Significance of Amplified Fragment Length Polymorphism in Identification and Epidemiological Examination of Candida Species Colonization in Children Undergoing Allogeneic Stem Cell Transplantation

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Candida albicans and non-C. albicans Candida species are increasingly being isolated from patients in high-risk categories, most notably, those who have undergone stem cell transplantation (SCT). Identification of the presence of non-C. albicans Candida species early in the course of the transplant procedure is important, as these species exhibit different sensitivities to the available antifungal treatments and cause mortality at rates that vary from those for C. albicans. Amplified fragment length polymorphism (AFLP) analysis has been shown to be a reliable method of reproducibly identifying medically important Candida species. We investigated the use of serial AFLP analysis of 54 routine surveillance cultures for the identification and epidemiological examination of Candida sp. colonization in five consecutive children undergoing allogeneic SCT. One child became colonized with a C. albicans strain and remained colonized with this strain during the whole admission period. Another child had persistent colonization with a C. albicans strain with striking variations in its AFLP patterns over time, which was considered indicative of microevolution. Candida dubliniensis, Candida lusitaniae, and Saccharomyces cerevisiae were identified in the three remaining patients, with two children being simultaneously and transiently colonized with different species. These findings show that colonization with yeasts during transplantation is a complex and dynamic interaction between the host and the organism(s). In our study three strains from eight separate time points were incorrectly identified as C. albicans by a rapid enzyme test. AFLP analysis of surveillance cultures allowed more accurate and informative epidemiological evaluations of pathogenic yeasts in children undergoing transplantation.

In patients undergoing allogeneic stem cell transplantation (SCT), fungal infections are an increasing cause of morbidity and are associated with a high mortality rate (16). Although Candida albicans is the most important cause of superficial candidosis and colonization of the gastrointestinal (GI) tract, at least 12 other members of the genus are recognized as human pathogens (13, 23). Infection tends to be derived from an individual’s own endogenous flora, with the GI tract acting as a reservoir of infection (8). Patients with impaired humoral and cell-mediated immunity, especially those undergoing SCT, are at risk of dissemination (26). Among pediatric recipients of bone marrow transplants, dissemination is rarely documented in those receiving prophylactic oral antifungal agents, even though colonization is common (9). The introduction of prophylactic oral fluconazole has led to clear reductions in the rates of both colonization and invasive infections with C. albicans (20, 26). However, in the last decade between 40 and 70% of cases of candidemia in adult patients with hematological malignancies and bone marrow transplant recipients have been reportedly caused by non-C. albicans Candida species (NACs) (13, 38). Similarly, in some pediatric oncology units, NACs are now the most common cause of candidemia, even in azole-naïve patients (19).

The levels of virulence and the consequent rates of mortality, as well as the sensitivities to the available antifungal agents, vary among the different species. The four most commonly isolated species are Candida parapsilosis (20 to 40% of all reported episodes of candidemia), Candida tropicalis (10 to 30%), Candida krusei (10 to 35%), and Candida glabrata (5 to 40%). However, other NACs have emerged, such as Candida lusitaniae, which has been documented in up to 8% of all cases (13). The occurrence of these species is associated with inherent or acquired resistance to fluconazole in up to 75% of all C. krusei infections (38) and 35% of all C. glabrata infections. Conversely, at present C. lusitaniae remains highly susceptible to azole antifungal agents (13, 24). Candida dubliniensis has emerged as an opportunistic pathogen closely related to C. albicans and shares many diagnostic characteristics with C. albicans but differs from C. albicans with respect to its epidemiology, virulence, and the ability to develop fluconazole resistance (7, 33). The inherent variability in the response to treatment and the associated mortality rates, which can exceed those of C. albicans infections, demand correct and timely identification. This calls for appropriate or species-directed treatment strategies for at-risk patients.

Molecular techniques, based on the demonstration of variations in the conserved DNA sequences in yeast genomes, have led to identification methods based entirely on the detection of naturally occurring DNA polymorphisms (6, 17, 27, 32, 35). These techniques have been applied in a limited manner to studies of Candida colonization in immunocompromised chil-

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taken with a calibrated loop to inoculate Sabouraud agar and malt extract agar. Buffered saline and further diluted to a 1:10 suspension, from which 104 colonies (containing penicillin at 20 U/ml and streptomycin at 40 mg/liter) and 2% malt extract broth were streaked onto Sabouraud dextrose agar medium. 

In the present study, fluorescent AFLP analysis was used to study the variations in medically important Candida species isolated from serial routine surveillance cultures of samples from children undergoing SCT and to assess the potential for a role of AFLP analysis in determining therapeutic interventions in these high-risk patients.

