A prospective analysis of the genotypic diversity and dynamics of the *Candida albicans* colonizing flora in neutropenic patients with *de novo* acute leukemia

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ABSTRACT

Background
Genotyping studies have shown heterogeneity of *Candida albicans* flora in patients with human immunodeficiency virus infection, with possible co-existence of multiple clones with distinct resistance patterns. We report the result of a prospective study aimed at investigating the dynamics and heterogeneity of *C. albicans* flora in patients with *de novo* acute leukemia.

Design and Methods
Between 2001 and 2003, 66 consecutive adults with newly diagnosed acute leukemia were monitored for *Candida* colonization. From 19 patients with repeated multi-site *C. albicans* colonization, eight were randomly selected and multiple isolates from each individual mucosal site were genotyped sequentially over time using microsatellite markers.

Results
Despite topical use of polyenes, 60.6% of the patients were colonized repeatedly and at multiple sites. Altogether, 2,730 peripheral samples were cultured, 379 (13.9%) of which yielded yeasts. *C. albicans* was the most common species recovered (68%). From eight randomly selected patients colonized with *C. albicans*, 429 isolates were genotyped. Seven patients carried a unique genotype which was identical in all body niches and over the period of study. In one case, minor genotypic differences were observed. None of the patients shared *C. albicans* clones with identical genotypic profiles. Candidemia occurred in one of eight patients and the blood strain genotype did not differ from those of colonizing isolates. The genotypic profile was not altered by topical and/or systemic use of antifungal agents in any of the patients.

Conclusions
In patients with *de novo* acute leukemia, genetic evolution of the colonizing *C. albicans* flora and selection of variants or replacement of the original strain upon antifungal drug pressure or nosocomial transmission are rare events.

Key words: *Candida albicans*, *de novo* leukemia, microsatellite, diversity, antifungal resistance

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Introduction

Bloodstream infections due to *Candida* species are an important cause of morbidity and mortality in hospitalized patients, with an attributable mortality ranging from 20-60%.[1,2] In the United States, *Candida* species are the fourth leading cause of hospital-acquired bloodstream infections.[3] Despite a recent increase in the incidence of candidemia due to non-*albicans* species of *Candida*,[4] *Candida albicans* still accounts for 50-70% of disseminated *Candida* infections.

Yeasts of the *Candida* genus normally reside as commensals of the mucosa and skin. In immunocompromised or intensive-care patients, these organisms may overcome host defenses, resulting in increased mucosal colonization and eventually invasion of the fungus into the bloodstream through epithelial and endothelial layers.[5] The main established risk factors for candidemia include prior mucosal colonization, use of central venous catheters and broad-spectrum antimicrobials, mucosal disruption and neutropenia.[6] Colonization of the digestive tract has been reported as a frequent event preceding bloodstream infection in both non-neutropenic and neutropenic patients.[7,8] Furthermore, invasive candidiasis occurs more frequently in patients colonized at multiple, non-contiguous anatomic sites[9,10] with an overall genetic similarity between strains isolated from the blood and peripheral sites of the same patient.[7,11,12] These observations strongly suggest endogenous acquisition as the main source of invasive candidiasis[8] and colonization of body sites is assumed to be an independent risk factor for candidemia and disseminated candidiasis.[10]

In patients with hematologic malignancies, particularly those affected by acute leukemia, *Candida* species blood stream infections represent a major threat.[13] Based on the concept that colonized mucosa are the main source of bloodstream dissemination, antifungal prophylaxis aimed at reducing the *Candida* mucosal flora has emerged as an attractive strategy in high-risk neutropenic patients.[14] However, a rise in colonization and bloodstream infections due to fluconazole-resistant species has been reported in neutropenic patients receiving fluconazole prophylaxis.[15-17] Furthermore, the emergence of *C. albicans* isolates with reduced susceptibility to fluconazole has been described in the course of long-term, low-dose exposure to fluconazole.[18] Interestingly in this context, molecular-relatedness studies have shown that multiple *C. albicans* sub-populations with distinct antifungal susceptibility patterns can coexist in patients with human immunodeficiency virus infection presenting with oral candidiasis.[19,20] Taken together, these observations suggest that clonal or species selection may emerge as a result of the diversity of the *Candida* colonizing flora exposed to the selective pressure of antifungal therapy.[21]

