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Incidence of Polyene-Resistant Yeasts Recovered from Clinical Specimens

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The development of resistance to amphotericin B and nystatin in yeast isolates was determined. Organisms recovered from patients on the oncology service, undergoing extensive chemotherapy for acute leukemia and bone marrow transplantation, were compared with yeasts recovered from patients on other services in the same hospital over a 7-month period. An agar dilution method was used to assay the susceptibility for each antibiotic; resistance was defined as a minimal inhibitory concentration of ≥2 μg/ml for amphotericin B and ≥16 μg/ml for nystatin. None of 625 isolates from 238 patients on non-oncology services demonstrated polyene resistance. Resistance only occurred in a subpopulation of oncology patients, in which 55 isolates (7.4%) from six patients (8.6%) exhibited polyene resistance. Resistant yeasts included *Candida albicans* (three strains), *Candida tropicalis* (one strain), and *Torulopsis glabrata* (two strains). All of the patients from whom resistant yeasts were recovered had experienced extensive chemotherapy with cytotoxic agents, granulocytopenia, and long-term treatment with both antibacterial and polyene antibiotics. Resistance to 2 μg of amphotericin B per ml and to 16 μg of nystatin per ml was associated with loss or marked depression of ergosterol in the cell membrane as measured by ultraviolet spectra. A significant incidence of polyene resistance in an oncology subpopulation was documented, suggesting a need for susceptibility testing in patients who are at high risk for development of drug-resistant fungal pathogens.

Although the polyene antibiotics amphotericin B and nystatin have been widely used therapeutically for 18 years, isolations of polyene-resistant strains of yeasts from clinical material have been rare (11). Resistant yeasts that have been recovered include *Candida tropicalis* (6, 17, 20, 24), *Candida krusei, Candida parakrusei* (*parapsilosis*) (20), *Candida albicans* (4), and *Candida lusitaniae* (17).

Although clinical isolates of polyene-resistant yeast have been rare, resistance has been induced experimentally through serial transfer of an isolate on media containing increasing concentrations of a polyene, a procedure which is referred to as "training" (12, 16, 21). Alternatively, mutant strains have been produced which exhibit a single gene mutation involving a discrete lesion in the sterol biosynthetic pathway (2, 3, 8, 23). In these laboratory studies, *Candida* spp. have varied widely in the ease with which polyene resistance could be induced (2, 5, 16) and in the degree of resistance manifested in the same laboratory systems (12). *C. albicans*, the most common isolate seen clinically, has proven to be most refractory to inducible resistance to polyenes. For this reason, it has been suggested that clinically significant resistance development is rare and of limited major therapeutic import (11). In keeping with this viewpoint, polyene susceptibility testing aimed at determining the likelihood of development of resistant strains in specific subpopulations has not been widely monitored.

The isolation of a polyene-resistant variant strain of *C. tropicalis* at this institution from a patient who had undergone homologous bone marrow transplantation (17), in conjunction with other recent reports of polyene-resistant clinical isolates (6, 18, 20), led us to study the incidence of yeasts resistant to the polyene antibiotics nystatin and amphotericin B in various patient populations, as described in this report.

MATERIALS AND METHODS

Previous laboratory and clinical studies have used a range of methods for evaluating resistance to polyene antibiotics and have differed in their criteria for resistance, as expressed in minimal drug concentrations required to inhibit the organism. To date, the majority of polyene-resistant fungi, both laboratory induced and clinically isolated, have been associated with the absence or decrease of 5,7-ene sterols, specifically ergosterol, in the membranes of resistant isolates (19). For this reason, and to correlate the type of isolate recovered in this study with those reported elsewhere, we assayed the sterol composition of selected isolates considered resistant in this surveillance study.

Organisms. All yeasts referred to in this study were isolated from clinical specimens submitted to the Mycology Laboratory, Microbiology Division, The Johns Hopkins Hospital, during a 7-month period. Specimens were processed without knowledge of hospital location or clinical source. Subsequently, isolates recovered from patients on the leukemia and bone marrow transplant services, all undergoing extensive therapy, were defined as the study group which was compared with all other patients (control group). All yeasts were identified by standard macroscopic and microscopic morphology, fermentation, and assimilation tests (7).

