Candidemia Surveillance in Brazil: Evidence for a Geographical Boundary Defining an Area Exhibiting an Abatement of Infections by *Candida albicans* Group 2 Strains[▽]

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Prospective population surveillance has been conducted for candidemia in Brazil (A. L. Colombo, M. Nucci, B. J. Park, et al., J. Clin. Microbiol. 44:2816–2823, 2006). In the present study, a total of 63 isolates from 61 patients, representing 11 medical centers from nine geographic regions, were characterized by multilocus sequence typing (MLST). A total of 48 unique profiles or diploid sequence types (DSTs) were observed, with nine new sequence types (STs) and 32 new DSTs. There were no apparent correlations between center/region and DST patterns. Subtypes were compared to those in a known characterized reference set, including a large database of strains obtained worldwide. Significantly, only one *C. albicans* group 2 isolate was found in our collection, although isolates from this particular group are commonly found worldwide. These data, combined with information from other previously reported studies, establish a statistically significant diminishment of group 2 strains in Central and South America, including Mexico and portions of the Southwestern United States.

The commensal yeast *Candida albicans* is a normal part of the human microflora. This species can be an agent of mucosal infections of healthy individuals and is capable of causing invasive disease, particularly when the host is debilitated or immunocompromised. Bloodstream infections due to *Candida albicans* are associated with significant morbidity and mortality (21), and its importance has resulted in the initiation of sentinel and population-based surveillance studies to determine the burden of disease and the incidence of antifungal resistance (9, 30, 32). Many corollary genetic analyses of *C. albicans* have also been undertaken (1, 10, 25), and our understanding of virulence and pathogenicity in this species has increased (21). *C. albicans* is diploid, with a primarily clonal mode of reproduction. However, there is evidence for a low level of recombination between strains, and either cryptic or parasexual meiosis has been suggested (24). Within a single strain there can be relatively high rates of nonhomologous mitotic recombination, translocation, and aneuploidy (24). These are thought to be the major underlying mechanisms responsible for the observed genetic diversity within the species (1, 24, 25).

Early cladistic studies by a variety of workers (16, 22) demonstrated three major groups, or clades, of strains within the species. Those studies used collections that were primarily from Northern Europe and the United States. More-recent studies using a variety of DNA markers have substantiated and extended these results (11). In particular, the use of the middle-repetitive element Ca3 demonstrated this tripartite population structure (23), and the groups were named groups 1 to 3. Group 1 strains are typically the majority of any defined population (15, 22, 23). Unlike groups 2 and 3, this group is positively correlated with the presence of a subtelomeric element (8).

Later analyses extended the number of strains to larger geographic regions, and it was observed that other clades were associated (but not exclusively) with Europe (termed group "E") and South Africa (termed group "SA") (2, 23, 26). To date, it has been assumed that groups 1 to 3 are ubiquitous and distributed worldwide, although the number of analyzed isolates from many discrete geographical regions has been too low to give statistical support for this concept.

More recently, multilocus sequence typing (MLST) has been developed for *Candida albicans*, using seven consensus "housekeeping" genes (3, 4, 19, 29). For each locus individually, alleles are assigned on the basis of single-nucleotide polymorphisms (SNPs) found within the sequenced region. Because *C. albicans* is diploid, there are three possibilities for each polymorphism: two homozygous and one heterozygous. By definition, a single base change defines a new allele, or sequence type (ST) (4, 18). The summation of each sequence type for the seven loci then collectively defines a diploid sequence type (DST) for a given strain. To date, there are between 100 and 200 known alleles for each locus and between 1,000 and 2,000 DSTs (17). In a recent study, Odds et al. used MLST in a cladistic analysis of 1,391 strains from a worldwide collection. Groups 1, 2, and 3 were again observed, along with group SA (termed group 4 in this study) (17). Clade E was divided into clade SA and a new clade (group 11), which contained 7.5% of the total isolates, and 96.7% of the total isolates could be assigned to one of 17 groups (17). The five largest clades assigned by MLST as groups 1 to 4 and 11 corresponded with

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up to 70% of *C. albicans* isolates found worldwide (19, 28, 29). In addition, Odds et al. scored strains for the presence of the IS*1* element as a corollary to MLST typing. Termed "ABC" typing, this simply refers to the presence, "A," absence, "B," or heterozygosity, "C," of the element.