Studies evaluating *Candida* colonization as a pre-requisite for candidemia have only been conducted at the single isolate level, *i.e.* by analyzing a unique colony among multiple colonies grown from a clinical sample. The real importance of the diversity of *C. albicans* subpopulations in neutropenic patients at high risk of candidemia and likely to receive antifungal drugs remains unknown. We, therefore, performed a longitudinal prospective study of the diversity of the *C. albicans* flora in patients with newly diagnosed acute leukemia undergoing several regimens of myelosuppressive treatment over a 6-month period. Altogether, 429 *C. albicans* clones representative of 59 positive samples collected from eight randomly selected patients with multisite repeated *C. albicans* colonization were genotyped to establish the dynamics of colonization by *C. albicans* subpopulations. The nature of the colonizing flora, the genotypic diversity and the effect of antifungal therapy on the dynamics of the *C. albicans* flora are discussed.

Design and Methods

Patients and conditions of hospitalization

Between October 2001 and October 2003, 130 adult patients with acute leukemia were treated in our center. Sixty-six patients with newly diagnosed leukemia were enrolled in this study, while 64 patients with progressive hematologic disease were excluded. All patients were scheduled to receive one to three regimens of a myelosuppressive treatment over a 6-month period. Patients were hospitalized in single reverse isolation rooms or in laminar airflow-protected rooms. Chest X-rays were taken twice a week. Axillary temperature was measured every 3 h. Microbiological investigations included daily blood cultures for the detection of hematogenous candidiasis, and cultures from the throat, nose, urine and stools twice a week as a screening for *Candida* mucosal colonization. Patients received oral amphotericin B (4×500 mg/day) or nystatin (3×1 million UI/day) for fungal decontamination of the digestive tract. Upon the first febrile episode (axillary temperature >38°C for longer than 3 h), empirical antibiotic therapy including a β-lactam plus an aminoglycoside was initiated after microbiological sampling and chest X-ray. Antibiotic therapy was subsequently modified according to the results of the cultures. When cultures were negative and fever persisted, vancomycin or teicoplanin was added at 48 h. Intravenous amphotericin B (1 mg/kg/day) was administered when either the fever persisted for 48 hrs. following the addition of glycopeptides or a subsequent episode of fever occurred in a patient with ongoing neutropenia. According to the results of cultures or on the occurrence of renal failure, the antifungal therapy was also modified. In both cases, an azole drug or liposomal amphotericin B was prescribed. All patients were given sterile alimentation during the period of aplasia.

Microbiological investigations

Blood samples and specimens from clinically infected foci were collected according to established clinical guidelines.[22] Blood samples were incubated in a BACTEC 9240 system (Becton Dickinson, Le Pont de Claix, France).

Samples from the throat, nose, urine and stools were cultured at 30°C for 48 h on Chromagar medium (Becton Dickinson). Yeast species were identified according to the color of colonies on Chromagar and their carbohydrate assimilation patterns (API 32C system, Bio-Mérieux, Marcy l'Etoile, France). For each sample harboring *C. albicans* on the primary culture, up to ten colonies (clones) were separately sub-cultured on Sabouraud dextrose agar for 24-48 h and stored at –20°C until genotyping. Mucosal colonization was considered *repeated* when samples were positive for *Candida spp*. at one or more anatomical sites on more than one occasion, and *multi-site* if samples were positive for *Candida* spp. in more than one anatomical site over the monitoring period.

