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Comparison of Four Molecular Typing Methods for Evaluating Genetic Diversity among *Candida albicans* Isolates from Human Immunodeficiency Virus-Positive Patients with Oral Candidiasis

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***Candida albicans* strain delineation by karyotyping, *Not*I restriction pattern analysis, hybridization with specific probe 27A, and PCR fingerprinting with the phage M13 core sequence were performed with 30 isolates from the oral cavities of 30 human immunodeficiency virus (HIV)-infected patients and 8 reference strains. Within the panel of clinical isolates, 20 were geographically related, although 10 isolates were susceptible to fluconazole and 10 isolates were resistant to fluconazole. The remaining isolates used in this study were fluconazole resistant and geographically unrelated. A composite DNA type was defined for each of the strains as the combination of types obtained by the four molecular methods. By this procedure, a great diversity of DNA types was found among isolates from the oropharynges of HIV-infected individuals with oral candidiasis. This diversity was not reduced when isolates were evaluated on the basis of whether they came from the same geographical locale and whether they were fluconazole resistant. These data refute the idea of a clonal origin for fluconazole-resistant strains among HIV-positive patients. Karyotyping was the least discriminatory method, yielding 19 DNA types among the 38 strains analyzed. Conversely, hybridization with the 27A probe showed a unique DNA pattern for each of the strains examined in this study. Our results demonstrate that at least two different molecular methods are needed for *Candida albicans* typing and that there is a great deal of strain variation within the species, irrespective of place of origin or antifungal resistance patterns.**

Oral candidiasis is the most common opportunistic infection in individuals infected with the human immunodeficiency virus (HIV), with *Candida albicans* being the most frequently isolated etiologic agent. Despite the reported increase (14, 15) in the number of fluconazole (FLZ)-resistant isolates of *C. albicans* from AIDS patients with recurrent oral candidiasis, questions related to the origin of FLZ resistance remain unanswered. In the last few years several reports (1, 12, 13, 17) have described studies in which molecular typing techniques have been used to delineate sequential isolates from a single patient suffering from recurrent oral candidiasis. Studies have demonstrated that two mechanisms of resistance acquisition may occur: persistence of the same strain throughout the infection or acquisition of a new, resistant strain. Because the whole oral yeast population was not adequately sampled and defined in those studies (1, 17), the last mechanism has not been unequivocally demonstrated, but preliminary data suggested that transmission of resistant strains may occur. Other workers (6, 20) report that a genetic relationship exists between commensal and pathogenic strains of *C. albicans* isolated from the oral cavities of individuals in the same geographical locale. However, most of those published reports described studies in which only one molecular typing method was used to delineate the genetic relationship between *C. albicans* isolates, and the use of only one method may not detect subtle changes in genomic structure. For example, Magee et al. (9) showed how the use of four different molecular approaches led to an increase in the number of DNA types identified in a group of 20 strains of *C. albicans* from patients in a single hospital.

Due to the above-mentioned increased numbers of FLZ-resistant *C. albicans* organisms isolated from the oral cavities of HIV-positive patients, an effort should be made to clarify whether the resistant population has a clonal origin, that is, if the genetic diversity of the resistant population is minimal compared with the genetic diversity of the sensitive one. In addition, it is important to know if there is a genetic relationship between the strains which are geographically related, regardless of their FLZ susceptibility.

In order to answer these questions, at least in part, we used four molecular approaches, i.e., electrophoretic karyotyping with a contour-clamped homogeneous electric field (CHEF) system, macrorestriction analysis of chromosomal DNA with *Not*I, hybridization of *Eco*RI-digested DNA with the 27A repeat sequence of *C. albicans*, and PCR fingerprinting with the phage M13 core sequence. With the exception of *Not*I macrorestriction analysis, these methods have already been demonstrated to be useful tools for the delineation of *C. albicans* strains (18, 19, 21).

(Part of this work was presented at the 35th Interscience Conference on Antimicrobial Agent and Chemotherapy, San Francisco, Calif., 17 to 20 September 1995 [5].)

MATERIALS AND METHODS

Strains. Thirty-eight *C. albicans* strains were included in the study. Thirty of them were isolated from the oral cavities of 30 HIV-positive patients. Culture, identification, and storage of strains were carried out by standard procedures (2).

