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Epidemiological Analysis of *Candida albicans* Strains by Multilocus Enzyme Electrophoresis

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Genotypic diversity in a collection of 98 isolates of *Candida albicans* was assessed by multilocus enzyme electrophoresis. Four of the 10 enzyme loci studied were polymorphic. The electrophoretic patterns observed were compatible with those expected for a diploid organism. The 98 isolates were assigned to 14 electrophoretic types, each of which was represented by from 1 to 21 isolates. Samples from various clinical sites of seven bone marrow transplant patients treated in the same unit within a 13-month period were obtained repeatedly. Three patients were found to be colonized with more than one strain. In one patient, flucytosine-resistant strains were isolated after systemic antifungal treatment was started. These isolates had electrophoretic types different from those of the strains that colonized the patient before treatment. There was no evidence that cross-infection between these patients occurred in the hospital. The population structure of *C. albicans* is discussed in regard to the multilocus genotype data.

The asexual diploid yeast *Candida albicans* produces a large spectrum of infections, including infections of the skin, oropharynx, esophagus, vagina, and urinary tract (16). *C. albicans* is also emerging as a major nosocomial pathogen, causing invasive disease in both immunocompromised and seriously ill immunocompetent patients (9). In the United States, a marked increase in *Candida* bloodstream infection rates has been reported (2, 11), and candidemia accounts for approximately 10 to 15% of all hospital-acquired blood infections (25). Mortality rates have been estimated to be 40 to 60% in patients with *C. albicans* fungemia. The epidemiology of nosocomial *Candida* infections often remains uncertain. There has been little documentation of cross-infections in the hospital setting (12).

As a consequence, interest in gaining a better understanding of the pathogenesis and epidemiology of *C. albicans* infections has risen in the last decade, leading to the development of a number of new methods that can be used to differentiate between strains (13, 19). Typing systems have usually relied upon phenotypic characteristics of the strains. Serotyping with rabbit antisera yields only two types (4, 5); profiles obtained by electrophoresis of whole cell proteins differentiate only between the two serotypes (19); biotyping and resistotyping permit the differentiation of many classes (14, 22, 23), but the reproducibility of these methods has been questioned (4, 19). Methods based on genotypic variation, such as electrophoretic karyotyping (1, 15) and restriction fragment length polymorphism (7, 10, 24, 28, 32, 35), have permitted a better and more reliable means of discriminating *C. albicans* strains.

Multilocus enzyme electrophoresis has been used to assess genetic diversity and population structure in a number of bacterial species (29) and has provided powerful marker systems for epidemiological investigations (6, 21). Electrophoretic variations in enzymes have been used only to a limited extent for differentiation of *Candida* spp. (3, 17, 18). Analysis of variations in eight enzymes among 37 isolates of *C. albicans* revealed 23 patterns (18). However, the value of the method for epidemiological purposes has not yet been evaluated (19).

We report here the results of an electrophoretic analysis of 10 enzyme loci in strains of *C. albicans* isolated from patients in Norway. Consecutive isolates recovered from seven patients in a bone marrow transplant unit within a 1-year period were compared to assess the stability of strains harbored by single individuals and possible cross-infection among patients.

MATERIALS AND METHODS

***Candida* strains.** A collection of 98 *C. albicans* strains was analyzed. Thirty-three strains were obtained from 14 microbiological laboratories situated in different parts of Norway; the strains were isolated from 31 different patients during the period from 1979 to 1990; two isolates obtained from blood and pus of the same individual and two isolates from the pus of another patient that differed in their resistance to fluconazole were compared. The clinical sources of the isolates were blood (12 isolates), wound (11 isolates), lung (3 isolates), urine, oropharynx, and pleural fluid (2 isolates each), and bile (1 isolate). Sixty-one strains were obtained from seven bone marrow transplant patients treated at the Norwegian Radium Hospital, Oslo, from February 1990 to March 1991. As a part of the microbiological surveillance, specimens from the mouth, groin, and feces of these patients were cultured twice a week during their stay in the bone marrow transplant unit, starting at the time of admission. From 4 to 13 isolates from each of these patients were tested. In addition, three strains obtained from culture collections (ATCC 24433, CBS 2668 = IFO 1388, CBS 562 = ATCC 18804 = IFO 1385) and a reference strain (H29) used for susceptibility testing of flucytosine were included.

