion

The molecular method provides the highest possible degree of precision for identification of the studied *Candida* spp. compared with the currently applied phenotypic techniques.

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