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Multilocus sequence typing of sequential *Candida albicans* isolates from patients with persistent or recurrent fungemia

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Multilocus sequence typing (MLST) is a useful tool to explore the phylogenetics and epidemiology of *Candida albicans* isolates recovered from cases of invasive candidiasis. The goal of this study was to determine whether the same or different strains were responsible for persistent or recurrent fungemia through the use of MLST and ABC typing on sequential *C. albicans* isolates from the same patient. We applied both typing methods to 21 *C. albicans* strains recovered from 8 patients with persistent or recurrent candidemia. The isolates were collected during a multicenter surveillance study in four public tertiary care hospitals in Brazil. Persistent candidemia was defined as two or more blood cultures positive for *C. albicans* on 2 or more separate days. Recurrent candidemia was defined as an episode of candidemia occurring at least 1 month after the apparent complete resolution of an infectious episode caused by *Candida* species. We observed that, except for one patient, all strains from the first and second samples of the same patient showed the same MLST diploid sequence type (DST), ABC type and susceptibility profile to antifungals. Three distinct strains, well discriminated by MLST, were found in the seven samples collected sequentially over 10 days from one patient. The strains from the first four samples were indistinguishable, the fifth and sixth were also indistinguishable but different from the first four and seventh samples. Significantly, the seventh strain was the only *C. albicans* clade 2 isolate found in our total collection involving 61 patients, although clade 2 is commonly found worldwide. To the best of our knowledge, this is the first study describing the recovery of three distinct *C. albicans* strains in the same patient with a persistent blood stream infection within a short period of time.

Keywords *Candida albicans*, multilocus sequence typing, MLST, recurrent candidemia

Introduction

Bloodstream infections due to *Candida* species are associated with significant morbidity and mortality [1]. The commensal yeast *C. albicans* remains the major species associated with *Candida* bloodstream infections. A normal component of the human microflora, this species is capable of causing superficial disease in immunocompetent hosts, but in immunocompromised individuals the organism can cause severe invasive disease [2–4].

During the past two decades great progress has been made in our understanding of the genetics of *C. albicans*, including its pathogenicity [3], host interactions [4], and drug resistance [5]. From a population perspective, we now

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have a better understanding of the underlying mechanisms of genetic exchange [6], recombination [7] and the creation of worldwide collections of strains. For example, in one recent study Odds *et al*. performed a cladistic analysis of 1,391 strains collected worldwide, describing at least 17 distinct clades [8]. Four of these are major groups that have been reported previously [9].

Recurrent and/or persistent fungal infections caused by *Candida* species usually involve oropharyngeal and vaginal candidiasis and to a lesser extent systemic disease. Studies describing recurrent candidemia [10–14] have attempted to determine whether the same or different strains are infecting patients for different periods of time. Other studies have focused on colonization versus infection where there is a tendency to match the strain causing persistent infection to the strains found at other anatomical sites such as skin or mucosa [15–17].

Multilocus sequence typing (MLST) analyzes fragments of housekeeping genes with sequencing lengths of approximately 400 to 600 base pairs (bp). This method has a high degree of resolution, can characterize large numbers of isolates rapidly and does not require the subjective interpretation of banding patterns. Moreover, the sequences can be stored and characterized in multiple formats, offering an unprecedented degree of portability and accessibility to all interested users [18,19].

Our goal was to determine whether the same or different strains of *C. albicans* were responsible for persistent or recurrent episodes of fungemia by using MLST and ABC typing on sequential *C. albicans* isolates from the same patient. ABC typing is based on the presence or absence of an intron in the 26S rDNA region, and it was used as a supplementary method to augment MLST studies [20,21].

Materials and methods

C. albicans isolates

We evaluated a total of 21 *C. albicans* strains recovered from eight patients with persistent (six cases) or recurrent (two cases) episodes of candidemia. The isolates were obtained from samples collected during a multicenter surveillance study performed in four public tertiary care hospitals in Brazil [22]. Persistent candidemia was defined as two or more blood cultures positive for *C. albicans* on 1 or more days apart despite antifungal therapy. Recurrent candidemia was defined as an episode of candidemia occurring at least 1 month after the initial diagnosis of fungemia treated with an antifungal drug, and caused by *Candida* species [10].