Genotyping of C. albicans by the analysis of microsatellite markers

Because of the high number of samples to process in our study, we randomly selected 22 patients with *de novo* acute leukemia. Eight of these presented repeated multi-site colonization with *C. albicans*. *C. albicans* clones were processed for DNA isolation as previously described.[23] Clones were genotyped using four microsatellite markers: one set of three loci referred to as *EF3, CDC3*, *HIS3*, located on chromosomes 5, 1 and 2, respectively,[24] and one additional marker referred to as *CAI*, located on chromosome 4.[25] Amplification reactions were performed using primer sequences and thermal cycling parameters described elsewhere.[24,25] Fragment size analysis of the polymerase chain reaction fragments was performed by automated fluorescent capillary electrophoresis as previously described.[23] To ensure reproducibility of the results, the reference strain ATCC 26278 was run as a control in each series. The results are expressed as the name of the locus tested and the length of the two alleles observed in base pairs.[26]

Susceptibility testing

The minimal inhibitory concentrations of amphotericin B and fluconazole were determined by the Etest method (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions with RPMI 1640 medium supplemented with 2% glucose.

Calculation of the genotypic diversity of the techniques

To calculate diversity, we used Simpson's unbiased index of diversity[27] by which diversity (λ) is defined as:

$$(1-\Sigma p_i^2)n/(n-1)$$

where p_i is the frequency of a particular multilocus genotype and n is the sample size. This diversity has a value that ranges from a minimum of 0, when all isolates have the same genotype, to a maximum of 1, when every isolate has a different genotype. The standard deviation of λ was calculated as:

$$SD=\sqrt{(2[2(n-2)\ \Sigma p_i^3 + \Sigma p_i^2 - (2n-3)(\Sigma p_i^2)^2]/n(n-1))}$$

Finally, this value indicates the discriminatory power of the technique.

Results

Patients' characteristics

During the study period, 130 patients received 214 courses of myelosuppressive treatment. Of these, 66 patients (37 males, 29 females) with *de novo* acute leukemia were included in the survey. The underlying diseases were acute myeloid leukemia in 56 patients and acute lymphoblastic leukemia in ten patients. The median age was 52.3 years (range, 18-81). These 66 patients received 118 courses of chemotherapy inducing prolonged neutropenia (median of two courses per patient; range, 1-4). A mean of 24±8 mucosal samples were obtained per patient and per hospital admission. Irrespective of the species, yeasts of the genus *Candida* were isolated at least once in 81 out of 118 hospital admissions. In 20 hospital admissions, the patients were colonized at the time of hospitalization, and in 61 after having been hospitalized for ≥72 h. The 81 hospitalization admissions during which *Candida* species were isolated were for 53 of the 66 patients. In 12 of these 53, colonization occurred at the time of their first hospital admission, whereas the other 41 were colonized after ≥72 h. Overall, 40 of these 53 patients displayed repeated multi-site colonization over one or more hospital admission, including 19 who displayed repeated multi-site colonization with the same *Candida* species.

Microbiological investigations

A total of 2,730 peripheral samples were collected, 379 (13.9%) of which grew yeasts in culture (Table 1). Despite oral therapy with polyenes, the samples from which positive cultures were most commonly obtained were the stools (38.1%). Among the 53 patients with at least one positive sample, *C. albicans* was isolated from 36 patients (alone = 23, associated with other *Candida spp.* = 13) of these 36 patients, 30 had repeated colonization with *C. albicans* at multiple peripheral sites over one or more hospital admissions (alone = 19, in association with other species = 11). The other species recovered were *C. glabrata* (n=13), *C. kefyr* (n=11), *C. parapsilosis* (n=7), *C. krusei* (n=6), *C. tropicalis* (n=3) and *C. lusitaniae* (n=1). In 16 out of 53 patients, two or more species were recovered from the positive samples. Two patients in our series had can-

Table 1. Distribution and recovery of *Candida* organisms from 5,475 clinical specimens (2,745 blood cultures + 2,730 peripheral samples) obtained from the 66 patients enrolled in this study.