Antifungal susceptibility testing of all isolates was performed by a broth microdilution adaptation of the reference method (11) which has previously been described in detail (10). We chose 20 isolates for which FLZ MICs were ≥8.0 μg/ml as the group of isolates with decreased susceptibility to FLZ and 10 isolates for which FLZ MICs were ≤0.5 μg/ml as the group of susceptible strains. These breakpoints were chosen according to recent suggestions (15). The isolates

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were classified into two groups according to their geographic origin. One group contained 20 geographically related strains isolated from 20 patients visiting the same clinic in Madrid; 10 of them were FLZ-resistant and the other 10 strains were FLZ-susceptible. The second group contained 10 FLZ-resistant strains that were geographically unrelated and that were obtained from 10 patients dispersed in different hospitals across Spain. A third group of eight well-characterized, unrelated *C. albicans* strains was included as controls for the techniques assessed: six of them were American Type Culture Collection (ATCC) organisms (ATCC 64551, ATCC 64548, ATCC 64550, ATCC 90028, ATCC 24433, and ATCC 76615) and the other two were kindly provided by D. R. Soll, University of Iowa (*C. albicans* 3153a and WO-1).

EKs. The method used to prepare plugs containing yeast chromosomal DNA was similar to that described previously (8). Electrophoretic karyotypes (EKs) were determined by pulsed-field electrophoresis on agarose gels with a CHEF system (CHEF-XA mapper; Bio-Rad, Alcobendas, Spain). The conditions of electrophoresis were selected to resolve the whole chromosome size range in a unique gel. A 1% gel was prepared with Pulsed Field Certified agarose (Bio-Rad); 1× TBE (90 mM Tris-borate, 2 mM EDTA) at 10°C was used as the running buffer. Electrophoresis was performed for 72 h at 3 V/cm, with pulse times ramped from 3 to 17 min. The reorientation angle was 106 degrees. Chromosomal preparations of *Hansenula wingei* (Bio-Rad) and *C. albicans* WO-1 were included as standards in each gel.

Restriction enzyme digestion of chromosome-sized DNA. A slice of the agarose insert (approximately one-quarter of the insert) was washed twice at room temperature (1 h per wash) with 1 ml of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (Sigma). The slice was rinsed for 30 min with 1 ml of TE buffer before placing it in a 2-ml microcentrifuge tube (with a flat bottom) with 0.5 ml of the restriction buffer recommended by the endonuclease manufacturer, and the tube was kept on ice for 30 min. The buffer was replaced by 0.2 ml of fresh restriction buffer with 10 U of *Not*I (New England Biolabs, Landerdiagnostico S.A., Madrid, Spain), and the tube was incubated overnight at 37°C. After digestion, the slice was rinsed with 0.5 ml of TE buffer and was applied to agarose gels.

Separation of large DNA fragments was performed by pulsed-field gel electrophoresis (CHEF-XA mapper; Bio-Rad). The gels were prepared at 1% with Pulsed Field Certified agarose (Bio-Rad). 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 14°C was used as the running buffer. Electrophoresis was performed for 20 h at 5.9 V/cm with an initial 10-s pulse time and a final 60-s pulse time. The reorientation angle was 120 degrees. DNA lambda concatemer markers (Bio-Rad) were included on both sides of the gels.

Hybridization with probe 27A sequence. Preparation of DNA was performed by a modification of the method of Scherer and Stevens (19). Cultures of *C. albicans* in YEPD (yeast extract-peptone-dextrose) and washes of the yeast cells were performed as described above for the preparation of plugs containing chromosomal DNA (8). Yeast cells were placed in 1 ml of 0.2 M Tris-HCl–0.1 M EDTA–1.2 M sorbitol (pH 9.1) containing 0.7% β-mercaptoethanol (Sigma-Aldrich, Alcobendas, Spain), maintained at room temperature for 10 min, and they were then rinsed twice with 1 ml of 20 mM citrate-phosphate (pH 5.6)–50 mM EDTA (pH 8.0)–0.9 M sorbitol, resuspended in 1 ml of the same solution with 10 μl of a stock of lyticase (Sigma-Aldrich) prepared at 12,500 U/ml in 50% (vol/vol) glycerol–0.01 M $NaPO_4$ (pH 7.4), and incubated at 37°C for 2 h. The resultant spheroplasts were washed twice with 1 ml of 0.01 M $CaCl_2$–0.01 M Tris-HCl–1 M sorbitol. Upon resuspension in 0.5 ml of 50 mM EDTA (pH 8), the spheroplasts were lysed by adding sodium dodecyl sulfate at a final concentration of 0.2% and incubating at 65°C for 30 min. Then, 50 μl of 5 M potassium acetate was added to the mixture and incubation was continued at 0°C for 60 min. After centrifugation, DNA was purified from the supernatant by extraction with phenol-chloroform-isoamyl alcohol (25:24:1; Sigma-Aldrich) and ethanol precipitation. The pellet was dried, resuspended in 50 μl of TE buffer, treated with RNase (100 μg/ml; Sigma-Aldrich) and proteinase K (50 μg/ml; Sigma-Aldrich), and repurified via a second phenol-chloroform-isoamyl alcohol extraction and precipitation.

