

Multilocus Sequence Typing of *Candida tropicalis* Shows the Presence of Different Clonal Clusters and Fluconazole Susceptibility Profiles in Sequential Isolates from Candidemia Patients in São Paulo, Brazil

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The profiles of 61 *Candida tropicalis* isolates from 43 patients (28 adults and 15 children) diagnosed with candidemia at two teaching hospitals in São Paulo, Brazil, were characterized by multilocus sequence typing (MLST). For the 14 patients who had bloodstream infections, 32 isolates were serially collected from their blood and/or catheters. Thirty-nine diploid sequence types (DSTs) were differentiated. According to the *C. tropicalis* MLST database (<http://pubmlst.org/ctropicalis/>), 36 DSTs and 23 genotypes identified from the 61 isolates had not previously been described. This report represents the first study to characterize sequential isolates of *C. tropicalis* from candidemia cases in South America. Microvariation in a single gene was found in the sequential isolates from 7 patients. The main polymorphisms occurred in the alleles of the *XYR1* gene, specifically at nucleotide positions 215, 242, and 344. Macrovariation in six gene fragments was detected in the isolates from 3 patients. eBURST analysis added two new groups to this study (groups 6 and 18). Additionally, susceptibility tests indicate that 3 isolates were resistant to fluconazole. No correlation was found between the DSTs and susceptibility to fluconazole and/or selective antifungal pressure. Two patients were sequentially infected with resistant and susceptible strains. MLST is an important tool for studying the genetic diversity of multiple/sequential isolates of patients with candidemia, allowing the comparison of our data with those from other regions of the world, as well as allowing an analysis of the genetic relationship among several clones in sequential isolates from the same or different candidemia patient sites (blood or catheter).

Infections caused by *Candida* spp. have been characterized as important causes of nosocomial infections and are associated with high morbidity and mortality. Additionally, these infections have been related to higher hospitalization costs because of longer hospitalization times and intensive care needs (1–8).

Candida tropicalis is one of the most frequently isolated species in invasive candidiasis, ranging from 4% to 24% of all candidemia cases, depending on the geographical area and other predisposing conditions, such as cancer, neutropenia, diabetes in adults and the elderly, and lung disease in pediatric patients (9–21).

In the ARTEMIS DISK Global Antifungal Surveillance Study, which covered the period from 1997 to 2007, Pfaller et al. (22) studied fluconazole *in vitro* susceptibility test results for 256,882 isolates of *Candida* spp. collected from 142 sites in 41 countries. Increased rates of isolation of *C. tropicalis* (5.4% to 8.0%) were noted. Overall, 90.2% of *Candida* isolates tested were susceptible to fluconazole. In addition, 91% of *C. tropicalis* isolates were fluconazole susceptible and 4.1% were resistant. Fluconazole resistance varies according to geographic region. The highest rates of resistance were observed in isolates from the Asia-Pacific region (6.5%). Europe presented 2.9% resistance and North America 4.4%. The lowest rates of resistance were observed from the Africa/Middle East region and Latin America, with both presenting 2.6% resistance.

Fluconazole resistance in *C. tropicalis* isolates has been described in Brazil, although it is a rare event. Colombo et al. (23) reported that from 2003 to 2004, in Brazilian hospitals, there were 712 cases of candidemia, with an incidence of 2.49 cases per 1,000

admissions. *Candida albicans* was the most common species (40%), followed by *C. tropicalis* (20.9%) and *C. parapsilosis* (20.5%). Fluconazole resistance occurred in 6 (0.8%) isolates (*C. krusei*, *C. glabrata*, and *C. albicans*). In addition, 27 cases (4%) were classified as having dose-dependent susceptibility to fluconazole (*C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. albicans*).

The largest Brazilian study of fungemia by *C. tropicalis* was published by Nucci and Colombo (18), with a total of 924 episodes of candidemia in 906 patients from 12 Brazilian institutions. *C. albicans* was the most frequent (384 cases, 41.5%), followed by *C. tropicalis* (188 cases, 20%) and *C. parapsilosis* (187 cases, 20%). The proportion of candidemia caused by *C. tropicalis* ranged from 15.7% to 25.8% among the 12 institutions. Antifungal resistance was a rare event and was restricted to one isolate of *C. albicans* and one of *C. tropicalis*.

In a multicenter study in São Paulo (Brazil), Da Matta et al. (24) analyzed 1,000 isolates of candidemia from 1995 to 2003. In the first period, from 1995 to 1999, *C. tropicalis* was the second

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most frequent species (74/299). In the second period, from 2000 to 2003, *C. tropicalis* was the third most frequent (169/701), whereas *C. albicans* and *C. parapsilosis* were the two most frequent. All *C. tropicalis* isolates were fluconazole susceptible.

According to Costa et al. (25), at the Clinics Hospital of the University of São Paulo Medical School (FMUSP) in São Paulo (Brazil), 86 fungemia patients, treated between 1994 and 1996, were described as having *C. albicans* (50%), *C. parapsilosis* (17%), and *C. tropicalis* (12%). The fungemia mortality rate was 70%. At the same hospital in 2006, Motta et al. (28) found an incidence of 1.87 candidemia cases/1,000 admissions. The main species isolated were *C. albicans* (52.2%), *C. parapsilosis* (22.1%), *C. tropicalis* (14.8%), and *C. glabrata* (6.6%). The *C. tropicalis* isolates were 100% susceptible to fluconazole.

Several molecular techniques with high discriminatory power and reproducibility have been described, such as multilocus sequence typing (MLST), which is based on the analysis of single-nucleotide polymorphisms (SNPs) in housekeeping gene fragments of up to 500 bp (as detected by molecular sequencing) (29–31). Fragments of the *C. tropicalis* genes *ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a* have already been analyzed (32, 33). Previous reports have indicated that MLST can provide additional information on the mechanisms of genetic variability, such as the persistence, replacement, and microevolution of strains in the hosts (30, 34, 35).

