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Investigation of the Sequence of Colonization and Candidemia in Nonneutropenic Patients

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Among neutropenic patients with hematologic malignancies, candidemia has been shown to arise typically from autoinfection after colonization. In patients without neutropenia, we examined the similarities of strains colonizing or infecting various body sites and those subsequently causing *Candida* bloodstream infections. Strain similarity was examined by karyotyping and restriction endonuclease analysis of genomic DNA (REAG) by using two restriction enzymes (*Sfi*I and *Bss*HII). The banding patterns of 42 isolates from 19 patients were independently evaluated in a blinded fashion by three observers. The interobserver reliability measured with a generalized kappa statistic was 0.59 for karyotyping, 0.84 for REAG with *Sfi*I, and 0.88 for REAG with *Bss*HII ($P < 0.001$ for each). REAG classified the initial colonizing or infecting isolate and subsequent blood isolates as identical in 16 patients (84%). The mean duration of colonization or infection prior to a positive blood culture was 5 and 23 days in patients infected with related and unrelated isolates, respectively ($P = 0.14$; 95% confidence interval = -14.5 to 50.5). Karyotyping results matched the REAG results for isolates from 14 of the 19 patients (74%). In patients infected with identical isolates, the initial isolate was most frequently recovered from the urine ($n = 5$) or vascular catheter tips ($n = 4$). In the five subjects with organisms showing disparate results between the methods, karyotyping revealed different banding patterns, whereas REAG suggested that the isolates were identical. *Candida* colonization or infection with an identical strain frequently precedes bloodstream infection in nonneutropenic patients. Future studies should evaluate whether patients at high risk for candidemia and who have vascular catheter or urine samples that are positive for a *Candida* on culture should be treated empirically.

In recent years, *Candida* spp. have emerged as important nosocomial pathogens. Between 1978 and 1984, the incidence of *Candida* bloodstream infections increased by 3- to 10-fold (6, 19). Over the past decade, the incidence of *Candida* bloodstream infections increased by two- to fivefold in U.S. teaching hospitals and by one- to fourfold in nonteaching hospitals (2, 26). Candidemia occurs most frequently in immunocompromised patients with an underlying malignancy or hematologic disorder (15, 16), severely ill burn patients, and patients in surgical and neonatal intensive care units (1, 3, 5, 22, 29, 30). Therefore, the incidence of candidemia is highest in tertiary-care referral hospitals. At our center, the incidence was 8.5/10,000 admissions in 1992. Candidemia is associated with a 57% crude and a 38% attributable (direct) mortality (34).

The clinical importance of *Candida* colonization at different body sites has been controversial. For example, some have argued whether strains colonizing the urine or stool subsequently cause candidemia. Among hematologic malignancy and bone marrow transplantation patients, Reagan et al. (23) demonstrated that colonizing and subsequently infecting strains of *Candida* spp. were identical in 94% of patients. In a carefully conducted case-control study, four independent risk factors for candidemia were identified after accounting for underlying disease: colonization by *Candida* spp. at various body sites, prior exposure to hemodialysis, central intravascular catheters, and previous exposure to antibiotics (35). Relatively little, however, is known about the molecular epidemiology of antecedent colonization and candidemia in nonneutropenic patients.

Since May 1987, our laboratory has routinely banked all *Candida* isolates from the bloodstream as well as any available *Candida* isolate from other clinical sites in the same patient. The purpose of the study described here was to evaluate the similarity of *Candida* bloodstream isolates and prior colonization or infecting isolates in nonneutropenic patients. Fingerprinting of the organisms was performed by karyotyping and restriction endonuclease analysis of genomic DNA (REAG) by using contour-clamped homogeneous electrophoretic field (CHEF) electrophoresis.

MATERIALS AND METHODS

Hospital and patients. The University of Iowa Hospitals and Clinics is a 900-bed teaching hospital and tertiary-care referral center, with approximately 200 beds designated for intensive care. Patients with *Candida* infections were identified retrospectively by reviewing the microbiology reports for isolates banked from May 1987 to July 1992. Neutropenic hematology-oncology and bone marrow transplant patients and those without a previous sample from another site that was positive for *Candida* spp. on culture before their blood sample was

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positive on culture were excluded from the study. During the study period, all *Candida* isolates were stored in sterile distilled water at ambient temperature. Isolates from patients included in the current study were retrieved from this isolate bank and were identified to the species level by using the API 20C system (Analytab Products, Plainview, N.Y.).