Species identification and in vitro *susceptibility testing*

Isolates were identified according to their microscopic morphology on cornmeal Tween 80 agar and by biochemical tests using the ID 32C system (BioMérieux AS, Marcy l'Etoile, France). Antifungal susceptibility tests were performed against fluconazole, voriconazole and 5-fluorocytosine using the broth microdilution assay according to the methodology recommended by the CLSI, document M27-A2 (2002) [23].

MLST and ABC typing

The methodology of MLST has been previously described in detail [24,25]. The seven gene loci, *AAT1*, *ADP1*, *ACC1*, *MPIb*, *SYA1*, *VPS13* and *ZWF1* were assigned from the MLST *Candida albicans* database (http://calbicans.mlst.net/) as sequence types (STs) defining unique sequences for pairs of alleles, and diploid strain types (DSTs) which defined unique combinations of genotypes.

ABC typing, based on the presence or absence of an intron in the 26S rDNA region, was used with all isolates employing the methodology described by McCullough *et al*. in 1999 [21] and using the same DNA as for MLST. Primers and PCR conditions were as previously described [21]. Fragments were electrophoresed on 2% agarose with ethidium bromide and visually scored for the presence, absence, or heterozygosity of the insertion element.

Definition of clades

To delineate clusters of closely related strains we applied the same unweighted *P*-distance cutoff value used by Odds *et al*. in 2007 [8]. The cutoff of 0.04 employed for *C. albicans* in that study was chosen because it separated clades 2 and 4, which were well discriminated by other typing methods [9]. DSTs were assigned according to the 17 clades found in reference [8], and were confirmed when representative DSTs from that study were included in a separate analysis (data not shown).

Results

All isolates originally identified as *C. albicans* underwent molecular subtyping using MLST and ABC typing. DNA sequencing of 373 to 491 bp fragments from the coding region of each of the *AAT1*, *ADP1*, *ACC1*, *MPIb*, *SYA1*, *VPS13* and *ZWF1* genes resulted in a data set of 2,883 nucleotides for each isolate. The seven genes sequenced for the 21 *C. albicans* strains isolated from eight patients exhibited a total of 63 variable sites. Table 1 shows the results of the ABC typing and DST patterns related to each isolate tested, including the new STs and DSTs found. The 21 isolates generated 10 unique DSTs. Two STs (ST 185 for VPS13 and ST 156 for ZWF1) and three DSTs (1162, 1169 and 1186) were novel when submitted and assembled into the MLST *C. albicans* consensus database (http://test1.mlst.net/).

Table 1 Results of MLST, ABC typing and antifungal susceptibility testing of 21 *Candida albicans* strains from 8 patients with persistent or recurrent candidemia.