MATERIALS AND METHODS

Patients. Five consecutive, nonselected children entered into the Leiden University Medical Center transplant program (all boys; age range, 3 to 15 years) were included in the study. The patient characteristics are summarized in Table 1. Children received standard myeloablative conditioning regimens. All but one child (patient 305) received in vitro T-cell modulation with antithymocyte globulin (ATG) and antilymphocyte globulin 2% sterile food and beverages. All patients received standard myeloablative conditioning regimens. All but one child (patient 305) received in vitro T-cell modulation with antithymocyte globulin (ATG) and antilymphocyte globulin 2% saline instead of radionucleotide labeling of primers means that a computer-based automated sequence analyzer can read the polyacrylamide gel electrophoretic patterns (29).

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Disease</th>
<th>Donor type</th>
<th>Conditioning</th>
</tr>
</thead>
<tbody>
<tr>
<td>499</td>
<td>14 yr 10 mo</td>
<td>ALL, 2CR</td>
<td>MUD T+</td>
<td>VP16, Cyclo, TBI, ATG</td>
</tr>
<tr>
<td>501</td>
<td>7 yr 8 mo</td>
<td>X-LPD</td>
<td>MUD T+</td>
<td>Bu, Cyclo, ATG</td>
</tr>
<tr>
<td>502</td>
<td>12 yr 5 mo</td>
<td>ALL, 3CR</td>
<td>MUD T+</td>
<td>Bu, Cyclo, ATG</td>
</tr>
<tr>
<td>503</td>
<td>3 yr 0 mo</td>
<td>X-LPD</td>
<td>MUD T+</td>
<td>Bu, Cyclo, ATG</td>
</tr>
<tr>
<td>505</td>
<td>8 yr 11 mo</td>
<td>ALL, 2CR</td>
<td>ID twin</td>
<td>VP16, Cyclo, TBI</td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphoblastic leukemia; X-LPD, X-linked lymphoproliferative disease; CR, complete remission; MUD, matched unrelated donor; T+, non-T-cell-depleted stem cells; ID, identical; Bu, busulfan (Bu-sulfox); Cyclo, cyclophosphamide; TBI, total-body irradiation; ATG, antilymphocyte globulin 2%; CR, complete second remission; 3CR, third complete remission; and VP16, etoposide.

RESULTS

After completion of the study, we found the isolates obtained from the preputial, vulval, and nose swab samples to be of limited additional benefit. Few isolates yielded Candida, and when Candida isolates were found, the results for the rectal (fecal) swab samples were identical to those for the preputial and vulval swab samples. No nasal Candida isolates were obtained. As such, a total of 54 clinical isolates of Candida obtained from the throat, rectal (fecal), and perineal sampling
sites form the basis of this study. A dendrogram of all clinical isolates together with the reference strains is depicted in Fig. 1. The AFLP patterns show that each specific species forms a distinct pattern. Known cophenetic values, as described previously (1), allowed genotypic species identification over the time course of the individual patient's transplant.

Although systemic dissemination of yeasts did not occur during the administration of total gut decontamination medi-
cation, all patients had positive surveillance cultures for Candida at various times during the transplant period. All strains isolated were phenotypically identified as C. albicans by the Murex assay. AFLP analysis recognized C. albicans, C. dubliniensis, and C. lusitaniae as well as Saccharomyces cerevisiae by comparison with known reference strains (Fig. 1). Sequence analysis of the D1/D2 domain of the 26S ribosomal DNAs of a selection of the yeast strains confirmed the data obtained by AFLP analysis. In this study, because of the positive Murex reaction, C. lusitaniae and C. dubliniensis isolates were initially not investigated with the API 32C system. However, when the results of AFLP analysis became available, strains of both species were investigated with and correctly identified by the API 32C system.

On admission, two patients (patients 501 and 505) were colonized with C. albicans and one patient (patient 503) was colonized with C. dubliniensis. Throat, fecal, and perineal swab samples from all three patients were positive, and colonization with these strains was maintained in all three patients throughout the study period. The two remaining patients (patients 499 and 502) became colonized with C. albicans shortly after admission, but the colonization was unrelated to the start of gut decontamination or systemic antibiotic administration. In addition, one of these children (patient 502) developed perineal colonization with C. lusitaniae. The transient presence of various additional yeasts species was documented simultaneously with the presence of C. albicans in two of the five children: in patient 502, C. lusitaniae was found at two locations in two episodes, and in patient 501, S. cerevisiae was found at one location in one episode. These episodes were of short duration, however, i.e., 2 and 3 weeks, respectively.

The Candida isolates were genotypically identifiable by AFLP analysis. They were distinct and specific to the individual patient, with the isolates from no two patients having exactly the same DNA fingerprint. For three patients, variations within the DNA fingerprint of the same Candida isolate were observed over the study period. The sequences of these isolates remained from 90 to 95% similar to those of the isolates in the original specimen. This was considered to reflect minor technical variations rather than true clonal evolution. Conversely, the C. albicans isolate from one patient (patient 505) showed a more striking variation over the study period. This was considered indicative of microevolution, which for the purpose of this study was defined as similarity of $<90\%$.