Molecular typing and DNA preparation. Ten colonies (>1 mm) of each *Candida* isolate from 48-h cultures on Sabouraud agar plates were incubated overnight at 37°C in 10 ml of YEPD broth (yeast extract, 10 g/liter; peptone, 10 g/liter; and D-glucose, 20 g/liter). The cells were packed by centrifugation (1,000 × *g* for 15 min), washed in sterile distilled water, and introduced into preweighed Eppendorf tubes. The pellets were suspended in 1 volume of 50 mM sodium EDTA (50E; pH 8.0). The cell titer was normalized by adding the volume (in microliters) equal to the weight of the cells (in milligrams). A 173-μl aliquot of this suspension was evenly mixed with 300 μl of 50E, 66.5 μl of lyticase (L5263; Sigma Chemical, St. Louis, Mo.), and 295 μl of 2% agarose (SeaPlaque GTG; FMC BioProducts, Rockland, Maine) that was previously melted and kept liquid at 56°C. Aliquots were placed in forms (Bio-Rad, Hercules, Calif.) that were incubated for 2 h at 37°C. The inserts were removed from the forms and were placed in 2 ml of a buffer of 10 mM Tris-HCl (pH 7.5), 400 mM EDTA, 1% Sarkosyl, 0.750 mg of proteinase K per ml (protease type XXVII; Sigma) and incubated overnight at 50°C. The inserts were washed six times in 50E and were stored at 5°C until use.

CHEF karyotyping. Electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (Bio-Rad) in 0.7% agarose (SeaKem GTG agarose; FMC BioProducts)–0.5× 100 mM Tris (pH 8.5)–100 mM boric acid–1.0 mM EDTA (TBE buffer) at 13°C and 150 V. The pulse interval was 120 s for 24 h and then 240 s for 36 h. *Saccharomyces cerevisiae* chromosome DNA molecular weight markers (Bio-Rad) were included in each gel as standards. After electrophoresis, the gels were stained with ethidium bromide, illuminated with UV light, and photographed.

REAG. For restriction endonuclease digestion, agarose inserts containing chromosome-sized DNA were prepared as described above and were placed into 100 mM Tris-HCl (pH 8.0) containing 5 mM magnesium chloride. After two washes with the buffer, the agarose inserts were digested overnight with 20 U of *Sfi*I or *Bss*HII (New England Biolabs, Beverly, Mass.), as recommended by the manufacturer.

Electrophoresis was performed with a CHEF-DRII pulsed-field electrophoretic system (Bio-Rad). Electrophoresis was performed in 1.0% agarose gel (SeaKem GTG)–0.5× TBE at 13°C and 200 V. The pulse interval was ramped from 5 to 90 s over 24 h. The 48.5-kb bacteriophage lambda DNA ladders (Bio-Rad) were included in each gel as molecular size standards. After electrophoresis, the gels were stained with ethidium bromide, illuminated under UV light, and photographed.

Analysis. Three observers, blinded to the origin of the isolate and the results of the other observers, examined the photographs of the ethidium bromide-stained gels to detect similarities and differences in banding patterns. All bands had to match exactly to classify isolates as identical; any difference in a major or a minor band was considered important. Banding patterns with ≥95% but less than 100% of the bands matching were termed "similar." Isolates with less than 95% of bands matching were considered different. Interobserver reliability was calculated with the generalized kappa statistic for measurement of agreement (37). Means were compared with a *t* test for independent samples after evaluation for equality of the variances by Levene's test. Alpha was set at 0.05, and all *P* values are two-tailed.

TABLE 1. Clinical characteristics of study patients

Patient code	Age (yr)	Sex[a]	Underlying disease[b]	Species
A	36	M	Pancreas and kidney transplant, diabetes mellitus	*C. albicans*
B	4	F	Acute lymphocytic leukemia	*C. albicans*
C	27	M	Motor vehicle accident, burn injury (52% BSA)	*C. albicans*
D	68	F	Transoral odontoid resection and tracheostomy, rheumatoid arthritis	*C. albicans*
E	87	M	Motor vehicle accident, intraventricular hemorrhage	*C. albicans*
F	58	F	Ovarian neoplasm, diabetes mellitus	*C. albicans*
G	52	F	Cervical carcinoma, post radiation cystitis and enteritis	*C. albicans*
H	76	M	Head and neck cancer, intraspinal abscess	*C. albicans*
I	78	F	Amyloidosis, stroke	*C. albicans*
J	27	M	Burn injury (90% BSA)	*C. albicans*
K	56	F	Biliary tract obstruction, pancreatitis	*C. albicans*
L	61	M	Pancreatitis	*C. albicans*
M	73	M	Aortic valve stenosis, CABG, diabetes mellitus	*C. albicans*
N	46	F	GI surgery, urinary diversion	*C. albicans*
O	52	F	Status post-GI surgery, myocardial infarction	*C. albicans*
P	70	M	Coronary artery disease	*C. albicans*
Q	80	M	Bladder carcinoma, cystectomy with ileal conduit	*C. tropicalis*
R	60	F	Cervical carcinoma, status post-pelvic exoneration	*C. parapsilosis*
S	69	M	Status post-oropharyngeal carcinoma, dysphagia	*C. tropicalis*