Although there have been several studies attempting to relate differences in geographic locations and virulences among *C. albicans* strains, clinical and pathogenic differences among isolates of different clades are still unclear (17). The present study was undertaken to determine the MLST groups and ABC types of bloodstream isolates from Brazil and to determine if there was a correlation between genotypes and geographic origins of the surveillance population.

MATERIALS AND METHODS

***C. albicans* isolates.** We evaluated *C. albicans* bloodstream isolates recovered from 61 randomly chosen patients with candidemia collected during a multicenter surveillance study of 11 public tertiary care hospitals, representative of the public health system of nine major cities in Brazil (5). These hospitals are located in the South, Southeast, and Central regions, including the metropolitan areas of Rio de Janeiro and São Paulo (5). Each isolate was obtained from a different patient, with the exception of three distinct strains isolated from one patient during the course of candidemia (5). These three strains were included in the study, for a total of 63 isolates.

Species identification. Isolates were identified according to their microscopic morphologies on cornmeal Tween 80 agar and by biochemical tests using the ID 32C system (bioMérieux AS, Marcy l'Etoile, France).

MLST and ABC typing. The methodology of MLST was described in detail previously (4, 18). Briefly, DNA was prepared from fresh cultures grown on Sabouraud dextrose agar using a MoBio (Carlsbad, CA) microbial DNA isolation kit according to the manufacturer's instructions. PCR primers and conditions were described previously in reference 4. PCR fragments were visually inspected by electrophoresis on 2% agarose prior to purification by Exosap-It treatment (USB, Cleveland, OH). Fragments were sequenced in both forward and reverse orientations using the original PCR primers. Sequencing was performed by using ABI (Carlsbad, CA) reagents and equipment according to the manufacturer's recommendations. DNA analysis was performed by using Sequencher software (Genecodes, Ann Arbor, MI), and each electrophoretic trace was visually examined for heterozygosities. The seven gene loci *AAT1a*, *ADP1*, *ACC1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b* 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. By definition, any nucleotide difference within the defined region of a locus is scored as a new ST and will also be scored as a new DST. Single-nucleotide polymorphisms (SNPs) from the diploid sequence were converted as a single sequence and concatenated as previously defined (18). Cladistic analysis was performed with Mega software, version 4 (28), and Phylip, version 4.0 (7). ABC typing, based on the presence or absence of an intron in the 26S ribosomal DNA (rDNA) region, was determined for all isolates as described previously by Lott et al. (14), using the same DNA as that used for MLST. Primers and PCR conditions were previously described (14). 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 previously by Odds et al. (17). The cutoff of 0.1 used for *C. albicans* in that study was chosen because it separated groups 2 and 4, which were well discriminated by other typing methods (22). eBURST analysis (http://eburst.mlst.net/v3/enter_data/single/) was performed under the criterion of strict clonality (6).

RESULTS

A total of 63 isolates from 61 patients were included in this study. For most patients, a single colony was observed and chosen from the primary culture plate. Multiple isolates were obtained from several patients, but subsequent analysis showed that the isolates were of the same MLST and ABC types. In these cases, a single representative isolate was included for the patient. There was no known epidemiological relationship among the patients.

