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Typing *Candida albicans* Oral Isolates from Human Immunodeficiency Virus-Infected Patients by Multilocus Enzyme Electrophoresis and DNA Fingerprinting

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A total of 189 *Candida albicans* isolates have been typed by multilocus enzyme electrophoresis. The results obtained confirm the clonal mode of reproduction of *C. albicans*. The *C. albicans* populations found in the oropharynx of human immunodeficiency virus (HIV)-infected patients, in the oropharynx of healthy carriers, or in association with invasive candidiasis could not be distinguished. No clone or group of clones could be associated with the appearance of clinical disorders or with a reduced in vitro susceptibility to the antifungal agent fluconazole. Multiple and sequential oral isolates from 24 HIV-infected patients were also typed by restriction enzyme analysis with the enzymes *Eco*RI and *Hin*fI and by use of the Ca3 repetitive probe. The results obtained by the combination of all three typing methods show that all but one patient each carried a unique major *C. albicans* clone in their oropharynx. The 21 patients with sequential isolates had the same *C. albicans* clones in their throats during recurrent oropharyngeal candidiasis episodes, independently of clinical status or of changes of in vitro susceptibility to fluconazole. Finally, several isolates of the same *C. albicans* clone found simultaneously in the oropharynx of a patient may present different levels of susceptibility to fluconazole.

Among the numerous AIDS-associated diseases, oropharyngeal candidiasis (OPC) is the most frequent, with up to 90% of HIV-infected patients suffering from it at one time or another (9, 47, 49). This clinical syndrome is not life-threatening, but it is painful and its recurrent nature makes it of importance for patients already fighting numerous other diseases. OPC is caused mainly by the commensal yeast *Candida albicans*. In spite of several studies using molecular typing methods, it is still unclear if *C. albicans* strains from the oral cavities of human immunodeficiency virus (HIV)-infected patients are the same as those from healthy persons (4, 13, 20, 29, 42, 53). Although most investigators have demonstrated a common clonal origin for pathogenic and commensal isolates, some of them have shown a reduced genetic diversity (42) or a change in the frequency of phenotypes or genotypes (4, 20) among pathogenic oral isolates in comparison with commensal ones. Thus, further studies are needed to clarify whether OPC-associated *C. albicans* represents a limited subgroup within the wider population of commensal strains. Because of its clinical efficacy and ease of use, fluconazole has become the antifungal agent of choice for the treatment of OPC (6). However, several recent reports have made the medical community aware of the development of clinical resistance to fluconazole therapy and of the appearance of resistant *C. albicans* isolates (34).

In order to better understand the epidemiology of OPC in AIDS and the development of resistance to fluconazole, molecular typing methods have been used to analyze the *C. albicans* strains found in OPC cases, with variable results. Some investigators have found that the oropharynx of HIV-infected patients is colonized mostly by a unique *C. albicans* strain and that recurrent OPC episodes are due to the same unique strain, regardless of antifungal treatment (24, 53). Others have reported that several distinct strains may simultaneously colonize the oropharynx of a patient or strain replacement may take place during the course of repeated OPC episodes in up to 45% of cases (1, 22, 28, 30, 39). Similarly, some authors found that fluconazole-susceptible and -resistant isolates from a patient generally belonged to the same type (23, 24, 33, 36). Others, however, found that in a significant proportion of cases, susceptible and resistant isolates from the same patient were of different types (2, 28, 39). These discrepancies may be related to methodological differences between the studies, either in the typing technique used, in the patient populations studied, or in the therapeutic protocols applied to the patients. It therefore was of interest to investigate this problem by another technique, not yet widely applied to OPC epidemiology, i.e., multilocus enzyme electrophoresis (MEE) or isoenzyme typing.

Besides strain delineation MEE allows the assessment of genetic diversity and of population structures (25). This technique has been widely used in bacterial epidemiology for years and was recently applied to the field of *Candida* epidemiology and genetics by several investigators (3, 5, 17, 31, 38). Using MEE, we first studied the diversity of *C. albicans* isolates from the oral cavities of HIV-infected patients in comparison with that of isolates from the oral cavities of healthy people and with *C. albicans* strains isolated in the course of invasive candidiasis in HIV-negative patients. In a second step, oral *C. albicans* isolates obtained during recurrent OPC episodes from HIV-infected patients were compared by MEE, restriction enzyme analysis (REA), and DNA fingerprinting with the repetitive Ca3 probe (43). Finally, the susceptible and resistant *C. albicans* strains isolated from several patients in connection with the development of clinical resistance to fluconazole were examined by the same three typing methods.

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MATERIALS AND METHODS

Patients. *C. albicans* isolates from 69 individuals were used for the present study. Oral *C. albicans* isolates taken from 15 persons during routine medical

examinations at their admission as medical personnel in our hospital in April 1993 were used as a reference population of *C. albicans* from healthy carriers. Oral isolates from 33 HIV-infected patients attending our university clinic between June 1990 and August 1994 were used as a representative population from HIV-infected *C. albicans* carriers. Of these, 12 patients never had clinical signs of OPC and 21 patients had OPC at the time of one or several samplings (20 patients) or between successive sampling dates (1 patient). Yeast isolates from 21 patients suffering from invasive candidiasis between March 1983 and March 1994 were used as a sample population for pathogenic isolates originating from sites other than the oral cavity. These latter isolates were from blood (14), intravenous catheters (2) or deep infections (5).

Isolation and identification procedures. For the isolation of yeasts from normally sterile sites, enrichment media (Septi-Chek blood culture bottles and thioglycolate broth; Becton Dickinson and Co., Cockeysville, Md.) and/or selective medium (Sabouraud dextrose agar containing 10 μg of gentamicin per ml and 100 μg of chloramphenicol per ml) was inoculated with the clinical samples and incubated under aerobic conditions at 30°C. Yeast cells were subcultured and isolated onto one Sabouraud dextrose agar plate with antibiotics or onto one Albicans ID plate (bioMérieux SA, Marcy l'Etoile, France), microscopically examined, and tested for germ tube formation in serum (16). All strains were secondarily tested on Albicans ID plates for their ability to form blue colonies typical for *C. albicans* on this indicator medium (27).

For the isolation of oral *C. albicans* from healthy persons and HIV-infected patients, a swab (Culturette; Becton Dickinson and Co.) was rubbed across the whole palate and on oropharyngeal lesions when present. For each specimen, one Sabouraud dextrose agar plate containing 10 μg of gentamicin per ml and 100 μg of chloramphenicol per ml and one tube of Sabouraud dextrose broth (Oxoid) with the same antibiotics were inoculated with the swabs and incubated under aerobic conditions at 30°C. After 2 days of incubation, the broth was subcultured onto one Sabouraud dextrose agar plate with antibiotics or one Albicans ID plate. Phenotypically distinct yeast colonies were isolated and identified as described above. Except when stated below, only one colony per sample was tested.

Yeast isolates. A total of 189 *C. albicans* isolates were examined and typed by one method or several methods. All the isolates were coded and blindly submitted for typing. All 15 healthy carriers and 21 patients with invasive candidiasis are represented by a single isolate. Among the 33 HIV-infected patients, 9 patients were represented by a single oral isolate and 24 by multiple oral isolates.

The 24 HIV-infected patients with multiple isolates were represented by 2 to 23 isolates (median, three oral isolates per HIV-infected patient). Three of these HIV-infected patients never had signs of OPC and had never received any fluconazole therapy. They are represented by 5, 10, and 10 *C. albicans* isolates, originating from distinct colonies on plates directly inoculated with a unique clinical sample without a previous enrichment step. Six of the 24 HIV-infected patients with multiple isolates are represented by single and multiple simultaneous isolates from successive samples (12 samples with two to eight isolates per sample). The remaining 15 of the 24 HIV-infected patients with multiple isolates are represented by single successive isolates. Thus, a total of 21 HIV-infected patients are represented by isolates from successive samples, and the interval between the first and the last samples ranged from 39 to 1,374 days (median, 263 days).

Testing of susceptibility to fluconazole. The in vitro susceptibility to fluconazole of all isolates was tested by an agar disk diffusion method described elsewhere (50). Results obtained with this method have been previously shown to correlate with those obtained by classical MIC broth dilution testing (7). On the basis of correlation with clinical response, the cutoff value separating susceptible from resistant isolates was set at an inhibition zone diameter of 25 mm (50).

MEE. Yeast isolates were grown overnight at 35°C on brain heart infusion agar (Difco, Detroit, Mich.) (eight plates per strain), scraped, and washed once in 0.9% NaCl. They were resuspended in 1.8 ml of lysate buffer (44) and subjected twice to ultrasound treatment for 40 s by using a microtip on a Soniprep 150 sonicator (Zivy SA, Oberwil, Switzerland) with maximal power and an amplitude of 18 μm. All the manipulations were made on an ice-water cooling bath. The resulting lysates were centrifuged for 30 min at 12,000 × *g* at 4°C, and the supernatants were frozen in aliquots at −80°C until use. Electrophoresis in starch gels was performed as described by Selander and coworkers (44). The 18 specific enzyme staining procedures and the corresponding buffer systems used are shown in Table 1. Bands on the gels were numbered in order of decreasing mobility. Each unique combination of electrophoretic migration patterns for the 18 loci examined results in an electrophoretic type (ET). The ETs were numbered arbitrarily without any relation to genetic relatedness. When possible, the allelic composition at the corresponding loci was determined according to general rules commonly accepted for diploid organisms (11, 25, 26).

Discriminatory power. The ability of MEE to discriminate between epidemiologically unrelated strains was assessed by use of Simpson's index of diversity (15). For this purpose, one isolate per patient was considered representative, except for one patient, who carried two clearly distinct strains. In three other cases with two ETs per patient differing for one or two alleles, only the predominant type was considered.

Cluster analysis. By using the NTSYS-PC program (Applied Biostatistics, Inc., Setauket, N.Y.), the similarity between ETs was expressed as the Dice coefficient of similarity based on the allele composition at the 16 genetic loci, allowing a clear allelic interpretation of the migration patterns. To visualize the relationships between ETs, a dendrogram was built using the unweighted pair-group method with arithmetic averages (UPGMA method) based on the matrix of Dice coefficients.