[a] M, males; F, female.
[b] BSA, body surface area; CABG, coronary artery bypass graft; GI, gastrointestinal.

RESULTS

During the study period, 107 patients whose blood was positive for *Candida* spp. on culture were identified; 19 patients (17.8%) met the inclusion criteria. The other patients were neutropenic and had a hematologic malignancy or were undergoing bone marrow transplantation (40.2%), did not have positive cultures of samples from another site before their positive blood culture (34.6%), or had different fungal species isolated from their blood and another body site previously (7.5%). Therefore, 42 *Candida* strains (*C. albicans* [n = 35], *C. tropicalis* [n = 5], *C. parapsilosis* [n = 2]) from 19 patients (Table 1) were available for molecular typing. The 19 patients represented 30% of the total number of nonneutropenic patients with candidemia during the study period.

The underlying diseases of the 19 patients included 6 (31%) with solid tumors, 1 (5%) who had received an organ transplant, 1 (5%) with acute lymphocytic leukemia prior to treatment, and 11 (58%) with prior surgery involving the gastrointestinal tract. Diabetes mellitus or prior antibiotic treatment were present in 16 and 89% of the study patients, respectively. None of the patients included in the study were neutropenic either prior to or at the time of candidemia. The interval between the isolation of *Candida* spp. from a blood culture and

another body site previously ranged from 1 to 31 days, with a mean of 7.8 days (median, 5 days). In patients in whom the isolate from a blood culture and isolates from other sites were identical by REAG, the interval between the isolations ranged from 1 to 11 days, with a mean of 5 days (median, 4 days). By contrast, nonidentical isolates from colonized or infected body sites occurred from 8 to 31 days earlier than the bloodstream isolate, with a mean of 23 days (median, 30 days). This mean difference in time from positivity for a prior *Candida* isolate was not statistically significant ($P = 0.14$; 95% confidence interval about the difference = -14.5 to 50.5), although the power to detect a difference was low, given the relatively few subjects with nonidentical isolate pairs. In the 16 patients with identical isolates from a blood culture and another site, the urine ($n = 5$) and catheter tips ($n = 4$) were the most frequent prior sites. Specimens from the respiratory tract ($n = 1$) and remote (≥30 days) tissue biopsy specimens ($n = 2$) were the sources of the initial isolates among the three patients with nonidentical isolate pairs by REAG.

In the analysis of the fingerprint patterns of the isolates, a high interobserver reliability was observed; the generalized kappa statistic was 0.59 for karyotyping, 0.84 for REAG with *Sfi*I, and 0.88 for REAG with *Bss*HII ($P < 0.001$ for each). The results of the overall interpretation (consensus call of the three observers) are given in Table 2. Overall, the method revealed 20 unique karyotypes. The karyotype banding patterns of initial site and subsequent bloodstream isolates (Fig. 1) were identical in 11 of 19 (57.9%) patients. In eight patients infected with either *C. albicans* ($n = 7$) or *C. parapsilosis* ($n = 1$), the karyotypes of the paired isolates did not match.

The relationships among isolates within a given patient determined by REAG (Fig. 2 and 3) were identical regardless of the restriction enzyme employed. The only difference observed in the REAG with the two different endonucleases was in the interpatient evaluation, e.g., whether a specific banding pattern was classified as unique or shared with another patient. By the REAG method, isolates from the initial site and blood cultures were shown to be identical in 16 patients (84.2%). In three patients with *C. albicans* infections, the paired isolates were different. Isolates from three patients (patients J, O, and P) were classified as identical with *Sfi*I, whereas their banding patterns after digestion with *Bss*HII classified them as different. Thus, the REAG method identified 16 unique patterns after digestion with *Sfi*I and 19 after digestion with *Bss*HII.