MLST has been used to establish the relationship between the DSTs with susceptibility profile to fluconazole and the trailing phenomenon (27, 32, 36). In Brazil, fluconazole is an effective systemic antifungal drug against most isolates of *C. tropicalis* (24). No studies in this country have reported on the MLST of *C. tropicalis* and its correlation with antifungal susceptibility tests.

The objective of this study was to use MLST to analyze the presence of genetic variation among sequential isolates from blood and catheter samples from the same patient and to characterize the fluconazole susceptibility pattern in patients with bloodstream infections caused by *C. tropicalis* at two teaching hospitals in São Paulo, Brazil, between 1998 and 2003.

MATERIALS AND METHODS

Case definition and *C. tropicalis* isolates. A total of 61 *C. tropicalis* isolates from 43 patients diagnosed with bloodstream infection and who were admitted to two hospitals (Clinics Hospital of the University of São Paulo Medical School [HCFMUSP] and the Clinics Hospital of the University of Campinas [UNICAMP]) and isolated from 1998 to 2003 were studied. In 14 patients with bloodstream infection, 32 isolates were serially collected from their blood and/or catheters. The cultures were initially identified by the mycology section at the HCFMUSP central laboratory using automated methods (Vitek, bioMérieux, Nürtingen, Germany) and carbon assimilation using an API20C strip (bioMérieux SA, Paris, France). The UNICAMP central laboratory used a Vitek system and a chromogenic method (CHROMagar *Candida* Microbiology, Paris, France). All of the isolates were reidentified using the ID 32C method (BioMérieux SA, Paris, France) for standardization. The yeasts were maintained on Sabouraud agar (Oxoid, Basingstoke, United Kingdom) and BHI (BHI-Difco) glycerol at -80°C until use. The standard strains of *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were used.

DNA extraction. DNA extraction was performed according to the method described by Buchman et al. (37). The samples were cultured in liquid Sabouraud medium (Oxoid, Basingstoke, United Kingdom), stirred for 48 h at 30°C and then treated with lyticase (Sigma-Aldrich). This procedure was followed by the addition of lysis buffer consisting of sorbitol, β -mercaptoethanol, and proteinase K (Invitrogen). A 1:1 mix-

ture of phenol and chloroform was used to perform the DNA purification. The DNA precipitation was performed with 3 M sodium acetate and 1 ml of cold ethanol. The DNA was resuspended in 100 ml of TE, measured, and stored at -20°C until use.

MLST typing. The MLST method and PCR amplification were performed as described by Tavanti et al. (33). Briefly, oligonucleotide primers were used for six gene fragments: *ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*. The PCR results were revealed using 2% agarose gel electrophoresis, which displayed a single band. The DNA was purified with GFX PCR and a gel band purification kit (GE Health Care Life Sciences, United Kingdom).

Forward and reverse sequencing of the fragments was performed with an ABI 3500 automated DNA sequencer (Applied Biosystems) using the original PCR primers and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's guidelines. The quality of each electropherogram was evaluated using Phred-Phrap software (38, 39), and consensus sequences were obtained using CAP3 software, which is available at <http://asparagin.cenargen.embrapa.br/phph/>.

The chromatograms were visually checked and scrutinized for heterozygosity. Subsequently, the SNPs in the six previously sequenced gene fragments were concatenated into a single sequence, as described by Tavanti et al. (33). The alignment was performed with the MEGA 5.0 (40) and BioEdit (<http://www.mbio.ncsu.edu/bioedit/page2.html>) software. The sequences were compared with others from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/tbl2asn2/>) and submitted to the *Candida tropicalis* MLST database (<http://pubmlst.org/ctropicalis/>), as described by Jolley et al. (41).

The new allelic profiles and new allele combinations (new DSTs) were submitted to the *C. tropicalis* MLST database. Heterozygosity was defined by the presence of overlapping peaks in the forward and reverse chromatograms. A haplotype analysis was subsequently performed. Microvariation (referred to as “microevolution”) was defined as minor genetic changes and minor variations in genetically determined strain types in surveys of multiple isolates from individual patients (30, 31). “Macrovariation” was defined as *C. tropicalis* strains with variations/genetic changes in five or six gene fragments.

Phylogenetic analysis. A phylogenetic analysis was obtained through the following clustering methods: unweighted pair group method with arithmetic averages (UPGMA), neighbor-joining, maximum parsimony, maximum likelihood, and Bayesian inference. Unrooted trees were created using MEGA 5.0 and MrBayes 3.2.1 software (<http://mrbayes.sourceforge.net/download.php>) and the BEAST software package (<http://beast.bio.ed.ac.uk/Programs>), which includes the programs BEAST 1.7.2, BEAUti, LogCombiner, TreeLog Analyzer, Tracer, and Figtree. The best evolutionary history was inferred using the maximum likelihood method, which is based on the Tamura-Nei model (42). The bootstrap consensus tree inferred from 2,000 replicates is taken to represent the evolutionary history of the analyzed taxa. A discrete gamma distribution was used to model the evolutionary rate differences among the sites (16 categories, $+G$ [discrete gamma distribution], parameter = 0.584). The analysis involved 39 nucleotide sequences. All of the ambiguous positions were removed for each sequence pair. There were 416 positions in the final data set. The evolutionary analyses were conducted in MEGA 5.0. Finally, the 267 sequences described in the *C. tropicalis* MLST database were inferred using Bayesian inference. The groups were numbered to be consistent with bootstrap values (43) greater than 70%. The discriminatory power was calculated using Simpson's index of diversity (44, 45). The ancestor-descendant relationship among the isolates was studied with the eBURST package, v3.0 (<http://eburst.mlst.net/>).