The strains were identified as *C. albicans* by the production of germ tubes, formation of clamydospores on cornmeal agar, and carbohydrate assimilation profiles (27) and were stored at −70°C in Greaves solution (8). Each isolate was coded and submitted blindly for multilocus enzyme electrophoresis.

Preparation of culture lysates. Isolates were inoculated into 100 ml of Sabouraud broth and incubated for 18 to 20 h

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TABLE 1. Allelic profiles in 14 ETs of 98 isolates of *C. albicans*

ET	No. of isolates	Alleles at the indicated enzyme loci[a]									
		MDH	G6P	PEP	LAP	ACO	IPO	IDH	PGM	6PG	GOT
1	3	1	1	2	1	1	1	1	1	1	1
2	5	1	2	2	1	1	1	1	1	1	1
3	4	1	2	2	2	1	1	1	1	1	1
4	20	1	2	2	1/2	1	1	1	1	1	1
5	21	1	1/2	2	1	1	1	1	1	1	1
6	4	1	1/2	1/2	1	1	1	1	1	1	1
7	2	2	1	2	1	1	1	1	1	1	1
8	4	2	2	2	1	1	1	1	1	1	1
9	13	2	1/2	2	1	1	1	1	1	1	1
10	9	1/2	1	2	1	1	1	1	1	1	1
11	2	1/2	2	2	1	1	1	1	1	1	1
12	2	1/2	2	2	1/2	1	1	1	1	1	1
13	1	1/2	1/2	2	1/3	1	1	1	1	1	1
14	8	1/2	1/2	2	1	1	1	1	1	1	1

[a] Heterozygotes having alleles 1 and 2 are noted 1/2, and those having alleles 1 and 3 are noted 1/3. Enzyme abbreviations: MDH, malate dehydrogenase; G6P, glucose 6-phosphate dehydrogenase; PEP, phenylalanyl-leucine peptidase; LAP, leucine aminopeptidase; ACO, aconitase; IPO, indophenol oxidase; IDH, isocitrate dehydrogenase; PGM, phosphoglucomutase; 6PG, 6-phosphogluconate dehydrogenase; GOT, glutamic oxaloacetic transaminase.

at 37°C with continuous agitation. Cell suspensions were harvested by centrifugation at 10,000 × *g* for 10 min. The yeast cells were mixed with glass beads in a mortar and frozen at −20°C. The cells were lysed by beating the frozen suspension with a pestle until liquid, freezing, and beating again. After centrifugation at 25,000 × *g* at 4°C for 20 min, the supernatants were stored at −70°C until they were used for electrophoresis.

Electrophoresis of enzymes. Starch gel electrophoresis and selective enzyme staining were performed by methods similar to those described by Selander et al. (29). The 10 enzymes assayed were malate dehydrogenase (MDH), glucose 6-phosphate dehydrogenase (G6P), phenylalanyl-leucine peptidase (PEP), indophenol oxidase (IPO), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), aconitase (ACO), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), and glutamic oxaloacetic transaminase (GOT). All 10 enzymes were electrophoresed in buffer system A (29).

Electromorphs of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Each isolate was characterized by its combination of alleles at the 10 enzyme loci, and distinctive allele combinations were designated electrophoretic types (ETs).

Susceptibilities to antifungal agents. Isolates from the bone marrow transplant patients were tested for their susceptibilities to amphotericin B and flucytosine by a microtiter broth dilution method (26). The culture media were yeast nitrogen broth with glucose and asparagine (YNB) for flucytosine and Casamino Acids medium for amphotericin B. Twofold serial dilutions of the two antifungal agents in the respective assay broths (50 μl) were added to the microtiter wells. The last well in each row was filled with 50 μl of drug-free medium to serve as the growth control. The antifungal agent concentration ranges were 0.06 to 4 mg/liter for amphotericin B and 0.06 to 64 mg/liter for flucytosine. The microtiter plates were stored at −70°C until use. The yeast inoculum was prepared from an overnight growth on YNB agar. Cells were suspended in sterile 0.9% saline, and the inoculum was standardized by using a spectrophotometer. The wells were inoculated with 50 μl of adjusted suspension (approximately 10^3 CFU). A control strain handled in the same way was included on each microtiter plate. Plates were incubated at 37°C, and the MIC end points were read at 48 h of incubation. The MIC for the control strain varied plus or minus 1 dilution step between assays. The MIC at the lower end of the dilution range was reported as <0.25 mg/liter. Isolates for which the amphotericin B MIC was >1 mg/liter or the flucytosine MIC was >8 mg/liter were defined as resistant.