TABLE 1. Electrophoresis buffers and specific enzyme staining procedures for MEE

Buffer system[a]	Specific enzyme	Reference
A	αG	25
	βG	25
	G6P	44
	LAP	44
	MDH	44
	PEP3	44
	PGI	44
B	CAT	11
C	IPO	44
G	ACO	44
	ALP	44
	FUM	44
	GOT	11
	HEX	44
	IDH	44
	MPI	44
	PEP2	44
	6PG	44

[a] For composition, see reference 44.

Statistical analysis of MEE results. Allele frequencies at each locus, genetic diversity (calculated as

$$1 - 1/K \sum_{L=1}^{K} \sum_{i=1}^{a} p_i^2,$$

where K is the number of loci and p is the frequency of the ith allele [54]), and observed and expected heterozygosity under Hardy-Weinberg equilibrium (12) were calculated by using the program FSTAT V-1.2 (10). Departure from random mating (12) was assessed within and among samples by means of Wright's fixation indices F_{is} and F_{st}, estimated by the method of Weir and Cockerham (55). Fixation indices F_{is} and F_{st} were tested per locus and overall for departure from 0 by using permutation procedures (10). Genotypic linkage disequilibria (54) expected in the case of a clonal mode of reproduction were tested with the program GENEPOP V-1.2 (32). For all these tests, only one representative isolate per patient was used, except for the patient with two clearly distinct types mentioned above.

REA. DNAs of the *C. albicans* strains were extracted by the method of Millon and coworkers (23). Five micrograms of DNA of each strain was digested for 3 to 4 h at 37°C with 15 U of the restriction enzyme *Eco*RI or *Hin*fI (GIBCO-BRL, Life Technologies, Inc., Gaithersburg, Md., and Biofinex, Praroman, Switzerland, respectively) under the conditions prescribed by the manufacturers. The digested DNA was electrophoresed in 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml in 1× Tris-borate-EDTA (TBE) buffer at 3 V/cm for 16 to 18 h at approximately 10°C. Patterns were read visually and confirmed by putting representative isolates side by side on the same gels.

Southern blot and DNA hybridization. The same procedure as for REA was used for electrophoresis of *Eco*RI-digested genomic DNA, except that 1× Tris-acetate-EDTA (TAE) buffer was used instead of TBE. Electrophoresis was halted when the bromphenol blue marker had migrated approximately 18 cm. Transfer was done by vacuum blotting using a GeneScreen plus Membrane (DuPont, Biotechnology Systems, Boston, Mass.) after depurination of the DNA. λ Ca3 DNA (43) was prepared from a liquid lysate with a λ kit (Qiagen, Chatsworth, Calif.) and radioactively labeled with ^{32}P by nick translation with a kit from Amersham (Amersham International plc, Little Chalfont, Buckinghamshire, England). Prehybridizations and hybridizations (overnight) were done at 65°C in 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) containing 5% dextran sulfate and 0.3% sodium dodecyl sulfate (SDS). The membranes were washed four times for 30 min each at 48°C in 2× SSPE containing 0.2% SDS. Kodak XAR5 films (Eastman Kodak Company, Rochester, N.Y.) were used for revelation of the hybridization patterns. Alternatively, a nonradioactively labeled probe was prepared with a RENAISSANCE random primer fluorescein dUTP labeling kit (DuPont, NEN, Boston, Mass.) and *Eco*RI-digested DNA from λ Ca3. In this case, a nucleic acid chemiluminescence reagent from the same manufacturer was used to reveal the hybridization patterns.

TABLE 2. Allele and electromorph compositions of the 52 ETs from 189 *C. albicans* isolates[a]

ET	ACO	αG	ALP	βG	CAT	FUM	GOT	G6P	IDH	LAP	MDH	MPI	PEP2	PEP3	PGI	6PG	HEX	IPO
1	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	2,6	6,6	3,3	3,3	2,2	7	1
2	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,3	1,1	2,6	1,7	3,3	3,3	2,2	7	1
3	3,3	2,2	2,2	4,6	3,3	1,3	1,1	2,2	1,2	3,3	1,5	2,6	3,6	5,5	3,3	2,2	2	1
4	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,3	1,5	6,6	6,6	5,5	3,3	2,2	9	1
5	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,3	1,1	6,6	6,6	3,3	3,3	2,2	7	1
6	3,3	2,2	2,2	2,5	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,6	3,3	0,0	3,3	2,2	9	1
7	3,3	1,1	1,5	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,6	6,8	3,3	3,3	2,2	9	1
8	3,3	1,1	5,5	7,7	3,3	1,3	1,1	2,2	1,2	3,3	1,1	1,3	3,10	2,2	3,3	2,2	2	1
9	3,3	1,1	5,5	1,1	3,3	1,3	1,1	2,5	1,2	2,2	1,1	6,6	6,6	3,3	3,3	2,2	4	1
10	3,3	1,1	5,5	4,6	3,3	1,3	1,1	2,5	1,2	3,4	1,1	4,6	3,6	3,5	3,3	2,2	8	1
11	3,3	1,1	5,5	2,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,8	6,6	2,3	3,3	2,2	4	1
12	3,3	1,1	3,3	2,2	3,3	1,3	1,1	5,5	1,2	3,3	1,5	3,6	6,10	4,5	1,3	2,2	9	1
13	3,3	2,2	2,2	4,4	3,4	1,3	1,1	5,5	1,2	3,4	1,1	2,6	6,6	3,3	3,3	2,2	7	1
14	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	2,2	3,6	3,3	3,3	2,2	7	1
15	3,3	2,2	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,3	3,8	2,3	3,3	2,2	9	1
16	3,3	2,2	2,2	9,9	3,3	1,3	1,1	5,5	1,2	3,3	1,1	4,4	9,11	3,3	3,3	2,2	9	1
17	3,3	2,2	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,2	3,8	2,3	3,3	2,2	3	1
18	3,3	1,1	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,7	4,4	3,5	3,3	2,2	3	1
19	3,4	1,1	5,5	2,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,8	6,6	2,3	3,3	2,2	4	1
20	3,3	2,2	2,2	9,9	3,3	1,3	1,1	5,5	1,2	3,3	1,1	4,4	11,11	3,3	3,3	2,2	9	1
21	3,3	3,3	3,3	2,2	3,3	1,3	1,1	2,5	1,2	3,5	1,1	2,4	8,9	2,3	3,3	2,2	3	1
22	3,3	2,2	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,3	3,8	2,2	3,3	2,2	3	1
23	3,3	2,2	4,4	2,5	3,3	1,3	1,1	5,5	1,2	3,3	1,1	6,6	4,6	2,5	3,3	2,2	9	1
24	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	2,6	6,6	3,3	3,3	2,2	9	1
25	3,3	2,2	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,3	3,8	2,3	3,3	2,2	5	1
26	3,3	3,3	3,3	4,4	3,3	2,4	1,1	2,5	1,2	3,3	1,1	2,3	3,8	2,2	3,3	2,2	3	1
27	3,3	1,1	0,0	2,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,8	6,6	3,3	3,3	2,2	4	1
28	2,3	1,1	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,6	6,8	3,3	3,3	2,2	9	1
29	3,3	2,2	3,3	4,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	2,3	3,3	2,2	3,3	2,2	9	1
30	3,3	1,1	5,5	3,5	3,3	1,3	1,1	2,5	1,2	3,4	1,1	4,6	4,6	3,5	3,3	2,2	6	1
31	3,3	1,1	5,5	2,4	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,8	6,6	3,3	3,3	2,2	4	1
32	3,3	1,1	3,3	1,1	3,3	1,3	1,1	5,5	1,2	3,3	1,1	4,6	6,6	3,5	3,3	2,2	9	1
33	3,3	2,2	5,5	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,5	2,6	6,6	5,5	3,3	2,2	10	1
34	3,3	2,2	5,5	8,8	3,3	1,3	1,1	2,2	1,2	3,3	1,1	1,3	3,10	2,2	3,3	2,2	2	1
35	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	6,6	6,6	3,3	3,3	2,2	9	1
36	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	2,6	2,7	3,3	3,3	2,2	7	1
37	3,3	1,1	3,3	0,0	3,3	1,3	1,1	5,5	1,2	3,3	1,1	4,6	6,6	3,5	3,3	2,2	9	1
38	3,3	1,1	5,5	9,9	3,3	1,3	1,1	2,5	1,2	3,4	1,1	4,6	4,6	3,5	3,3	2,2	6	1
39	3,3	2,2	5,5	4,4	3,3	1,3	1,1	1,5	1,2	3,3	1,1	2,3	3,8	3,3	3,3	2,2	1	1
40	3,3	3,3	3,3	3,5	3,3	1,3	1,1	2,5	1,2	3,3	1,1	3,6	3,8	2,2	3,3	2,2	9	1
41	3,3	1,1	3,3	4,4	3,3	1,3	1,1	5,5	1,2	3,3	1,1	6,6	6,6	3,3	3,3	1,1	9	1
42	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	3,3	1,1	2,6	6,6	3,3	3,3	2,2	7	1
43	3,3	2,2	3,3	4,4	3,3	1,3	1,3	5,5	1,2	3,3	5,5	6,6	2,7	5,5	3,3	2,2	9	1
44	3,3	2,2	5,5	7,7	3,3	1,3	1,1	2,2	1,2	3,3	1,1	1,3	10,10	2,2	3,3	2,2	2	1
45	3,3	2,2	3,3	4,4	3,3	1,3	1,1	5,5	1,2	3,5	1,1	3,6	6,6	5,5	3,3	2,2	9	1
46	3,3	1,1	2,2	1,1	3,3	1,3	1,1	2,5	1,2	3,3	1,1	6,8	6,6	2,2	3,3	2,2	4	1
47	3,3	2,2	5,5	4,4	3,3	1,3	1,1	2,2	1,2	3,3	1,1	2,3	4,6	2,3	3,3	2,2	2	1
48	3,3	2,2	6,6	4,4	3,3	1,3	1,1	5,5	1,2	3,4	1,1	2,6	6,6	3,3	3,3	2,2	7	1
49	3,3	2,2	2,2	4,4	3,3	1,3	1,1	5,5	1,2	1,3	1,1	4,5	6,6	3,3	3,3	2,2	9	1
50	3,3	1,1	4,4	9,9	3,3	1,3	1,1	2,5	1,2	3,4	1,1	4,6	4,6	5,5	3,3	2,2	6	1
51	3,3	1,1	5,5	6,8	3,3	1,3	1,1	5,5	1,2	1,3	1,1	3,6	3,3	3,5	3,3	2,2	7	1
52	3,3	1,1	3,3	0,0	3,3	1,3	1,1	5,5	1,2	3,3	1,1	6,6	6,6	3,5	3,3	2,2	9	1

[a] Numbers separated by a comma represent alleles at the corresponding loci (for example, the code 5,5 for G6P in ET52 means that isolates of this ET were homozygous for allele 2, whereas code 2,5 of ET50 corresponds to a heterozygote genotype with alleles 2 and 5). For the enzymes HEX and IPO, no interpretation of the migration profiles at the allele level was possible, and the numbers represent the migration pattern as a whole.

