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Microsatellite Length Polymorphism for DNA-Based Typing of *Candida albicans* Isolated from HIV Positive Patients in Tehran, Iran

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Abstract

Background: The importance of *Candida albicans* as the most common cause of fungal infections in humans is undeniable. Genotyping methods have been developed as useful tools to differentiate between fungal strains isolated from various infections. Several molecular typing methods have been described for *C. albicans*, and fragment length analysis of microsatellites called microsatellite fragment length polymorphism (MLP) is one of the most accurate genotyping methods.
Objectives: The present study aimed at evaluating the genetic diversity and genetic relationships among *C. albicans* isolates recovered from HIV-positive patients with oral candidiasis in Iran using MLP.
Methods: We analyzed 30 isolates of *C. albicans* obtained from HIV-positive patients in Tehran. Genotypes of *C. albicans* isolates associated with oropharyngeal candidiasis were determined using microsatellite length polymorphism analysis. Three loci including EF3, CDC3, and HIS3 were amplified using multiplex PCR. After amplification, the product was run on an automated single-capillary genetic analyzer, and band sizes (known as alleles) were calculated with gene scan mapper.
Results: PCR MLP typing of the 30 isolates under study yielded 27 different profiles, and the discriminatory power index was obtained as 0.993. Ten alleles and 18 different combinations were detected for the EF3 gene, 7 alleles and 18 combinations for the EF3 gene, and 10 alleles and 14 combinations for the HIS3 gene. Only 2 isolates were homozygous in all the 3 loci. To identify the origin of superficial infections in 6 patients, *C. albicans* isolates from the superficial as well as oral samples were simultaneously genotyped. Results showed the identity of genotypes in 4 of these patients. For 1 patient, the *C. albicans* genotype of the nails was different from the genotype observed in the oral cavity, which raised the possibility of an exogenous source for the superficial infection. Also, there were changes at only 1 or 2 alleles, which represented microevolution in some isolates.
Conclusions: The high variation of genotypes throughout the population of *C. albicans* suggested that the microsatellite fragment length polymorphism using multiplex PCR-based system provided high-speed genotyping, indicating its usefulness in molecular epidemiological evaluation.

Keywords: Oral Candidiasis, Microsatellite Length Polymorphism, HIV-Positive Patients, *Candida albicans*

1. Background

In recent years, *Candida albicans* has emerged as an important fungal pathogen and a common opportunistic agent in immunocompromised patients. *Candida albicans* has led to the emergence of both superficial and deep infections in the past 3 decades (1-3). Considering the rising prevalence of immunosuppression, long-term hospitalization, and invasive medical treatments, *Candida* species have become a major group of opportunistic pathogens causing infections in humans. Prevention and treatment of candidiasis are of importance considering the prevalence of nosocomial *Candida* infections (4). Recurrent or persistent infections caused by *Candida* species are common, especially among patients with vaginal and oropharyngeal candidiasis (OPC); nonetheless, such infections have also been reported in patients with urinary tract infections (5, 6).

Through genotyping of *C. albicans*, we can reach the following goals: 1) determining the relationship between commensalism and infection; 2) identifying nosocomial candidiasis to determine strains associated with the out-

break of infection; 3) distinguishing epidemic from sporadic or endemic strains; 4) identifying the etiology of infection, strain transmission, and acquisition routes; 5) examining the genetic diversity of isolates from a carrier; 6) determining recurrent infections to find virulent strains; 7) evaluating the emergence of drug-resistant strains; and 8) assessing the population diversity, structure, and dynamics (7, 8). Typing methods are recognized as helpful tools in differentiating strains causing recurrent infections (7). Despite the availability of different typing methods for *C. albicans* (eg, PCR-RFLP, AFLP, and MLST), microsatellite length polymorphism (MLP) is known as a highly efficient method of fragment length analysis of microsatellites (9, 10), with high reproducibility and discriminatory power (11).