Isolates originally identified as *C. albicans* by standard methods underwent molecular subtyping by using MLST and ABC typing. DNA sequencing of 373- to 491-bp fragments from the coding region of each of the genes *AAT1a*, *ADP1*, *ACC1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b* resulted in a data set of 2,883 nucleotides for each isolate. The seven genes sequenced for the 63 *C. albicans* strains exhibited a total of 73 variable sites, representing 2.6% of total sites. A total of 48 unique profiles or diploid sequence types (DSTs) were observed, with 9 new sequence types (STs) and 32 DSTs. New STs were found in the *AAT1* (two new sequence types) *ACC1* (one), *ADP1* (one), *VPS13* (one), and *ZWF1* (four) loci. No new STs were observed for the *MPIb* and *SYA* loci. For eight of the nine new STs, new combinations of previously described nucleotide polymorphisms were observed. For *AAT1* ST109, there was a new mutation in addition to a new combination of known polymorphisms. This was a G→A second-position nonsynonymous transition, Gly→Asp, at position 299 relative to the reference start position (4). The remaining 23 new DSTs were a result of new combinations of existing STs. New STs and DSTs are shown in Table 1.

For previously described diploid sequence types, DST69 was the most common DST isolated. DST69 was found in isolates from 10 patients (16.5%) in six medical centers from six different cities: Brasília (three strains), Curitiba (two strains), Federal University of Rio de Janeiro (UFRJ) in Rio de Janeiro (two strains), Ribeirão Preto (one strain), São José do Rio Preto (one strain), and Hospital do Servidor Público Estadual (HSPE) in São Paulo (one strain). DST24 was the second most isolated DST and was found in isolates from four patients (6.5%): two in Brasília, one in Campinas, and one at HSPE in São Paulo. Isolates from three other patients had DSTs that were represented twice in the population.

Polymorphisms for all seven loci were concatenated and used for both distance matrix and parsimony-based approaches in a cladistic analysis. In both cases, the general topologies of the derived dendrograms were similar but not identical. Both parsimony and neighbor-joining (including the unweighted-pair group method using average linkages [UPGMA]) algorithms clustered most group 1 isolates together. A representative dendrogram based on UPGMA for the 63 total strains is given in Fig. 1. For group assignments, similar matrices that included reference sequences from the 17 previously described groups were constructed. We observed that by using a previously defined *P* value cutoff of 0.1 (18), all but one isolate could be assigned (isolate designated a "singleton"). This *P* value cutoff could discriminate the two strains assigned to groups 2 and 4 (Fig. 1). Thirty-five of the 63 isolates (55%) were found to be group 1 isolates. Groups 8 and 3 were the next most abundant, each containing approximately 13% of the population. We observed that 50 isolates (82%) were of ABC type A, while types B and C were found for 16.5% and 1.5% of the isolates, respectively. A large percentage of group 1 isolates were of type A, consistent with previous suggestions that these isolates were related by descent (Fig. 2) (11).

For group 1 isolates, we were interested in determining whether relationships of clonal descent could be inferred. An eBURST analysis was performed by using all group 1

TABLE 1. Unique genotypes from 11 tertiary care hospitals in Brazil

Isolate	ST at locus[a]:							DST[b]	ABC type	Clade[c]
	AAT1	ACC1	ADP1	MPIb	SYA	VPS13	ZWF1			
1	54	31	10	36	66	**185**	111	1162	B	16
2	55	14	4	54	6	6	15	1171	A	8
3	13	13	**85**	6	50	32	15	1163	B	3
4	20	3	6	2	51	36	5	1172	A	1
5	2	5	5	12	2	6	**154**	1164	A	1
6	25	7	6	3	34	24	37	1173	A	8
7	70	14	6	4	35	10	8	1174	B	4
8	2	8	2	2	2	6	5	1175	A	1
9	42	5	5	19	2	6	5	1176	A	1
10	2	5	5	2	2	6	**154**	1165	A	1
11	13	10	15	2	2	37	15	1177	B	3
12	33	14	38	2	78	122	5	1178	B	8
13	2	2	6	2	2	6	5	1179	A	1
14	**108**	5	5	2	2	6	5	1166	A	1
15	60	3	3	3	3	39	95	1180	A	9
16	47	14	57	28	44	30	**136**	1167	A	17
17	47	14	57	28	44	30	5	1181	A	17
18	60	3	3	3	3	39	3	1182	A	9
19	2	5	2	4	2	24	5	1183	A	1
20	*109*	7	10	4	65	110	**155**	1168	A	S
21	2	5	5	27	2	71	5	1184	A	1
22	2	2	5	4	2	6	5	1185	A	1
23	13	7	15	6	77	55	**156**	1169	B	3
24	35	2	4	4	36	4	4	1186	A	2
25	2	7	6	3	6	27	37	1187	A	8
26	21	17	21	19	27	27	22	1188	B	12
27	31	5	6	2	2	6	5	1189	C	1
28	13	**74**	15	6	7	37	5	1176	A	3
29	47	14	52	28	44	30	6	1190	A	17
30	8	5	2	2	2	24	5	1191	A	1
31	13	11	15	15	50	37	15	1192	A	3
32	47	3	52	28	44	4	6	1193	A	17

