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Species Identification and Strain Typing of *Candida* Isolates by PCR-RFLP and RAPD-PCR Analysis for Determining the Probable Sources of Nosocomial Infections

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Abstract

Background: Since the incidence of nosocomial *Candida* infections have been increasing in parallel with the raising in the number of patients involving in predisposing factors, determining the sources and the ways of acquisition of infection is necessary as an efficient strategy for controlling the diseases. The aim of this study is identification and strain typing of *Candida* strains isolated from hospitals to facilitate tracing the sources of infections in hospitalized patients in addition to assess the discriminatory power of some random primers by using RAPD analysis.

Methods: Samples were collected from patients who were hospitalized in oncology, intensive care unit (ICU), and organ transplants wards of the Shohadaye-Tajrish Hospital affiliated to Tehran University of Medical Sciences, Tehran, Iran, and their environments. The yeasts were isolated on CHROMagar Candida. Species identification was performed by PCR-amplification of ITS1-5.8SrDNA–ITS2 region followed by restriction digestion with the enzyme *Msp*I. To determine the probable origin of *Candidia* infections, in case of each patient whose the clinical and relevant environmental isolates were identified as the same species, a set of eight primers namely number 1 to 8, were applied in RAPD-PCR to find out the possible homogeny or variation within the isolated strains.

Results: One hundred and four *Candida* strains were identified. The most common species was *C. albicans* (57.5%) followed by *C. tropicalis* (13.5%), *C. glabrata* (12.5%), *C. parapsilosis* (8.65%), *C. famata* (3.8%), *C. krusei* (1.9%), *C. guilliermondii* (0.96%). and *C. lusitaniae* (0.96%). While the source of infection for three patients were not determined by RAPD analysis, interpretable results from RAPD-typing of *Candida* species isolated from 8/18 of cases implied that the infections might originate from the exogenous sources. Moreover, according to the function of each primer, primer No. 1 was determined as a best primer for typing of *Candida albicans* strains.

Conclusion: The species of yeast isolates were determined by PCR-RFLP. It was found that RAPD assay can point out the genomic variability within the *Candida* species. Besides, the method could show a probable relationship between acquired infections and their sources.

Keywords: *Candida*; Identification; PCR-RFLP; RAPD typing

Introduction

Although members of the genus *Candida* are considered as harmless commensal organisms in healthy individuals, they frequently are responsible for inducing life-threatening systemic infections under certain conditions.[1,2] As a matter of fact, among human pathogenic fungi, the genus *Candida* has a dominant role in afflicting the hospitalized patients with systemic life-threatening infections.[1,3] Nosocomial *Candida* bloodstream infections have been considered as an increasing cause of mortality, with an estimated

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range of 25-38% in predisposed hospitalized patients despite appropriate treatments.[4,5] In particular, bloodstream infection due to the *Candida spp.* has been documented in different countries with similar spectrum of 3.0 - 4.4 per 100,000 patients/day.[6] In present, *Candida* species are the fourth most common microorganisms causing nosocomial blood stream infection in hospitalized patients.[5,7]

For appropriate antifungal therapy, accurate identification of the isolates at the species level is necessary, because of the following reasons: i) A significant trend of either intrinsic or acquired decreasing susceptibility of some species to antifungal agents, ii) Emerging of new species, iii) Rising in the number of non-*Candida* species as considerable pathogenic agents in various nosocomial infections and iv) Persistence of some species such as *Candida parapsilosis* in hospital environment which allows the microorganism to colonize on skin of patients and form biofilms on plastic venous catheters.[5,8-10] On the other hand, there is a need for recognizing the main source of the infections; whether it is endogenous or acquire exogenously from other patients or even health care workers.[5,11] Therefore, precise identification of the strains at the species and sub-species levels are highly demanded to perform epidemiological investigations as well as for controlling the outbreaks.