Antifungal susceptibility testing. The stock solutions of antifungal agents, inocula, and plates were prepared according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) document M27-A2/A3 (46, 47). The results were read visually and using a spectrophotometer (Versa Max tunable microplate reader) with a wavelength of 530 nm. The MICs of fluconazole were defined as the lowest concentration of the

drug that was able to inhibit the growth of at least 50% of the organisms. The cutoff points were defined according to the standards established for *C. tropicalis* by CLSI document M27-A3/S3. The trailing phenomenon was analyzed, and when there was doubt between trailing and fluconazole resistance, the susceptibility tests were repeated using RPMI at a pH of 5.0.

RESULTS

Among the 61 isolates, 48 were isolated from the blood and 13 from catheter tips. The study included 9 patients (no. 1, 2, 3, 4, 6, 8, 9, 13, and 14) whose blood cultures and central venous catheter tip cultures were both positive and 5 patients with more than one positive blood culture. The positivity interval between the cultures ranged from the same day to 12 days, with a median of 6 days. Isolates 17, 19, 20, and 26 came exclusively from catheter tips. The patient admission units included pediatrics (23%), adult and pediatric intensive care (18%), gastrointestinal surgery (14.75%), trauma surgery (8.2%), neurology and neurosurgery (8.2%), emergency (6.6%), gastroenterology clinic (6.6%) and "other." Investigating the hypothesis of an outbreak was not possible because the isolates were not from the same unit and were not obtained during the same period of time. The data from the 43 patients with sequential isolates are shown in Table 1.

Of the 14 patients with sequential isolates, three had positive blood cultures despite the use of antifungals (patients 1, 2 and 11). Patients 1, 7, and 11 maintained the catheter despite the candidemia. Patients 7 and 11 died. The treatment was started prior to the blood culture only for patients 1 and 4, based on the diagnosis of candidemia. In our study, no antifungal prophylaxis had been prescribed during the 6 months prior to the candidemia.

In the sequencing analysis, the sizes of the DNA fragments that were obtained for each of the six genes studied ranged from 370 bp to 525 bp. A total of 543 cases of heterozygosity were detected: Y = C+T (59.5%), R = A+G (22.3%), W = A+T (13.6%), M = A+C (1.5%), K = G+T (2.2%) and S = G+C (0.9%). In the six analyzed gene fragments, 154 polymorphic sites were identified (20 in *ICL1*, 27 in *MDR1*, 39 in *SAPT2*, 34 in *SAPT4*, 19 in *XYR1*, and 15 in *ZWF1a*).

The *C. tropicalis* MLST database identified 23 new alleles, specifically in *ICL1* (3 new), *MDR1* (6 new), *SAPT2* (3 new), *SAPT4* (1 new), *XYR1* (7 new), and *ZWF1a* (3 new). The most frequently detected genotypes were the following: genotype 1 in 45 isolates (73.8%) in *ICL1*; genotype 7 in 21 isolates (34.43%) and genotype 24 in 17 isolates (27.87%) in *MDR1*; genotype 4 in 27 isolates (44.26%) and genotype 3 in 22 isolates (30.07%) in *SAPT2*; genotype 7 in 26 isolates (42.62%) and genotype 11 in 13 isolates (21.3%) in *SAPT4*; and genotype 6 in 20 isolates (32.79%), genotype 3 in 15 isolates (23%) and genotype 4 in 12 isolates (19.67%) in *ZWF1a*. The *XYR1* gene presented a great variety of genotypes (a total of 24).

Regarding the DSTs, 39 were found in the 61 isolates and were submitted to the *C. tropicalis* MLST database on April 2, 2012 (Table 1). Of the 39 DSTs, types 124, 134, and 203 were previously known. DST 232 was the most frequently observed (isolates 1c, 6b, 11b, and 14a [UNICAMP] and isolates 33, 42, and 43 [HCFMUSP]). Interestingly, five of the seven isolates with DST 232 were obtained from children. DST 251 was the second most frequently observed (isolates 27, 30, 36, 39, and 41 [HCFMUSP]).

Minor genetic changes characterized as variations in a single genotype (microvariation) were more common in the *XYR1* gene fragment: 1a versus 1c, 5a versus 5b, 6a versus 6b, 8a versus 8b,

9a = 9b versus 9c, 10a versus 10b, 12a versus 12b (Table 2). Differences in a single nucleotide occurred among isolates 8a versus 8b, 9a = 9b versus 9c, 10a versus 10b, and 12a versus 12b. As an example, isolate 8a (genotype 52) was different from isolate 8b (allele 84) because of a loss of heterozygosity at position 215. Multiple variations in the same gene occurred between isolates 1a and 1c (3 nucleotide differences), 5a and 5b (5 nucleotide differences), and 6a and 6b (3 nucleotide differences). The main polymorphisms in the *XYR1* gene occurred at positions 215, 242, and 344 (Table 2). Genetic changes in two gene fragments were found between isolates 6b and 6c in *MDR1* (a single variation) and *XYR1* (3 nucleotide differences), between 13a and 13b in *SAPT2* (4 nucleotide differences) and *XYR1* (a single variation), and between 14a and 14b in *MDR1* (a single variation) and *XYR1* (3 nucleotide differences).

Greater differences (macrovariation) occurred in isolates from 6 patients (no. 1, 2, 3, 7, 11, and 12), particularly those from patients 3, 7, and 11, which displayed differences in all six gene fragments.

All isolates had sufficient growth for MIC determination using the CLSI method. In the majority of the cases (95%), the CLSI method categorized an isolate as susceptible (MIC \leq 4 μ g/ml). A MIC greater than or equal to 32 μ g/ml was observed in at least one isolate from patients 5 (5b, DST 124), 12 (12c, DST 256), and 25 (DST 238) (Table 3). Patient 5 had a good response to treatment with fluconazole, despite a MIC of 64 μ g/ml.