Patient # (PC or RC)[a]	Isolate n°	Center	Collected date	Genotypes							DST	ABC Typing	Clade	MIC (μg/mL)		
				AAT1	*ACC1*	*ADP1*	*MPIb*	*SYA1*	*VPS13*	*ZWF1*				Fluconazole	5- Fluorocitosine	Voriconazole
P1 (PC)	1	01	14 Mar 2003	54	31	10	36	66	185[b]	111	1162[c]	B	16	0.125	0.125	0.03
	2	01	1 Apr 2003	54	31	10	36	66	185[b]	111	1162[c]	B	16	0.125	0.125	0.03
P2 (PC)	77	03	9 Apr 2003	21	17	21	19	27	83	22	601	C	12	0.125	0.125	0.03
	78	03	11 Apr 2003	21	17	21	19	27	83	22	601	C	12	0.125	0.125	0.03
P3 (PC)	192	03	6 Oct 2003	8	5	5	2	2	6	5	572	A	1	0.25	2	0.03
	193	03	9 Oct 2003	8	5	5	2	2	6	5	572	A	1	0.125	2	0.03
P4 (PC)	306	12	15 Jun 2004	2	5	5	2	2	6	5	69	A	1	0.25	2	0.06
	307	12	19 Jun 2004	2	5	5	2	2	6	5	69	A	1	0.25	1	0.03
P5 (PC)	314	12	20 Jul 2004	2	5	5	2	2	24	5	24	A	1	0.25	2	0.03
	315	12	22 Jul 2004	2	5	5	2	2	24	5	24	A	1	0.125	2	0.03
	316	12	23 Jul 2004	2	5	5	2	2	24	5	24	A	1	0.125	2	0.03
	317	12	25 Jul 2004	2	5	5	2	2	24	5	24	A	1	0.125	2	0.03
	318	12	26 Jul 2004	13	7	15	6	6	55	156[b]	1169[c]	B	3	0.125	0.5	0.03
	319	12	28 Jul 2004	13	7	15	6	6	55	156[b]	1169[c]	B	3	0.125	0.5	0.03
	320	12	29 Jul 2004	35	2	4	4	36	4	4	1186[c]	A	2	0.5	2	0.03
P6 (PC)	378	12	15 Mar 2004	2	5	5	2	2	6	5	69	A	1	0.5	2	0.03
	379	12	25 Mar 2004	2	5	5	2	2	6	5	69	A	1	0.5	2	0.06
P7 (RC)	349	12	1 Nov 2004	2	2	5	2	2	6	5	277	A	1	0.125	0.125	0.03
	350	12	6 Dec 2004	2	2	5	2	2	6	5	277	A	1	0.125	0.125	0.03
P8 (RC)	275	9	9 Aug 2004	2	5	5	2	2	6	5	69	A	1	0.5	2	0.03
	276	9	28 Oct 20 04	2	5	5	2	2	6	5	69	A	1	0.125	2	0.03

[a]PC: Persistent Candidemia – RC: Recurrent Candidemia.
[b]New genotypes.
[c]New Diploid Sequence Types (DSTs).

Figure 1 illustrates the UPGMA dendrogram based on MLST data, showing that these *C. albicans* strains were assigned to clades 1, 2, 3, 12 and 16. Isolates from 6 patients were assigned to clade 1. With one exception, all pairs of isolates collected from the first and second samples of the same patient showed the same MLST diploid sequence type (DST), ABC type and susceptibility profile to antifungals.

The seven clinical isolates of Patient P5 that were recovered from samples collected sequentially within 10 days after starting therapy, showed 3 distinct DSTs (24, 1169 and 1186), as illustrated in Table 1. This patient had several samples sequentially collected for culture because she had been enrolled in a clinical trial with an investigational antifungal drug. All seven isolates remained susceptible to all antifungal drugs tested. The isolates from patient P5 were assigned to three different clades. The first four samples were included in clade 1, the fifth and sixth were assigned to clade 3. Significantly, the seventh strain recovered from that patient was the only *C. albicans* strain found in clade 2.

For ABC typing, type A isolates were found to be the most frequent, followed by type B. We did not find any type C isolates (Table 1). ABC typing and MLST results showed that 15 strains from five patients were type A and distributed in clades 1 and 2. One patient (P5) had different strains of type A and those isolates were assigned to clades 1 and 2. This patient also had another strain typed as B and assigned to MLST clade 3. In patients P1 and P2 we observed type B clades 16 and 12, respectively.

Table 1 also summarizes the minimal inhibition concentrations (MICs) of three antifungal agents against multiple *C. albicans* strains isolated from eight patients matched by DSTs and ABC types. For the susceptibility profile, all *C. albicans* strains were susceptible to fluconazole, voriconazole and 5-fluorocytosine. Pairs of isolates from five of the six patients with persistent candidemia and two patients with recurrent candidemia exhibited limited MIC variability and remained susceptible to all drugs tested. The latter group had two isolates each, collected more than 30 days apart.