RESULTS

Overall genetic diversity. In the collection of 98 isolates examined, 4 of the 10 enzyme loci (MDH, G6P, PEP, and LAP) were polymorphic for two or three alleles (Table 1; Fig. 1). The remaining loci were monomorphic, and the mean number of alleles per locus was 1.50. The pattern of allozymes was compatible with that expected for a diploid organism. Heterozygotes at the PEP and LAP loci showed only two bands (monomeric enzyme), while heterozygotes at the MDH and G6P loci showed three bands, suggesting dimeric proteins. At the MDH locus, two-band patterns instead of the three bands that would be expected for a dimeric enzyme in a heterozygote were observed in four culture lysates; three of these cultures were of samples from the same individual. Repeated single-colony isolation from the stock culture led to the finding of a single band for the MDH locus, suggesting that a mixture of two strains had existed in the samples. Strains with two-band patterns at the LAP and PEP loci were submitted to repeated single-colony isolation. The patterns were stable, showing that the stock cultures were pure.

Fourteen distinctive ETs were identified over the 10 loci (Table 1). The mean genetic diversity per locus among ETs (29) was 0.130, and the average number of heterozygous loci per ET was 0.10. Table 2 provides the observed and expected (Hardy-Weinberg) frequencies of the different genotypes at the four polymorphic loci among 42 isolates, which included only 1 isolate of each ET from each patient, and among the 14 ETs. No significant deviation from expected values was seen.

ET diversity in strains from unrelated patients. The four reference strains belonged to ET-4 (ATCC 24433), ET-5 (CBS 562 and CBS 2668), and ET-11 (H29). The 33 clinical isolates obtained from different Norwegian laboratories were

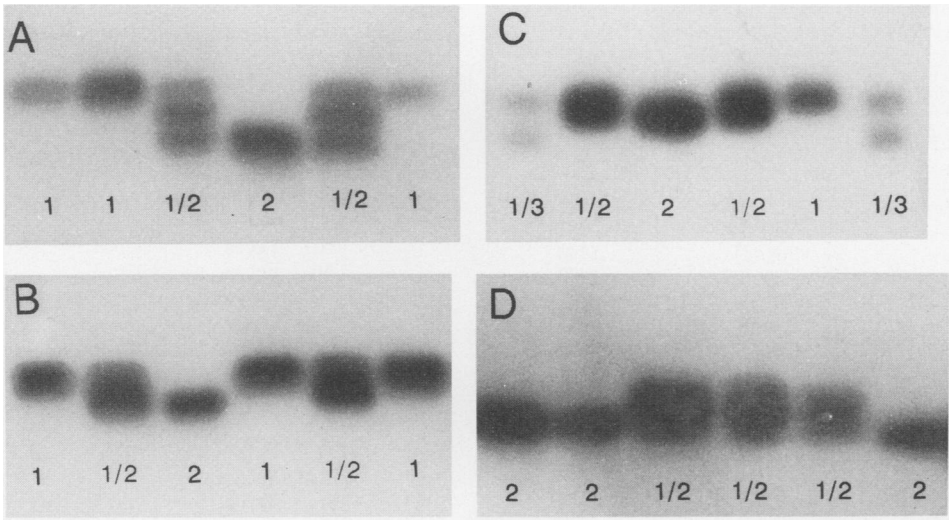

FIG. 1. Homozygotes and heterozygotes at four polymorphic loci in *C. albicans*. Gels were stained for MDH (A), G6P (B), LAP (C), and PEP (D).

divided into 11 different ETs (Table 3), each represented by from one to six isolates. The two isolates from the pus and blood of the same patient were identical (ET-7), as were the two isolates from the pus of another patient (ET-12); the isolates from the latter patient differed in their susceptibilities to fluconazole (MICs, 3.125 and 12.500 mg/liter, respectively).

Isolates with the same ET recovered from different patients originated essentially from different microbiological laboratories and were often collected several years apart. No association between ETs and the clinical site of isolation was observed; isolates from blood were assigned to seven of the ETs.