Despite a similar number of unique karyotype patterns, the results matched the overall REAG results for only 14 of the 19 patients (74%). The isolates from five patients whose initial site and blood isolates had different karyotypes appeared to be identical when REAG was done with both enzymes (see Fig. 1 to 3, patients J and K).

DISCUSSION

Rates of nosocomial bloodstream infections have increased markedly over the last decade, particularly because of the increased number of infections caused by coagulase-negative staphylococci, *Candida* spp., *Staphylococcus aureus*, and enterococci. In 1975, *Candida* spp. accounted for less than 3% of nosocomial bloodstream infections (20) but are currently responsible for approximately 10% (34). Risk factors involved in the pathogenesis of candidemia have been identified in numerous studies (1, 4, 5, 11, 13, 14, 22, 24, 29, 30, 35). Case-control studies that use multivariate analysis to control for underlying diseases have convincingly demonstrated that colonization by *Candida* spp. at various body sites, prior exposure to hemodialysis, central intravascular catheters, previous exposure to antibiotics, and azotemia are independent risk factors for candidemia (4, 11, 13, 35). Until recently, it was suggested that candidemia arises more or less exclusively from autoinfection caused by colonizing organisms in the intestine. The availability of molecular typing methods has allowed investigators to confirm this hypothesis (23, 27). Furthermore, unique strains colonize the patient, persist, and are frequently responsible for subsequent infection or relapse (9, 31). Additionally, a number of documented outbreaks of *Candida* infections have highlighted the importance of transmission of *Candida* spp. from an exogenous source to the patient (4, 7, 10, 18, 28, 32, 33, 36).

Our study, in which we used the REAG method for fingerprinting, demonstrated identical colonizing or infecting and blood culture isolates in the majority (84%) of the patients analyzed. Reagan et al. (23) found a slightly higher frequency of identical colonizing and infecting strains among hematologic malignancy and bone marrow transplant patients than we found in the current study. Importantly, the isolates compared by Reagan et al. (23) were routinely obtained as part of an ongoing surveillance program in severely immunocompromised patients. In contrast, most of the isolates from nonneutropenic patients in the present study were from cultures of clinical samples taken during sporadic epidemiologic surveillance or when an infection was suspected. However, our study population represented one-third of all nonneutropenic patients with candidemia during the study period. Furthermore, Reagan and colleagues (23) reported that matching strains could be found even after an interval of up to 432 days. In our patients, the interval between a positive culture of a sample from another site and a positive blood sample culture was inversely related to the likelihood of finding identical colonizing and blood culture isolates. The fact that most of the isolates in our study were not obtained during ongoing surveillance may have created a selection bias that precludes a widespread generalization to all nonneutropenic patients. However, the high proportion of study patients with identical colonizing or infecting isolates prior to bloodstream infection supports the clinical view that the bloodstream infection frequently arises endogenously.

The difference in interval (days) between the previous isolation of *Candida* spp. from a body site and a blood culture in patients in whom the blood culture and other site isolates were identical was approximately one-fifth that in patients in whom the isolates were different. However, given the relatively small number of subjects, this difference did not reach statistical significance. The two patients whose tissue biopsy specimens were infected with *C. albicans* were among three patients colonized or infected with a different strain prior to the development of candidemia. The extended time interval was long enough to allow recolonization and infection with a second strain, or the patients had been colonized with strains that were not isolated in the current study. Isolation of *Candida* spp. from tracheal secretions alone did not predict a subsequent bloodstream infection with the same strain. Among the patients with matching *Candida* strain pairs, the organisms were isolated initially most frequently from urine or vascular catheter tips. Since the isolates were not routinely obtained as part of an ongoing surveillance program, it is difficult to draw firm conclusions about this time interval. The observed differences in the interval between colonization or infection and subsequent bloodstream infection and initial isolation of *Candida* from a body site emphasize the fact that colonization of certain body sites (respiratory tract and remote tissue biopsy specimens) may infrequently result in candidemia with that same strain, despite long periods of follow-up. However, the

TABLE 2. Results of karyotyping and REAG of isolates from a blood culture and other body sites