[a] Numbers refer to assigned alleles (STs) (http://calbicans.mlst.net/). Boldface type indicates new STs from existing SNPs. Italic type indicates a new mutation.
[b] Numbers refer to assigned DSTs.
[c] S, singleton.

isolates from this study and several previously reported strains that had DSTs unique to Brazil (http://test1.mlst.net /earth/maps/). These results are shown in Fig. 2. We had initially observed that several of the DSTs observed in this study appeared to be similar. For example, DST1167 and DST1181 differ only by a *ZWF1* ST. A closer examination revealed that this could be explained by a loss-of-heterozygosity (LOH) event in DST1181. Likewise, DST1164 and DST1165 share a unique *ZWF1* ST, and their difference can be explained by an LOH in the *MP1b* locus. We also found examples where previously reported DSTs were related to unique DSTs from this study. As shown in Fig. 2, the analysis of clonal relatedness demonstrated that 17 of the 25 unique profiles clustered into one clonal cluster. DST1167 and DST1181 were not included. However, as expected, DST1164 and DST1165 were included, as were Brazilian DSTs 1373 and 1400 from a previous study. DST69 was calculated to be the progenitor of the cluster.

DISCUSSION

Comparatively few prospective studies of candidemia have been conducted in Brazil, and the corresponding collection of