Approximately 2 μg of purified DNA was digested with 20 U of *Eco*RI (Pharmacia Biotech, S.A., Madrid, Spain) overnight at 37°C. DNA fragments were separated through a 0.8% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) at 4 V/cm for 4 h. The DNA fragments were transferred to a nylon membrane (Hybond-N^+; Amersham Iberica, Madrid, Spain) with a vacuum blotting system (VacuGene XL; Pharmacia Biotech) by standard procedures (16). The probe used, probe 27A, is an *Eco*RI fragment of *C. albicans* DNA cloned into a pUC18 plasmid that was kindly supplied by S. Scherer, University of Minnesota, Minneapolis. Labelling of the probe with fluorescein (enhanced chemiluminescence [ECL] random prime labelling system), hybridization, stringency washes, and detection by chemiluminescence on blue light-sensitive autoradiography film (ECL detection system) were performed according to the manufacturer's instructions (Amersham Iberica).

PCR fingerprinting. About 50 ng of DNA (obtained as described above) served as the template for PCR fingerprinting analysis. PCRs were performed with the core sequence of phage M13 primer (5′-GAGGGTGGCGGTTCT-3′) (22) under conditions previously tested with *C. albicans* (21). The amplified fragments were separated through a 1.3% agarose gel by electrophoresis at 4 V/cm for 16 h.

Analyses of the EKs, restriction patterns, and PCR profiles were performed by visual inspection of the stained agarose gels or autoradiographs. To interpret the pulsed-field gels, two isolates were judged to be different if their EKs or *Not*I restriction patterns had different numbers of bands or, if they had the same number of bands, their bands had different migration rates. The PCR fingerprints and hybridization patterns of two given strains were considered different if any readily detectable band did not match.

RESULTS

General results of molecular typing by four different molecular methods. The DNA patterns obtained by each of the four molecular methods used in this study with 38 strains are summarized in Table 1. Figures 1 through 4 illustrate the results obtained by electrophoretic karyotyping, macrorestriction analysis with *Not*I, hybridization with 27A probe, and PCR fingerprinting, respectively, within the panel of 10 FLZ-resistant strains of the same geographical origin.

Molecular typing of 30 clinical isolates and 8 reference strains by resolving chromosome-sized DNA molecules into EKs showed 19 distinct karyotypes, recognized on the basis of the number and migration of DNA bands. In general, it was more difficult to discriminate between the patterns for smaller chromosomes than between the patterns for larger ones, and subjective criteria often had to be used to delineate differences between various EK patterns. A fuzzy band could sometimes be observed between the two largest bands of an EK pattern (Fig. 1, lanes 2, 4, and 5). This has been reported to be one of the two homologous subunits of the ribosomal DNA-bearing chromosome (named chromosome R). It is considered a highly variable characteristic by electrophoretic karyotyping, and is unsatisfactory for discriminating between strains of the same taxon (9). So, we did not consider this fuzzy band in the strain delineations by karyotyping. Digestion of chromosomal DNA with *Not*I yielded patterns with large fragments (about 10 bands with sizes ranging from less than 50 to roughly 800 kb), which were resolved by pulsed-field gel electrophoresis. A total of 22 distinct *Not*I restriction patterns (N patterns) were observed. The combination of EKs and N patterns permitted us to define 35 different molecular types among the 38 strains studied.

Southern blot hybridization with the probe 27A yielded patterns (H patterns) which were different for all 38 strains, and the analysis of PCR fingerprints of 38 strains gave a total of 34 distinguishable patterns (M patterns).

Genetic diversity among the group of 20 geographically related isolates. Within the subgroup of 10 FLZ-resistant strains of the same geographical origin, 5 EKs, 7 N patterns, 10 H patterns, and 8 PCR profiles were found. Data for this group of strains (strains 11 to 20) are summarized in Table 1, and banding patterns are illustrated in Figures 1 through 4. In Fig. 1 it can be seen that strains CNML-1518 (lane 1) and CNML-586 (lane 9) shared karyotype EK1; strains CNML-1548, CNML-1403, and CNML-736 (lanes 5, 7, and 10, respectively) shared karyotype EK6; and strains CNML-1444, CNML-1727, and CNML-1354 (lanes 4, 6, and 8, respectively) displayed karyotype EK10. When *Not*I restriction patterns were compared (Fig. 2), strains in lanes 2 to 6 and 10 were observed to have markedly different N patterns. In contrast, the strains in lanes 1, 7, 8, and 9 had the same number of bands, with very similar band mobilities, and their respective N patterns differed only in the brightness of the double band of about 200 kb. These four N patterns were considered a single DNA type, and this type was named N9. When the results of karyotyping and *Not*I macrorestriction digestion were combined, it was apparent that strains CNML-1518 (Fig. 1 and Fig. 2, lane 1) and CNML-586 (Fig. 1 and Fig. 2, lane 9) displayed not only a unique EK but also the same N pattern (DNA type EK1 N9);