These observations showed no evident relationship to a particular diagnosis or previous treatment. All children received systemic broad-spectrum antibiotics, including vancomycin, which was unrelated to the isolates temporally or to the species identified.

Of the three children who received oral fluconazole (at 2 to 3 weeks postadmission), one (patient 501) developed transient colonization with S. cerevisiae and another (patient 502) was colonized with C. lusitaniae. The third patient (patient 503) had persistent C. albicans colonization, but no additional organisms were identified. All Candida isolates except those from patient 501 remained sensitive to fluconazole (MICs, 0.19 to 0.25 mg/liter); 5 days after the start of therapy patient 501 excreted fluconazole-resistant C. albicans (MIC, 64 mg/liter) in the feces.

These results are summarized in Fig. 2. As isolates from the preputium or vulva did not differ from those from the rectum (feces) and no Candida strains were isolated from any nasal samples, results for samples from these sites are not included.
DISCUSSION

In our study, conventional surveillance cultures demonstrate that colonization with *Candida* spp. is extremely common, despite the administration of oral nonabsorbable antifungal agents. All children were colonized with *Candida* during the course of transplantation. No child had documented systemic yeast infections, and as such, the effect of the total gut decontamination was clinically acceptable. Molecular techniques can also be useful for the identification of yeasts in the clinical setting, and as expected, AFLP analysis proved to be more discriminative than conventional phenotypic identification of *Candida* species in all of our patients. As considerable overlaps in admission dates and treatment durations occurred among the study patients, phenotypic identification alone would have raised the concern of nosocomial spread. However, molecular identification by AFLP analysis confirmed that no cross-infection occurred among the members of the study group. Colonization with more than one species of *Candida* at a specific site can exist in as many as 40% of patients with hematological malignancies (21, 22). However, the literature on this topic dates from the late 1980s, when molecular typing techniques were not available on a large scale. The results for our small study group concur with the published data that colonization with multiple species is not infrequent. Colonization in individual patients has been described as clonal in origin for *C. albicans*, but small genetic rearrangements can arise by microevolution (14). However, we were not able to demonstrate a frequent occurrence of microevolution in this study. This may reflect the relatively short duration of the study, in which, although microevolution of yeasts might have taken place, microevolution was not a major feature. What was not apparent in our study was any relationship of colonization to any form of treatment administered. Host immunity is an important determinant of infection in transplant patients and thus may have played a more crucial role in species evolution and viability than any of the therapeutic interventions.

Thus, AFLP analysis offers important new epidemiological insights into the process of colonization of the GI tract with *Candida* in immunocompromised children. We have shown, even in these few patients, that this is a highly dynamic process. Our patients, even those children who were azole naïve, transiently demonstrated colonization with multiple *Candida* species, reflecting the complexity of host factors in children undergoing SCT. These children are often predisposed to colonization before their transplant episode, as seen for the children with positive cultures on admission to the Leiden University Medical Center prior to any transplant-related intervention. The underlying illness or the treatments received, inclusive of steroids and/or chemotherapy, may influence the risk of colonization but, as was demonstrated in our patients, not the development of colonization with additional and multiple yeasts, often simultaneously.

Our study confirms that the emergence of NAC species may go undetected if one relies on phenotypic identification alone. Although the germ-tube test is a simple and economic method for the rapid identification of *C. albicans*, false-positive reactions can occur with *C. tropicalis* and *C. parapsilosis* (2). Alternative tests based on enzymatic methods, such as the Murex test, have been developed (3). The sensitivities and specificities of these commercial tests for the identification of *C. albicans* and NAC yeasts were greater than 97% (9). As the germ-tube test is time-consuming and laborious and due to the misidentification of *C. tropicalis* and *C. parapsilosis*, we have used the Murex assay for the routine laboratory identification of *C. albicans* since 1999. We use this assay for the presumptive identification of *C. albicans* for yeasts isolated from patients with invasive infections. In these circumstances, a definite identification is performed with the API 32C system. However, when yeasts are isolated from surveillance cultures of samples from hematology patients, the Murex assay is not followed by a test with the API 32C system, unless the physician is considering treatment intervention.

The Murex enzyme test misidentified *C. lusitaniae* and *C. dubliniensis*. Although *C. albicans* has been reported to be the only species that expresses both N-acetyl-β-D-galactosaminidase and l-proline aminopeptidase, *C. dubliniensis* was not included in the published evaluation of the test (4, 10). Only six isolates of *C. lusitaniae* were tested, and they were reported to be negative. We therefore advise that *C. albicans*-like strains isolated from blood or other sterile compartment be investigated further by additional phenotypic assays (e.g., assays for β-glucosidase) (30) or DNA-based methods.