Susceptibility test. An agar dilution replicate plate method was employed for antifungal susceptibility testing. Sabouraud dextrose agar containing the concentrations of polyene antibiotic to be tested was dispensed in 35-ml volumes into 10-by-10-cm gridded petri dishes. Plates were used within 24 h of preparation. Inocula from 48- to 72-h cultures on Sabouraud dextrose agar were suspended in 3 ml of sterile distilled water and further diluted to match the turbidity of a standard containing 1×10^6 to 2×10^6 heat-killed *C. albicans* cells per ml. Using a Steers replicating device, 0.01 ml of the suspension (approximately 1×10^4 to 2×10^4 cells) was spotted onto the surface of each antibiotic-containing medium. Amphotericin B and nystatin (dissolved in sterile distilled water and 5% dimethyl sulfoxide) were tested at 0-, 0.5-, 1-, and 2-μg/ml concentrations and 0-, 4-, 8-, and 16-μg/ml concentrations, respectively. Plates were examined after 24 h of incubation at 35°C. The minimal inhibitory concentration was defined as the lowest concentration of antibiotic that inhibited growth. Control isolates of *Candida stellatoidea* (Squibb SZ2211) and *C. tropicalis* (J.H.H. Z613) with known minimal inhibitory concentrations were included on each plate in each run. A resistant yeast was defined as an organism requiring a minimal inhibitory concentration of 2 μg of amphotericin B per ml and 16 μg of nystatin per ml or greater.

Sterol analysis. For sterol analysis, isolates were grown in 100 ml of Sabouraud dextrose broth in 500-ml flasks with stirring at 37°C for 48 h. The cells were collected by centrifugation at 3,000 rpm for 10 min, washed once in sterile physiological saline, and recentrifuged. A 50-mg sample of acetone-dried cells was saponified at 80°C for 30 min with 3 ml of 40% alcoholic KOH in tubes (16 by 100 mm) with Teflon-lined caps. The tubes were brought to room temperature, 1 ml of distilled water and 5 ml of *n*-hexane were added to each, and then the tubes were inverted 20 times. The hexane layers were removed for measurements of absorption spectra. These spectra were measured in a Beckman Spectrophotometer model 26 from 200 to 350 nm along with the spectrum of control samples of ergosterol (Sigma).

RESULTS

A total of 1,372 yeast isolates, including *C. albicans, C. tropicalis, Torulopsis glabrata, Candida parapsilosis, Saccharomyces* sp., *C. krusei, Trichosporon* sp., *Candida guilliermondii,* and *Candida pseudotropicalis,* were recovered from 308 patients during the 7-month study period. The 625 isolates recovered from the 238 control patients were compared with the 747 isolates recovered from the 70 study patients. The distribution and numbers of individual species recovered were remarkably similar in the two patient populations (Table 1). Resistance occurred only in yeasts recovered from study patients (Table 2). Of the 747 isolates from this group, 55 (7.4%) were resistant to amphotericin B at or above 2 μg/ml and to nystatin at or above 16 μg/ml. All 55 were recovered from 6 of the 70 patients, 8.6% of this total.

Resistant yeasts included the most commonly isolated species, *C. albicans* (three strains), *C. tropicalis* (one strain), and *T. glabrata* (two strains). The resistant isolates were recovered from surveillance cultures of the respiratory tract (20 isolates), gastrointestinal tract (16 isolates), and urinary tract (12 isolates) (Table 3). In one case, a resistant *C. tropicalis* was recovered from the lung tissue of a patient at autopsy. In patients from whom serial specimens could be obtained, resistant yeasts were isolated

TABLE 1. *Yeast isolates recovered during the survey period*

Organism	Control patients		Oncology patients	
	No. of isolates	No. of patients	No. of isolates	No. of patients
C. albicans	337	132	516	51
C. tropicalis	197	68	100	18
T. glabrata	50	29	92	7
C. parapsilosis	27	16	6	5
Saccharomyces sp.	2	1	16	7
C. krusei	9	4	6	3
Trichosporon sp.	2	2	4	3
C. guilliermondii	1	1	4	4
C. pseudotropicalis	0	0	3	3
Total	625	253[a]	747	101[a]

[a] More than one species of yeast was recovered from some patients.

for periods of up to 32 days. All patients who yielded polyene-resistant yeasts had previously yielded polyene-susceptible isolates of the same species on multiple occasions. Three of the patients continued to be colonized through two successive hospitalizations. The time interval between the initial isolation of the susceptible isolates and the initial resistant isolates ranged from 10 to 129 days (a median of 20 days and a mean of 43 days).

The results of the sterol analyses showed that there was a marked depression of the ergosterol content of the polyene-resistant isolates. Scanning ultraviolet spectra from 200 to 300 nm of ergosterol alone confirmed absorption peaks at 271, 281, and 293 nm, with a shoulder occurring at 261 nm. An example of the change in spectrum of susceptible and resistant *C. albicans* is presented in Fig. 1. Comparison of the optical density at 281 nm of resistant species and control polyene-susceptible yeasts for each species indicated a 74 to 85% decrease in ergosterol content in all three species (Table 4). The resistance of strains to 2 μg of amphotericin B per ml and to 16 μg of nystatin per ml correlated with changes in membrane sterol monitored by spectrum analysis. Resistance has remained stable for a year in those isolates that have been retested.