TABLE 1. Characteristics of oral cavity-derived isolates and reference strains and summarized results of four genotyping techniques

Geographical relation, isolate no., and isolate[a]	Geographical origin	Date of isolation (mo-yr)	FLZ-MIC (μg/ml)	EK	N pattern	H profile	M profile	Type[b]
Geographically unrelated strains								
1. CNML-1208	Ferrol	2-1994	8.0	EK1	N13	H13	M23	1
2. CNML-282	Pontevedra	6-1992	32.0	EK7	N11	H9	M18	2
3. CNML-341	Hospitalet	6-1992	>128.0	EK8	N4	H30	M10	3
4. CNML-589	Sevilla	1-1993	32.0	EK9	N1	H10	M19	4
5. CNML-1113	Villajoyosa	12-1993	16.0	EK10	N1	H31	M21	5
6. CNML-468	Mostoles	11-1992	64.0	EK10	N12	H11	M20	6
7. CNML-1186	Covadonga	1-1994	8.0	EK10	N14	H12	M22	7
8. CNML-1383	Huesca	4-1994	16.0	EK11	N1	H14	M24	8
9. CNML-152	Vigo	2-1992	128.0	EK12	N10	H15	M25	9
10. CNML-1026	Madrid	10-1993	8.0	EK13	N9	H16	M26	10
Geographically related strains								
11. CNML-1518	Madrid	3-1994	16.0	EK1	N9	H17	M10	11
12. CNML-586	Madrid	1-1993	>128.0	EK1	N9	H22	M16	12
13. CNML-736	Madrid	4-1993	64.0	EK6	N4	H23	M17	13
14. CNML-1403	Madrid	1-1994	16.0	EK6	N9	H33	M14	14
15. CNML-1548	Madrid	6-1994	128.0	EK6	N15	H20	M11	15
16. CNML-1727	Madrid	9-1994	64.0	EK10	N8	H21	M15	16
17. CNML-1354	Madrid	12-1993	8.0	EK10	N9	H37	M17	17
18. CNML-1444	Madrid	2-1994	8.0	EK10	N18	H19	M13	18
19. CNML-1402	Madrid	1-1994	16.0	EK14	N17	H18	M12	19
20. CNML-1689	Madrid	8-1994	8.0	EK15	N16	H32	M13	20
21. CNML-116	Madrid	11-1993	0.25	EK1	N1	H38	M4	21
22. CNML-550	Madrid	1-1993	0.25	EK1	N4	H25	M2	22
23. CNML-896	Madrid	6-1993	0.12	EK1	N4	H29	M9	23
24. CNML-1294	Madrid	2-1994	0.25	EK1	N22	H28	M4	24
25. CNML-945	Madrid	7-1993	0.12	EK6	N6	H35	M6	25
26. CNML-859	Madrid	6-1993	0.12	EK6	N21	H27	M3	26
27. CNML-1031	Madrid	9-1993	0.12	EK16	N4	H34	M5	27
28. CNML-417	Madrid	10-1992	0.25	EK17	N7	H26	M7	28
29. CNML-511	Madrid	11-1992	0.06	EK18	N19	H24	M1	29
30. CNML-683	Madrid	2-1993	0.12	EK19	N20	H36	M8	30
Reference strains								
31. ATCC 64551				EK1	N1	H1	M27	31
32. ATCC 24433				EK1	N7	H7	M33	32
33. ATCC 64548				EK2	N2	H2	M28	33
34. ATCC 64550				EK2	N3	H3	M29	34
35. 3153a				EK3	N4	H4	M30	35
36. WO-1				EK4	N5	H5	M31	36
37. ATCC 90028				EK5	N6	H6	M32	37
38. ATCC 76615				EK6	N8	H8	M34	38

[a] Isolate number from the Centro Nacional de Microbiología/Levaduras (CNML) collection of fungi.
[b] The composite type of each isolate was identified with a number.

however, strains CNML-1548, CNML-1403, and CNML-736, considered identical on the basis of their karyotypes, were found to have different N patterns (Fig. 2): CNML-1548 (lane 5) had a double band of about 800 kb, while CNML-1403 and CNML-736 (lanes 7 and 10, respectively) had only one band of this size. On the other hand, the double band of roughly 200 kb observed in CNML-1548 and CNML-1403 (lanes 5 and 7, respectively) did not appear in strain CNML-736 (lane 10). Strains CNML-1444, CNML-1727, and CNML-1354 (lanes 4, 6, and 8, respectively, in Fig. 1 and 2) were also distinguishable by their N patterns.