Earlier epidemiological studies of nosocomial *Candida* infections have used a variety of phenotypic characteristics to reflect genetic diversity and strain identity.[12,13] These phenotypic methods include diagnostic kits such as API, automated diagnostic systems like Vitek which are based on physiological properties and the methods rely on biotyping e.g. enzyme profiles and serological agglutination reactions.[13] Phenotyping systems are usually time-consuming and provide limited data for differentiation between unrelated isolates.[5] Moreover, some *Candida* species are able to switch between numbers of phenotypes spontaneously, so their phenotypic properties are unstable.[14] In contrast, genotyping approaches are capable of detecting differences directly in the genetic information. They are less vulnerable to variations due to growth condition and phenotypic switching and also have several advantages over phenotyping techniques like speed, accuracy, and higher discriminatory power.[5] These DNA-based methods include pulse-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), Real-Time PCR, amplified fragment length polymorphism (AFLP), sequencing, DNA microarray, microsatellite typing, and more recently multi locus sequence typing (MLST) and single-nucleotide polymorphism (SNP) analysis.[1,2,5,7,12,15,16]

The random amplified polymorphic DNA (RAPD) technique relies on the application of short arbitrary primers that anneal to multiple genomic sites using low temperature conditions. This method detects strain polymorphisms and does not depend on prior knowledge of species-specific sequence.[3,13]

The first aim of this study was species-identification of the *Candida spp.* isolated from different forms of infection, some locations where this fungus is likely to be obtained as normal flora and patients' environment. The second and the main goal was strain typing of the relevant isolates to facilitate tracing of probable sources and routes of transmission of infection to hospitalized patients by using RAPD analysis.

Materials and Methods

About four hundreds fungi isolates (including yeasts and moulds) were collected from clinical specimens consisting of urine, surgical wounds, bedsores, sputum and others, and some locations where *Candida* was considered as normal flora such as skin, oral and nasal cavities. The isolates were obtained from oncology, intensive care unit (ICU), and organ transplants wards at Shohadaye-Tajrish Hospital. Equal numbers of samples were collected from surrounding environment of patients including air, medical devices, clothes, catheters and health care workers. Yeast isolates were sub-cultured on CHROMagar *Candida*, incubated at 35 □ C for 2-4 days and harvested yeast colonies were stored at -20°C until further use.

Genomic DNA was extracted using glass bead disruption.[17] Briefly, 300 mg of 0.5 mm diameter glass beads, 300 µg of lysis buffer (100 mM Tris-Hcl pH 8, 10 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate (SDS), 2% triton X-100) and 300 µl of phenol chloroform-isoamyl alcohol (25:24:1) were added to about 1-5 mm^3 of a fresh yeast colony in a 1.5 ml tube. The samples were shacked vigorously for 5 min, centrifuged for 5 min at 5000 rpm, and the supernatant was transferred to a fresh tube. The supernatant was extracted again with chloroform and DNA was precipitates by adding the equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The solution was vortexed and incubated for 10 min at -20°C and centrifuged for 15 min at 12000 rpm.

The precipitant was washed with cold 70% ethanol, dried in air, dissolved in 50 µl of double distilled water and stored at -20°C till used for PCR.

Fungal-specific universal primer pairs were used to amplify internal transcribed spacer 1(ITS1)-5.8S rDNA-ITS2 regions in all yeast isolates. The amplification reaction was performed in a final volume of 25 µl containing 1 µl of extracted genomic DNA (about 20 ng), 1.25 U of *Taq* DNA polymerase, 0.3 mM of each deoxynucleoside triphosphate mix (dATP, dTTP, dGTP, dCTP), 0.4 µM of each forward ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers, 1.5 mM of $MgCl_2$, and 2.5 µl of 10X PCR buffer. PCR was carried out in a thermal cycler (Corbett Research, Australia) with the following temperature profile: 1 cycle of 5 min at 95°C, followed by 30 cycles of 45s at 94°C, 1 min at 55°C and 45s at 72°C and a final extension step at 72°C for 5 min. Each amplification products was loaded onto 1% agarose gel and run in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.3) at 80V for 1 h. The products were detected by staining with ethidium bromide (0.5 µg/ml) and photographed.

RFLP was performed according to the method described by Mirhendi *et al*.[18] to identify the most medically important *Candida* species. To achieve species-specific discriminatory patterns, a 5 µl aliquot of PCR products was digested with 5 U of *Msp*I (Fermentas, Lithuania) in a final volume of 15 µl at 37 □ C for 2.5 h. The restriction fragments were separated on 2% agarose gel electrophoresis in TBE buffer for about 2.5 h at 80 V and visualized by staining with 0.5 µg/ml of ethidium bromide.