RESULTS

MEE patterns. The results of MEE typing are summarized in Table 2. Each migration pattern was coded in order to be representative of its allelic interpretation. For example, a code of 2,2 for glucose 6-phosphate dehydrogenase (G6P) corresponds to a homozygote for allele 2, whereas a code of 2,5 represents a heterozygote with alleles 2 and 5 at this locus. Among the 18 enzymes examined, two (isocitrate dehydrogenase [IDH] and indophenol oxidase [IPO]) showed monomorphic patterns for the 189 *C. albicans* isolates tested (Table 2). The number of migration patterns (electromorphs) per enzyme ranged from 1 to 15 (mean, 5.2). Three enzymes presented a few strains with no demonstrable activity (alkaline phosphatase [ALP], β-glucosidase [βG], and leucyl-proline peptidase [PEP3]). Five enzymes showed three distinct bands for heterozygotes compatible with a dimeric structure (glutamic-oxalacetic transaminase [GOT], G6P, malate dehydrogenase [MDH], PEP3, and phosphoglucose isomerase [PGI]), whereas nine other enzymes (aconitase [ACO], ALP, βG, catalase [CAT], fumarase [FUM], IDH, leucine aminopeptidase [LAP],

TABLE 3. Typing results for multiple oral *C. albicans* isolates from 24 HIV-infected patients

Patient	Isolate	Interval between first and following isolates (days)	CD4 cells/mm^3	Clinical sign[a]	Fluconazole inhibition zone diam (MIC)[b]	MEE ET	REA type[c]		Southern Ca3 profile[d]
							*Eco*RI	*Hin*fI	
1	5383.01	0	198	Asymptomatic	48	32	A9	N	3a
	5383.02	0	198	Asymptomatic	48	32	A9	N	3b
	5383.03	0	198	Asymptomatic	47	32	A9	N	3c
	5383.04	0	198	Asymptomatic	47	32	A9	N	3a
	5383.05	0	198	Asymptomatic	48	32	A9	N	3c
	5383.06	0	198	Asymptomatic	48	37	A9	N	3d
	5383.07	0	198	Asymptomatic	48	32	A9	N	3d
	5383.08	0	198	Asymptomatic	46	32	A9	N	3c
	5383.09	0	198	Asymptomatic	48	32	A9	N	3b
	5383.10	0	198	Asymptomatic	49	32	A9	N	3a
2	5401.01	0	642	Asymptomatic	47	33	A10	L	1a
	5401.02	0	642	Asymptomatic	47	33	A10	L	1b
	5401.03	0	642	Asymptomatic	47	33	A10	L	1c
	5401.04	0	642	Asymptomatic	48	33	A10	L	1c
	5401.05	0	642	Asymptomatic	47	33	A10	L	1c
3	2665.01	0	649	Asymptomatic	47	36	A5	F	2a
	2665.02	0	649	Asymptomatic	47	36	A5	F	2a
	2665.03	0	649	Asymptomatic	48	36	A5	F	2a
	2665.04	0	649	Asymptomatic	47	36	A5	F	2a
	2665.05	0	649	Asymptomatic	48	36	A5	F	2a
	2665.06	0	649	Asymptomatic	47	36	A5	F	2a
	2665.07	0	649	Asymptomatic	47	36	A5	F	2a
	2665.08	0	649	Asymptomatic	47	36	A5	F	2a
	2665.09	0	649	Asymptomatic	48	36	A5	F	2a
	2665.10	0	649	Asymptomatic	48	36	A5	F	2a
4	C1	0	2	Asymptomatic	40	1	A2	F	19a
	C31	115	ND[e]	OPC	41	1	A4	F	19a
5	C98	0	33	OPC	45	34	B4	P	17a
	C101	126	44	OPC	26	35	A8	(J,K),M	18a
	4639.01	156	55	OPC	31 (1.5)	35	ND	ND	18d
	4639.02	156	55	OPC	29	35	ND	ND	18b
	4639.03	156	55	OPC	47 (0.5)	35	A8	ND	ND
	4639.04	156	55	OPC	48	34	ND	ND	ND
	4639.05	156	55	OPC	46	34	ND	ND	ND
	4639.06	156	55	OPC	30	35	ND	ND	ND
	4639.07	156	55	OPC	30	35	ND	ND	ND
	4639.08	156	55	OPC	31	35	ND	ND	ND
	C103	163	ND	Not symptomatic	42	34	B4	P,(R)	17a
	7277.01	194	ND	OPC	29	35	ND	ND	ND
	7277.02	194	ND	OPC	46	34	ND	ND	ND
	7277.03	194	ND	OPC	31	35	ND	ND	ND
	7277.04	194	ND	OPC	31	35	ND	ND	ND
	7277.05	194	ND	OPC	30	35	ND	ND	ND
	7277.06	194	ND	OPC	47	34	ND	ND	ND
	7277.07	194	ND	OPC	48	34	ND	ND	ND
	7277.08a	194	ND	OPC	48	34	B4	ND	ND
	7277.08b	194	ND	OPC	30	35	A8	ND	ND
	C122	201	ND	Not symptomatic	27	35	A8	(J,K),M	18b
	C123	240	26	OPC	41	35	A8	(J),M	18c
6	C42	0	662	Asymptomatic	41	24	A1	J,(N)	20a
	C47	170	637	Asymptomatic	41	24	A2	J	20b
7	C62	0	67	Not symptomatic	44	27	B11	H,N	24a
	C65	57	ND	Not symptomatic	48	27	B11	H,N	24b
	C68	277	104	Not symptomatic	48	27	B11	H,N	24c
8	C60	0	13	Asymptomatic	42	25	B10	O	22a
	C63	39	ND	OPC	42	25	B10	O	22b
9	C61	0	339	Not symptomatic	41	26	B4	J	21a
	C64	32	ND	OPC	42	26	B14	C,J	21b
	C66	114	393	Not symptomatic	38	26	B14	C,J	21c

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TABLE 3—*Continued*

Patient	Isolate	Interval between first and following isolates (days)	CD4 cells/mm^3	Clinical sign[a]	Fluconazole inhibition zone diam (MIC)[b]	MEE ET	REA type[c]		Southern Ca3 profile[d]
							*Eco*RI	*Hin*fI	
10	C86	0	11	Not symptomatic	46	11	B1	N,P,(S)	23a
	C87	29	ND	Not symptomatic	44	11	B1	N,P,(S)	23a
	C16	96	ND	NK[f]	45	11	B1	N,(P)	23b
	C88	166	5	OPC	49	11	B1	N,P,(S)	23c
	C89	193	ND	OPC	51	11	B1	N,P,(S)	23c
	C90	199	ND	OPC	40	11	B1	N,P,(S)	23d
11	C45	0	32	Not symptomatic	40	12	B2	L,P	4a
	C53	119	1	Not symptomatic	32	12	B2	L,P	4a
	C18	125	ND	OPC	34	12	B2	L,P	4b
	C46	167	ND	OPC	23	12	B2	L,P	4a
12	C38	0	9	OPC	47	1	A5	F	5a
	C41	402	1	OPC	13	1	A5	F	5b
13	C78	0	67	Not symptomatic	44	28	B12	N,P,T	25a
	C83	101	10	OPC	40	28	B12	N,P,T	25a
	C84	122	ND	Not symptomatic	42	28	B12	N,P,T	25a
	C79	165	ND	OPC	39	28	B12	N,P,T	25c
	C72	212	4	Not symptomatic	24	28	B12	N,P,T	25b
	C85	324	4	OPC	24	28	B12	N,P,T	25e
	C108	344	ND	Not symptomatic	17	28	B12	N,P,T	25d
	C110	349	ND	OPC	22	28	B12	N,P,T	25d
	C109	352	ND	OPC	13	28	B12	N,P,T	25d
	C107	358	ND	OPC	23	28	B12	N,P,T	25d
	C111	372	ND	OPC	24	28	B12	N,P,T	ND
	C93	379	ND	OPC	13	28	B12	N,P,T	25e
	C112	395	ND	OPC	19	28	B12	(N;P),T	25f
	C118	395	ND	OPC	18	28	B12	N,P,T	25e
	C119	395	ND	OPC	17	28	B12	N,P,T	25e
	C113	406	ND	OPC	6	28	B12	N,P,T	25e
	C120	406	ND	OPC	6	28	B12	N,P,T	25d
	C121	406	ND	OPC	6	28	B12	N,P,T	25d
	C114	427	ND	OPC	6	28	B12	N,P,T	25d
	C99	447	3	OPC	22	28	B12	N,P,T	25g
	C115	516	ND	OPC	6	28	B12	N,P,T	25f
	C116	516	ND	OPC	6	28	B12	N,P,T	25h
	C117	516	ND	OPC	6	28	B12	N,P,T	25i
14	C23	0	95	NK	44	13	A5	F	9a
	C32	190	4	OPC	31	13	A5	F	9a
	C36	250	41	OPC	22	13	A5	F	9a
	C39	384	32	OPC	16	13	A5	F	9a
15	C33	0	319	OPC	46	15	B4	(B),J	10a
	C34	385	ND	OPC	40	15	B4	J	10b
	C26	879	4	OPC	6	15	B4	(B),J	10b
	C75	919	ND	OPC	6	29	B4	(B),J	10a
	C82	1,024	ND	OPC	19	15	B4	(B),J	10c
16	C50	0	ND	OPC	45	2	A5	F	6a
	C44	228	17	NK	21	2	A5	F	6b
17	C27	0	9	OPC	46	16	B3	G	11a
	C37	605	1	OPC	32	16	B3	G	11b
	C57	697	1	OPC	6	16	B3	G,(O)	11c
	C40	745	ND	OPC	6	20	B3	G,(O)	11d
	C58	793	1	OPC	6	16	B3	G,(O)	11e
18	C43	0	59	OPC	39	21	B9	L,N	12a
	C48	202	12	OPC	15	21	B9	L,N	12b
	C56	263	ND	OPC	6	21	B9	L,N	12b
19	C92	0	312	Asymptomatic	51	31	B1	(I),K,N	7a
	C96	1,188	12	OPC	30	31	B1	(I),K,N	7b
	5072.II	1,369	ND	OPC	18	31	ND	ND	ND