When the hybridization patterns of the geographically related FLZ-resistant strains were compared, a single H pattern could be seen for each strain (Fig. 3). The pair of strains CNML-1518 and CNML-586 which showed the same pattern by the two first DNA analytical methods (EK1 N9) had very dissimilar H patterns, differing by more than one hybridization band (lanes 1 and 8, respectively, in Fig. 3). The PCR fingerprints of the strains of this group are presented in Fig. 4. Two patterns were repeated; the pattern named M13 was found in strains CNML-1689 and CNML-1444 (lanes 4 and 5, respectively), and pattern M17 was found in strains CNML-736 and CNML-1354 (lanes 9 and 10, respectively). However, CNML-1689 and CNML-1444 could be distinguished by the other methods used. Looking at the EKs of these two strains in Fig. 1, we concluded that they are clearly different: CNML-1689 (lane 3) displayed a unique karyotype, with an extra chromosomal band at approximately 0.4 Mb. The karyotype of strain CNML-1444 (lane 4) was EK10, which was more commonly found among the FLZ-resistant strains. CNML-1689 and CNML-1444 also differed in their N patterns (lanes 3 and 4, respectively, of Fig. 2), which matched at only two bands, one at 200 kb and the other one at 400 kb. The hybridization patterns of CNML-1689 and CNML-1444 were clearly different (lanes 4 and 5, respectively, of Fig. 3). The other two strains showing a single PCR pattern, strains CNML-1354 and

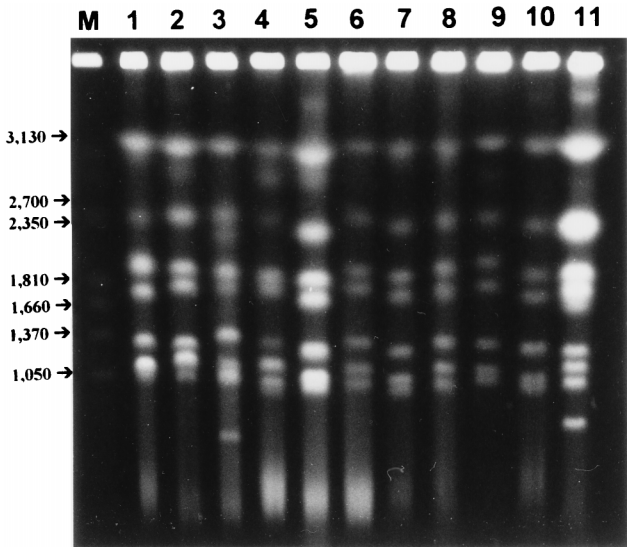

FIG. 1. Electrophoretic karyotypes of 10 geographically related FLZ-resistant isolates. Sizes (in kilobase pairs) are denoted on the left. Lane M, *H. wingei* chromosomes (Bio-Rad); lane 11, *C. albicans* WO-1 reference strain; lane 1, CNML-1518; lane 2, CNML-1402; lane 3, CNML-1689; lane 4, CNML-1444; lane 5, CNML-1548; lane 6, CNML-1727; lane 7, CNML-1403; lane 8, CNML-1354; lane 9, CNML-586; and lane 10, CNML-736.