Fragment size polymorphism (FSP) of both ITS1 and ITS2 regions[19] was used for identification of the strains which were remained as unknown species. Briefly, the forward (ITS1) and reverse (ITS2: 5'-GCTGCGTTCTTCATCGATGC-3') primers and the forward (ITS3: 5'-GCATCGATGAAGAACGCAGC-3') and ITS4 primers were used for amplification of ITS1 and ITS2 regions, respectively. The amplicons of each region for each individual yeast isolate were mixed and subjected to agarose gel electrophoresis. Species identification was based on the unique pattern for each species.

Totally, eight oligonucleotides i.e. primers 1 (5'-GGGGGTTAGG-3'), 2 (5'-GGTGTAGTGT-3'), 3 (5'-GTATTGGGGT-3'), 4 (5'-GGTTCTG GCA-3'), 5 (5'-AGGTCACTGA-3'), 6 (5'-AAGGATCAGA-3'), 7 (5'-CACATGCTTG-3'), and 8 (5'-TAGfATCAGA-3') were used in this study. Each amplification reaction was performed in a final volume of 25 µl containing 1 µl of genomic DNA, 1.25 U of *Taq* DNA polymerase, 0.3 mM of each four deoxynucleoside triphosphate,1.5 mM of $MgCl_2$, 0.4 µM of each individual primer and 2.5 µl of 10X PCR buffer. PCR was carried out with the following program: 1 cycle of 5 min at 95° C, followed by 40 cycles of 45s at 95°C, 1.5 min at 32°C and 1.5 min at 72°C and a final extension step at 72°C for 8 min. Amplified DNA fragments were run onto 1.2% agarose gel electrophoresis in TBE buffer at 100V for 2 h. The products were detected by staining with ethidium bromide and photographed.

Results

One hundred and four yeast strains were isolated from different clinical and environmental samples and identified by PCR-RFLP method. Consequently, the identified isolates were divided into three groups 1) The yeasts isolated from clinical specimens including urine, surgical wounds, sputum and bedsores (clinical samples), 2) The yeasts isolated from body sites where *Candida* species were normal flora or can be considered as colonization, such as nasal and oral cavity, sputum and skin and 3) the yeasts isolated from patients' surrounding areas including air, catheters, clothed, medical devices and healthcare workers' hands.

The ITS1-5.8SrDNA-ITS2 was successfully amplified in all *Candida* isolates representing a single PCR product of about 380-880 base pairs. Figure 1 shows agarose gel electrophoresis of the examples of PCR products of *Candida* species isolated from different sources. Digestion of amplicons by the restriction enzyme *Msp*1 generated fragments with different sizes. Each *Candida* isolate was identified according to the RFLP patterns regarding already described data.[16] Figure 2 indicates the agarose gel electrophoresis of PCR-RFLP for some examples of *Candida* isolates. The size of fragments complies with the expected sizes. As restriction digestions of the PCR amplicons were not discriminatory in all testing isolates fragment size polymorphism of both ITS1 and ITS2 regions was considered for their identification. By the method unidentified, *Candida* species were diagnosed as. *C. parapsilosis*, *C. famata* and *C. lusitaniae* (data not shown). Table 1 indicates the prevalence of *Candida* species isolated from three distinct sources. *C. albicans* was the most frequent species with prevalence

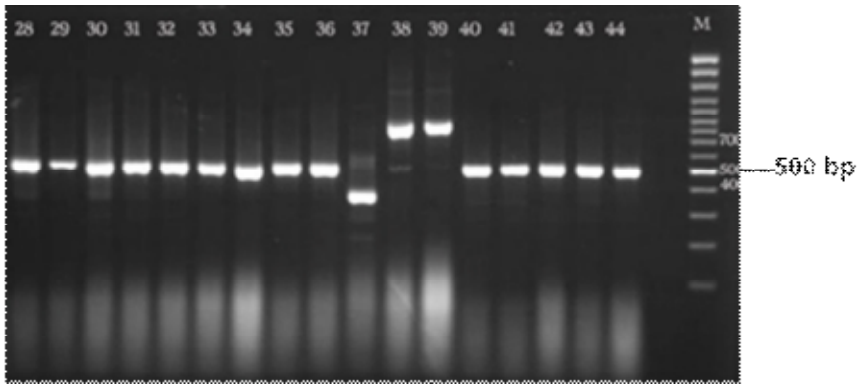

Fig. 1: Agarose gel electrophoresis of ITS-PCR products of the examples of *Candida* species isolated from different samples from hospital specimens. NC: negative control. M: 100 bp molecular size marker