ET diversity in the strains isolated from bone marrow transplant patients. Eight ETs were identified among the 61 isolates from seven bone marrow transplant patients treated at the Norwegian Radium Hospital over a 13-month period (Table 4). In each of four patients (patients 4 through 7), only isolates with the same ET were identified, but each patient harbored strains of a different ET. The stays of these four patients in the unit overlapped. Patient 6 harbored strains with the same ETs for over 4 months, and indistinguishable isolates were obtained from the patient's throat, axilla, feces, and urine.

Of the remaining patients, two were colonized by strains with two ETs, and one (patient 1) was colonized with strains with three ETs. This last patient harbored strains of ET-4 in the throat, groin, and feces for about 3 weeks, and then strains of ET-3 were isolated from consecutive fecal samples for another 3 weeks. At the time of autopsy, which occurred

TABLE 2. Observed and expected genotype frequencies at four polymorphic enzyme loci in *C. albicans*

Enzyme locus[a]	Genotype[a]	Frequency in isolates[b]		Frequency in ETs	
		Observed	Expected[c]	Observed	Expected
MDH	1	0.595	0.545	0.429	0.369
	2	0.119	0.069	0.214	0.154
	1/2	0.286	0.386	0.357	0.477
G6P	1	0.167	0.154	0.214	0.154
	2	0.381	0.369	0.429	0.369
	1/2	0.452	0.477	0.357	0.477
PEP	1	0	<0.001	0	0.001
	2	0.976	0.976	0.929	0.930
	1/2	0.024	0.024	0.071	0.069
LAP	1	0.762	0.755	0.714	0.675
	2	0.024	0.014	0.071	0.020
	3	0	<0.001	0	0.001
	1/2	0.190	0.207	0.143	0.235
	1/3	0.024	0.021	0.071	0.059
	2/3	0	0.003	0	0.010

[a] See footnote *a* of Table 1 for enzyme abbreviations and allele designations.
[b] Only one isolate of each ET per patient (42 isolates) was used for the calculations.
[c] Expected genotype frequencies are calculated as the square of the allele frequency for homozygote genotypes and twice the product of the allele frequencies for the heterozygotes.

TABLE 3. ET distribution among 33 isolates of *C. albicans* recovered from 1979 to 1990 in 14 laboratories in Norway

ET	No. of isolates	No. of laboratories	Clinical site (no. of isolates)	Year of isolation
1	3	3	Blood (2)	1982, 1983
			Pus (1)	1990
2	5	4	Blood (2)	1981, 1987
			Bile (1)	1985
			Pus (1)	1990
			Urine (1)	1990
4	4	4	Blood (2)	1980, 1990
			Pleural fluid (1)	1985
			Pus (1)	1984
5	6	4	Blood (2)	1985, 1986
			Lung (1)	1987
			Urine (1)	1990
			Pus (1)	1982
			Mouth (1)	1986
7	2[a]	1	Blood (1)	1986
			Pus (1)	1986
9	2	2	Blood (1)	1987
			Pus (1)	1990
10	2	2	Pus (2)	1990
11	1	1	Lung (1)	1986
12	2[a]	1	Pus (2)	1990
13	1	1	Lung (1)	1989
14	5	4	Blood (2)	1979, 1990
			Throat (1)	1989
			Pus (1)	1989
			Pleural fluid (1)	1990

[a] Two isolates from the same patient.

just a few days after the last fecal sample was obtained, a strain of ET-4 was again isolated from the lung, while strains of a different ET, ET-14, were obtained in small quantities from the bone marrow, kidney, and spleen. In patient 2, strains of ET-5 were first demonstrated in the throat and feces, and then strains of ET-4 were found in the throat and groin. In samples taken on day 317, an isolate of ET-4 was identified in the groin and a strain of ET-5 was identified in the feces. Patient 3 first harbored an isolate of ET-8 in the throat, and then in subsequent samples, ET-4 strains were recovered from the throat, feces, and urine. Culture lysates of samples taken from this patient on days 237 and 258 had an exceptional MDH banding pattern, which suggested that strains with two different ETs (ET-4 and ET-8) colonized the same clinical site at the same time.