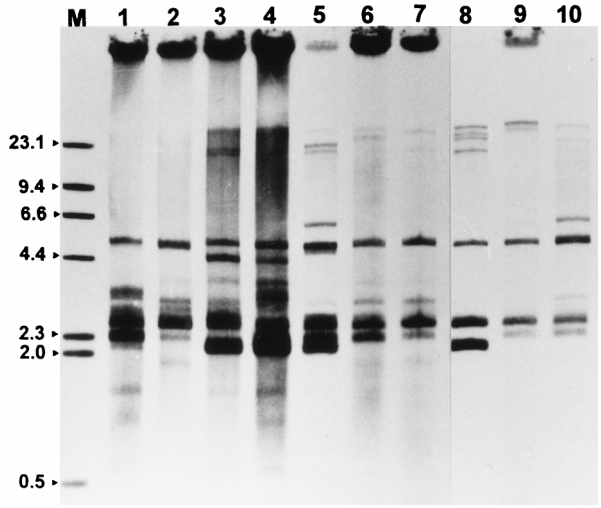

FIG. 3. Hybridization profiles obtained with the 27A probe of genomic DNA from 10 geographically related FLZ-resistant isolates. Sizes (in kilobase pairs) are denoted on the left. Lane M, size marker (fluorescein-labelled *Hin*dIII digest of bacteriophage lambda; Amersham); lane 1, CNML-1518; lane 2, CNML-1548; lane 3, CNML-1402; lane 4, CNML-1689; lane 5, CNML-1444; lane 6, CNML-1403; lane 7, CNML-1727; lane 8, CNML-586; lane 9, CNML-736; lane 10, CNML-1354.

CNML-736, displayed different EKs (differing in the relative mobilities of the two smallest chromosomal bands; lanes 8 and 10, respectively, in Fig. 1) and different N patterns: CNML-1354 (Fig. 2, lane 8) had a double band in the molecular mass range of 150 to 200 kb, while CNML-736 (Fig. 2, lane 10) had a single band of this size. The hybridization patterns presented by CNML-736 and CNML-1354 (lanes 9 and 10, respectively, in Fig. 3) differed by more than one hybridization band.

Among the subgroup of 10 FLZ-susceptible isolates from the same geographical locale, 6 EKs, 8 N patterns, 10 H patterns, and 9 PCR profiles were obtained. Data for this group of strains (strains 21 to 30) are summarized in Table 1. Karyotype EK1 was displayed by strains CNML-1116, CNML-550, CNML-896, and CNML-1294. Another pair of strains, strains CNML-945 and CNML-859, shared karyotype EK6. The macrorestriction patterns obtained for this set of isolates allowed strains CNML-550, CNML-896, and CNML-1031 to be grouped into a unique DNA type (N4 pattern), while each of the remaining strains of this panel had its own N pattern. Two of these strains, strains CNML-550 and CNML-896 (strains 22 and 23, respectively, in Table 1), were also found to have the same EK, so these isolates were identified as EK1 N4. The hybridization profiles obtained among the group of 10 FLZ-susceptible isolates of the same geographical origin were different for each strain, and the isolates previously identified as being of a single DNA type by either one or two methods were distinguishable on the basis of their H patterns. A single PCR profile was obtained for each of the FLZ-susceptible isolates with a common origin except for the pair CNML-1166 and CNML-1294 (strains 21 and 24, respectively, in Table 1), which showed the same karyotype and PCR profile (EK1 M4). Considering the results obtained for the whole group of 20 strains isolated in the same hospital, the karyotype most commonly found was EK1 (a total of six strains showed this karyotype); this was followed by EK6 (five strains) and EK10 (three strains). Among the macrorestriction patterns, four strains shared pattern N9 and four strains shared pattern N4. There were no repetitions in the hybridization profiles shown by this group of 20 isolates, while 3 PCR fingerprints (M13, M17, and M4) were shared by pairs of strains.

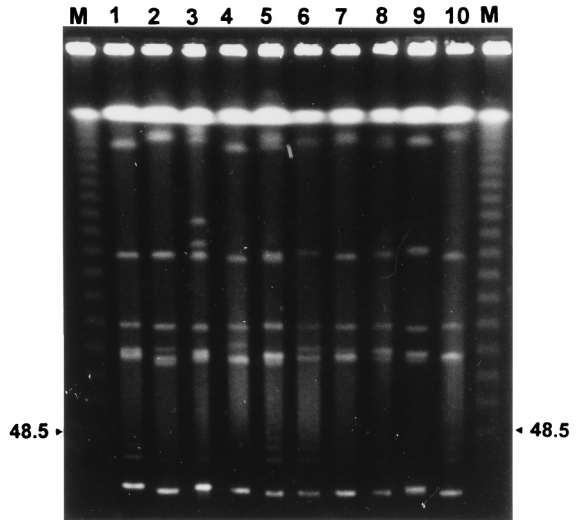

FIG. 2. *Not*I restriction patterns of chromosomal DNAs from 10 geographically related FLZ-resistant isolates. Sizes (in kilobase pairs) are denoted on both sides. Lanes M, size markers (bacteriophage lambda concatemer ladder; Bio-Rad). Isolates (lanes 1 to 10) are arranged in the same order as in Fig. 1: lane 1, CNML-1518; lane 2, CNML-1402; lane 3, CNML-1689; lane 4, CNML-1444; lane 5, CNML-1548; lane 6, CNML-1727; lane 7, CNML-1403; lane 8, CNML-1354; lane 9, CNML-586; lane 10, CNML-736.