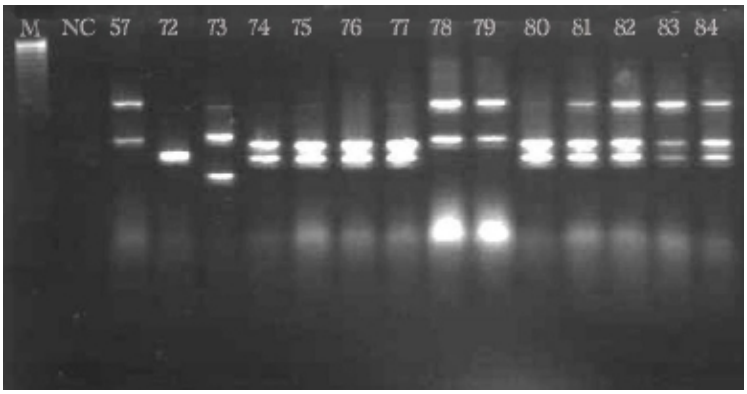

Fig. 2: Restriction digestion of PCR products of *Candida* strain with the enzyme *Msp*1. Lane 57, 78 and 79 identified as *C. glabrata*. Lane 72: *C. krusei*, Lane 73: *C. tropicalis*. Lanes No 74-77 and 80-84: *C. albicans*, NC: Negative control. M: 100bp molecular size marker.

Table 1: Prevalence of *Candida* species isolated from different reservoirs.

Species	Sources		
	Group 1 (Clinical specimens)	Group 2 (Normal flora)	Group 3 (Patients' environment)
C. albicans	18	15	27
C. tropicalis	3	8	3
C. parapsilosis	1	5	3
C. krusei	1	0	1
C. glabrata	3	3	7
C. famata	1	1	2
C. guilliermondii	0	0	1
C. lusitaniae	0	0	1
	27	32	45

of 66.6%, 46.8% and 60.0% in group 1, 2 and 3, respectively. *C. glabrata* was identified in 15.5% of the environmental strains, whereas *C. tropicalis* was in the second position in case of group 2 with the frequency of 25%. In group 1, after *C. albicans*, both *C. glabrata* and *C. tropicalis* were identified in 11.1% of cases equally. The less frequent species were *C. guilliermondii* and *C. lusitaniae* and each consisted 2.2% of cases that isolated only in group 3.

From the total of 104 isolates identified as *Candida* species, only in 18 cases, the same given species were isolated from both clinical samples and body sites as normal flora or/and from patients' surrounding areas. In other words, to make a reasonable

relationship between endogenous and exogenous reservoirs and fungal infection, only the strains associated to those 18 cases allowed us to assess the probability of a relationship between the infections and its suspected source(s).

Table 2 shows information about identified strains from 18 patients and their respective origin of infections. Similar RAPD profiles were generated by primers No 2, 3, 4 and 8 such that they were unlikely to be suitable primers with enough discriminatory power for typing of *Candida* strains. Therefore they were not studied any more. However, another four primers could successfully produce a total of 36 variable electrophoretic RAPD patterns from the testing strain genomes.

In 8/13 patients in whom we identified *C. albicans* from clinical specimens and different reservoirs, distinguishable RAPD patterns were revealed by using primers No 1, 5 and 6 within strains from clinical specimens and environmental reservoirs. The data support the strong possibility of exogenous-originated infections. Despite application of four different primers, the source of infection of two patients (No. 27 and 18) remained unknown. In the case of patient No. 27, the identified strains isolated from environmental and clinical samples revealed very similar electrophoretic patterns by using primer No. 1, 5 and 7, suggesting exogenous sources for the infection; while the outcome of using primer 6 did not ascertain that it had been an exogenous-source of infection. In patient No. 18, isolated strains of urine and patient's environment did not demonstrate a relevant RAPD patterns. On the other side, no *C. albicans* strain was isolated from skin, nasal and oral cavity as normal flora. Taken together, an apparent contradiction was observed between results. As a consequence, a definite origin was not found for the infection of those two patients.