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TABLE 3—*Continued*

Patient	Isolate	Interval between first and following isolates (days)	CD4 cells/mm^3	Clinical sign[a]	Fluconazole inhibition zone diam (MIC)[b]	MEE ET	REA type[c]		Southern Ca3 profile[d]
							*Eco*RI	*Hin*fI	
	5072.III	1,369	ND	OPC	26	31	ND	ND	ND
	C97	1,374	ND	OPC	19	31	B1	I,K,N	7c
20	C91	0	11	Not symptomatic	26	30	A3	O	8a
	C94	200	6	OPC	25	30	A3	O	8a
	C95	358	5	Not symptomatic	18	30	A3	O	8a
	6372.I	389	ND	OPC	14	30	ND	ND	ND
	6372.V	389	ND	OPC	20	30	ND	ND	ND
21	C70	0	70	OPC	50	2	A5	F	14a
	C76	328	118	Not symptomatic	34	2	A5	F	14b
	C80	328	118	Not symptomatic	40	2	A5	(A),F	14c
	C9	435	7	Not symptomatic	37	2	A5	F	14b
	C73	577	3	OPC	26	2	A5	F	14b
	C81	577	3	OPC	27	2	A5	F	14b
	C77	587	ND	OPC	24	2	A5	F	14b
	C74	587	ND	OPC	30	2	A5	F	14b
22	C49	0	38	OPC	36	22	B7	J,Q	13a
	C55	112	33	OPC	24	22	B7	J,Q	13b
	C71	127	ND	OPC	26	22	B7	J,Q	13b
23	C52	0	304	OPC	42	23	B8	N	15a
	C51	118	307	Not symptomatic	19	23	B8	N	15a
	C67	158	ND	OPC	47	23	B8	N	15a
	C59	309	227	Not symptomatic	45	23	B8	N	15b
24	C100	0	13	OPC	45	11	B13	(F)Q,R	16a
	C104	0	13	OPC	32 (0.32)	11	B13	(F)Q,R	16b
	7014.I	0	13	OPC	52 (0.125)	11	ND	ND	ND
	C105	6	ND	Not symptomatic	24 (2)	11	B13	(F)Q,R	16c
	C106	6	ND	Not symptomatic	37	11	B13	(F)Q,R	16b
	7438.I	6	ND	Not symptomatic	49 (0.19)	11	ND	ND	ND
	C102	44	15	OPC	17	11	B13	(F)Q,R	16d

[a] Asymptomatic, without OPC signs at the time of sampling or earlier; not symptomatic, having had OPC episodes earlier but not having symptoms at the time of the corresponding sampling; OPC, having OPC signs at the time of sampling.
[b] The inhibition zone diameters (in millimeters) were obtained with the agar disk diffusion test, and the values in parentheses are MICs (in micrograms per milliliter) obtained by E-test.
[c] For the types obtained with the enzyme *Eco*RI, each combination of one letter and one number represents a different migration profile. For the types obtained with the enzyme *Hin*fI, each letter or combination of letters corresponds to a different migration profile; for the profiles obtained with *Hin*fI, parentheses represent differences related to bands of weak intensity.
[d] For the Ca3 hybridization profiles, the letters represent the variants of the main types (numbers) described in the text.
[e] ND, not determined.
[f] NK, not known.

mannose phosphate isomerase [MPI], and leucyl-glycyl-glycin peptidase [PEP2]) showed two bands for heterozygotes and were considered monomeric. For two enzymes (α-glucosidase [αG] and 6-phosphogluconate dehydrogenase [6PG]), only single bands were observed under the electrophoresis conditions used and no conclusions concerning their structure could be drawn.

Fifty-two different combinations of electromorphs (ETs) were observed among the 18 enzyme loci tested (Table 2). One ET (ET1) was observed for 14 different patients, another ET (ET11) was observed for 3 patients, and six ETs (ET2, ET8, ET24, ET31, ET34, and ET44) were observed for 2 patients each (Table 3). The remaining 44 ETs were found for single patients only. Among the 24 HIV-infected patients with multiple isolates, only 4 showed more than one ET (two ETs each; Table 3). In three of these four cases (patients 1, 15, and 17), the ETs found for each patient differed in only one (ETs 16 and 20) or two (ETs 32 and 37 and ETs 15 and 29) alleles. None of these differences were confirmed by the two other typing methods (Table 3). For the fourth patient with two ETs (patient 5), differences were observed for 15 alleles and the strains were also clearly distinguished by the other methods.

Genetic diversity. For the 16 enzyme loci interpreted at the allele level (IPO and hexokinase [HEX] were not interpretable), the number of alleles per locus ranged from 1 to 10 (mean number of alleles per locus, 4.3). The effective number of alleles, however, varies between 1 and 4. The genetic diversity is an index of the quantity of polymorphism at the loci studied (54). It is 0 for monomorphic loci and closer to 1 for highly polymorphic loci. The overall genetic diversity was 0.35, with the different polymorphic loci ranging from 0.014 to 0.723. Six of the 16 loci had one major allele at a frequency of >95% (ACO, CAT, GOT, MDH, PGI, and 6PG). This left us with 10 polymorphic loci (Table 4). The number of alleles per locus, effective number of alleles, and genetic diversity among individuals for these 10 loci as well as overall are presented in Table 4.

Expected and observed frequencies of heterozygotes. Departure from random mating was tested by using the fixation index F_{is} as a statistic: an F_{is} of 0 means that there is no departure

TABLE 4. Number of alleles, genetic diversity, and Wright's fixation indices for 10 polymorphic loci

Locus	No. of alleles/locus		Genetic diversity	Index (significance)[a]	
	Total	Effective		F_{st}	F_{is}
αG	3	2	0.493	−0.036 (NS)	1.000 (+)
ALP	7	3	0.685	−0.010 (NS)	0.980 (+)
βG	10	2	0.575	−0.017 (NS)	0.674 (+)
FUM	4	2	0.514	−0.001 (NS)	−0.946 (−)
G6P	3	2	0.414	−0.005 (NS)	0.148 (NS)
IDH	2	2	0.500	0.000 (NS)	−1.000 (−)
LAP	5	2	0.358	0.004 (NS)	−0.144 (NS)
MPI	8	4	0.723	0.000 (NS)	−0.149 (−)
PEP2	10	3	0.614	−0.002 (NS)	0.381 (+)
PEP3	5	2	0.557	−0.011 (NS)	0.541 (+)
Overall[b]			0.3504	−0.007 (NS)	0.194 (+)

[a] F_{st} and F_{is}, Wright's fixation indices estimated by the method of Weir and Cockerham (55). Statistical significance is indicated as follows: NS, not significant; +, highly significant for a deficit in heterozygotes; −, highly significant for an excess of heterozygotes.

[b] For all 16 loci interpreted at the allele level.

from random mating, an F_{is} of >0 means that there is a deficit of heterozygotes, and a negative F_{is} means an excess of heterozygotes. Of the 10 polymorphic loci, 5 showed a highly significant deficit of heterozygotes (αG, ALP, βG, PEP2, and PEP3) and 3 showed a highly significant excess (FUM, IDH, and MPI). For IDH, the only observed genotype was 1,2, and for FUM, all the observed genotypes were 1,3 heterozygotes apart from one ET with genotype 2,4 (Table 2). On the other hand, not a single heterozygote at the αG locus was observed, and all but one genotype were homozygotes at ALP. Only two polymorphic loci did not show departure from Hardy-Weinberg equilibrium (G6P, LAP).

Genotypic disequilibrium. Test results are presented in Table 5 for the loci showing sufficient variability (αG, ALP, βG, G6P, LAP, MPI, PEP2, and PEP3). IDH and FUM are represented by too few genotypes for the test to have any meaning. All but one pair of loci showed a strong linkage disequilibrium between loci.

Genetic relationships between ETs. With the allelic composition of the ETs for 16 enzyme loci, a matrix of similarity was built, using the similarity coefficient of Dice. The dendrogram based on this matrix using the UPGMA method and graphically summarizing the relationships between ETs is shown in Fig. 1. No division of the population into clearly distinct clusters is visible. As shown in the dendrogram, no subpopulation of *C. albicans* clones seems to be associated with HIV-infected patients or with OPC signs in these patients (Fig. 1). Similarly, no specific subpopulation of clones could be associated with invasive candidiasis or a particular origin of samples. *F*-statistics analysis confirms this result. Table 4 reports F_{st} per locus as well as overall, together with their significance. F_{st} is an estimate of the level of subdivision among populations. An F_{st} of 0 means that there is no subdivision, whereas an F_{st} of 1 means that populations are completely isolated genetically. Of the 10 polymorphic loci, none showed significant departure from the null hypothesis of no differentiation among the three populations. Since the amount of sexual reproduction in *C. albicans* seems to be very low, if not nonexistent (reference 31 and this study), each genotype at a locus could be considered a marker (e.g., genotype 1,1 = allele 1, genotype 1,2 = allele 2, and genotype 2,2 = allele 3). The whole datum set was reencoded this way, and a molecular analysis of variance (8) was performed, with the distances between ETs being 1− (the similarity coefficient of Dice). The calculated statistic, ϕ_{st}, is an F_{st}-like statistic which accounts for distances between ETs. The pattern obtained from this analysis confirms the results of the *F*-statistics and UPGMA analyses. The observed ϕ_{st} is −0.011, clearly not significant.