Genetic diversity among the group of 10 geographically unrelated FLZ-resistant isolates. Within the panel of 10 FLZ-resistant strains isolated from patients at 10 different hospitals that were not in the same geographical area, 8 EKs, 8 N patterns, 10 banding patterns with the probe 27A, and 10 PCR fingerprints were found. Data for this group of strains (strains 1 to 10) are summarized in Table 1. Analysis of karyotypes grouped three strains, strains CNML-1113, CNML-468, and

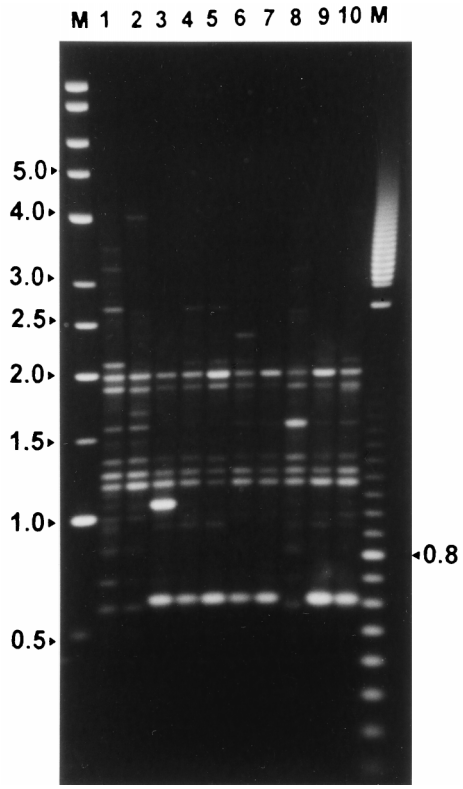

FIG. 4. PCR fingerprinting analysis with the core sequence of phage M13 primer of 10 geographically related FLZ-resistant isolates. Sizes (in kilobase pairs) are denoted on both sides. Lanes M, size markers: 100-bp ladder (right) and kilobase DNA marker (left) (Pharmacia). Isolates (lanes 1 to 10) are arranged in the same as in Fig. 3: lane 1, CNML-1518; lane 2, CNML-1548; lane 3, CNML-1402; lane 4, CNML-1689; lane 5, CNML-1444; lane 6, CNML-1403; lane 7, CNML-1727; lane 8, CNML-586; lane 9, CNML-736; lane 10, CNML-1354.

CNML-1186, into a single type (EK10); karyotype EK10 was also found among the group of FLZ-resistant strains from a single hospital in Madrid analyzed previously in this study (strains 11 to 20 in Table 1). From the DNA band patterns obtained by macrorestriction analysis, strains CNML-589, CNML-1113, and CNML-1383 were considered to have the same pattern (pattern N1). Hybridization with probe 27A and by the PCR-based method enabled us to distinguish all 10 geographically unrelated FLZ-resistant isolates. No coincident results by more than one technique were seen between any two isolates of this group.

Genetic analysis of control strains. The genotypes obtained for each of the control strains (strains 31 to 38) are summarized in Table 1. Within this panel of strains, 6 EKs, 10 N patterns, 10 H patterns, and 10 M patterns were found. Strains ATCC 64551 and ATCC 24433 presented karyotype EK1, which was the most common karyotype among the *C. albicans* isolates surveyed in this study. Moreover, the type strain, ATCC 64551 (strain 31 in Table 1), shared not only the karyotype but also the N pattern (EK1 N1) with FLZ-susceptible isolate CNML-1116 (strain 21 in Table 1) obtained from a patient in Madrid. Of the remaining reference strains, only ATCC 64548 and ATCC 64550 shared a common karyotype (EK2). Nevertheless, the eight control strains were clearly distinguished by analyzing their macrorestriction patterns, hybridization patterns with probe 27A, and PCR fingerprints.

DISCUSSION

The origins of FLZ-resistant isolates from HIV-infected patients with recurrent episodes of thrush remain to be ascertained. Studies that have used molecular typing to investigate the epidemiology of FLZ resistance in this fungal infection have shown that the recurrent isolate often appears to have the same DNA type as the initial one, as shown by restriction endonuclease analysis with *Bss*HII followed by pulsed-field gel electrophoresis (1), CHEF analysis (12, 17), and DNA probe analysis (13). In addition, those studies have concomitantly found the occurrence of FLZ-resistant isolates which displayed DNA patterns different from those of FLZ-susceptible strains isolated from a single patient. In this latter case, if exogenous acquisition of FLZ-resistant strains was the main mechanism for resistance, the genetic diversity within a group of FLZ-resistant isolates would be reduced compared with that for a similar group of FLZ-susceptible isolates. However, this mechanism of FLZ resistance remains a hypothesis because, to our knowledge, the genetic diversity of the population of FLZ-resistant strains has not been analyzed. Almost all of these studies were based on only one molecular technique; however, when more than one molecular typing system has been used to evaluate *C. albicans* strains, a more accurate strain delineation has been achieved (9). Although comparisons of the usefulness of different DNA-based methods for the delineation of *C. albicans* strains isolated either from patients with oral candidiasis (7) or from other clinical settings (3) have recently been published, no one method has been defined as the more suitable one.