Of the total of 14 patients with sequential isolates, one patient received no treatment and 11 received antifungal agents. Amphotericin B (given to eight patients) was the most common. Fluconazole was given to two patients, and a combination of fluconazole and amphotericin B were used in one patient. It is important to note that the antifungal drugs were prescribed at doses recommended by existing protocols of each hospital at that time.

Additionally, two patients (5 and 12) were simultaneously infected by resistant and susceptible isolates. For patient 5, isolate 5b (DST 124) was considered resistant to fluconazole, and 5a (DST 239) was considered susceptible to fluconazole. For patient 12, isolate 12c (DST 256) was resistant to fluconazole, and 12a (DST 238) and 12b (DST 248) were considered fluconazole susceptible. DSTs 124, 238, and 256 were not exclusive to the resistant isolates. As shown in Table 3, isolates 20 (DST 124), 16 (DST 256), 7b, 12, and 26 (DST 238) were all susceptible to fluconazole.

Phylogenetic analysis. The genetic distance between the strains of *C. tropicalis* included in this study was illustrated by the construction of an unrooted dendrogram. The clustering methods chosen for the phylogenetic analysis were maximum likelihood and Bayesian inference. The result of the phylogenetic analysis of the six gene fragments is displayed in the concatenated haplotypes shown in Fig. 1. The discriminatory power was 97.4%.

The 61 sequences of the six gene fragments in the present study were classified into two major groups (I and II) and one singleton group, supported by a 99% bootstrap value. Group I was divided into 3 subgroups, with 57 isolates: subgroup A, with 24 isolates (39.3%), 22 from UNICAMP and 2 (isolates 31 and 34) from HCFMUSP; subgroup B, with 12 isolates (19.7%), 8 from HCFMUSP and 4 from UNICAMP; and subgroup C, with 21 isolates (34.4%), 6 from HCFMUSP and 15 from UNICAMP. The singleton group consists of isolate 28. Group II consists of isolates 3b, 7a, and 18 (Fig. 1). The fluconazole-resistant isolates remained distant in the dendrogram. Isolates 12c and 25 were clustered in

TABLE 1 Details of 61 *C. tropicalis* isolates tested by MLST, listed in order of their DST, showing genotypes for six DNA fragments sequenced

Patient/ location	Age	Isolate	Sample	Date (mo/day/yr)	Ward	Genotype						DST	eBURST group
						<i>ICL1</i>	<i>MDR1</i>	<i>SAPT2</i>	<i>SAPT4</i>	<i>XYR1</i>	<i>ZWF1a</i>		
1/U	44 yr	1a	Blood	3/24/03	ICU	1	24	3	7	53	6	239	4
		1b	Blood	3/28/03	ICU	1	7	4	11	4	3	234	6
		1c	CVC	3/31/03	ICU	1	24	3	7	24	6	232	4
2/U	79 yr	2a	CVC	7/25/02	Gastrosurgery	1	7	4	11	50	3	236	6
		2b	Blood	7/30/02	Gastrosurgery	3	58	4	13	76	1	265	Singleton
3/U	9 yr	3a	Blood	6/25/02	Pediatric	1	29	4	1	51	1	249	Singleton
		3b	CVC	6/25/02	Pediatric	28	84	28	7	86	28	258	Singleton
4/U	9 mo	4a	CVC	4/21/03	Pediatric ICU	3	4	4	17	77	4	264	Singleton
		4b	Blood	4/21/03	Pediatric ICU	3	4	4	17	77	4	264	Singleton
5/U	71 yr	5a	Blood	7/13/02	Trauma surgery	1	24	3	7	53	6	239	4
		5b	Blood	7/24/02	Emergency	1	24	3	7	48	6	<u>124</u>	4
6/U	2 yr	6a	Blood	6/17/02	Pediatric	1	24	3	7	53	6	239	4
		6b	CVC	6/17/02	Pediatric	1	24	3	7	24	6	232	4
		6c	Blood	6/17/02	Pediatric	1	87	3	7	89	6	261	Singleton
7/U	2 yr	7a	Blood	9/2/02	Pediatric	29	48	29	48	87	30	267	Singleton
		7b	Blood	9/8/02	Pediatric	1	7	4	6	52	4	238	1
8/U	65 yr	8a	Blood	9/8/01	Gastroclinic	1	7	1	6	52	4	237	1
		8b	CVC	9/10/01	Gastroclinic	1	7	1	6	84	4	243	1
9/U	36 yr	9a	CVC	1/15/00	Neurosurgery	10	58	4	8	31	3	246	18
		9b	Blood	1/15/00	Neurosurgery	10	58	4	8	31	3	246	18
		9c	Blood	1/17/00	Neurology	10	58	4	8	3	3	247	18
10/U	44 yr	10a	Blood	12/15/02	Gastrosurgery	1	7	4	11	19	3	241	6
		10b	Blood	12/22/02	Gastroclinic	1	7	4	11	51	3	235	6
11/U	9 yr	11a	Blood	9/12/01	Pediatric ICU	3	7	4	6	52	4	203	1
		11b	Blood	9/24/01	Pediatric	1	24	3	7	24	6	232	4
12/U	58 yr	12a	Blood	10/28/02	Internal medicine	1	7	4	6	52	4	238	1
		12b	Blood	10/31/02	Gastrosurgery	1	7	4	6	84	4	248	1
		12c	Blood	10/31/02	Gastrosurgery	1	1	18	1	31	1	256	3
13/U	64 yr	13a	Blood	10/14/02	Gastroclinic	3	7	4	6	84	4	245	1
		13b	CVC	10/17/02	Gastrosurgery	3	7	1	6	52	4	134	1
14/U	3 yr	14a	Blood	1/3/01	Trauma surgery	1	24	3	7	24	6	232	4
		14b	CVC	1/3/01	Trauma surgery	1	87	3	7	89	6	261	Singleton
15/U	69 yr	15	Blood	1/11/01	ICU	1	24	3	7	90	6	262	4
16/U	11 mo	16	Blood	2/13/01	Pediatric	1	1	18	1	31	1	256	3
17/U	70 yr	17	CVC	2/4/02	Pneumology	1	7	4	6	67	6	244	Singleton
18/U	25 yr	18	Blood	2/5/02	Oncology	30	85	30	7	88	29	259	Singleton
19/U	72 yr	19	CVC	2/19/02	Hemodialysis	1	49	18	1	31	1	266	3
20/U	1 yr	20	CVC	5/19/02	Gastrosurgery	1	24	3	7	48	6	124	4
21/U	9 yr	21	Blood	6/25/02	Pediatric	3	39	3	7	90	7	268	Singleton
22/U	27 yr	22	Blood	8/5/02	Neurology	1	24	3	7	47	6	240	4
23/U	65 yr	23	Blood	9/25/02	Emergency	1	87	3	7	89	6	261	Singleton
24/U	17 yr	24	Blood	12/14/02	Trauma surgery	3	1	4	11	19	1	255	Singleton
25/U	62 yr	25	Blood	3/4/03	Trauma surgery	1	7	4	6	52	4	238	1
26/U	72 yr	26	CVC	3/28/03	Gastrosurgery	1	7	4	6	52	4	238	1
27/H	65 yr	27	Blood	3/30/01	Emergency	1	7	4	11	76	3	251	6
28/H	5 yr	28	Blood	2/1/01	Pediatric	3	86	12	11	73	3	260	Singleton