bloodstream isolates presented here can be studied from a population perspective. First, we observed that one-third (22/63) of the strains could be classified as group 1 strains. This was not unexpected, in that group 1 strains have been shown to be a significant percentage of all worldwide geographic communities (15, 20). Second, we observed that slightly over half (33/63) of the strains represented new DSTs and that a majority of these were derived from new combinations of existing STs. Again, we do not believe that this is unexpected. Under the assumption that STs behave as alleles in the population (i.e., freely associating), then if *C. albicans* is undergoing any recombination, there will be a significant reshuffling of alleles, and corresponding new DSTs will be formed. We have observed that for group 1 strains, some newly described DSTs appear to be related. For example, DST1164 and DST1165 share unique *ZWF1* STs (Table 1), and the difference in their genotypes can be explained by an LOH in the *MP1b* locus. We also observed that some new group 1 DSTs appear to be related to previously described DSTs unique to Brazil (Fig. 2). Interestingly, DST69 was selected to be the progenitor of the clonal cluster. This DST is the most common genotype found in group 1 isolates and is the most commonly found DST worldwide (17). We believe that this lends additional support
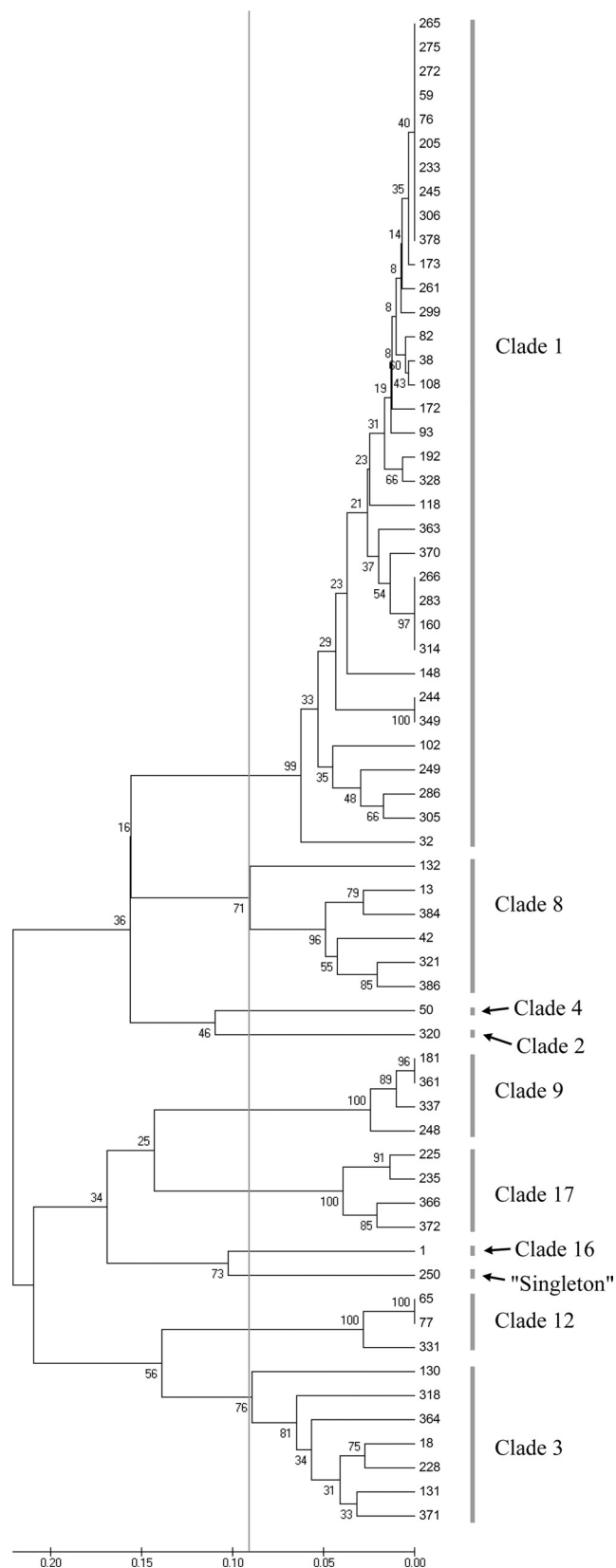
FIG. 1. UPGMA dendrogram showing uncorrected *p* distances for 63 *Candida albicans* strains typed by MLST. The dashed line shows the cutoff at a *P* value of 0.1, used to delineate clades. Bootstrap values are shown at the nodes (*n* = 1,000). Clade (group) assignments are given at the right.
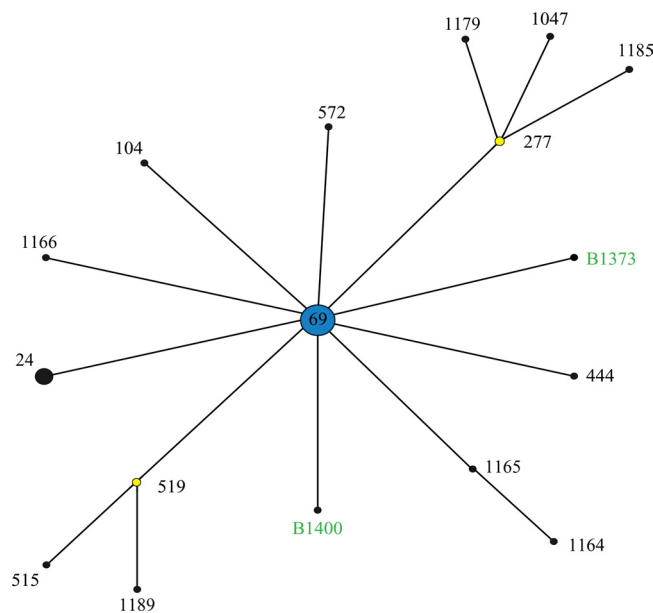

FIG. 2. eBURST analysis of group 1 strains from this study and previously observed DSTs unique to Brazil. The blue circle (DST69) represents the predicted progenitor of the clonal cluster, and yellow circles represent secondary progenitors. Sizes are proportional to the frequency in the population. Green numbers represent DSTs reported in a previous study (17).

to the concept that many Brazilian group 1 strains are derived from a common ancestral type.