Various epidemiological studies have confirmed the reproducibility and efficacy of MLP analysis. As a PCR-based method, MLP typing uses high repeat variability in microsatellite sequences (described as tandem repeats of 2 - 6 nucleotides). Microsatellite markers include a definite pair of primers flanking a particular microsatellite site inside the genome. For amplifying specific loci, fluorescence-labeled primers are used, and the allele length is measured via gel electrophoresis of PCR products with an automatic sequencer. In general, MLP is a highly discriminative method for *C. albicans* typing. Nevertheless, the microsatellite marker affects its resolving power. In the *C. albicans* genome, different polymorphic microsatellite loci have been described (eg, CDC3, EF3, HIS3, ERK1; 2NF1, CCN2, EFG1, CPH2, CAI; and CAIII-CAVII). A combination of markers on different chromosomes from the same typing system facilitates a more precise classification of *C. albicans* with MLP (10, 12, 13).

2. Objectives

In this study, we aimed at examining the genetic diversity and relatedness among isolates of *C. albicans*, collected from HIV positive patients with oral candidiasis, using microsatellite fragment length polymorphism.

3. Methods

A total of 30 *C. albicans* isolates were collected from patients with HIV and OPC in Tehran, Iran. Moreover, *C. albicans* was isolated from 8 out of 30 patients with recurrent OPC in 2 different episodes. To identify the origin of cutaneous and nail infections in six patients, *C. albicans* isolates, as well as isolates related to OPC were analyzed. *C. albicans* isolates were collected from CHROMagar Candida and Sabouraud dextrose agar media after incubation for 72 hours at 35°C. Phenotypic identification was confirmed using different tests including chlamydospore production, colony morphology on CHROM agar, and carbohydrate assimilation using rapid yeast identification system (Remel Inc., USA) (14, 15).

For DNA extraction, a physicochemical method was applied. A portion of the yeast colony was washed with phosphorous-buffered saline containing 50 mM EDTA and 0.5% SDS. Afterwards, the freeze-thawing method with glass beads was applied to disrupt the yeasts. After centrifugation for 2 minutes at 12,000 g, lysis buffer (500 mL) was added to the precipitate, followed by incubation for 10 minutes. Potassium acetate buffer (150 μL, pH = 4.8; 28.5 mL of distilled water; 60 mL of 5 M potassium acetate; 11.5 mL of glacial acetic acid) was then added to the tube and vortexed for a short period.

Centrifugation (2 minutes at 12,000 g) was used to remove the precipitated proteins and cell debris. The supernatant was transferred to a microtube and centrifuged for 2 minutes at 12,000 g. After decanting the supernatant to a new microtube (1.5 mL), isopropyl alcohol was added with an equal volume. The tube was briefly mixed via inversion and centrifuged for 2 minutes at 12,000 g. After removing the supernatant, the DNA pellet was washed 3 times in 70% ethanol (300 μL). The supernatant was discarded after centrifugation for 1 minute at 12,000 g. Then, DNA was dried and dissolved in distilled water (50 μL).

To determine DNA concentration and its purification, optical density (OD) was read and run on agarose gel. To amplify the 3 loci, multiplex PCR was performed in a reaction volume of 50 μL, containing 5 mM of $MgCl_2$, 1X PCR buffer, 0.2 mM of each dNTP, 1.25 U of Taq gold polymerase (Applied Biosystems), 5 pmol of EF3 (Chromosome 5) primers, and 2 pmol of CDC3 (Chromosome 1), and HIS3 (chromosome 2) primers. The microsatellite markers were amplified with the primers (Table 1), and different dyes were used to label a primer from each set. The EF3 antisense primer was stained using fluorescein amidite (FAM), CDC3 sense primer was labeled with hexachlorofluorescein (HEX), and HIS3 sense primer was labeled with NED (16). The PCR included initial denaturation at 95°C for 10 minutes, followed by 30 cycles at 95°C for 30 seconds, at 55°C for 30 seconds, and at 72°C for 60 seconds, followed by a final extension at 72°C for 30 minutes. After amplification, 2 μL of the PCR product was added to formamide (20 μL) and 6-TAMRA Genescan 500 size standard (0.5 μL, Ap-