(Continued on following page)

TABLE 1 (Continued)

Patient/ location	Age	Isolate	Sample	Date (mo/day/yr)	Ward	Genotype							eBURST group
						<i>ICL1</i>	<i>MDR1</i>	<i>SAPT2</i>	<i>SAPT4</i>	<i>XYR1</i>	<i>ZWF1a</i>	DST	
29/H	32 yr	29	Blood	7/4/99	ICU	1	17	3	7	1	3	242	Singleton
30/H	66 yr	30	Blood	3/6/01	Emergency	1	7	4	11	76	3	251	6
31/H	70 yr	31	Blood	1/18/00	Vascular surgery	3	4	4	7	77	7	254	Singleton
32/H	40 yr	32	Blood	4/16/01	Neurology	1	7	4	11	76	7	263	6
33/H	16 yr	33	Blood	1/29/99	Hematology	1	24	3	7	24	6	232	4
34/H	15 yr	34	Blood	4/7/98	Pediatric	1	83	1	11	2	3	253	Singleton
35/H	40 yr	35	Blood	12/11/99	Burns unit	1	24	3	7	24	7	233	4
36/H	2 yr	36	Blood	9/14/00	Pediatric	1	7	4	11	76	3	251	6
37/H	18 yr	37	Blood	9/24/01	Pediatric	1	24	3	7	47	6	240	4
38/H	23 yr	38	Blood	3/12/98	Hematology	1	82	3	7	24	6	250	4
39/H	28 yr	39	Blood	4/3/01	Gastrosurgery	1	7	4	11	76	3	251	6
40/H	70 yr	40	Blood	3/17/00	Burns unit	1	66	3	7	9	7	252	Singleton
41/H	75 yr	41	Blood	7/29/98	Nephrology	1	7	4	11	76	3	251	6
42/H	30 yr	42	Blood	10/20/98	Emergency	1	24	3	7	24	6	232	4
43/H	15 yr	43	Blood	4/18/01	Gynecology	1	24	3	7	24	6	232	4

H, HCMUSP; U, UNICAMP; CVC, central venous catheter; DST, diploid sequence type. FCZ, fluconazole. The alleles/genotypes and a new representative from each of the surrounding 23 alleles are shown in bold. Underlining indicates resistant DSTs; italic type indicates previously described DSTs.

subgroup A and separated by a 99% bootstrap value. The isolates from patient 5 were placed in subgroup C. The DSTs (234, 236, 235, 241, and 251) associated with genotypes (*ICL1*, 1; *MDR1*, 7; *SAPT2*, 4; *SAPT4*, 11; *ZWF1a*, 3; and *XYR1*, variable) were grouped in subgroup B on the dendrogram. We tried to correlate the results with the original hospital, clinic, year, age, and susceptibility to antifungal drugs unsuccessfully.

The isolates from Brazil, when analyzed by the Bayesian inference clustering method, were grouped into a variety of possibly existent clades. Figure 2 shows the arrangement of the isolates with the addition of the analysis of all 267 DSTs obtained from the *C. tropicalis* MLST database. The distribution of the DSTs from the study is shown in the tree. Isolates 3b, 7a, and 18 are clustered and distant from the other isolates.

