Sequences Type Analysis of *Candida albicans* Isolates from Iranian Human Immunodeficiency Virus Infected Patients with Oral Candidiasis

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Abstract- The growing number of immunocompromised individuals has increased the incidence of infections caused by Candida species during the recent decades. Typing of C. albicans on the basis of DNA sequences at multiple loci has greatly advanced our knowledge about the epidemiology and phylogeny of candidiasis. The aim of this study was to evaluate the diversity, and genetic relationships among C. albicans isolates obtained from HIV patients in Iran using multilocus sequence typing (MLST) method. We analyzed 25 C. albicans isolates obtained from HIV positive patients referred to Iranian Research Center for HIV/AIDS. After diagnostic test and DNA extraction C. albicans isolates were typed using the original MLST scheme explained previously include of six loci: ACC1, VPS13, GLN4, ADP1, RPN2, and SYA1. Fifty one (2.17%) nucleotide sites were found to be polymorphic; all were found to be heterozygous in at least one isolate. For the 25 clinical isolates, 22 diploid sequence types were defined by the genotypes identified from the six loci. The MLST data suggest a relatively high level of divergence in the population structure of C. albicans isolated from HIV infected patients. These findings indicate that in these patients there is a favorable context for the growth of potential pathogenic C. albicans. We found no association between fluconazole resistance, highly active antiretroviral therapy (HAART) receiving and either sequence type or group.

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Introduction

Candida albicans is the most common *Candida* species associated with opportunistic fungal infection and human commensalism. The increased incidence of infections caused by *Candida* species during the recent decades has been documented. The growing number of immunocompromised individuals in the population as a result of the human immunodeficiency virus (HIV) pandemic and the increased use of immune suppressive therapy in cancer and organ transplant patients are the reasons for this status (1). The typing methods used to characterize *C. albicans* isolates in epidemiological studies are very diverse. They include restriction fragment length polymorphism analysis, Southern blot hybridization with discriminating probes, electrophoretic karyotyping, and randomly amplified polymorphic DNA analysis. Results are laboratory dependent, which precludes inter laboratory comparison of infecting isolates.

Typing of *C. albicans* on the basis of DNA sequences at multiple loci has greatly advanced our knowledge about the epidemiology and phylogeny of candidiasis. Multilocus sequence typing (MLST) is a method based on the analysis of nucleotide polymorphisms of the sequences of approximately 450-500bp internal fragments (loci) of housekeeping genes. For each housekeeping locus, the different sequences present within a bacterial species are assigned as distinct alleles, and for each isolate, the alleles at each of the sequenced loci define an allelic profile or sequence type. Each isolate of a species is therefore unambiguously characterized by a series of integers which correspond to

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the alleles at the housekeeping loci studied. A major advantage of MLST over other typing methods is that sequence data can be easily compared between laboratories, thus permitting the exchange of molecular typing data via the internet for global epidemiology (2,3).

Multilocus sequence typing is becoming a widely used approach to microbial isolate differentiation for epidemiological purposes (2-5). For *C. albicans*, two central internet databases have been set up for deposition and analysis of *C. albicans* MLST data from any global source (http://calbicans.mlst.net and http://mlst.net). In few years ago this MLST system was based on fragments of six *C. albicans* genes ACC1, ADP1, GLN4, RPN2, SYA1, and VPS13. Today's study of *C. albicans* MLST involved seven *C. albicans* genes includes ACC1, ADP1, SYA1, VPS13, AAT1a, MPIb, and ZWF1b (3). In this study we used six set up of MLST genes.

Materials and Methods

Isolates of *C. albicans*

We analyzed 25 *C. albicans* isolates obtained from HIV positive patients referred to Iranian Research Center for HIV/AIDS (IRCHA, Imam Khomeini Hospital, Tehran University of Medical Science); they were patients who came to receive treatment in April 2007. Except two cases, all patients had clinical sign of oral candidiasis. The isolates were cultured on Sabouraud dextrose agar and CHROMagar *Candida* directly and incubated for 72h at 30˚C. Identification was confirmed based on colony color on CHROMagar, chlamydospore production and carbohydrate assimilation (RapID yeast plus). For all isolates diagnosed as *C. albicans* a PCR method; as described previously; were carried out for differentiation of *C. albicans* from probable *C. dubliniensis* (6).