plied Biosystems). The samples were run on an ABI XL 370 genetic analyzer after denaturation at 95°C for 120 seconds and were placed in an ice bath. Moreover, the GeneMapper Version 5 (Applied Biosystems) was used to determine the band size (allelic number) (13, 16, 17).

4. Results

There were I or 2 bands based on the multiplex PCR analysis of each microsatellite marker and isolate. As *C. albicans* is a diploid pathogen, every marker had a single locus, and every band was attributed to an allele. Accordingly, a profile of 6 alleles characterized every isolate. Overall, 27 different profiles were obtained via PCR MLP typing of 30 isolates (discriminatory power: 0.993) (Figure 1). There were 10 alleles and 18 combinations, 10 alleles and 14 combinations, and 7 alleles and 18 combinations for EF3, HIS3, and EF3 genes, respectively. Heterogeneity was observed in allele frequency. In a particular locus, the most probabilistic number of repeats indicated an association with the increase or decrease in the number of repeats. Nevertheless, the alleles were not normally distributed, and some were overrepresented. Furthermore, most isolates were heterozygous inside at least 1 locus, whereas only 2 isolates were homozygous in all the 3 loci (Table 2). Although the haploidy of these isolates was not definite, they were described as homozygous.

To identify the origin of cutaneous and nail infections among 6 patients, *C. albicans* isolates from the culture, as well as isolates related to oral cavity infections were genotyped. The genotypes were identified in 4 of these patients. The genotype of *C. albicans* from the nails differed from the genotype of oral cavity in 2 patients and was considered as an exogenous source of superficial infection. To evaluate microevolution and change the isolates in recurrent OPC, *C. albicans* was isolated from the culture of 8 patients and genotyped in 2 different episodes of disease in different intervals. The genotypes were identical in 6 patients. In addition, changes in only 1 or 2 alleles, which represent microevolution (evolutionary changes within a species or small group of organisms, especially over a short period), were observed in some isolates (Table 3).

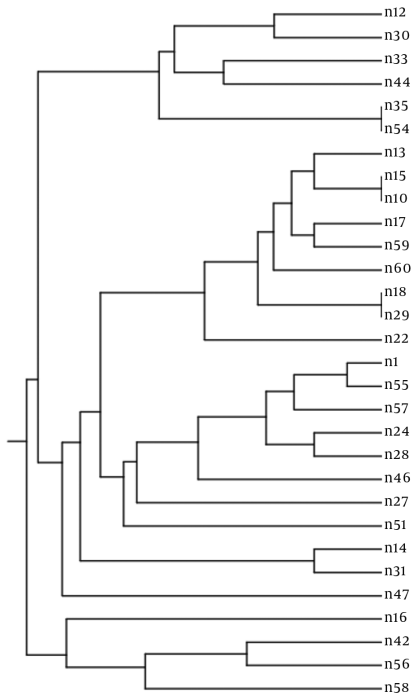

Figure 1. Genetic Diversity of 30 Dependent *C. albicans* Isolates Collected from HIV Positive Patients Via MLP Typing

5. Discussion

Candida albicans is capable of causing severe infections, particularly in immunocompromised patients. Consequently, researchers have attempted to develop accurate methods to discriminate between isolates. Understanding the relationship between the strains involved in *Candida* infections is strongly associated with the development of proper treatment methods and adds to the available epidemiological knowledge. Epidemiological studies have applied different typing methods for characterizing *C. albicans* isolates including electrophoretic karyotyping, southern blot hybridization using discriminating probes, RFLP, and random polymorphic DNA amplification. Microsatellites are common markers for differentiating between microorganisms, given their potential application and high diversity in automated tests besides the simplicity of PCR amplification and interpretation of the results (18, 19). *Candida albicans* isolates contain many loci, and accordingly, several markers have been developed for them (16, 19). However, it seems that most of microsatellites in coding regions have low levels of heterozygosity, although the use of multiplex PCR and the evaluation of more loci increase the discriminatory power (20, 21).