Strains of ET-4 were identified in all three patients with multiple ETs, but these ET-4 strains were not obtained concomitantly.

Antifungal susceptibility. All 61 isolates from the bone marrow transplant patients were susceptible to amphotericin B (MICs, from <0.25 to 1 mg/liter), and 57 were susceptible to flucytosine (MICs, from <0.25 to 2 mg/liter). The four strains resistant to flucytosine were isolated from patient 1, and all were of ET-3. These strains also appeared to be less

TABLE 4. ET and antibiotic susceptibilities of 61 *C. albicans* isolates from seven bone marrow transplant patients

Patient no.	Sample date[a]	Clinical site of isolation	ET	MIC (mg/liter)	
				Amphotericin B	Flucytosine
1	1	Throat	4	<0.25	0.50
	1	Feces	4	0.25	1.00
	8	Groin	4	<0.25	0.50
	19	Throat	4	0.25	0.50
	22	Feces	4	<0.25	1
	29	Feces	3	1	32
	35	Feces	3	1	32
	44	Feces	3	1	32
	49	Feces	3	0.50	64
	54	Lung[b]	4	<0.25	2
	54	Bone marrow[b]	14	0.50	0.25
	54	Kidney[b]	14	0.25	0.25
	54	Spleen[b]	14	<0.25	<0.25
2	293	Feces	5	<0.25	<0.25
	301	Throat	5	<0.25	<0.25
	307	Groin	4	0.25	0.25
	312	Throat	4	<0.25	0.25
	317	Groin	4	<0.25	<0.25
	317	Feces	5	0.25	0.50
	321	Throat	5	0.25	<0.25
3	184	Throat	8	<0.25	<0.25
	212	Feces	4	0.25	<0.25
	213	Throat	4	0.50	<0.25
	218	Urine	4	0.25	<0.25
	229	Throat	4	0.25	<0.25
	229	Feces	4	0.25	<0.25
	237	Throat	4	<0.25	<0.25
	237	Feces	8	<0.25	<0.25
	258	Throat	8	<0.25	<0.25
	307	Feces	8	<0.25	<0.25
4	373	Throat	5	<0.25	<0.25
	373	Feces	5	<0.25	<0.25
	386	Feces	5	<0.25	0.25
	386	Throat	5	<0.25	0.25
	393	Groin	5	<0.25	<0.25
	400	Feces	5	<0.25	<0.25
	400	Throat	5	<0.25	<0.25
	407	Throat	5	<0.25	<0.25
	408	Feces	5	<0.25	<0.25
5	348	Throat	6	<0.25	<0.25
	372	Throat	6	<0.25	<0.25
	376	Throat	6	<0.25	<0.25
	395	Throat	6	<0.25	<0.25
6	215	Throat	9	<0.25	<0.25
	237	Throat	9	0.25	<0.25
	254	Feces	9	0.25	0.50
	265	Axilla	9	0.50	<0.25
	272	Throat	9	0.50	0.50
	294	Feces	9	0.50	0.50
	300	Axilla	9	0.50	<0.25
	303	Throat	9	0.25	<0.25
	338	Feces	9	0.50	<0.25
	338	Urine	9	0.50	<0.25
	352	Throat	9	0.25	<0.25
7	343	Feces	10	<0.25	0.25
	348	Throat	10	<0.25	<0.25
	359	Throat	10	<0.25	0.25
	372	Throat	10	<0.25	0.50
	372	Groin	10	<0.25	0.50
	383	Throat	10	<0.25	0.50
	383	Groin	10	<0.25	0.50

[a] Sample dates are given as number of days counted from the first sample.
[b] Sample taken at autopsy.

susceptible than the majority of the other strains to amphotericin B (Table 4).

DISCUSSION

Several typing methods that can be used to differentiate among strains of *C. albicans* have been developed for epidemiological purposes. Both the phenotypic and genotypic methods introduced thus far have been hampered by the lack of diversity of the species and have shown only moderate discriminatory power (13). Through the application of multilocus enzyme electrophoresis, 94 isolates from patients in Norway and 4 reference strains were assigned to 14 multilocus enzyme genotypes. The genetic diversity in the population was low, with only 4 of 10 enzyme loci being polymorphic and with there being an average of 0.10 heterozygous locus per ET. Many of the ETs, however, were represented at a relatively high frequency, thus providing a variation sufficient for using multilocus enzyme electrophoresis in epidemiological studies of *C. albicans*.