TABLE 2 Similarity of 32 selected isolates of *C. tropicalis*^a

Patient	Isolates compared	Genotyping difference	Nucleotide difference(s)	Similarity (%)	eBURST result
1	1a and 1c	<i>XYR1</i> : 53–24	215, C-Y; 242, C-Y; 344, T-Y	99.19	4
	1a/1c and 1b	5 alleles	5 alleles		4 versus 6
2	2a and 2b	5 alleles	5 alleles		6 versus singleton
3	3a and 3b	6 alleles	6 alleles		Different singleton
4	4a and 4b	Identical	Identical	100	Identical singleton
5	5a and 5b	<i>XYR1</i> : 53–48	11, Y-T; 14, Y-T; 215, C-Y; 242, C-Y; 344, T-Y	98.65	4
6	6a and 6b	<i>XYR1</i> : 53–24	215, C-Y; 242, C-Y; 344, T-Y	99.19	4
	6a = 6b and 6c	<i>MDR1</i> : 24–87	7, T-Y	99.76	
	6a and 6c	<i>XYR1</i> : 53–89	14, Y-C; 344, T-Y	99.46	4 versus singleton
	6b and 6c	<i>XYR1</i> : 24–89	14, Y-C; 215, Y-C; 242, Y-C	99.19	
7	7a and 7b	6 alleles	6 alleles		Singleton versus 1
8	8a and 8b	<i>XYR1</i> : 52–84	215, Y-C	99.73	1
9	9a = 9b and 9c	<i>XYR1</i> : 31–3	242, C-Y	99.73	18
10	10a and 10b	<i>XYR1</i> : 19–51	344, Y-T	99.73	6
11	11a and 11b	6 alleles	6 alleles		1 versus 4
12	12a/12b and 12c	5 alleles	5 alleles		1 versus 3
	12a and 12b	<i>XYR1</i> : 52–84	215, Y-C	99.73	1
13	13a and 13b	<i>SAPT2</i> : 4–1	55, W-T; 262, G-R; 310, R-A; 421, C-Y; 215, C-Y	99.06	1
		<i>XYR1</i> : 84–52		99.73	
14	14a and 14b	<i>MDR1</i> : 24–87	7, T-Y	99.76	4 versus singleton
		<i>XYR1</i> : 24–89	14, Y-C; 215, Y-C; 242, Y-C	99.19	

^a Isolates were obtained from 14 patients with fungemia, according to identical patterns generated by sequencing the fragments of six genes (*ICL1*, *MDR1*, *SAPT2*, *SAPT4*, *XYR1*, and *ZWF1a*) and determining the differences in the genotypes. All gene fragments were submitted to the official MLST website (<http://pubmlst.org/ctropicalis/>). Isolates from patients 1 to 14 are from UNICAMP. In the "Genotyping difference" column, italicized entries represent the different respective genotypes of the compared isolates (e.g., "*XYR1*: 53–24" in the first row indicates that 1a has *XYR1* genotype 53 and 1c has *XYR1* genotype 24).

TABLE 3 Relationship among DST, alleles, and fluconazole susceptibility testing

DST or allele	Isolate(s)	
	S	R
DSTs		
124	20	5b
256	16	12c
238	7b, 12a, 26	25
Alleles		
<i>ICL1</i>		
3	2b, 11a, P13, 21, 24, 28, 31	
1	P1, 2a, 3a, 5a, P6, 7b, P8, P10, 11b, 12a, 12b, P14, 15–17, 19, 20, 22, 23, 26, 27, 29, 30, 32–43	5b, 12c, 25
<i>MDR1</i>		
1	16, 24	12c
7	1b, 2a, 7b, P8, P10, 11a, 12a, 12b, P13, 17, 26, 27, 30, 32, 36, 39, 41	25
24	1a, 1c, 5a, 6a, 6b, 11b, P14, 15, 20, 22, 33, 35, 37, 42, 43	5b
<i>SAPT2</i>		
1	P8, 13b, 34	
3	1a, 1c, 5a, P6, 11b, P14, 15, 20–23, 29, 33, 35, 37, 38, 40, 42, 43	5b
4	1b, P2, 3a, P4, 7b, P9, P10, 11a, 12a, 12b, 13a, 17, 24, 26, 27, 30–32, 36, 39, 41	25
18	16, 19	12c
<i>SAPT4</i>		
1	3a, 16, 19	12c
6	7b, P8, 11a, 12a, 12b, P13, 17, 26	25
7	1a, 1c, 3b, 5a, P6, 11b, P14, 15, 18, 20–23, 29, 31, 33, 35, 37, 38, 40, 42, 43	5b
<i>XYR1</i>		
31	9a, 9b, 16, 19	12c
48	20	5b
52	7b, 8a, 11a, 12a, 13b, 26	25
<i>ZWF1a</i>		
1	2b, 3a, 16, 19, 24	12c
4	P4, 7b, P8, 11a, 12a, 12b, P13, 26	25
6	1a, 1c, 5a, P6, 11b, P14, 15, 17, 20, 22, 23, 33, 37, 38, 42, 43	5b

DST, diploid sequence type; P, total isolates from the same patient. The DSTs are those of isolates 5b, 12c, and 25, which had MICs of >32 $\mu\text{g/ml}$ for fluconazole (resistant [R]) or a MIC of <16 $\mu\text{g/ml}$ (susceptible [S]).

eBURST analysis. Among the 267 DSTs from the *C. tropicalis* MLST database, eBURST software found 28 groups and 131 solitary DSTs, known as singletons. Of the 39 DSTs observed in the study, 24 DSTs were grouped into five groups (groups 1, 3, 4, 6, and 18), and 15 DSTs were classified as singletons. Group 1 comprised 27 DSTs, 7 from this study. Isolate 13b (DST 134) assumed the central position of this group. Group 3 included 10 DSTs, 2 from this study. Group 4 comprised 9 DSTs, 7 from this study. Groups 6 and 18 are new groups added from this study comprising only DSTs identified only in the present work. Among the singletons, isolates 3b, 7a, and 18 remained far from the other DSTs according to the ancestry and phylogenetic analyses.

DISCUSSION

This study is the first MLST analysis to characterize the sequential isolates of *C. tropicalis* candidemia in South America and accounted for 36 new DSTs and 23 alleles that were incorporated into the official database (2 April 2012; <http://pubmlst.org/ctropicalis/>), allowing the comparison of our data with that from other countries. We compared both the phenotypic (through *in*

vitro antifungal susceptibility testing) and genotypic patterns of the samples found in two large cities and analyzed the DNA polymorphisms—microvariations and macrovariations—in patients with candidemia who had sequential isolates.

Until July 2012, the *C. tropicalis* MLST database had a total of 401 isolates worldwide and 267 DSTs compared with 2062 DSTs for *C. albicans* (<http://calbicans.mlst.net/>). The rate of the description of new alleles in our study was lower than the number of DSTs found, as observed by Odds and Jacobsen (31) in *C. albicans*, suggesting that there might be a tendency toward the conservation of these alleles and an increase in the combination of them. Our data reinforce their same theory for *C. albicans*.