All six resistant strains were isolated from patients receiving extensive chemotherapy for acute myelogenous leukemia (three patients), chronic myelogenous leukemia in blast crisis (one), chronic lymphocytic leukemia (one), and aplastic anemia (bone marrow transplant) (one). The six patients had received a total of 10 courses of chemotherapy. Two with acute myelogenous leukemia had received three separate courses each. All had experienced long periods of hospitalization with granulocytopenia and systemic antibacterial antibiotic and amphotericin B therapy, with five of the six receiving oral nonabsorbable antibiotics consisting of nystatin, polymyxin B or colistin, and vancomycin or paramomycin (Table 5). During hospitalization, four of the six patients developed systemic fungal infections, two with *C. tropicalis* and two with *T. glabrata*. Two of the fungal infections proved to be fatal. Three of the four additional patients who harbored resistant fungi died of infections with *Staphylococcus epidermidis* (one patient) or *Klebsiella pneumoniae* (two patients).

TABLE 2. *Polyene-resistant yeast isolated from 70 study patients*

Organism	No. of isolates (%)	No. of patients (%)
C. albicans	27 (3.6)	3 (4.3)
C. tropicalis	3 (0.4)	1 (1.4)
T. glabrata	25 (3.4)	2 (2.9)
C. parapsilosis	0 (0)	0 (0)
Saccharomyces sp.	0 (0)	0 (0)
C. krusei	0 (0)	0 (0)
Trichosporon sp.	0 (0)	0 (0)
C. guilliermondii	0 (0)	0 (0)
C. pseudotropicalis	0 (0)	0 (0)
Total	55 (7.4)	6 (8.6)

DISCUSSION

Although sporadic instances of the emergence of resistant yeast in a clinical setting have been reported, the incidence of resistance in clinical isolates reported to date has been surprisingly low (10, 11). This can be attributed to a lack of routine susceptibility testing of polyenes based on the impression that yeasts are universally susceptible to these antifungal agents. In a recent study of 864 clinical isolates, Safe et al. (20) found only 3 (0.4%) that could be classified as resistant. Two of the three, *C. krusei* and *C. parapsilosis*, are not frequently encountered and are of relatively low pathogenicity. In contrast, the 1,372 yeast isolates recovered from clinical material in this study yielded an overall incidence of 4%, a 10-fold difference. However, the primary observation was that the emergence of polyene-resistant yeasts occurred only in a specific patient population, severely compromised patients undergoing treatment for acute leukemia and aplastic patients with bone marrow transplantation. In separating the leukemia and aplastic patients from all other patients, the anticipated low incidence (none) among patients on other hospital services changed to an unex-

TABLE 3. *Resistant organisms: sites of recovery and length of isolation*

Patient no.	Organism	Sites recovered (no. of specimens)	Persistence of resistant isolates (days)
1	*C. albicans*	Respiratory (2), stool (2)	11
2	*C. albicans*	Urine (2)	1
3	*C. albicans*	Respiratory (9), stool (11), urine (1)	32
4	*C. tropicalis*	Respiratory (1), stool (1), lung, autopsy (1)	1
5	*T. glabrata*	Respiratory (8), stool (7), urine (2)	21
6	*T. glabrata*	Urine (8)	6

pectedly high incidence (8.6%) among leukemia and transplant patients. In reviewing recent reports of clinically isolated polyene-resistant yeasts, four of the six strains were obtained from immunocompromised hosts, including two patients with aplastic anemia (17, 20), one with leukemia (18), and one with a neutrophil deficiency (6). All but one of the six cases developed resistant strains after receiving therapy with a polyene antibiotic.

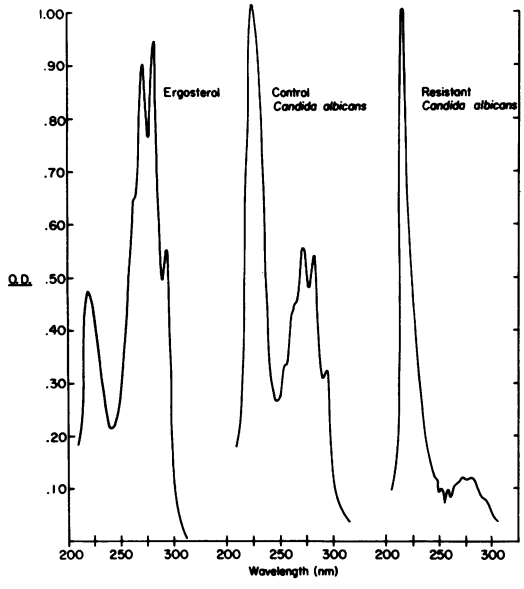

FIG. 1. *Ultraviolet spectra of the non-saponifiable fractions of a susceptible isolate of C. albicans, a resistant isolate of C. albicans, and a control solution of ergosterol.*