Antifungal susceptibility

The minimum inhibitory concentrations (MICs) of fluconazole for the *C. albicans* isolates were determined by the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute document M44-A as described previously (7-8).

Amplification & nucleotides sequence determination

After DNA extraction (6), *C. albicans* isolates were typed using the original MLST scheme explained previously by Bougnoux *et al.* include of six loci: ACC1, VPS13, GLN4, ADP1, RPN2, and SYA1 (4). No amplification will obtain with the CaACC1 and CaGLN4 oligonucleotides when the DNAs of the non-*C. albicans* species were used as templates. PCRs were carried out in a standard PCR mixer of 50 µl reaction volume containing about 10 ng of extracted DNA, 5 µl of 10x PCR buffer, a 2.5 mM concentration of $MgCl_2$, a 0.2 mM concentration of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1.25 U of pfu DNA polymerase (Fermntase). The PCR program started with an initial denaturation step of 5 min at 95°C, continued by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min, with a final extension step of 5 min at 72°C (9). The amplified fragments were sequenced by using the same primers as those used in the initial amplification. After a purification step by column chromatography, sequencing of both strands were performed using the ABI 3730xl and were analyzed (bidirectional sequencing reactions with automated capillary sequencing equipment).

MLST data analysis

The data of six loci for all strains were stored and analyzed with BioEdit sequence alignment editor software version 7.0.5.3 and Mega4 software (10). The sequencing chromatograms were visually checked, and the polymorphic nucleotide sites (homozygous or heterozygous SNPs) within these sequences were identified. Heterozygosity was identified when two peaks in a sequencing chromatogram coincide with equal heights approximately half the size of neighboring peaks.

For each gene, distinct alleles were identified and numbered using the *C. albicans* MLST website (http://mlst.net), also allowed us to compare the strains to those previously typed by this method, and sited in the database(11). Combination of a set of six allelic numbers defines a diploid sequence type (DST). Genetic relatedness between the strains was evaluated by constructing an unweighted-pair group method with arithmetic averages dendrogram, which compares genotypic profiles (12-13).

Stability and reproducibility of the MLST method

The stability and reproducibility of the sequence data at each MLST locus were assessed by carrying out the sequence analysis in duplicate on two randomly selected isolates. For each locus and isolate, the duplicate DNA extractions, PCRs, and sequencing reactions were carried out independently. Resulting sequence duplicates for each isolate were compared to each other (3).

Result

The results of molecular typing, antifungal susceptibility testing is shown in Table 1. Data were obtained from 25 isolates fully sequenced in duplicate. The positions of the polymorphic nucleotide sites, the different genotypes identified at each of the six loci, and their frequency of detection among the 25 of *C. albicans* isolates are shown in table 2.

A total of 2354 bp from the 6 MLST loci (ACC1, VPS13, GLN4, ADP1, RPN2, and SYA1) were sequenced in each of the 25 isolates.

Table 1. Genotyping results for *Candida albicans* from oral cavity of HIV infected individuals