Reproducibility and discriminatory power of MEE. The reproducibility of results obtained by MEE was tested with nine isolates belonging to nine different ETs, for which MEE typing was independently done twice with freshly prepared cell lysates. In all nine cases, the same ETs were obtained. The index of discrimination of Hunter and Gaston (15) based on 70 epidemiologically unrelated strains tested for 18 enzyme loci was 0.96.

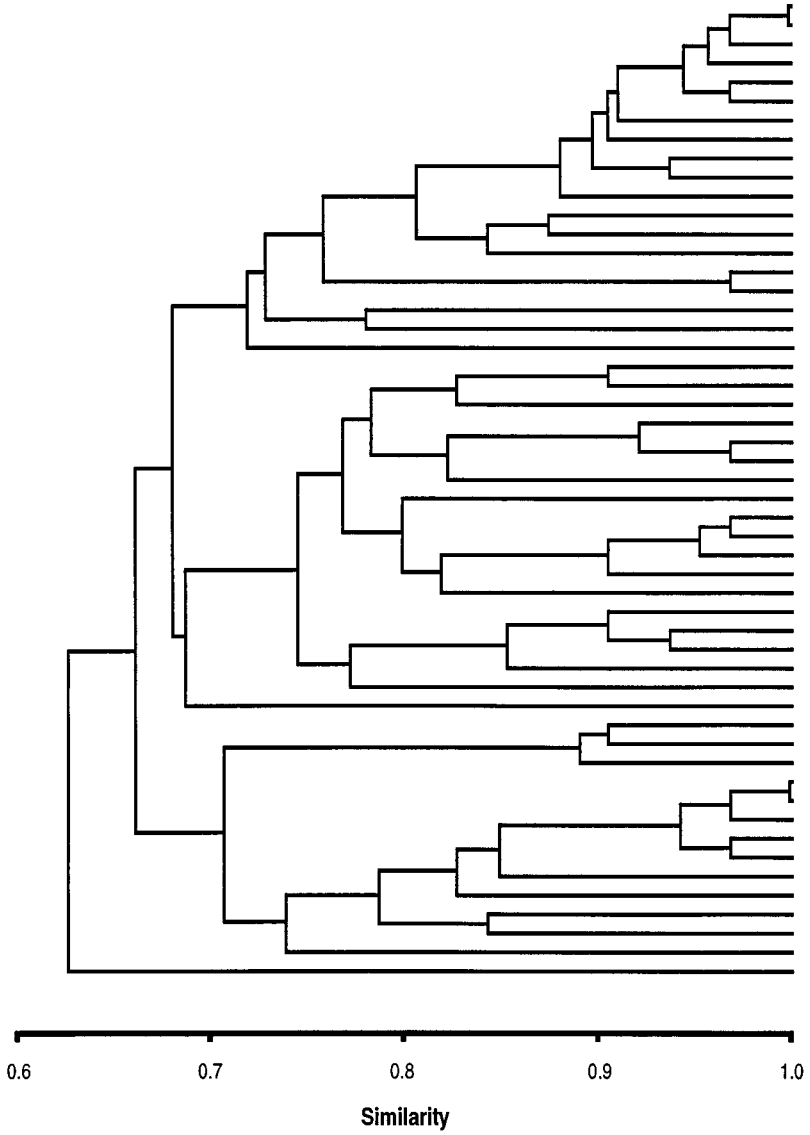
Typing of sequential oral *C. albicans* isolates from HIV-infected patients. Sequential oral *C. albicans* isolates from 21 patients were typed (Table 3). Eighteen patients carried only one ET over the sampling time (median, three sampling dates per patient; median time between first and last sample, 246 days). For two patients with five isolates each (793 and 1,024 days between first and last samples), one isolate differed from the others in one (PEP2, patient 17) or two (PEP2 and PEP3; patient 15) alleles of the 18 loci examined. The last patient (patient 5) simultaneously harbored two clearly distinct ETs differing at eight enzyme loci (Tables 2 and 3).

The REA patterns of the same sequential isolates obtained with the enzymes *Eco*RI and *Hin*fI broadly confirmed the MEE results. Only minor differences in the patterns could be observed within sequential isolates of the 18 patients with unchanged ETs (examples in Fig. 2). These were related to the appearance or disappearance of low-intensity bands or to changes in intensity of a few bands. These differences were reproducibly obtained when the REA procedure was repeated with freshly prepared DNA (52 different isolates were typed two to four times by REA with freshly extracted DNA). In one case only (patient 9), one clearly visible band was completely missing with both enzymes in the restriction patterns of one of three isolates (Fig. 2). The general patterns of these three isolates, however, were very similar, and all belonged to the same ET unique to this patient. When the REA patterns of oral isolates from different patients were compared, most isolates belonging to distinct ETs also showed differences in their REA patterns. In one case only, isolates with identical REA profiles belonged to three different but closely related ETs (patients 12 and 21 and patient 16, ET1, ET2, and ET13; Table 3). Conversely, isolates belonging to a unique ET but originating from two patients had consistently different REA patterns

TABLE 5. Probability of independence of loci[a]

Locus	Probability						
	αG	ALP	βG	G6P	LAP	MPI	PEP2
ALP	0.00						
βG	0.00	0.00					
G6P	0.00	0.00	0.00				
LAP	0.01	0.00	0.01	0.00			
MPI	0.00	0.00	0.00	0.00	0.00		
PEP2	0.03	0.00	0.00	0.00	0.19	0.00	
PEP3	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Genotypic linkage disequilibria were tested by using the program GENEPOP V-1.2 (32). Only the eight loci with enough polymorphism to give statistically significant results have been used. A number close to 0 represents a strong linkage disequilibrium, whereas a number close to 1 represents a clear lack of linkage disequilibrium. All but one pair (LAP-PEP2) show a strong disequilibrium.



C.albicans electrophoretic types and characteristics of isolates and patients

N° ET	Oral isolates: HIV-infected patients: Number of patients	Presence of OPC	Resistance to Fluconazole	Oral isolates: HIV-negative patients: Number of patients	Invasive candidiasis: Number of patients (origin)
1	5	+	+	6	3 (b/d)
24	1	-	-	1	-
13	1	+	+	-	-
42	-	-	-	-	1 (b)
5	-	-	-	-	1 (b)
35	1	+	-	-	-
14	1	-	-	-	-
48	-	-	-	-	1 (b)
2	2	+	+	-	-
36	1	-	-	-	-
49	-	-	-	-	1 (b)
4	-	-	-	-	1 (b)
33	1	-	-	-	-
45	-	-	-	-	1 (b)
16	1	+	+	-	-
20	1	+	+	-	-
3	-	-	-	-	1 (b)
6	-	-	-	-	1 (d)
23	1	+	+	-	-
7	-	-	-	-	1 (d)
28	1	+	+	-	-
18	1	-	-	-	-
32	1	-	-	-	-
37	1	-	-	-	-
52	-	-	-	1	-
41	-	-	-	1	-
9	-	-	-	-	1 (b)
11	2	+	+	-	1 (b)
19	1	-	-	-	-
31	1	+	+	-	1 (d)
27	1	-	-	-	-
46	-	-	-	-	1 (b)
10	1	+	-	-	-
30	1	+	-	-	-
38	-	-	-	1	-
50	-	-	-	-	1 (b)
51	-	-	-	1	-
12	1	+	+	-	-
8	1	+	-	-	1 (d)
44	-	-	-	1	1 (b)
34	1	+	-	1	-
15	1	+	+	-	-
25	1	+	-	-	-
17	1	-	-	-	-
22	1	+	+	-	-
29	1	+	+	-	-
39	-	-	-	1	-
47	-	-	-	-	1 (b)
26	1	+	-	-	-
40	-	-	-	1	-
21	1	+	+	-	-
43	-	-	-	-	1 (b)

FIG. 1. Genetic relationships among 52 ETs of *C. albicans*. The dendrogram was generated by the UPGMA method from a matrix of pairwise coefficients of similarity based on electrophoretically demonstrable allelic variation at 16 enzyme loci. ET numbers are the same as in Tables 2 and 3. Four HIV-infected patients carrying two different ETs in their throat are represented twice. For ETs associated with OPC and asymptomatic stages, only the OPC is indicated. For ETs with isolates resistant and susceptible to fluconazole, only the resistant ones are indicated. b, isolates originating from the blood or an intravenous catheter; d, isolates originating from a deep infection.

(patients 10 and 24, ET11). No correlation between REA patterns and the minor differences in MEE patterns found for sequential isolates from patients 15 and 17 could be observed (Table 3; Fig. 2). Two clearly distinct restriction patterns could be found in isolates from patient 5 (Fig. 2), thus confirming the major differences observed with MEE (Table 3).