In this study, we assessed the discriminatory abilities of four molecular typing methods directed at different targets of genomic DNA for typing clinical isolates and control strains of *C. albicans*. Because there are no standard rules for interpreting the differences observed between the DNA profiles obtained by any of these methods, we defined a composite DNA type on the basis of the combination of types obtained by the four methods (EK, N, H, and M types). Application of this typing method to a panel of 20 clinical isolates (both FLZ-susceptible and FLZ-resistant strains) of the same geographical origin and another panel of 10 FLZ-resistant clinical isolates of diverse geographical origins revealed a single DNA type for each of the 30 clinical isolates analyzed; that is, none of the composite DNA types was shared by two individual strains. When a group of eight control strains was similarly typed, each isolate could again be assigned to its own unique DNA type. Previous studies (4) reported that the level of discrimination reached by any of the four methods used in this work was not an artifact. These four approaches recognized three strains isolated from cerebrospinal fluid samples of a patient with AIDS as belonging to the same DNA type, while another strain isolated from the oral cavity of this patient was genetically different.

Electrophoretic karyotyping was shown to be the least discriminatory technique, yielding 19 karyotypes among the 38 strains studied, although many of these distinctions were subtle and required more than one gel to clearly ascertain them. Application of EKs alone suggested an apparently reduced genetic diversity between both FLZ-resistant and FLZ-susceptible isolates that are geographically related (strains 11 to 30 in Table 1), clustering strains of this large group into three main EK groups (EK1, EK6, and EK10). The macrorestriction digests with *Not*I identified 22 distinct N patterns. These patterns were easier to interpret than the EKs because of the better resolution of the DNA bands achieved by this technique. In addition, pulsed-field gel electrophoresis was shorter with the

digested DNA than with the intact DNA molecules (20 versus 72 h). Although the use of both EKs and N patterns could be very useful because the DNA extraction procedure is the same for both methods, the level of discrimination achieved with these two methods was not very satisfactory. For example, a possible common source could be considered for two pairs of clinical isolates if DNA type was defined only by their EKs and N patterns. Furthermore, clinical isolate CNML-1116 could not be distinguished from reference strain ATCC 64551 by their EKs and N profiles. Differentiation between these pairs of strains was accomplished by the addition of the other methods. The hybridization method with probe 27A proved to be the most discriminatory of the four techniques. It provided distinct patterns for each of the strains analyzed. We confirmed prior observations (9, 19) that use of the 27A probe to hybridize blots with whole digested DNA can differentiate between the vast majority of isolates by one or more bands. The PCR-based method gave results comparable to those of Southern blot hybridization with probe 27A, but with a slightly decreased level of discrimination, although PCR still had a higher discriminatory ability than pulsed-field gel electrophoresis-based methods. By PCR, 34 distinguishable profiles were detected among the 38 strains. In summary, differences between strains with identical karyotypes were detected by PCR and fingerprinting with probe 27A. This result demonstrates that electrophoretic karyotyping may not reflect subtle genomic variations. Somewhat similar results were obtained by macrorestriction analysis of chromosomal DNA.

As a general recommendation, we do not consider the discrimination obtained by electrophoretic karyotyping or macrorestriction analysis of chromosomal DNA good enough for any of these methods to be used alone. In contrast, Southern blotting with the probe 27A and, to a minor extent, PCR fingerprinting have been shown to be powerful typing tools.

In conclusion, a great genetic diversity among *C. albicans* isolates from HIV-infected patients was found. No identical genotypes were found in the group of geographically related isolates, regardless of whether the isolates were FLZ sensitive or resistant. Our results demonstrate that FLZ-resistant strains do not represent a group of organisms that is genetically distinguishable from FLZ-susceptible strains in the same geographical area, nor do FLZ-resistant and FLZ-susceptible isolates from a limited geographical area form a genetically distinguishable group compared with either subgroup from diverse geographical origins throughout Spain. That genetic diversity among strains either resistant or sensitive to FLZ refuted the idea of a clonal origin for the FLZ-resistant isolates among HIV-positive patients.

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