The analysis of 30 independent isolates recovered from HIV-positive patients with 3 loci (CDC3, EF3, and HIS3) identified 27 different genotypes (discriminatory power

Table 1. Details of Microsatellite Markers Applied in the Molecular Analysis of *C. albicans*

Locus, Chromosome	Gene Product	Primer	Sequence 5′ - 3′	Dye	Repeat Type	Size Range
EF3, chr 5	Elongation factor 3	EF3F	TTTCCTCTTCCTTTCATATAGAA	FAM	TTTC-TTC	120 - 141
		EF3R	GGATTCACTAGCAGCAGACA			
CDC3, chr 1	Cell division cycle protein	CDC3F	CAGATGATTTTTTGTATGAGAAGAA	HEX	AGTA	112 - 140
		CDC3R	CAGTCACAAGATTAAAATGTTCAAG			
HIS3, chr 2	Imidazole glycerol	HIS3F	TGGCAAAAATGATATTCCAA	NED	TTG	224 - 245
		HIS3R	TACACTATGCCCCAAACACA			

Table 2. Demographic Data and Genotypes of 30 Clinical *C. albicans* Isolates Collected from HIV Positive Patients

Isolates Number	Isolates Origin	Fragments Length (Bp) Identified By Gene Scan						Final Genotype	Fluconazole resistance	Gender	CD4 Count	HAART
		EF3 marker		CDC3 marker		HIS3 marker						
		Allele1	Allele2	Allele1	Allele2	Allele1	Allele2					
N1	OPC	129	136	116	116	150	162	GT9	DD	M	< 200	No
N10	OPC	129	138	116	128	154	154	GT1	S	F	< 200	No
N12	OPC	123	123	120	120	154	154	GT2	S	F	< 200	No
N13	OPC	129	138	116	116	154	154	GT3	S	F	< 200	No
N14	OPC	129	137	124	128	150	167	GT4	DD	F	< 200	No
N15	OPC	129	138	116	128	154	154	GT5	S	M	< 200	No
N16	OPC	129	129	116	116	162	162	GT6	S	M	< 200	No
N17	OPC	129	141	116	128	154	154	GT7	R	M	< 200	No
N18	OPC	129	138	128	128	154	154	GT8	S	M	< 200	No
N22	OPC	138	138	116	140	154	154	GT10	S	M	< 200	No
N24	OPC	123	129	112	116	150	162	GT11	R	M	< 200	Yes
N27	OPC	123	129	116	124	180	180	GT12	R	M	< 200	Yes
N28	OPC	123	129	112	116	150	162	GT11	S	M	200 - 400	No
N29	OPC	129	138	128	128	154	154	GT13	R	M	200 - 400	No
N30	OPC	120	123	120	120	154	154	GT14	S	F	200 - 400	No
N31	OPC	129	137	124	128	150	166	GT15	S	M	200 - 400	Yes
N33	OPC	124	136	120	124	154	192	GT16	R	M	200 - 400	Yes
N35	OPC	123	137	120	124	154	166	GT17	S	F	200 - 400	Yes
N42	OPC	120	128	116	124	162	162	GT18	S	F	200 - 400	Yes
N44	OPC	124	136	120	120	154	172	GT19	S	M	200 - 400	Yes
N46	OPC	129	136	116	116	149	162	GT20	S	M	200 - 400	Yes
N47	OPC	124	138	112	116	149	149	GT21	S	M	200 - 400	Yes
N51	OPC	123	138	116	116	179	179	GT22	S	F	> 400	Yes
N54	OPC	123	137	120	124	154	166	GT23	R	M	> 400	Yes
N55	OPC	129	136	116	116	150	162	GT9	R	M	> 400	Yes
N56	OPC	120	120	124	124	162	162	GT24	R	M	> 400	Yes
N57	OPC	129	140	116	116	150	162	GT25	S	M	> 400	Yes
N58	OPC	120	125	124	124	147	166	GT26	DD	M	> 400	No
N59	OPC	129	135	116	128	154	154	GT27	S	M	> 400	Yes
N60	OPC	129	141	116	116	154	154	GT7	R	M	> 400	Yes