C. parapsilosis was reported in one case and no relationship was found between endogenous source and acquired infection by using all four primers. In other words, identical RAPD banding patterns were observed within strains isolated from surrounding area and from clinical specimen. In the case of *C. tropicalis*, one of two cases isolated from both oral cavity and urine generated identical patterns, whereas the same species which obtained from patients' environment showed different RAPD patterns, therefore, it is more likely that the infection originated from endogenous sources. However in the second patient, it is suggestive that the strains which were responsible for elucidating the disease have been originated from surrounding areas. The same species of *C. glabrata* were identified only in one case whose strains were

Table 2: Information about identified strains from all 18 patients who were subjected to RAPD analysis and their respective origins of infection and type strains of *C. albicans* obtaining from RAPD analysis by using primer No1.

Number of patients	Identified species isolating from two distinct reservoirs and clinical specimens	The probale origin of infections	Number of strain types using primer No 1			
			A	B	C	D
1	*C. albicans*	Exogenous	3	0	0	0
3	*C. albicans*	Endogenous	3	0	0	0
5	*C. parapsilosis*	Exogenous				
7	*C. albicans*	Exogenous	3	0	0	0
9	*C. albicans*	Exogenous	3	0	0	0
11	*C. albicans*	Endogenous	5	0	0	0
14	*C. tropicalis*	Exogenous				
15	*C. albicans*	Endogenous	3	0	0	0
16	*C. albicans*	Exogenous	4	1	0	0
18	*C. albicans*	Unknown	1	0	1	0
19	*C. albicans*	Exogenous	2	0	1	0
21	*C. crusei*	Unknown				
24	*C. albicans*	Endogenous	4	0	0	0
25	*C. tropicalis*	Endogenous				
26	*C. albicans*	Exogenous	3	0	0	0
27	*C. albicans*	Unknown	3	0	0	0
29	*C. glabrata*	Endogenous				
31	*C. albicans*	Endogenous	0	0	0	3

isolated from both endogenous reservoir and clinical specimen, only one strain-specific RAPD banding patterns was generated using all four primers. In the patient No. 21, healthcare hands and bedsore wounds were regarded as two diverse sources where *C. krusei* was identified. The obtained electrophoretic patterns were remarkably similar in size and design using primer No. 1, confirming the exogenous source of infection for *C. krusei*-associated disease, whereas, the patterns achieving from RAPD analysis with the other three primers have not met the same results, and thus have not complied with results getting from primer No. 1. No certain origin was identified in this case.

Figures 3-6 demonstrated examples of RAPD variations of identified *Candida* species by using primers No. 1, 5, 6 and 7. Regardless to strain sources, several bands with different sizes and variable electrophoretic patterns were observed using all four primers demon-

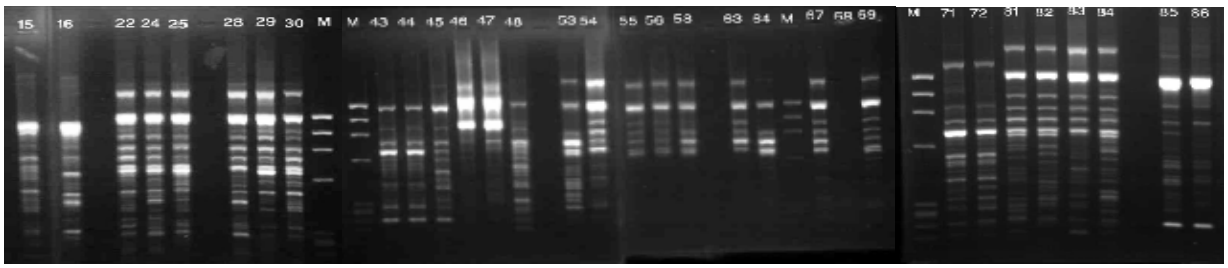

Fig. 3: RAPD typing patterns with *Candida* species by using primer No 1. Patient No 5 (strains 15 and 16): *C. parapsilosis*. Patient No 7 and 9 (strains 22-25 and 28-30): *C. albicans* type (A). Patient No 14 (strains 43-48): *C. tropicalis* type (a) and (b). Patient No 16 (strains 53-58): *C. albicans* type (A) and (B). Patient No 18 (strains 63 and 64): *C. albicans* type (A) and (C). Patient No 19 (strains 67-69): *C. albicans* type (A) and (C). Patient No 21 (strains 85 and 86): *C. krusei*. Patient No 24 (strains 71, 72 and 81-84): *C. albicans* type (A)