Perhaps the most interesting aspect of the present study is the distribution of strains within groups. Groups 5 to 17 are considered minor components of the population hierarchy and are found in various degrees in different geographic populations (15, 20). However, in populations from North America, Europe, and Asia, groups 1 to 4 comprise the large majority of the population (up to 80%) (15). In particular, group 2 strains comprise approximately 20% of the worldwide collection, and there is evidence that they are even more prevalent in Western Europe (17, 20). This clade is present in Japan and additional worldwide locations, including the northern United States (27). In the present study, only one group 2 strain was identified. This strain was obtained from a patient with persistent candidemia and no known travel history outside South America (5). Presently, there are 100 isolates in the database (http://calbicans.mlst.net.) from South America, Central America, and Mexico, and of these, there has been only one other group 2 isolate described (from Chile). Using a chi-square statistic and an average probability of 20%, the derived χ^2 value was 18.5, with a *P* value of <0.0001, showing that the South American frequency matches the worldwide frequency. Using a more conservative Fisher's exact test, the *P* value was 1.6×10^{-5} (two tailed), and this would be in the expected range. However, when the results of this study are added to the database, the combined probability (chi square) is now a χ^2 value of 35, and with the Fisher's exact test, the *P* value was 1.5×10^{-8}, showing that the South American frequency matches the worldwide frequency. Thus, there is now very strong evidence that a geographic abatement exists for group 2 in this region of the world.

Some earlier studies might shed light on how far north this abatement might extend. Pujol and coworkers examined populations from the United States using the repetitive element Ca3 and observed that no group 2 strains were found in a collection of 29 isolates in Texas, including Dallas and Galveston (23). However, other studies involving microsatellite allelic frequencies (13) suggested that group 2 strains are present in northern California. We therefore hypothesize that the abatement zone may extend through the Southwestern United States. This suggests some form of selection, and the boundary may imply a climate zone. This seems improbable, however, considering the known epidemiology of the organism. *C. albicans* is not known to exist in the environment for long periods, only transiently during person-to-person transmission. Perhaps a better explanation may be that there is some host factor that is prevalent in the population and leads to a diminishment of a strain type. These factors could possibly include specific HLA types or Toll-like receptor alleles. Unlike group 1 strains that contain group-specific genetic elements and where there has been a suggestion that these may represent more successful colonizers (8, 33), relatively little is known about group 2 strains. There is genetic evidence that *C. albicans* strain groups have been separated by several million years (12).

Phenotypic differences among *C. albicans* clades have been described previously (15). The most evident example concerns the resistance to flucytosine, where it is confined to group 1 isolates. In our study, the seven isolates showing flucytosine MICs of >4 µg/ml were classified as clade 1 type A (data not shown). Also, resistance to terbinafine has been found to be related to isolates in clade 1 and, to a lesser extent, isolates in clade 3 (17). Previously reported studies also described a higher proportion of salt-tolerant isolates in clade 1, very low whole-cell acid phosphatase activity among *C. albicans* clade 2 strains, and statistically significant interclade differences in numbers of midrepeat sequences in some members of the ALS and HYR gene families, which encode cell surface proteins (17).

Finally, the isolations of *C. albicans* strains recovered from human patients and animals do not seem to follow the same pattern, as demonstrated in two studies where the distributions of clades recovered from both groups were significantly different (19). Future studies may help resolve questions pertaining to the evolution of strain groups in this human-commensal species as well as help address the issues surrounding the source(s) of infection (colonization), virulence, and geographic location.

ACKNOWLEDGMENTS

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