Patient code	Isolate/ lane no.	Origin	Interval (days)	Result by karyotyping	Result by REAG with *Sfi*I[a]	Result by REAG with *Bss*HII[a]
A	1	Blood		a	1	1
	2	Urine	8	a	1	1
B	3	Blood		b	2	2
	4	Stool	4	b	2	2
C	5	Blood		a	3	3
	6	Tracheal	11	a	3	3
	7	Catheter	1	a	3	3
D	8	Blood		c	4	4
	9	Tissue biopsy	30	d	5	5
E	10	Blood		e	6	6
	11	Catheter	1	f	6	6
F	12	Blood		g	7	7
	13	Urine	6	g	7	7
	14	Urine	1	g	7	7
G	15	Blood		h	3	3
	16	Tracheal	8	i	8	8
H	17	Blood		j	9	9
	18	Tissue biopsy	31	k	10	10
I	19	Blood		k	11a	11
	20	Sputum	3	k	11a	11
J	21	Blood		l	11a	12
	22	Urine	1	k	11a	12
K	23	Blood		k	11a	11
	24	Urine	11	m	11a	11
L	25	Blood		n	12	13
	26	Urine	5	n	12	13
	27	Bile	4	n	12	13
M	28	Blood		o	13	14
	29	Pleural fluid	8	p	13	14
N	30	Blood		k	14	15
	31	Tracheal	4	k	14	15
O	32	Blood		k	11b	16a
	33	Catheter	2	k	11b	16b
P	34	Blood		k	11a	16b
	35	Catheter	1	k	11a	16b
Q	36	Blood		q	15	17
	37	Ascites	8	q	15	17
	38	Ascites	8	q	15	17
R	39	Blood		r	14	18
	40	Ascites	3	s	14	18
S	41	Blood		t	16	19
	42	Ascites	4	t	16	19

[a] The differences denoted a or b are insufficient to classify the isolates as different (sharing <95% of bands) rather than similar (sharing ≥95% but <100% of bands).

presence of a *Candida* sp. at other sites (urine or catheter tip) may be associated with subsequent candidemia within relatively short periods of time.

Patients with vascular catheters are at a significantly increased risk for fungal infections (14, 35). This may be due to the unique surface properties of *Candida* spp., which allow it to invade the host by attaching to plastic surfaces and evade the host immune system (12, 25). Some investigators argue that

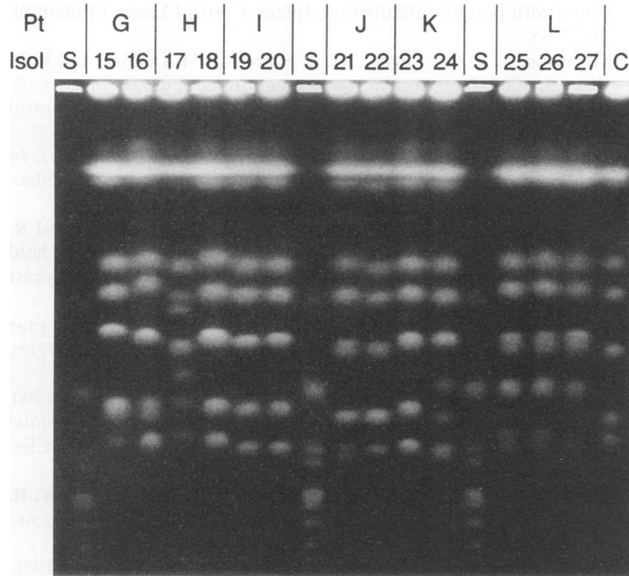

FIG. 1. CHEF karyotype. Shown are the blood culture and other site isolates from patients G to L. Note that the patterns of isolates from patients I and L appear to be identical. The karyotype pattern denoted k (see Table 2) is seen in isolates 18, 19, 20, 22, and 23 of patients H, I, J, and K, respectively. Note that isolates 21 and 22 (patient J) appeared to be different because of the spacing of the banding patterns. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