No	Gender	Sign	FLCZ	HAART	Hospitalized	Geographical	Allelic numbers						DSTs
							CaACC1	CaVPS13	CaGLN4	CaADP1	CaRPN2	CaSYA1	
CAIR1	F	Thrush	S	No	Out patient	Tehran	2	76	19	24	13	37	DST1
CAIR2	M	Thrush	R	Yes	In patient	Tehran	4	73	10	24	3	11	DST2
CAIR3	M	Thrush	R	Yes	Out patient	Kermanshah	2	33	13	6	17	12	DST3
CAIR4	M	Thrush	S	No	Out patient	Tehran	10	12	11	22	10	9	DST4
CAIR5	M	Thrush	R	Yes	Out patient	Karaj	10	50	11	10	13	9	DST5
CAIR6	M	Thrush	R	Yes	Out patient	Eslamshahr	1	15	2	4	9	37	DST6
CAIR7	M	Esophagitis	R	Yes	In patient	Tehran	5	14	11	42	10	9	DST7
CAIR8	M	Esophagitis	R	Yes	In patient	Mashhad	2	33	13	6	17	12	DST3
CAIR9	M	No sign	S	Yes	Out patient	Tehran	4	67	10	3	17	17	DST8
CAIR10	M	Thrush	S	Yes	Out patient	Tehran	5	11	8	15	4	9	DST9
CAIR11	M	Thrush	R	Yes	In patient	Tehran	5	14	10	15	4	9	DST10
CAIR12	M	Esophagitis	R	Yes	In patient	Tehran	5	11	10	15	4	6	DST11
CAIR13	M	Cheilitis	R	Yes	Out patient	Tehran	2	33	13	6	17	6	DST12
CAIR14	M	Cheilitis	R	Yes	Out patient	Tehran	5	11	10	15	4	9	DST13
CAIR15	M	Thrush	S	Yes	In patient	Tehran	4	73	19	1	3	17	DST14
CAIR16	M	Thrush	R	Yes	In patient	Tehran	3	67	13	2	10	11	DST15
CAIR17	M	Cheilitis	R	No	Out patient	Tehran	5	58	10	37	4	6	DST16
CAIR18	M	Thrush	S	No	Out patient	Tehran	4	73	10	1	3	11	DST17
CAIR19	F	Thrush	R	No	Out patient	Malayer	4	94	10	3	17	17	DST18
CAIR31	M	Thrush	S	Yes	Out patient	Malayer	4	73	8	1	3	11	DST19
CAIR32	M	No sign	S	No	Out patient	Tehran	4	73	10	6	3	11	DST20
CAIR33	M	Cheilitis	S	No	Out patient	Tehran	2	33	13	6	17	12	DST3
CAIR34	M	Esophagitis	S	Yes	In patient	Mashhad	4	67	10	17	17	17	DST21
CAIR37	M	Esophagitis	R	Yes	In patient	Tehran	5	14	11	5	10	9	DST22
CAIR46	M	Thrush	R	Yes	Out patient	Tehran	5	14	10	15	4	9	DST10

F: Female, M: Male, DST: diploid sequence type, FLCZ: Fluconazole

Table 2. Positions of polymorphic nucleotide sites and number of amino acid changes at the six loci used for MLST

Locus	Size of amplicon	No. of genotype in this study	Nucleotides polymorphic sites	Number of polymorphic amino acid
Ca*ACC1*	407	6	9,281,317,392 **(4)**	4
Ca*VPS13*	403	12	49,88,134,212,217,241,281,282,320,322, 326,327,334,370 **(14)**	9
Ca*ADP1*	404	13	40, 46, 109, 125,166, 205,215,225,232,263,282, 395 **(12)**	9
Ca*GLN4*	443	6	25,127,387,391,393 **(5)**	4
Ca*RPN2*	306	6	19,34,46,49,55,76,130,274 **(8)**	6
Ca*SYA1*	391	7	1, 25, 61, 100, 142, 160, 185, 351 **(8)**	7

Fifty one (2.17%) nucleotide sites were found to be polymorphic; all were found to be heterozygous in at least one isolate. The number of polymorphisms per locus was 4 in the ACC1 locus, followed by 5 in the GLN4 locus, 8 in the RPN2a and SYA loci, and 12 in the ADP1 locus, and 14 in the VPS13 locus. Polymorphic amino acids per locus were 4 in the ACC1 and GLN4 locus, followed by 6 in the RPN2 locus, 7 in the SYA1 locus, and 9 in the ADP1 and SYA1 loci. The number of genotypes identified for each of the six loci

investigated ranged from 6 (ACC1, GLN4, RBP2), 7 (SYA1), 12(VPS13) to 13 (ADP1) genotypes per locus. Among the six fragments sequenced, ACC1 gave the highest discriminatory ratio, yielding 6 different genotypes from just 4 polymorphic sites. We couldn't find any new genotype for adding to old MLST data base on Internet. In table 1, details are given of the DST for each isolate, together with the origin and anatomical source. For the 25 clinical isolates, 22 DSTs were defined by the genotypes identified from the six loci. For the present sample of 25 isolates, the discriminatory power of the MLST scheme for typing *C. albicans*, calculated using a numerical index was 88. *C. albicans* is almost always diploid as isolated, and evidence has been obtained for heterozygosity of complementary chromosomes in the present study none of the isolates was 100% homozygous. Figure 1 showed the similarities of 25 *C. albicans* isolates determined by MLST with 6 gene fragments.