The limitations of this study are represented by the interpretation of heterozygosity in diploid microorganisms (30, 31, 49) and the evaluation of only DNA fragments and not the genome as a whole (33). In both participating hospitals, great genotypic diversity was detected among the isolates. MLST was able to detect microvariation and macrovariation in the nucleotide polymorphisms of the gene fragments studied in the isolates of 13 of the 14 patients who had sequential isolates. Microvariation in a single

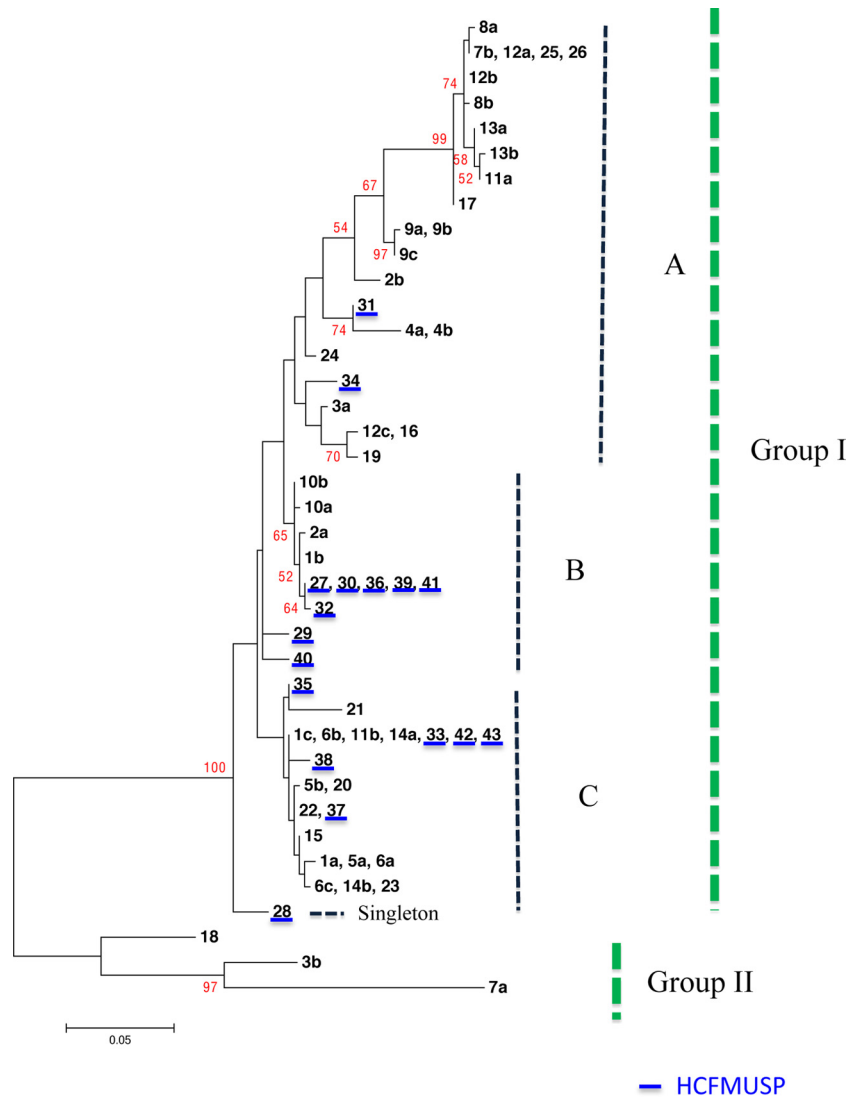


FIG 1 Maximum-likelihood dendrogram generated from the MLST of six gene fragments for the 61 isolates of *C. tropicalis* from HCFMUSP and UNICAMP. The dendrogram shows two groups, I and II, and a singleton. Group I was divided into three subgroups (A, B, and C). The HCFMUSP isolates are underlined in blue. Group II comprised isolates 7a, 3b, and 18.

gene fragment occurred more frequently in the sequential isolates from seven patients. These minor genetic changes occurred at 1 to 5 nucleotide positions. Macrovariation occurred in the isolates from 6 patients, 1, 2, 3, 7, 11, and 12, who presented differences in at least five sequenced gene fragments. Genetic changes in two gene fragments were found in 3 patients. Our results were consistent with those of Jacobsen et al. (49), who found 2 of 18 paired isolates of *C. tropicalis* with multiple losses of heterozygosity in a single gene and 2 pairs with differences in multiple genes. Of the 5 other isolates from the same source, 2 of 4 genes presented homozygous and heterozygous differences, and the fifth isolate showed a complete loss of heterozygosity in five of the six sequenced genes. During a prospective surveillance study in adult ICU patients, in specimens from different anatomical sites of the same patient, Chen et al. (32) found several cases of heterozygosity changes in single and multiple genes among 11 patients who had *C. tropicalis* isolates.

Da Matta et al. (26) analyzed 21 *C. albicans* isolates from 8 patients diagnosed with persistent or recurrent candidemia in a multicenter surveillance study. Except for one patient with 3 different DSTs, all of the isolates from the remaining 7 patients showed the same DST, the same ABC type and the same susceptibility to antifungal agents. In another study, Bougnoux et al. (50) used MLST to demonstrate that 32 *C. albicans* isolates were responsible for candidemia and 118 isolates for candiduria in an ICU. Of the 9 patients with candidemia and candiduria, only one presented isolates of different DSTs, and these isolates differed at one locus, which suggested that they were evolutionarily related. In our study, the sequential isolates from 13 of 14 patients had different DSTs (which was not a rare event), and sequential isolates from two patients presented different fluconazole susceptibility profiles.