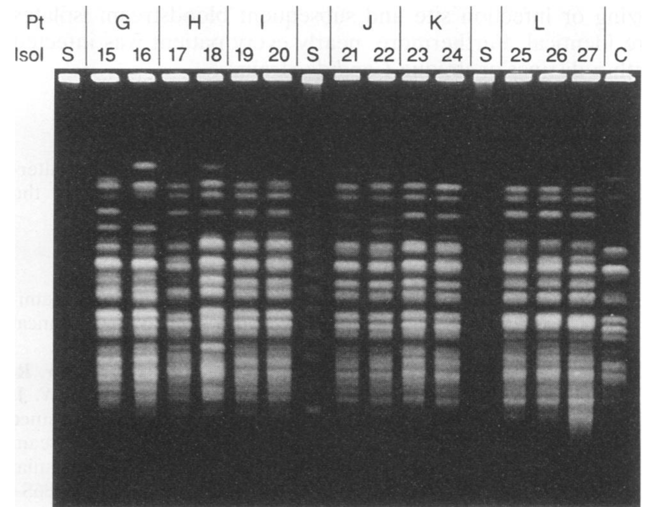

FIG. 3. REAG with *Bss*HII. Shown are blood culture and other site isolates from patients G to L. Banding patterns of paired isolates of subjects I to L appeared to be identical, as demonstrated previously by REAG with *Sfi*I. The overall (within-subject) evaluation was identical for REAG patterns with *Sfi*I and *Bss*HII. A difference between the two methods was seen only in the between-subject evaluation. Note that the banding patterns of isolates 21 and 22 (from patient J) do not match the banding patterns of isolates from patients J and K, as seen by REAG with *Sfi*I. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

patients with catheter tip cultures positive for a *Candida* sp. require antifungal therapy, although this point is controversial. The safety and efficacy of early antifungal treatment of patients with a positive urine or vascular catheter tip culture and suspected fungal infections in preventing *Candida* sp. bloodstream infections could be evaluated prospectively.

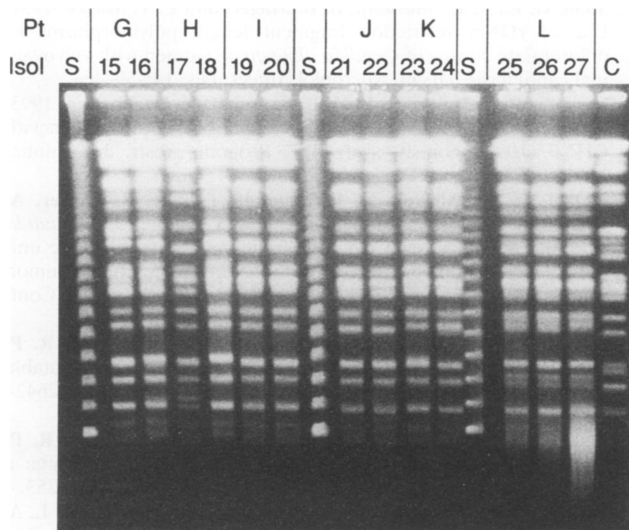

FIG. 2. REAG with *Sfi*I. Shown are blood culture and other site isolates from patients G to L. Banding patterns of paired isolates of patients I to L appeared to be identical, including isolates from patients J and K with karyotype patterns that appeared to be different (see Fig. 1). The REAG pattern with *Sfi*I denoted 11a (see Table 2) was shared by patients I, J, and K. Abbreviations: Pt, patient; Isol, isolate number; S, molecular size standard; C, *C. albicans* control strain.

With four exceptions, each patient was infected by his or her own unique *Candida* strain, suggesting no clustering or transmission of a predominant strain. The discriminative value of standard CHEF gel electrophoresis of *C. albicans* has been demonstrated (17, 21). However, data from two prior studies (8, 17) suggest that karyotyping may be of limited value for the discrimination of *C. tropicalis* isolates. The three typing methods used in the current study showed the identity of the *C. tropicalis* strains in patients Q and S. Compared with the more easily interpretable banding patterns of REAG, the interpretation of standard CHEF gel electrophoresis may be difficult because of the fewer number of bands. In our study, the initial site and bloodstream isolates from five patients had different patterns by karyotyping, yet they had identical patterns after endonuclease digestion. The reason for this finding is unclear.

Both methods resulted in relatively high levels of agreement between observers, i.e., greater than that expected by chance alone. The kappa statistic demonstrated the greatest level of agreement between the observers for the two REAG methods. We would suggest the routine use of a statistical method, such as the kappa statistic, to compare different methods quantitatively in molecular typing studies.

REAG appeared to be a reliable method for delineating strains of *Candida* spp. with high discriminatory abilities, regardless of whether *Bss*HII or *Sfi*I was used as the restriction enzyme. Since the cost per unit is considerably lower for *Sfi*I, this enzyme should be considered in typing situations in which a single restriction enzyme is needed.

In summary, we found that in the majority of patients who develop disseminated *Candida* sp. infections, the initial colo-

nizing or infection site and subsequent bloodstream isolates are identical. Furthermore, nearly every patient was infected with a distinct or unique *Candida* strain.

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