	N. (%) of specimens		
Clinical sample	*Positive for Candida species*	*Negative for Candida species*	*Total*
Nose	1 (0.1)	772 (99.9)	773 (28.3)
Throat	116 (14.9)	661 (85.1)	777 (28.5)
Urine	79 (11.3)	621 (88.7)	700 (25.6)
Stools	183 (38.1)	297 (61.9)	480 (17.6)
Total	379 (13.9)	2,351 (86.1)	2,730 (100)
Blood culture	3 (0.1)	2,742 (99.9)	2,745 (100)

didemia (one with two positive blood cultures for *C. glabrata* and one with one positive blood culture for *C. albicans*) (Table 1). In both cases, *Candida* colonization at multiple peripheral sites with the same species preceded the candidemia. In comparison, among 64 patients with non-*de novo* acute leukemia receiving myelosuppressive treatment during the same period, there were seven cases of candidemia during 96 episodes of neutropenia (compared with two during 118 episodes in the study group, p=0.045, χ^2 test). *C. albicans* was isolated in only one case in these patients; the other species were *C. glabrata* (n=1), *C. kefyr* (n=2) and *C. tropicalis* (n=3).

Genotyping of sequential C. albicans strains

Among 22 randomly selected patients with *de novo* acute leukemia, strains from eight patients, representative of the 19 patients who had multi-site colonization with *C. albicans* alone over one or more hospital admissions, were processed as described above (Figure 1). Altogether, 429 clonal isolates were obtained from 59 positive samples (*i.e.* a mean of 7.3 colonies per positive sample). To assess the discriminatory power of our technique, we had previously applied this molecular typing method to 174 *C. albicans* strains originating from non-related individuals (*data not shown*).[23,28] Using Simpson's unbiased index of diversity, the genotypic diversity of our genotyping tool was 0.998±0.0010.

Seven patients carried a unique multilocus genotype which was identical in all body niches and over the period of study (Table 2). In one case, (patient #4), among 52 clones tested, minor genotypic differences were encountered in two clones obtained from a throat sample during the patients' second hospitalization, suggesting microevolution. None of the patients shared *C. albicans* clones with identical genotypic profiles. Disseminated candidiasis occurred in one patient (patient #2): the genotype of the blood strain did not differ from those of colonizing isolates, confirming endogenous origin as the source of dissemination (Table 2). Six out of eight patients received one or more systemic antifungal regimens. The genotypic profile of the colonizing flora was altered upon systemic antifungal therapy in only one patient (patient #4). However, the change in the genotypic profile found in this patient was not associated with a change in susceptibilities to amphotericin B and fluconazole (Table 3). In all the other patients, the stability of the genotypic profile observed over the different hospital admissions was associated with stable susceptibilities of *C. albicans* to amphotericin B and fluconazole over the period of study. Patients #1 and 8

Table 2. Genotyping of *C. albicans* isolates obtained from eight patients with *de novo* acute leukemia, showing a remarkable stability of allelic combinations over time and at various body sites, for a given individual.

	Allelic combinations				N. of clones with identical allelic combinations / total n. of clones genotyped
Pt. n.	Locus EF3	Locus HIS1	Locus CDC3	Locus CAI	
1	126*/126	166/210	117/125	243/270	121/121
2	131/139	198/198	117/117	225/246	38/38
3	136/146	154/154	117/129	234/234	26/26
4	126/135	171/210	117/125	240/240	50/52
	126/135	171/210	117/117	240/240	2/52
5	126/135	246/246	117/125	246/246	123/123
6	126/135	170/210	117/125	243/243	16/16
7	126/135	162/214	117/129	246/246	23/23
8	126/135	162/202	117/129	246/246	30/30

*Allele size given as number of base pairs.

Table 3. Minimal inhibitory concentrations (MIC) for amphotericin B and fluconazole as tested with the Etest method. For each patient, two clones from the initial positive sample, as well as two clones from the last positive sample harboring *C. albicans* over the hospital admissions, were tested.