Discussion

MLST has been used in the analysis of the epidemiology and evolutionary phylogenetics of many bacterial and fungal pathogens. There are schemes published for five *Candida* species including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei* and *C. tropicalis* [19]. We applied MLST to our collection of *C. albicans* from bloodstream infections to confirm if one single strain or multiple strains

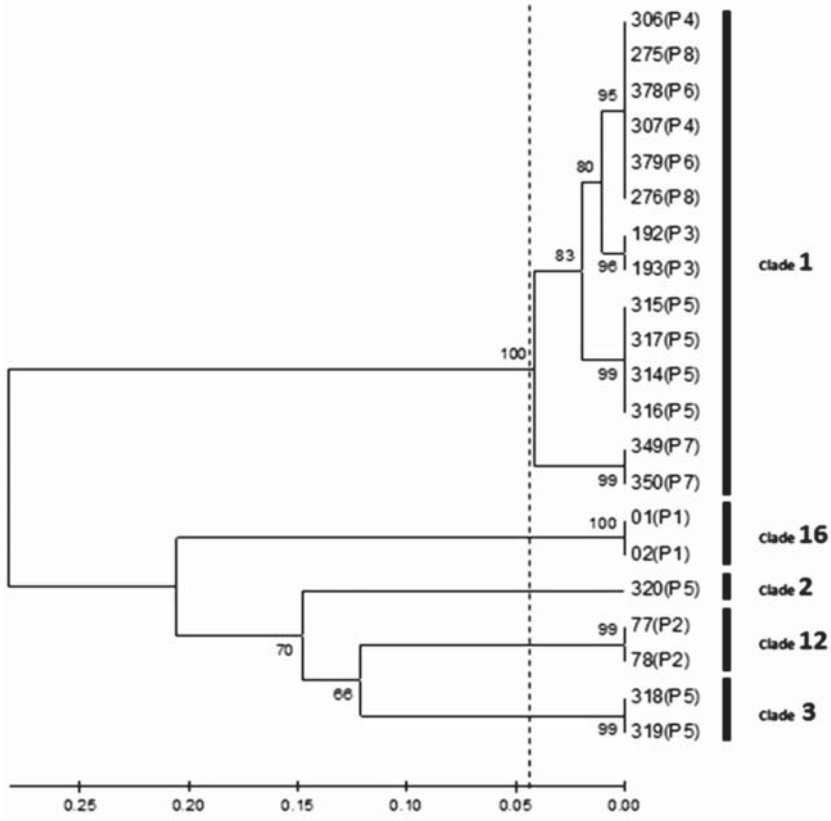

Fig. 1 UPGMA dendrogram for 21 *Candida albicans* isolates from 8 patients (in parentheses) with candidemia typed by MLST. Uncorrected P-distances are shown in the horizontal line. The dashed line shows the cutoff at a P distance of 0.04, used to delineate clades as suggested by Odds *et al*. [8].

could cause persistent or recurrent candidemia. This particular genotyping tool was able to discriminate 10 different multilocus genotypes among 21 clinical isolates from eight patients and corroborates the hypothesis that although most of the time there is a single strain, there can be multiple strains involved during an infection. In addition we observed that ABC typing was predominantly represented by type A which was found in six of eight patients, as has been seen in several studies [21,25,26].

A recent study showed *C. albicans* clade 1 as the most common clade among a large assemblage of strains collected worldwide [8]. Their results revealed that 40% and 50% of *C. albicans* isolates from South America and the United States were clade 1, respectively. Despite the limited number of clinical strains evaluated in our study, isolates from 6 of 8 patients were classified as clade 1, while isolates from two patients were placed in clades 12 and 16. Surprisingly, three distinct strains assigned to clades 1, 2 and 3 were recovered from patient P5 over a 10-day period.