In contrast to our study, Shin et al. (51) demonstrated that among 41 sequential *C. glabrata* isolates from 15 patients with persistent candidemia, the sequential isolates had the same se-

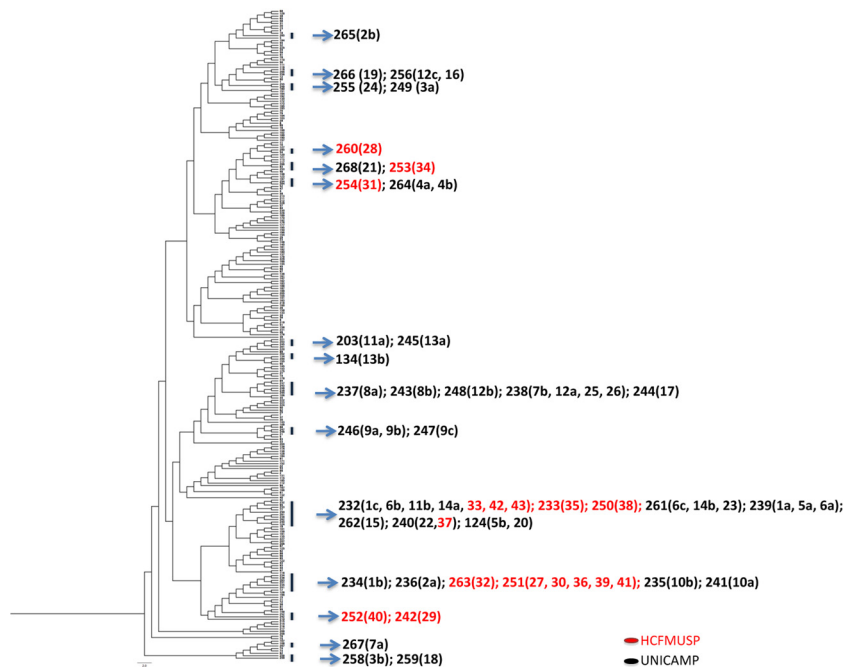


FIG 2 Bayesian inference dendrogram generated from the MLST polymorphic sites, concatenated and converted in haplotype form for 267 DSTs of *C. tropicalis*. The analysis includes the 61 isolates of *C. tropicalis* from UNICAMP and HCFMUSP that were submitted on 2 April 2012. The HCFMUSP isolates are in red.

quence type by MLST. In addition, they confirmed that acquiring azole resistance during the course of candidemia under the pressure of azole therapy is possible. In a study examining *C. glabrata* bloodstream and noninvasive isolates, Lott et al. (48) found no significant association between the sequence type and fluconazole resistance, with the MLST possessing low discriminatory power.

According to previous studies, variations in the isolates from the same patient could be explained by cross-contamination during the transport and storage times (33) or by polymorphisms that are related to antifungal-drug use (52). The present study was conducted in one of the laboratories from HCFMUSP, and the isolates have been monitored since 1998 to exclude the possibility of contamination. UNICAMP isolates were maintained in the Mycology Laboratory of the Clinics Hospital of UNICAMP from 2001 to 2003. All of the isolates were directly transferred to the Medical Investigation Laboratory (LIM 48), where they were immediately reidentified and confirmed as *C. tropicalis* before the beginning of the analyses. Since then, the isolates have been maintained in a unique laboratory and were carefully monitored during this study. DNA extraction was performed. Although contamination within the laboratory may have occurred, it is unlikely. In the present work, MLST was able to elucidate aspects of the differences between the sequential isolates from blood and catheter samples. Among isolates from patients whose blood and catheter cultures were both positive, the isolates from patients 2 and 3 deserve a special mention. In these patients, macrovariation was observed between the isolates from the blood and catheter, suggesting that each isolate apparently had no relationship with the isolate from the other site. In our study, we could not determine whether there was more than one clone in the host in the blood, the catheter, or both.

No relationships between antifungal MICs and defined DSTs and/or genotypes were observed in our study. Fluconazole resis-

tance was found in only 3 of 61 isolates. Although the blood and/or catheter isolates presented different patterns by MLST and different susceptibility profiles in our study, no relationship between the antifungal MICs and defined DSTs and/or genotypes was observed. The relationship between the DSTs and the antifungal resistance profile or trailing phenomenon has been described. Chen et al. (32) found that in *C. tropicalis*, DST 164 in MLST group II was associated with a high MIC of flucytosine. In other studies, Chou et al. (27) and Li et al. (36) found an association between DST 140 and a reduced susceptibility to fluconazole. Chou et al. (27) also reported an association between the DSTs and the *C. tropicalis* trailing phenomenon. Additionally, Li et al. (36) described several alleles associated with low MICs of fluconazole (alleles 3 of *ICL1*, 9 of *MDR1*, 1 of *SAPT2*, 3, 6, and 10 of *SAPT4*, 48 of *XYR1*, and 7 of *ZWF1a*). In our study, we found no isolate with DST 140, and there was an association of allele 48 (*XYR1*) and allele 6 (*SAPT4*) with fluconazole-susceptible and fluconazole-resistant isolates, respectively.

In conclusion, MLST is an important tool for studying the genetic diversity of multiple/sequential isolates of patients with candidemia, particularly with respect to the polymorphisms in gene fragments. This technique allowed us to compare our data with data from other regions of the world and added new groups to the MLST database. It also allowed us to compare the genetic profiles of isolates from different sites, such as blood and catheters, and sequential isolates from patients with candidemia. Fluconazole resistance was found in only 3 of 61 isolates, making any correlative analysis of DST with drug resistance impossible. In addition, the phylogenetic and eBURST analyses also suggested that there was no correlation between the DSTs and the susceptibility to fluconazole, and the selective pressure of antifungal drugs most likely exerted no influence. Prospective studies will be nec-

essay to assess the relationship between genetic variability and the pathogenicity of *C. tropicalis* isolates.

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We declare no conflicts of interest in the presentation of these findings.

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