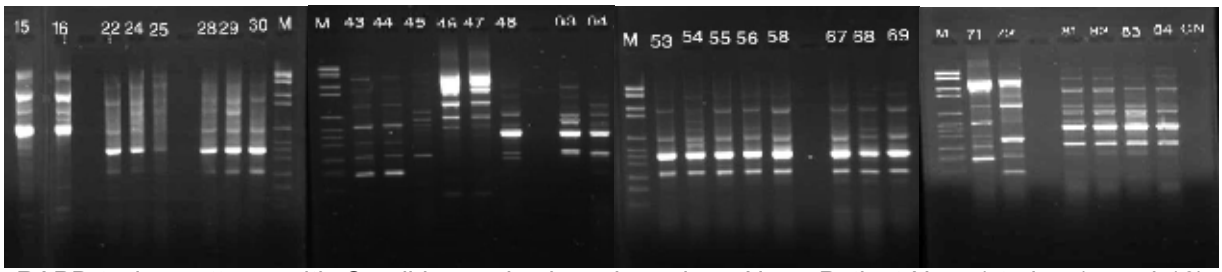

Fig. 4: RAPD typing patterns with *Candida* species by using primer No 5. Patient No 5 (strains 15 and 16): *C. parapsilosis*. Patient No 7 and 9 (strains 22-25 and 28-30): *C. albicans* type (A). Patient No 14 (strains 43-48): *C. tropicalis* type (a), (b) and (c). Patient No 16 (strains 53-58): *C. albicans* type (A) and (B). Patient No 18 (strains 63 and 64): *C. albicans* type (B). Patient No 19 (strains 67-69): *C. albicans* type (A) and (B). Patient No 21 (strains 85 and 86): *C. krusei*. Patient No 24 (strains 71, 72 and 81-84): *C. albicans* type (A).

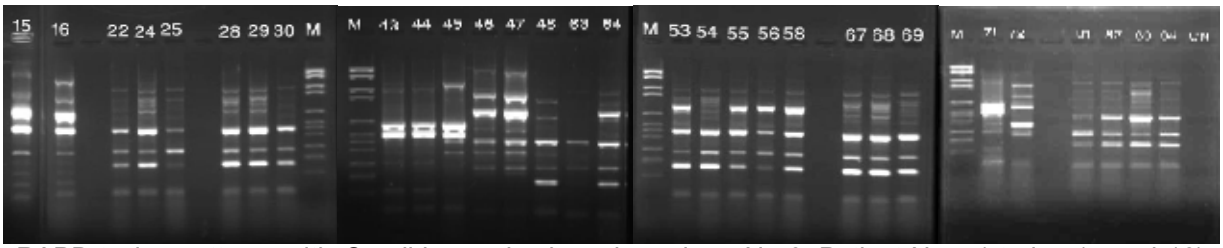

Fig. 5: RAPD typing patterns with *Candida* species by using primer No 6. Patient No 5 (strains 15 and 16): *C. parapsilosis*. Patient No 7 and 9 (strains 22-25 and 28-30): *C. albicans* type (A). Patient No 14 (strains 43-48): *C. tropicalis* type (a) and (b). Patient No 16 (strains 53-58): *C. albicans* type (A) and (B). Patient No 18 (strains 63 and 64): *C. albicans* type (A) and (B). Patient No 19 (strains 67-69): *C. albicans* type (B). Patient No 21 (strains 85 and 86): *C. krusei*. Patient No 24 (strains 71, 72 and 81-84): *C. albicans* type (A).