*C. parapsilosis*, *C. tropicalis*, and *C. glabrata* are among the most common NACs found in SCT recipients but, perhaps unexpectedly, were not present in our study population. What is striking is the occurrence of the less common variants that colonized almost all of our patients at various times during the transplant episode.

*C. dubliniensis* has emerged as an opportunistic pathogen commonly associated with recurrent oral candidiasis in human immunodeficiency virus-positive patients (11, 31) and has only recently been reported to occur in association with disseminated infections in patients with hematological malignancies and those undergoing bone marrow transplantation (15). A retrospective analysis of all *Candida* isolates in immunocompromised children concluded that 0.5% of all *Candida* fungemias are attributable to *C. dubliniensis* (3).

*C. lusitaniae* is an infrequent cause of fungemia, causing approximately 2 to 8% of cases of fungemia in adults with cancer. Its emergence is associated with the use of oral polyenes in susceptible patients (13), which existed in all our patients. These NACs show variable sensitivities and may be resistant to amphotericin B but at present remain susceptible to fluconazole (18, 24, 28). However, in a recent study of 67 *C. lusitaniae* isolates from blood cultures, only 1 strain was found to have a high level of resistance to polyenes (MIC, ≥8 mg/liter). All 67 strains tested were susceptible to fluconazole and voriconazole (24). *C. lusitaniae* is a species that is frequently reported in the literature to be capable of developing resistance to amphotericin B during the course of treatment. Consequently, persistent colonization with *C. albicans*-like strains during selective gut decontamination with oral amphotericin B should alert one to the possibility of *C. lusitaniae*.

Candidemia caused by *C. dubliniensis* and *C. lusitaniae* has been reported previously. The children colonized with the NACs were highly vulnerable to dissemination during the transplant procedure. It is known that prolonged neutropenia following delayed engraftment or treatment with steroids for GvHD increases the risk of dissemination (26). Our patients all
engraft successfully, with neutrophil regeneration at a median of 21 days post-SCT and with no clinical evidence of GvHD. This may have contributed to the control of infection and the lack of fungemia.

*S. cerevisiae* is an ascomycetous yeast that is widely distributed in nature and may colonize the GI tracts of healthy humans. Although the pathogenic nature of this organism had been questioned, it is now evident that virulent strains can be isolated from clinical specimens in association with dissemination and clinical disease in immunocompromised patients, including those undergoing SCT (40).

The relevance of this emerging pathogen in pediatric patients undergoing allogeneic SCT is unknown and until now has not been documented. The complexity of the immune manipulation required for successful engraftment is known to increase the risk of fungal infection (25), and further study of the relevance of these pathogens in pediatric SCT patients is required.

Our study highlights the pitfalls of the sole reliance on phenotypic methods, such as the Murex assay, for the identification of *Candida* species. Molecular techniques have the benefit of being able to determine the true extent of NAC yeast colonization and infection in high-risk patient populations. This was especially useful in the light of our findings of simultaneous colonization with multiple *Candida* species. Although many of the NACs are intrinsically more resistant to fluconazole treatment, our study had too few patients for it to be possible to study conclusively the effects of the prophylactic or preemptive use of oral azole therapy. The two children who received oral fluconazole and who developed NAC yeast colonization did so prior to the use of fluconazole. These strains were not resistant to fluconazole and were successfully eradicated after azole treatment. Resistance did, however, develop in one patient colonized with *C. albicans*; and although, interestingly, the resistance was associated with the presence of a DNA variation, as determined by AFLP analysis, it is unlikely that it was the cause of resistance. Resistance to azoles is known to require a much more complex genetic shift than is represented by our findings.

The evolving importance of NAC infections in high-risk patients and the need to develop species-directed treatment modalities require the incorporation of a reliable and universally acceptable method of identification. With the limited data set available, the sources or origins of these NAC yeasts could not be determined. However, our data suggest that the use of AFLP analysis within this setting could provide valuable insights into the identities of fungal pathogens. The AFLP technique offers a very high degree of flexibility, and the AFLP procedure is very easy to perform. By AFLP analysis, a fingerprinting result can theoretically be obtained within 8 h. Another advantage of AFLP analysis is the potential to automate virtually the entire procedure. The disadvantages associated with AFLP analysis are that it depends on expensive computer software and that the patterns obtained are not easy to exchange between different laboratories because of the uniformity of the equipment that is required. Standardization of this technique will certainly be required in order to allow interlaboratory comparisons of AFLP data. At present, the expense and expertise required would probably limit the routine application of AFLP analysis in the clinical setting. AFLP analysis of medically important *Candida* species is, however, a robust epidemiological research tool. It proved to be capable of identifying various *Candida* species present concurrently in children at high risk of infection and allowed an increased understanding of the dynamics of fungal colonization.

REFERENCES