TABLE 4. *Sterol analysis of resistant and control yeasts by ultraviolet absorption*

Organism	OD[a] at 281 nm	
	Resistant	Control
C. albicans	0.09	0.34
C. tropicalis	0.06	0.40
T. glabrata	0.19	1.03

[a] OD, Optical density.

The emergence of polyene resistance occurred in three species of yeast, *C. albicans, C. tropicalis,* and *T. glabrata.* Of the nine species of yeasts recovered, which were relatively equivalent in distribution between the study and control groups, these three were the most frequently isolated and are considered the most pathogenic among the species listed. Although polyene resistance has been reported in clinical isolates of *C. albicans* (4) and *C. tropicalis* (17, 20), this is the first report, to our knowledge, of resistance in *T. glabrata.*

Previous reports on mutant polyene-resistant yeasts have indicated that the primary mechanism of polyene resistance is associated with blocks in the sterol synthetic pathway which result in accumulations of some intermediate that has a polyene affinity lower than the wild-type sterol, ergosterol (3, 8, 14, 19, 22). Since 5,7-ene sterols, primarily ergosterol, are a major determinant of polyene susceptibility, less ergosterol in the membranes of resistant strains would result in fewer polyene-sterol binding sites, decreased pore formation, and resultant cytoplasmic leakage (1, 13, 25). Recent characterization of the membrane sterols of polyene-resistant clinical isolates by Woods et al. (24) and Safe et al. (20) have confirmed these observations of laboratory-induced resistance. The polyene-resistant yeasts in this study demonstrated a marked decrease in ergosterol content as compared to polyene-susceptible control isolates of the same species. Sterol analysis indicated the probable mechanism of resistance in our clinical isolates, and it also correlated and confirmed the definition of resistance used in this study, amphotericin B at 2 μg/ml and nystatin at 16 μg/ml. Although a total membrane analysis and comparison has not as yet been completed, the decrease in ergosterol content in our isolates supports previous reports of the mechanisms of resistance to polyene antibiotics.

All patients developing relatively resistant yeasts were first colonized with a susceptible strain of the same species. This, in conjunction with the separation of these patients in both time and location during hospitalization, sug-

TABLE 5. *Summary of the six patients from whom resistant yeasts were recovered*

Characteristic	Patients	Days		
		Range	Median	Mean
Hospitalization	6/6	30–84	51	50
Granulocytopenia[a]	6/6	19–84	34	37
Antibacterial antibiotics	6/6	30–81	39	40
Amphotericin B	6/6	9–36	22	23
Oral nonabsorbable antibiotics	5/6	0–77	33	33

[a] ≤1,000 leukocytes per mm.

gests the development of resistance from endogenous flora rather than aquisition from an exogenous source. In these six patients, infection with the resistant strain was only documented in patient 4, in which both postmortem culture and histology confirmed fungal disease. This finding tends to support the findings of Athar and Winner (2), Hamilton-Miller (9), and Drutz and Lehrer (6) that resistant isolates of fungi have reduced pathogenicity and altered physiological characteristics. The clinical significance of the isolation of polyene-resistant fungi, particularly in a severely compromised host who shows continued disease and microbial persistence on therapy, requires extensive further review.

Examination of the study patient population offers several possible explanations for the emergence of resistant yeasts in this group as compared to the control hospital population. All of the study patients experienced extended periods of hospitalization, granulocytopenia, therapy with cytotoxic agents (known mutagens), and extended antibiotic therapy. As pointed out by Pappagianis et al. (18), the use of cytotoxic drugs should be considered as a contributing factor to development of resistance. The long-term utilization of broad-spectrum antibiotics in conjunction with a nonexistent granulocyte response permits the rapid overgrowth of fungi in this group. When this finding is coupled with subsequent long-term exposure to both oral and systemic polyenes, one is reminded of the "training" experiments of Littman et al. (16), Sorensen et al. (21), and Bodenhoff (5). All of these factors, cytotoxic drug therapy, granulocytopenia, and antibiotic and polyene therapy, may have contributed singly or in combination to the incidence of resistant yeasts in the test patient population. Further studies are required to determine the exact relationship of these factors to the development of polyene resistance, and the relationship of resistance to significant disease in humans.

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