Persistent candidemia was observed in six patients in this study. *Candida albicans* strains from five of these patients exhibited the same MLST genotypes throughout the period of study. Similarly, in one molecular epidemiological analysis of bloodstream isolates of *C. albicans*, Shin *et al*. observed that in serial bloodstream isolates from 11 patients, all strains from each patient had the same PFGE pattern [12].

In another study, Shin *et al*. used PFGE and MLST to examine changes in genotypes among 41 sequential *C. glabrata* isolates from 15 patients with persistent candidemia over periods of up to 36 days [14]. The sequential isolates had small differences in electrophoretic karyotypes in 40% of patients, yet were of the same sequence type by MLST. As MLST is used to differentiate strains by detecting nucleotide changes, one possible limitation is that it analyzes only certain fragments of the DNA and not the whole genome. In the near future it may be possible to analyze larger genomic fragments or entire genomes [19].

Through MLST it has been suggested that microvariation may be related to gain or loss of sequence heterozygosity (LOH) in one or more of the seven genes used in this technique [20,27–28]. LOH may result from chromosome deletion or loss, recombination and/or gene conversion events [20]. In this study none of the sequential isolates recovered during bloodstream infection showed evidence of microvariation by MLST, although it is possible that sampling over longer periods could have demonstrated this phenomenon.

It is noteworthy that we documented persistent candidemia in a patient (P5) from whom three distinct *C. albicans* strains were collected from the bloodstream within a period of 10 days. Concerning this patient's strains we should note that (i) we do not believe that microvariation occurred since there were large differences in all genotypes by MLST and ABC typing and the strains were widely separated in the UPGMA dendrogram, assigned to three different clades, (ii) there is no evidence of inadvertent misidentification during the patient's stay because more than one sample was obtained on each collection day (except for the last day), and (iii) although not definitive, it is possible that the patient could have carried all three strains during hospitalization.

Another intriguing point is the assignment of the last isolate from patient P5 to clade 2. Clade 2 has been found in Africa and the UK, and is seen in up to 20–30% of isolates included in a recent worldwide study [8]. This is the first study describing a bloodstream infection by a *C. albicans* strain assigned to clade 2 in Brazil. These data are part of a larger study involving 74 *C. albicans* strains from 61 patients [D. A. da Matta *et al*. unpublished data].

Recurrent candidemia was defined as an episode of candidemia occurring at least 1 month after the initial diagnosis of fungemia. Two patients with recurrent candidemia included in our current study showed strong evidence that an indistinguishable strain was involved in both episodes. This was also described in other studies where identical *Candida* strains of various species were found responsible for different episodes of candidemia through the use of subtyping [10,13]. Neofytos *et al*. reported three episodes of recurrent fungemia caused by *C. parapsilosis* in a cancer patient. Molecular testing confirmed that all episodes occurring within an 8-month period were caused by the same strain [13]. Clancy *et al*. investigated five patients with recurrent candidemia and the isolates were characterized by restriction enzyme analysis of genomic DNA (REAG) and inter-repeat PCR (IR-PCR). Each recurrent episode of candidemia was caused by the same *Candida* species as the initial episode [10].

Conversely there are studies involving recurrent candidemia that describe different strains relating to different episodes of candidemia [11,14]. Shin *et al*., by applying electrophoretic karyotyping to sequential *C. parapsilosis* isolates from patients with recurrent fungemia, observed that index and recurrent episodes of fungemia were caused by different strains in six of the 17 patients [11]. One possibility is that events such as loss of heterozygosity may result in a misinterpretation of strain type.

To the best of our knowledge, this is the first study describing a bloodstream infection with three distinct *C. albicans* strains in the same patient within a short period of time. We note that this appears to be a rare occurrence, since there have been no previous studies relating to candidemia as described in patient P5. The exposure to more than one *C. albicans* strain could be due to the colonization of the gastrointestinal tract by several unrelated strains, arising in the bloodstream as a consequence of multiple abdominal surgeries documented in this particular patient. Although the current study has only a limited number of isolates, we observed persistent

or recurrent candidemia caused by the same *Candida albicans* strain.

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