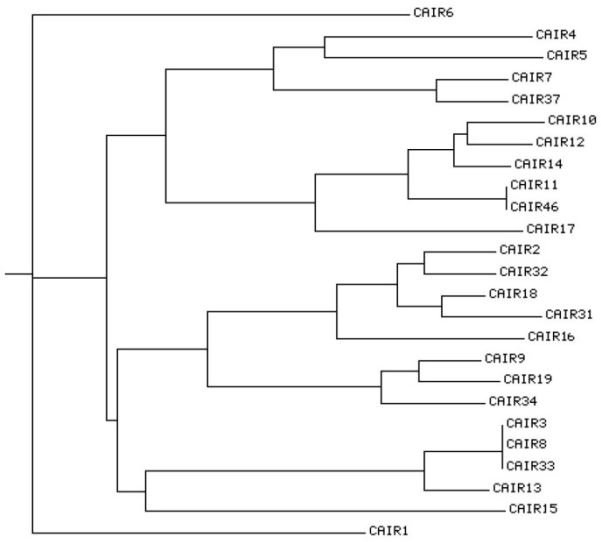

Figure 1. Dendrogram indicating the similarities of 25 *C. albicans* isolates determined by MLST with 6 gene fragments

Discussion

MLST is based on variability within particular housekeeping genes due to mutation or recombination events; thus, it provides many genetic types per locus and these can be utilized to define the allelic profile or sequence type and determine the relatedness of strains (14).

Our information is limited regarding the genotypes of *C. albicans*. There have been no reports based on MLST method for *Candida* species in Iran.

The aim of this study was to evaluate the diversity, and genetic relationships among *C. albicans* isolates recovered from the outpatient clinic in HIV patients in Iran. For this purpose, we selected 25 oral samples from these patients. MLST was used to evaluate the diversity and genetic relationships between 25 *C. albicans* isolates recovered from oral cavity of HIV patients. The data obtained in this study will contribute to our attempt to establish a central genetic database of fungal pathogens in Iran.

Sequence-based typing such as MLST data greatly facilitates data exchange and international standards. The MLST data suggest a relatively high level of divergence in the population structure of *C. albicans* isolated from HIV infected patients. These findings indicate that in these patients there is a favorable context for the growth of potential pathogenic *C. albicans*. Other studies showed that in some individuals mixed types were found in some instances; strains undergo microvariation within their host, often involving changes in zygosity of diploid allele pairs; and strains show strong, though imperfect, geographical associations (15,16).

These isolates could be assigned to 22 unique DSTs, indicating significant redundancy within our sample. As mentioned above, a significant number of strains with identical DSTs were observed in our study (25 isolates corresponding to 22 DSTs). Additionally, we observed 2 instances where isolates with identical DSTs were shared by several patients (two or three individuals). Moreover, we observed the occurrence of isolates that different at one or two loci only and that were likely to have evolved from a common ancestor.

The high level of sequence variation throughout the population of *C. albicans* suggests that MLST may be ideal for local epidemiological studies. We failed to find an association between hospitalized patients, outpatients and their genotype. Finally, we found no association between fluconazole resistance, highly active antiretroviral therapy (HAART) receiving and either sequence type or group. However, our results do not exclude the possibility that the capacity to become resistant through drug exposure is a function of Sequence Type or group (3,17). Sequencing-based methods in our country due to high costs have not been considered. Nevertheless further studies on *C. albicans* strains from the Iran region are needed to understand the epidemiology of *C. albicans* in Iran.

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