Serotyping, electrophoretic karyotyping, and DNA restriction fragment length polymorphism studies have shown that isolates recovered from one or several clinical sites of the same patients are usually identical (1, 5, 10, 32). Soll and coworkers (31), however, identified two *C. albicans* strains in monitoring a single bone marrow transplant patient. In the present study we observed that four of seven bone marrow transplant patients from whom samples from different clinical sites were repeatedly obtained over periods of from 1 to 4 months harbored strains of a single ET. Thus, in some patients, ETs may persist over time and strains of the same ET may colonize multiple anatomic sites. Three patients, however, were colonized with more than one strain. In two of these patients, colonization of the same site at a given time by more than one strain was also likely. It would thus be valuable to isolate and analyze multiple colonies from each sample to ascertain its homogeneity. Strains of three different ETs were recovered from patient 1. At the time of hospitalization, the patient was colonized with strains of ET-4. Treatment with amphotericin B was started at day 19. Ten days later, *Candida* strains isolated from the feces of the patient were clearly resistant to flucytosine and MICs of amphotericin B for the strains were greater than those for the previously isolated strains. Development of resistance during antibiotic therapy may result from genetic mutation of the previously colonizing strains or selection of new strains with higher levels of resistance. Multilocus enzyme electrophoresis revealed that the resistance phenotype was associated with strains of a new ET. Simultaneous mutations leading to changes in LAP, resistance to flucytosine, and decreased susceptibility to amphotericin B are unlikely. Thus, our results suggest that it was not the previously colonizing strain that developed antifungal resistance but, rather, the fact that either slightly more resistant strains present in low frequency in the fecal flora before treatment were selected or colonization with a new strain occurred. In contrast, using electrophoretic karyotype and isoenzyme patterns, Merz et al. (20) recently reported that acquisition of amphotericin B resistance by isolates of *C. lusitaniae* resulted from mutation(s) of the strains that formerly colonized the patients.

ET-14 isolates were found from several clinical sites of patient 1; they were found in small amounts and only at the time of autopsy. It may be questioned whether these *Candida* isolates were responsible for the death of the patient or represented a postmortem colonization.

There was no evidence of cross-infection between patients treated in the transplant unit within a 13-month period. Strains of only two ETs were found in more than one patient, and the patients were not concomitantly colonized. Infection from hospital personnel has been suggested in other studies (13), but it seems unlikely that the strains isolated from these bone marrow transplant patients were transmitted by long-term carriers working in the unit; patients 2 and 4 both harbored ET-5 strains upon arrival, and the same was true for ET-4 in patient 1.

An overrepresentation of certain DNA types among urogenital isolates compared with those among strains from other clinical sites has been reported previously (28, 32), as has a difference in serotype distributions among blood culture isolates from patients differing in immune status (5). No difference in the enzyme genotypes of the strains colonizing the immunosuppressed patients and those obtained from other patients was detected. Strains of six of the eight ETs detected in the bone marrow transplant patients were also identified in two or more patients outside the unit.

While most bacterial species (30) and many medically important eukaryotic microorganisms (34) have been found to have a clonal population structure, the clonality of *C. albicans* has been questioned (33) on the basis of the study of Lehmann et al. (18). Those investigators identified 17 patterns of isoenzymes in 37 isolates, but they provided no interpretation of the patterns observed, especially regarding the numbers of genes involved or the structure of hybrid molecules. In the present study, 14 ETs were distinguished among 94 isolates of *C. albicans* recovered from 38 patients from Norway. With the allelic variation observed, all expected genotypes were identified, no deviation from Hardy-Weinberg equilibrium was detected, and there was no disproportion in multilocus combinations. A criterion that has been used extensively to support clonality in bacteria is the recovery of strains identical in multilocus genotypes over extended periods of time and large geographic areas. Some multilocus genotypes of *C. albicans* were identified over many years in Norway, and the American Type Culture Collection reference strain isolated in the United States and two other reference strains originally from Japan were ET-4 and ET-5, respectively, the two most frequently identified ETs in the Norwegian strains. Thus, while the data presented here do not provide strong evidence of a clonal population structure, additional studies are warranted before rejecting clonality in *C. albicans*.

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