The Ca3 hybridization profiles of the sequential isolates were also in agreement with the MEE results. The profiles of the isolates from each patient were highly similar (examples in Fig. 3 and 4). Minor differences within successive isolates from all but three patients were observed (patients 4, 14, and 20; Table 3 and Fig. 3 and 4). Such variations were also found in isolates belonging to ETs not found in any other patients (Table 3). These differences in hybridization profiles were related mainly to bands larger than 7.9 kb (Fig. 3 and 4). In spite of these frequent minor variations, the constant bands of the patterns found in isolates from each patient allowed them to be distinguished from the isolates of all the other patients typed with the Ca3 probe in this study (Table 3). For patient 5, two clearly distinct hybridization profiles could be found in the

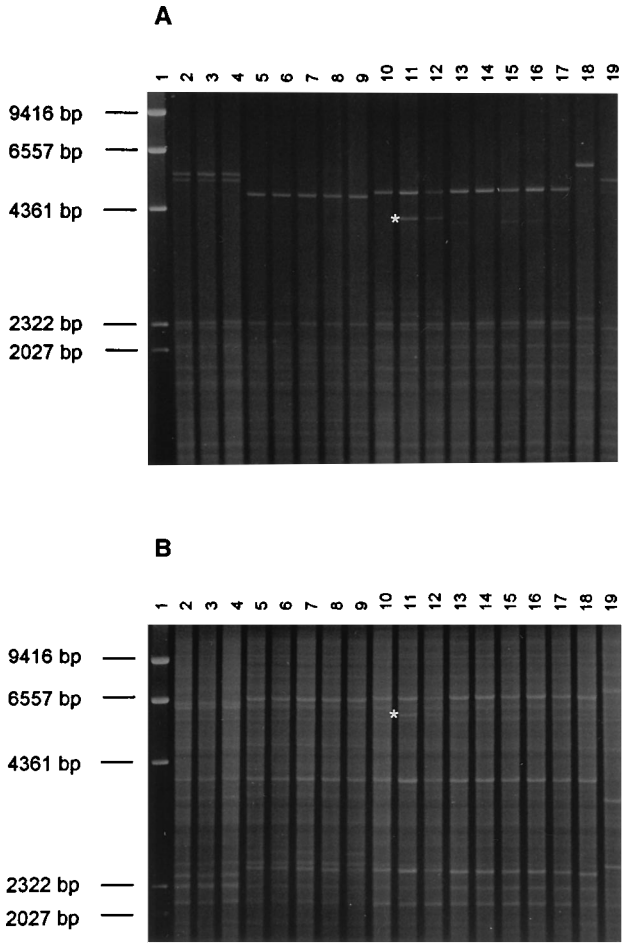


FIG. 2. REA patterns of 18 oral *C. albicans* isolates from five HIV-infected patients (Table 3) obtained with the enzymes *Hin*fI (A) and *Eco*RI (B). Lanes 1, λ *Hin*dIII marker; lanes 2 to 4, isolates C43, C48, and C56 from patient 18; lanes 5 to 9, isolates C27, C37, C57, C40, and C58 from patient 17; lanes 10 to 12, isolates C61, C64, and C66 from patient 9; lanes 13 to 17, isolates C33, C34, C26, C75, and C82 from patient 15; and lanes 18 and 19, isolates C98 and C101 from patient 5 in Table 3 (the two main types found in this patient). Note that a clear decrease of in vitro susceptibility to fluconazole was observed in the three isolates from patient 18 (lanes 2 to 4). Isolates C40 and C75 differed in their ETs from the other isolates of the respective patients, 17 and 15. Isolate C61 (lane 10) is missing a band in both *Hin*fI and *Eco*RI patterns (star) in comparison with the other isolates from patient 9 (lanes 11 and 12).

successive isolates which correlated with the types found by the two other methods.

***C. albicans* subtypes during development and recurrence of OPC.** Two patients (8 and 19) without previous known OPC episodes or antifungal therapy and without OPC signs at the time of the first sampling (312 and 13 CD4 cells per mm^3) carried isolates of the same MEE and REA types during the following OPC episodes. Similarly, for nine patients with recurrent OPC (patients 5, 9 to 11, 13, 20, 21, 23, and 24), isolates indistinguishable by MEE and showing only minor differences in their REA and Ca3 hybridization patterns could be isolated from the oral cavity during the OPC episodes and the remission phases in between (Table 3; Fig. 3). No association of specific Ca3 subtypes with the presence or absence of OPC signs within each patient was visible (Table 3).

***C. albicans* subtypes and changes in susceptibility to fluconazole.** The changes in susceptibility in sequential isolates as evidenced by changes of the inhibition zone diameters for the fluconazole agar disk diffusion test are reported in Table 3. Of the 21 patients with sequential isolates, 14 showed a significant change in the susceptibility to fluconazole of their *C. albicans* isolates, and three or more isolates from 12 of them (patients 11, 13 to 15, and 17 to 24) were examined. In four (patients 11, 14, 18, and 19), a gradual decrease in susceptibility to fluconazole of the isolates tested could be observed (Table 3). In three other patients (13, 23, and 24), a decrease as well as an increase in susceptibility to fluconazole of the sequential isolates was evidenced. No change in ETs, REA profiles, or Ca3 hybridization patterns could be associated with these variations in susceptibility to fluconazole (Table 3; Fig. 4). No relationship between a particular level of susceptibility to fluconazole and a specific Ca3 hybridization subtype could be seen for each patient (Table 3; Fig. 4). The same Ca3 hybridization patterns could be associated with significantly different levels of susceptibility to fluconazole in several patients (patients 11, 13 to 15, 18, and 23; Table 3 and Fig. 4).

Patients with multiple simultaneous isolates. From three patients without any signs of OPC at the time of sampling or earlier and not previously treated with antifungal agents, several *C. albicans* colonies originating from one clinical sample were isolated and typed (patient 1, 10 colonies; patient 2, 5 colonies; and patient 3, 10 colonies). For two patients, the isolates within each patient were indistinguishable by MEE and REA. For the first patient, the 10 isolates examined also had identical Ca3 hybridization profiles (Fig. 5), and for the second, three closely related profiles could be found. For the third patient, nine clones were indistinguishable by MEE and the 10th differed only in its lack of βG activity. However, all 10 clones from that patient showed identical REA patterns and very closely related Ca3 hybridization profiles (Fig. 5). In all three cases, the ETs found were unique to each patient. The CD4 counts of these patients at the time of sampling were 198, 642, and 649 cells per mm^3, respectively.

For three other patients previously treated with fluconazole (patient 13, ET28; patient 21, ET2; and patient 24, ET11), pairs (five) or triplets (three sets) of isolates taken from the same clinical samples were examined. All the clones within pairs or triplets were indistinguishable by MEE or REA (except for a few minor bands in the *Hin*fI profiles, as mentioned for sequential isolates). Identical or very closely related Ca3 hybridization profiles were also obtained for clones within the pairs or triplets (Table 3).

For a seventh patient (patient 5, ET34 and ET35) also previously treated with fluconazole, 18 isolates from two clinical samples (nine isolates each) were examined. In both samples, two clearly distinct ETs differing at eight enzyme loci could be found simultaneously (Table 3). These two ETs could also be clearly distinguished by REA and by hybridization with the Ca3 probe (Table 3; Fig. 2). The CD4 count of this patient was already at 55 cells per mm^3 at the first sampling date.

The inhibition diameters found in the fluconazole susceptibility test for the isolates from the three untreated patients mentioned above were almost identical (47 to 49 mm). Similarly, for two patients (patients 21 and 13) previously treated with fluconazole, all the isolates within the triplets showed the same or very similar inhibition diameters (Table 3). However, for two other previously treated patients (patients 24 and 5), isolates from the same sample and indistinguishable by MEE and REA showed significantly different inhibition diameters (Table 3). The latter results were confirmed by testing the isolates with the E-test for fluconazole (AB BIODISK, Piscataway, N.J.) (Table 3).

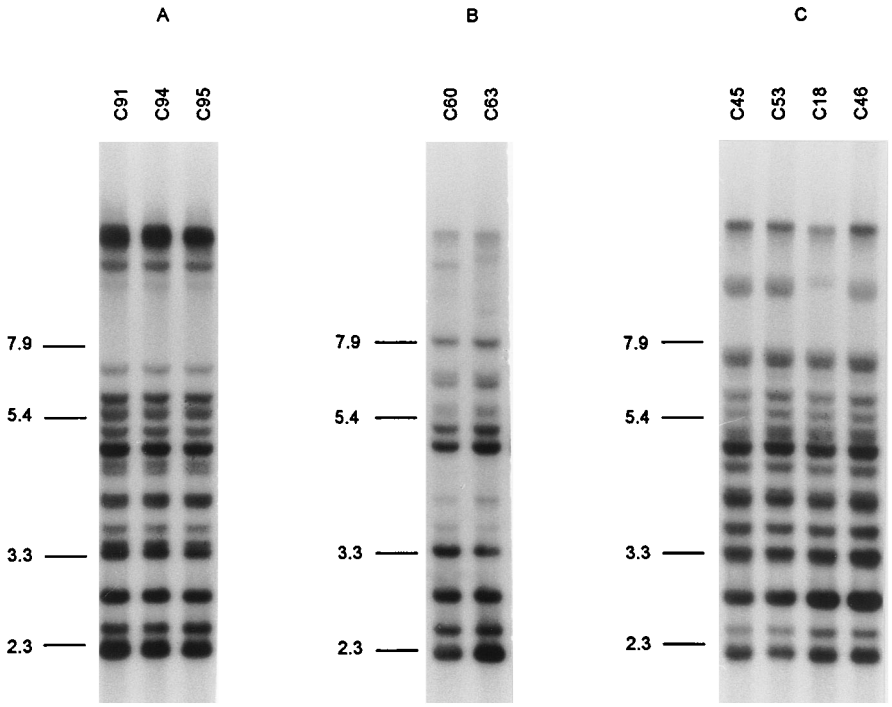


FIG. 3. Ca3 hybridization patterns of multiple successive oral *C. albicans* isolates from patients 20 (A), 8 (B), and 11 (C). These successive isolates were associated with changes in the clinical status of the patients (Table 3). For patient 11, a clear decrease in susceptibility to fluconazole was also observed in these successive isolates. The positions of reference molecular size markers (in kilobases) are shown to the left of each gel.