Abbreviations: DD, dose dependent susceptible; OPC, Oropharyngeal candidiasis, R, resistant S, susceptible.

of 0.993). The results of multiplex PCR regarding genetic relatedness discriminated 27 out of 30 (90%) strains, which otherwise would have been considered similar. Microevolution was reported due to minor changes in the strain genotypes including changes in only 1 allele, which could be described in a single mutational step. Using this method to assess recurrent infection isolates shows similar scenarios as previously described. Among 8 recurrent cases, 6 were related to a similar strain, and 2were attributed to a similar strain undergoing microevolution (One allele was changed.). Furthermore, MLP could detect microevolutionary changes and was useful in identifying microevolution after environmental stress. Moreover, it can improve therapeutic procedures in patients due to the recurrence of refractory fungal infections. The primers, analysis techniques, and allele nomenclature for microsatellite typing systems should be standardized. Therefore, for interlaboratory comparisons, these impor-

Table 3. Comparison of 8 Pairs of Isolates from Recurrent Oral Collected at Different Time from Same Patients Underlying Alleles Related to Change in *C. albicans* Genotypes

Patients/Episode	Length, bp Determined by PCR Analysis					
	EF3		CDC3		HIS3	
	Allele1	Allele2	Allele1	Allele2	Allele1	Allele2
P1						
Episode1	129	138	116	128	154	154
Episode2	129	138	116	128	154	154
P2						
Episode1	129	138	116	116	154	154
Episode2	129	138	116	128	154	154
P3						
Episode1	120	123	120	120	154	154
Episode2	120	123	120	120	154	154
P4						
Episode1	120	128	116	124	162	162
Episode2	120	120	124	124	162	162
P6						
Episode1	123	137	120	124	154	166
Episode2	123	137	120	124	154	166
P7						
Episode1	124	136	120	124	154	192
Episode2	124	136	120	124	154	192
P8						
Episode1	129	141	116	116	154	154
Episode2	129	141	116	116	154	154

tant issues need to be considered. Moreover, the development of a global public database similar to MLST is essential for the collection of microsatellite allele data. The present study can help develop a genetic database of fungal pathogens in Iran. A relatively high divergence was found in the structure of *C. albicans* among HIV-positive patients. The isolates were classified into 27 genotypes, showing major redundancy in isolates from clinical samples.

In this study, various strains with similar MLP genotypes were reported (30 isolates from 27 genotypes) (Figure 1). We found isolates, which were only different in 1allele and might have evolved from a common ancestor. No association was found between fluconazole resistance and genotypes. However, to determine the molecular epidemiology of *C. albicans* in Iran, further research is required on *C. albicans* strains in other regions of the country. Based on the present findings, potentially pathogenic *C. albicans* have the opportunity to overgrow in the context of HIV infection. In some previous studies, mixed types were detected in some patients. In the host, the strains undergo microvariation, which often involves alterations in zygosity of diploid allele pairs, but show inadequate geographical relatedness (2, 22, 23).

6. Conclusions

Considering the high variability of genotypes in the population of *C. albicans*, microsatellite fragment length polymorphism analysis with multiplex PCR facilitates high-speed genotyping for molecular epidemiological evaluation.

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