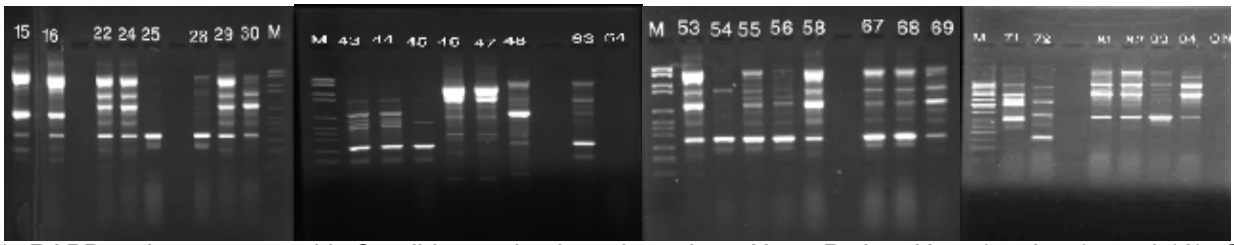

Fig. 6: RAPD typing patterns with *Candida* species by using primer No 7. Patient No 5 (strains 15 and 16): *C. parapsilosis*. Patient No 7 and 9 (strains 22-25 and 28-30): *C. albicans* type (A). Patient No 14 (strains 43-48): *C. tropicalis* type (a), (b) and (c). Patient No 16 (strains 53-58): *C. albicans* type (A). Patient No 18 (strains 63 and 64): *C. albicans* type (A). Patient No 19 (strains 67-69): *C. albicans* type (A). Patient No 21 (strains 85 and 86): *C. krusei*. Patient No 24 (strains 71, 72 and 81-84): *C. albicans* type (A).

strating different discriminatory power for each primer. Totally, thirteen different types were obtained for *C. albicans* strains. Primers No. 5, 6, and 7 generated only three different patterns while four distinguishable different patterns were observed within strains using primer No. 1 suggesing the highest degree of discriminatory power for this primer. The predominant strains were grouped as type A comprising 37 strains out of total 43 *C. albicans* strains. In contrast to *C. albicans*, in *C. tropicalis*, primers No. 5 and 7 yielded more variable RAPD patterns such that three variable banding patterns in different sizes that were produced by the primers, whereas primers No. 1 and 6 revealed only two distinguishable electrophoretic patterns. The most common strains were grouped as type (a) comprising 5 strains out of overall 8 *C. tropicalis* strains (data not shown).

Discussion

Since the incidence of nosocomial *Candida* infections have been constantly increasing in parallel with the raising in the number of patients involving in predisposing factors, determining the sources and the ways of acquisition of infection is more likely to be an efficient strategy to prevent both affliction and expansion of the diseases.[5,20,21]

In the present study, we chose a sort of random primers such that the strains could be discriminated from each other. To determine the sources, those patients whose isolated strains were the same, both from their clinical samples and the other reservoirs, should be appointed. Only 18 out of 27 cases represented the identical species from the reservoirs and the source of infection in remaining 9 patients were left unknown. The RAPD analysis used in this study was successfully able to grouping isolates from various kinds of sources to find probable predominant strain considering as the causing agents of the fungal disease. Additionally, RAPD assay could make a reasonable relationship between acquired infections and their sources. The study raises the possibility that at least some infections were originated from the exogenous sources. Hence, complying the health care rules is a necessity even in modern hospitals. However, we were not able determine the source in three patients (No. 18, 21 and 27). Owing to this fact that choosing the most efficient primers is the golden passkey to overcome the limitations, the ambiguous results obtained from these three patients may pose the lack of enough degree of discriminatory power of primers to delineate some *Candida* strains. Therefore seeking for such primer or sets of combined primers which can effectively distinguish between the strains should be taken into consideration in hope that the assay would completely discriminate the *Candida* strains isolating from health care settings. Furthermore, there is a strong possibility that infections may originate either from other parts of body (as normal flora) or other material and medical devices which have not been examined. So that, missing of some strains seems to be inevitable.

In spite of the limitations to strain delineation in *Candida* species, development of efficient, high discriminatory and easy to interpret RAPD method is not certainly unforeseen by applying some smart details such as applying a variety sets of primers and comparing various conditions.[12] In view of the results, a combination of several typing methods may be required to strain determination in *Candida* infections. This is supported by other investigators too.[12,22-24] The notable point is that cross-transmission may be one of probable routes of acquisition of nosocomial

Candida-related infections. Unfortunately, hospitalization itself have been now regarding as a remarkable predisposing factor which can facilitate affliction especially in individuals who suffer from some stages of acquired or inherited immunodeficiency.

As the conclusion, the species of yeasts isolated from various clinical and environmental samples were determined by PCR-RFLP. We also found that RAPD assay can point out the genomic variability within the *Candida* species and the method can show a probable relationship between acquired infections and their sources. More studies are still required in this field.

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