Patient	N. of clones tested	Sample harboring C. albicans	Sampling date	MIC Fluconazole		MIC Amphotericin B	
				Geometric mean (µg/mL)	Range (µg/mL)	Geometric mean (µg/mL)	Range (µg/mL)
1	2	Initial sample	02/04/2002	0.125	-	0.11	0.064 - 0.19
	2	Last sample	05/06/2003	0.125	-	0.19	-
2	2	Initial sample	16/06/2003	0.19	-	0.094	-
	2	Last sample	25/06/2003	0.19	-	0.047	-
3	2	Initial sample	12/11/2001	0.19	-	0.25	-
	2	Last sample	17/04/2002	0.19	-	0.5	0.25 - 1
4	2	Initial sample	17/12/2001	0.5	-	0.094	-
	10*	Sample with variable genotypic clones	20/02/2002	0.5	-	0.087	0.047 – 0.094
	2	Last sample	11/04/2002	0.5	-	0.094	-
5	2	Initial sample	11/09/2003	0.19	-	0.11	0.064 – 0.19
	2	Last sample	22/01/2004	0.19	-	0.125	-
6	2	Initial sample	06/02/2003	0.125	-	0.047	-
	2	Last sample	06/02/2003	0.125	-	0.047	-
7	2	Initial sample	30/10/2003	0.094	-	0.125	-
	2	Last sample	12/11/2003	0.094	-	0.125	-
8	2	Initial sample	16/09/2003	0.064	-	0.064	-
	2	Last sample	16/09/2003	0.064	-	0.064	-

*Ten clones including the two clones with genotypic differences were tested.