DISCUSSION

C. albicans is a very widespread facultative human pathogen which can be isolated from many body locations in a significant proportion of healthy people (35). Several different clinical pictures are associated with this yeast, including candidemia, abscesses, postoperative infections (mainly after intraperitoneal surgery), and most frequently vaginitis and oropharyngeal candidiasis, particularly in HIV-infected patients (35). The condition of the patient is probably the major factor governing the development of a clinical candidiasis, which is usually associated with immunodeficiencies or other predisposing factors (52). However, as recently shown for many other microbial pathogens, one cannot exclude that some strains or groups of strains are more prone than others to be involved in clinical disorders. The eventuality that particular strains may present a tropism for specific body locations or that some strains are associated with specific patient populations or clinical pictures has been suspected by some authors (46). Similar phenomena have been recently evidenced in other microbial pathogen populations (45). One of the aims of the present work was to address this problem and to see if some *C. albicans* clones may present particular characteristics of clinical relevance.

Among the numerous typing methods developed to differentiate *C. albicans* strains, only a few have the ability to reliably assess the genetic relatedness between isolates. MEE, or isoenzyme typing, is one of them. This method not only distinguishes *C. albicans* strains with a relatively high power of discrimination (14), but also allows assessment of the structure of the population under study, its mode of reproduction under natural conditions, and its genetic diversity (25, 26). MEE is therefore particularly well suited to answer questions about the population genetics of *C. albicans* and was used as the main technique for the present study.

Both enzyme loci found as monomorphic in the present work (IDH and IPO) had also been previously found to be monomorphic by other investigators (5). The other monomorphic loci described by those authors (ACO, GOT, phosphoglucomutase [PGM], and 6PG) were only poorly polymorphic in the present study. The higher average genetic diversity found here among ETs (0.35) than what has been previously reported (0.13 [5]) may rely on differences in electrophoresis conditions used, allowing the detection of more alleles (several different buffer systems) and on possible differences in the respective populations examined. Similarly to results of previous MEE studies (5, 31), our results with typical heterozygote migration patterns in some enzymes are consistent with the generally accepted diploid nature of the yeast *C. albicans* (40).

The clonal propagation of *C. albicans* had been difficult to assess in the past by molecular methods, and no definitive answer to this problem could be found (5, 48). The fixation indices F_{is} (12) obtained in the present work are in agreement with the lack of sexual reproduction observed in this species. In addition, the patterns obtained with IDH and FUM are certainly not what is expected under random mating. If sexual reproduction (and therefore recombination) occurs, then the different loci should assort independently at each reproduction, unless they are all located on a small segment of the same chromosome (unlikely). On the other hand, if there is no recombination, then the different loci should be in strong disequilibrium. Our results show clear evidence of disequilibrium among all pairs of loci but one. Thus, our data clearly support an asexual and clonal nature of *C. albicans* and confirm those recently obtained by other investigators (31). The clonal nature of *C. albicans* evidenced here also represents a necessary prerequisite for a simple use of molecular typing methods in epidemiological tracing.

In order to test whether some particular yeast populations may be associated either with HIV-infected patients or with

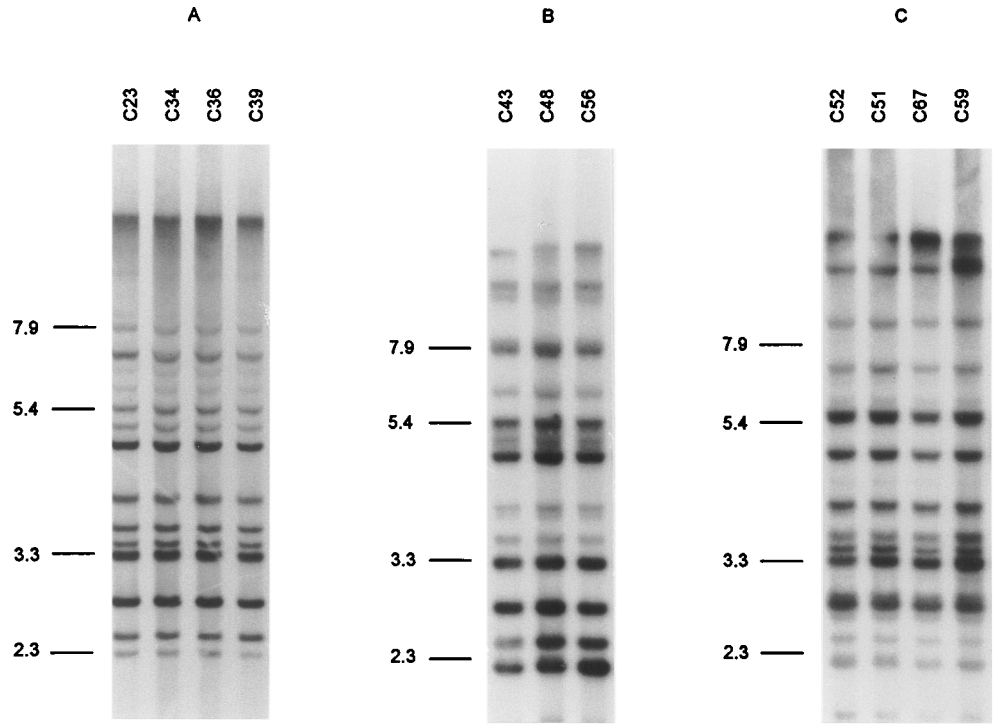


FIG. 4. Ca3 hybridization patterns of multiple successive oral *C. albicans* isolates from patients 14 (A), 18 (B), and 23 (C). The successive isolates from patients 14 and 18 showed a regular decrease in susceptibility to fluconazole (Table 4). Isolate C51 was the only one with a clearly reduced susceptibility to fluconazole in patient 23. The positions of reference molecular size markers (in kilobases) are shown to the left of each gel.

invasive candidiasis, by MEE we compared oral *C. albicans* isolates from HIV-infected patients with those from healthy persons and with those isolated in the course of invasive candidiasis. The clustering of isolates by the UPGMA method showed no particular grouping correlated with any specific origin. Similar results were obtained when *F*-statistics were applied; no significant differences could be found between the three populations mentioned above. Molecular analysis of variance also confirmed this hypothesis. These results are in agreement with those of other investigators using DNA fingerprinting, who showed that no particular strain is associated with HIV-infected patients and that *C. albicans* populations from the oral cavities of HIV-infected and HIV-negative persons have a common clonal origin (53). However, some researchers using serotyping or fingerprinting with the Ca3 probe have suggested that changes in the *C. albicans* population may occur during or after HIV seroconversion (4, 42). This seems not to be the case in the HIV-infected population examined in the present study. Furthermore, no cluster of strains was associated with invasive candidiasis in this study. Thus, our results show that no *C. albicans* clone is linked with any particular characteristic of clinical relevance so far. However, this result must be confirmed with a larger set of isolates and with *C. albicans* populations from other clinical settings, such as vaginitis cases. As can be seen in Fig. 1, the oral *C. albicans* isolates found in association with clinical signs of OPC did not belong to a limited cluster of closely related clones within the population studied. Similarly, the oral isolates showing a clearly reduced in vitro susceptibility to fluconazole were not restricted to a small group of genetically related clones.

The high frequency of isolates of ET1 in all three populations examined here also shows that this particular clone is specifically associated with neither healthy carriage nor clinical signs of candidiasis. Other authors have also observed major clusters of closely related isolates within the *C. albicans* population that they examined (13, 31). No clear explanation for the widespread overrepresentation of such clones has been given. It may be hypothesized that they are better adapted than other clones to life on or in the human body. They may also be more easily transmitted between humans than other clones.

The results blindly obtained by MEE for 145 isolates from 24 HIV-infected patients showed that 23 of the patients harbored a unique, major *C. albicans* clone during the period of observation. Only one patient among the 24 simultaneously harbored two clearly distinct clones. This result was confirmed by REA and DNA fingerprinting. For 3 of these 24 patients, one isolate belonged to a minor variant of the major ET. The differences between the major ETs and their respective variants could easily be explained by the lack of expression for one or two of the 36 alleles examined within the same clone. Minor modifications of the isoenzymes profiles over time during in vitro storage of *C. albicans* strains have previously been described (18). The appearance of minor variants of an ET within three patients in the present study probably relies on the same mechanism. These differences found with MEE were not confirmed with the two other typing methods used, and it must be assumed that all the isolates from these three patients belonged to the same clones.

In spite of differences in the discriminatory powers of the three methods, the MEE results were in broad agreement with those obtained by REA using the enzymes *Eco*RI and *Hin*fI (21, 41) and with those of DNA fingerprinting with the moderately repetitive Ca3 probe (43). When REA was used, minor but reproducible variants could also be observed within the isolates of some patients. They may be the consequence of the relatively frequent rearrangement in the *C. albicans* genome