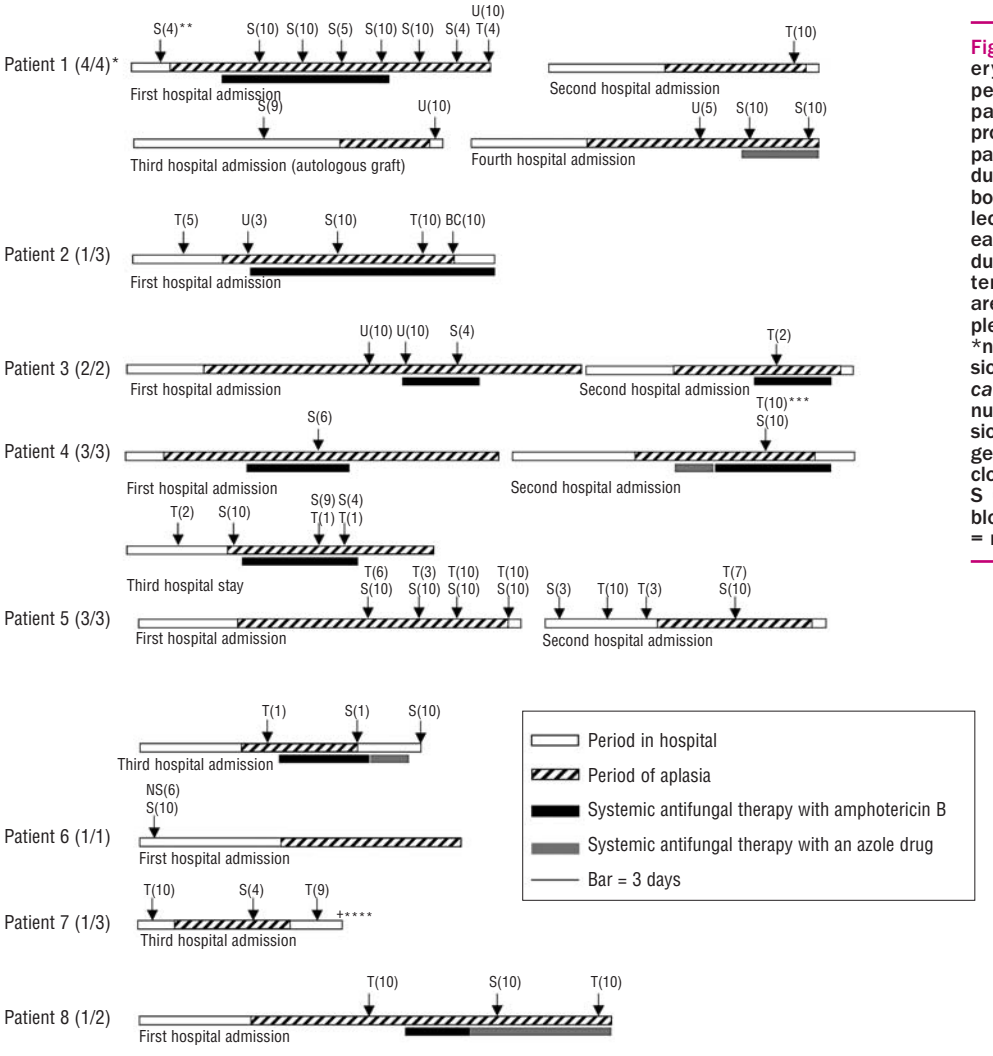

Figure 1. Sequential recovery of *C. albicans* from peripheral sites of eight patients enrolled in the prospective study. For each patient, hospital admissions during which samples harboring *C. albicans* were collected are presented. For each hospital admission, the duration of aplasia and systemic anti-fungal therapy are reported. îPositive sample harboring *C. albicans*; *number of hospital admissions during which *C albicans* was isolated/ total number of hospital admissions; **Number of clones genotyped; ***Variable clones obtained; ****Death; S = stool; T = throat; BC = blood culture; U = urine; NS = nasal swab.

developed oral mucitis (grade 1 and 2, respectively) that was treated with acyclovir without specific microbiological investigations.

Discussion

This is the first study addressing the diversity and dynamics of the *C. albicans* flora in patients with hematologic malignancies who are likely to receive topical and/or systemic antifungal therapy. Our data suggest that the genetic evolution of the colonizing *C. albicans* flora and the selection of variants or replacement of the original strain upon antifungal drug pressure or nosocomial transmission are rare events in neutropenic patients with *de novo* acute leukemia.

Strategies for antifungal prophylaxis in hematology patients receiving intensive cytotoxic chemotherapy are based on the principle of reducing mucosal colonization to limit the risk of systemic invasion. In our study group, the strategy consisted in oral administration of topical polyenes. Systemic antifungal drugs were only added when persistent or subsequent episodes of fever occurred in a patient with ongoing neutropenia. Using this approach, 80.3% of the patients harbored *Candida* species at least once and repeated multi-site colonization occurred in 60.6% of the patients. Previous studies performed in neutropenic patients reported frequencies of *Candida* colonization ranging from 20-73%[29-31] depending on the microbiological monitoring and the antifungal prophylactic schedules. The percentage of colonized patients in our study was, therefore, high despite the use of oral polyenes. The efficacy of topical prophylactic approaches in reducing the fungal load of mucosa in neutropenic patients is a matter of controversy.[32,33] This may in part reflect the fact that in their natural environment, *i.e.* the gastro-intestinal tract, *Candida* cells are commonly organized in biofilms that exhibit resistance against antifungal agents including amphotericin B.[34]

In our series 66 patients with *de novo* acute leukemia, candidemia was diagnosed in only two, an incidence that agrees with recent studies reporting values ranging from 1.2-4.9% in patients with acute leukemia undergoing prophylaxis with polyenes and/or azoles.[13,30,35] The relatively

high rate of colonization with *Candida* in our study was not, therefore, associated with a high incidence of candidemia, supporting the efficacy of our strategy in its ultimate goal of preventing invasive candidiasis. However, in patients with progressive hematologic disease, the incidence of candidemia was significantly higher, with an increase in the incidence of non-*albicans* species of *Candida*, suggesting that our conclusions do not necessarily apply to all groups of neutropenic patients.

As described elsewhere,[30,31] the most common *Candida spp.* identified in our study was *C. albicans* (68%), followed by *C. glabrata* (24.5%) and a few species classically isolated in neutropenic patients, including *C. parapsilosis*, *C. krusei*, *C. tropicalis* and *C. lusitaniae*. The relatively high frequency of *C. kefyr* (20.7%) agrees with a recent study reporting *C. kefyr* as an emerging species in neutropenic leukemia patients.[36] Resistance to amphotericin has been reported among *C. kefyr* isolates.[37] The relative frequency of this species in our series could, therefore, result from the systematic administration of topical amphotericin B and subsequent emergence of strains of *C. kefyr* with reduced susceptibility to this drug.

The persistence of *C. albicans* as a frequent colonizing organism despite topical antifungal prophylaxis raises the question of whether long-term therapy with topical and/or systemic antifungal agents could lead to the selection or mutagenic generation of drug-resistant fungal populations. In neutropenic leukemia patients, two previous studies examined the dynamics of *Candida* colonization on a long-term multi-site basis.[12,38] Both reported persistence of a genotypically constant strain in most of the patients by genotyping a unique colony recovered from a positive sample, assuming that a single colony was representative of the whole colonizing population in one anatomical site at a given time. However, the diversity of *C. albicans* subpopulations was not systematically examined. To address this question, we used a molecular typing method based on four microsatellite markers separately shown to be suitable for epidemiological studies.[24,25] These markers are located on different chromosomes, are stable over many generations, and are highly discriminative. Bretagne *et al.* observed a discriminatory power of 0.97 using a combination of the *EF3*, *HIS3* and *CDC3* microsatellites while Sampaio *et al*. obtained a similar value using *CAI* alone. When combining the four microsatellites for typing a series of 174 unrelated strains of *C. albicans*,[23,28] we obtained a discriminatory power of 0.998, making this method even more suitable for epidemiological studies.

We used this method to genotype 429 clonal isolates sequentially obtained from eight patients newly diagnosed with acute leukemia. For all positive samples, up to ten colonies were genotyped separately to establish a genotypic map of the colonizing flora. In all but one patient (patient #4), all clones were genotypically identical over the period of study, irrespective of the anatomical site and the treatment protocols. In patient #4, two clones originating from the same sample presented minor genotype differences suggestive of microevolution. In other studies, *C. albicans* colonization in individual patients has been reported as clonal in origin with small genetic rearrangements that could have arisen from microevolution.[11,39] Our data strongly suggest that such a genetic evolution is rare among neutropenic *de novo* acute leukemia patients undergoing several regimens of myelosuppressive treatment over a period of up to 6 months.

The genetic microevolution observed in two clones in patient n. 4 was not associated with a modification in susceptibilities to amphotericin B and fluconazole. Likewise, when randomly selecting two clones from the first and the last positive sample for each of the eight patients, no variation in susceptibility to amphotericin B and fluconazole was observed. Altogether, there was no evidence in favor of the selection of resistant clones or the replacement of the original strain upon antifungal drug pressure in our study, suggesting that these events are rare in neutropenic leukemia patients receiving topical amphotericin B along with clear indications for systemic antifungals over a period of up to 6 months. This is in contrast with studies conducted in patients with human immunodeficiency virus infection,[20,40] which found that different subpopulations of colonizing or infecting strains could co-exist at a given time in the same patient and exhibit different patterns of susceptibility to antifungal drugs. We believe that such a discrepancy could reflect the different populations of patients studied (*i.e.* neutropenic patients with *de novo* acute leukemia *vs.* patients with immunodeficiency virus infection) that generally receive distinct antifungal protocols.

In conclusion, in this population of patients, the approach of clinical mycology laboratories, which consists in characterizing a unique colony of the *C. albicans* mucosal flora, provides a valid basis for evaluation when systemic antifungal therapy is required. Finally, current data support the efficacy of the prophylactic strategy we use in limiting the emergence of resistant *C. albicans* strains in neutropenic patients with *de novo* acute leukemia.

Authorship and Contributions

IL and EF, participated under the responsibility of DC, in the clinical diagnosis, and collection and analysis of the clinical data; CL'O, PS and AL collected the samples and performed the cultures, the molecular typing studies and MIC measurements; AJ and CL calculated the discriminatory power of the typing method and participated in the design of the study; OV was responsible for the mycology diagnosis; FD, DC and AB were responsible for the conception and design of the study, analysis and interpretation of the overall data and clinico-biological summary, and drafting the first version of the article. All the co-authors listed were involved in the revision and correction of the manuscript and approved the final version of the article.The authors reported no potential conflicts of interest.

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