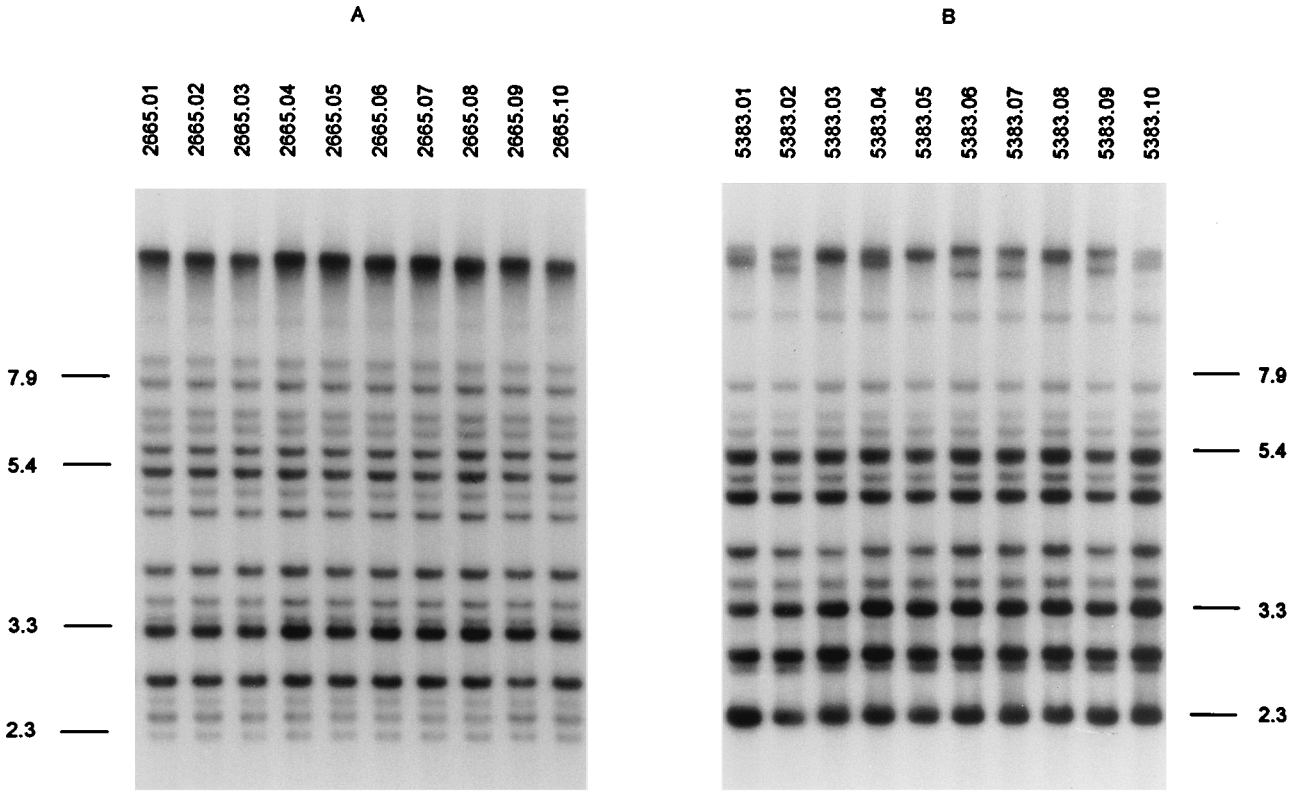


FIG. 5. Ca3 hybridization patterns of multiple simultaneous oral *C. albicans* isolates from patients 3 (A) and 1 (B). The positions of reference molecular size markers (in kilobases) are shown.

described by some researchers (37). With one distinct major type per patient for 23 patients and two types for the 24th patient, DNA fingerprinting with the Ca3 probe was clearly the most discriminatory typing method. However, many more variants could be distinguished among the multiple isolates of each patient when the Ca3 probe was used than with the two other methods. Minor but frequent variations in the Ca3 profiles observed within a clone have been described previously (19). Similarly to what was observed in the present study, these variations have been shown to occur mainly in the high-molecular-weight fragments. They seem to rely on the presence of an RPS repetitive element in the target sequence homologous to the C1 fragment of the Ca3 probe. This relatively rapid evolution of the Ca3 patterns within a clone previously led Soll and coworkers (46) to empirically set a cutoff value in the similarity coefficients below which *C. albicans* clones cannot be reliably distinguished. Fortunately, the isolates of each patient examined in the present study could be clustered together with confidence by the presence of distinctive bands. Together with the MEE and REA results, this criterion was considered sufficient to conclude that all but one patient with multiple isolates each harbored a unique major *C. albicans* clone in their throats. Our results obtained by three typing methods and showing the relative frequent appearance of variants within a clone stress the necessity of using at least two different methods simultaneously to obtain a clear and reliable identification of clones when typing *C. albicans* isolates in epidemiological studies.

The reproducibility of the results obtained by MEE has not been tested here on a broad scale, but the few isolates tested in a blinded fashion in duplicate gave clearly reproducible results. In addition, the fact that the MEE results with multiple isolates from HIV-infected patients blindly tested were in agreement with those of two other independent methods is a strong argument for the reproducibility of MEE. The only three discrepancies observed between MEE and the other methods were reproducibly obtained and must be attributed to effective changes in the genotypes (mutations) or in the phenotypes (lack of expression of some alleles) rather than to a lack of experimental reproducibility of MEE.

The discriminatory index (15) of 0.96 obtained with 70 epidemiologically unrelated isolates in the present study is slightly higher than what had been previously described for MEE (14). This may be a result of the more complex electrophoresis conditions used here, allowing the distinction of more electromorphs than in other studies, and the higher number of enzyme loci examined. With a probability of >95% of differentiating epidemiologically unrelated isolates, MEE is clearly a very powerful tool for typing *C. albicans* in epidemiological studies.

For six of seven patients tested by MEE, only one *C. albicans* clone could be evidenced per patient among multiple simultaneous oral isolates. Thus, our results confirm the observation of other researchers (13, 24, 53), showing that the vast majority of patients carry one unique or only one major *C. albicans* clone in their oral cavities. HIV-infected patients may sometime carry several *C. albicans* clones in their oropharynx, but this seems to represent only a minor part of the population studied. This result is also supported by the fact that only one *C. albicans* clone per patient was found in 20 of 21 patients with sequential oral isolates. It is not clear why some other investigators have found a much higher proportion of patients simultaneously carrying several strains in their oropharynx (21, 28, 39). In the present study, three of the seven patients men-

tioned above and carrying only one oral *C. albicans* clone had no known previous episode of OPC or prior antifungal treatment. Two of these three patients had CD4 counts above 600 cells per mm^3. Thus, our results suggest that neither antifungal therapy nor advanced AIDS status may be the reason for the presence of one unique *C. albicans* clone in the oropharynx of the majority of HIV-infected patients. The simultaneous and continuous presence of two distinct *C. albicans* clones in the oral cavity of one patient previously treated with antifungal agents and with very low CD4 counts also supports this conclusion. In contrast, it could be hypothesized that a deficient immunity may facilitate the simultaneous colonization of the oropharynx of the patient by several distinct strains. Among the 18 patients with multiple *C. albicans* isolates and CD4 counts lower than 100 cells per mm^3 examined in the present study, only 1 simultaneously harbored more than one *C. albicans* strain. Thus, our results do not support the latter hypothesis, suggesting that factors other than the immune status of a patient may influence the colonization of the oropharynx by one or several *C. albicans* strains.

The present results show that clones found before, between, and during OPC episodes in HIV-infected patients are the same. This does not preclude the possibility that a change in colonizing oral *C. albicans* clones may take place early during the development of HIV seropositivity (42). In fact, the two patients who were sampled before their first OPC, and who harbored the same *C. albicans* clone during subsequent OPC episodes, already had decreased CD4 counts at the time of the first sampling (312 and 13 CD4 cells per mm^3) and may have had changes in colonizing oral *C. albicans* earlier. However, the fact that the ET distributions in healthy oral carriers and in HIV-infected patients are similar does not corroborate this hypothesis. Our observation that HIV-infected patients keep the same oral *C. albicans* clone during and between OPC episodes is in agreement with the data obtained by some but not all other investigators. Changes in oral *C. albicans* strains have been shown in a significant proportion of cases in several studies (2, 28, 39). The reasons for the discrepancy between the results of different groups concerning this phenomenon are not clear. However, the regimen of therapy for the treatment of OPC may play an important role in this context. As mentioned by Powderly and collaborators (30), who showed a strong statistical association between change in *Candida* strains during recurrent OPC episodes and use of suppressive antifungal therapy, the continuous isolation of the same strain during recurrent OPC episodes may be related to the lack of complete eradication of this strain by antifungal therapy. The total eradication of *C. albicans* from the oral cavity apparently obtained in certain cases (39) may facilitate the colonization by a new strain. Several patients from the present study had been treated with relatively low single doses of fluconazole until the appearance of clinical resistance (51). Thus, the therapy used for these patients may not have been sufficient in most cases to eliminate the *C. albicans* from their oral cavities. This may also have been the case in the study of Millon and coworkers (23), in which low-dose therapy was used as a first step. No changes in *C. albicans* strains could be detected by those authors. This is in contrast with two other studies in which changes in oral *C. albicans* strains were observed at relatively high frequencies. However, in those two studies, the antifungal therapy used higher doses than in the former studies and for a longer period (28, 39). These observations are in agreement with the results of Powderly and coworkers (30).

We could not find evidence of any change of *C. albicans* clones in patients during the development of in vitro resistance to fluconazole. Again, this is in agreement with the results obtained by some (23, 33), but not all, authors. The reasons for the changes during the development of resistance to this antifungal agent described in several reports (1, 2, 28, 39) may be the same as for the changes in the course of OPC recurrences. The results of our study confirm that the development of resistance to fluconazole can take place within a clone and that the replacement of a susceptible clone by another, unrelated resistant one is not a necessary or frequent event, at least in certain populations.

Ca3 fingerprinting shows that no particular subpopulation within a clone is associated with clinical OPC episodes or with asymptomatic carriage between OPC episodes. In addition, no correlation between Ca3 subtypes and levels of susceptibility to fluconazole could be observed. Different Ca3 subtypes may show a decreased susceptibility to fluconazole and seem to be recovered randomly during the development of resistance.

Our results also show that isolates of the same clone but presenting significantly different levels of susceptibility to fluconazole can be found simultaneously in the throats of some HIV-infected patients. Multiple in vitro subcultures without fluconazole have shown that these differences in phenotypes are stable and do not represent transitory adaptation to the presence of fluconazole (data not shown). This phenomenon may be the reason for some unexplained and irregular variations in susceptibility to fluconazole observed in a few sequential *C. albicans* isolates in the present study.

In conclusion, our results show and confirm that the yeast *C. albicans* has a clonal mode of reproduction in natural populations and that there is no particular *C. albicans* subpopulation associated with HIV-infected patients or with invasive candidiasis. The vast majority of HIV-infected patients examined here carried only one major *C. albicans* clone in their oral cavities. The patients harbored the same *C. albicans* clones during and between recurrent OPC episodes. Development of in vitro resistance to fluconazole in these patients was not associated with replacement of *C. albicans* clones. Finally, subpopulations of *C. albicans* presenting different levels of susceptibility to fluconazole and belonging to the same *C. albicans* clone may be found in the oral cavities of HIV-infected patients during the development of resistance to this widely used antifungal agent.

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