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Contribution of Molecular Typing Methods and Antifungal Susceptibility Testing to the Study of a Candidemia Cluster in a Burn Care Unit

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We investigated a cluster of cases of *Candida* septicemia diagnosed in four burn patients. Twenty clinical isolates of *Candida albicans* and two of *Candida parapsilosis*, plus eight isolates of *C. albicans* recovered from nurses' clothes, were analyzed by antifungal susceptibility testing and three genotyping methods (restriction fragment length polymorphism analysis with *Eco*RI and *Hin*fI, arbitrarily primed PCR, and karyotyping). The high MICs of the azoles for all of the *C. albicans* isolates tested suggest either a natural resistance of the endogenous flora or the transmission of isolates with acquired resistance. The genotyping methods demonstrated the involvement of four different strains, cross-infections with one *C. albicans* strain and one *C. parapsilosis* strain, and identity between some of the strains from the patients and nurses. The origins of the strains remain unclear. Our results show that the use of a combination of at least two different methods such as those used in the present study is recommended for *C. albicans* typing.

Candida septicemia is now recognized as a major nosocomial infection (3). Among the species identified, *Candida albicans* ranks first, but other species such as *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, and *Candida parapsilosis* are frequently cited as emerging colonizing flora or pathogens during the sustained use of antifungal agents (10). The major factor predisposing an individual to candidemia is neutropenia, but severely burned patients are also at risk, especially when central venous catheters and broad-spectrum antibiotics are required (12). The poor prognosis and the increasing frequency of *Candida* septicemia have modified therapeutic and prophylactic approaches (14). Diagnosis is hampered by the delayed positivity of blood cultures and by the lack of reliable tests for the detection of soluble antigens or antibodies. Treatment is often unsuccessful because of the underlying condition of the patient and of the absence of truly fungicidal agents. *Candida* septicemia is therefore still associated with at least a 50% mortality rate (10). Although most of the cases are related to the endogenous flora, some have been proved to be caused by isolates coming from the environment (16, 23). The contamination of infusions (25) or the hands of the health care workers (7, 16, 17) has been shown to represent potential exogenous sources of *Candida* spp. The differences in the pathogenesis of the infection require different but not exclusive prophylactic approaches. For cases related to the endogenous flora, decreasing the risk for translocation of isolates from the gut by the prescription of prophylactic antifungal agents or at least surveying the digestive flora is appropriate. For cases related to contamination, reinforcement of sanitary measures is mandatory (14).

Nosocomial infections cannot be completely avoided, and their occurrence in clusters always raises the question of a common source. This was the case when four patients were diagnosed with *Candida* septicemia within 3 months in a burn care unit. Since most of the clinical isolates have been stored and since some of the environmental isolates recovered at the time of the diagnosis were also available, we decided to study their relatedness. In vitro antifungal susceptibility to four major antifungal agents and three DNA typing methods were used for strain delineation.

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MATERIALS AND METHODS

Patients. The patients' characteristics are given in Table 1. The four female patients were hospitalized immediately after the accident in the Burn Care Unit at Hôpital Saint Antoine in Paris. The Burn Care Unit is a small ward composed of 10 patient rooms (Fig. 1). The wounds of all four patients were excised, and all four patients underwent auto- or allo-skin transplantation because of the extent and severity of the lesions. The severity of the burns was judged on the basis of their localization and on a scale ranging from 0 (no burn) to 400 (entire surface of the body burned) expressed in units burn standard according to the following formula: UBS = TBSA + (3 × FTBA), where TBSA is total burn surface area and FTBA is full thickness burn area. The patients were bathed every other day in the same room located on the ward. The tub was washed after each patient was bathed and was sterilized with bleach. Cases of septicemias caused by gram-positive or -negative bacteria occurred early after the patients were admitted and required prolonged treatments with various antibiotics. The risk factors for candidemia were multiple (bacteremias requiring broad-spectrum antibiotics and the use of central venous catheters and a urinary catheter but no neutropenia), and the patients had various underlying diseases (Table 1). The diagnosis of *Candida* septicemia was based on blood cultures that were repeatedly positive for *C. albicans* (three or more cultures more than 24 h apart) together with cultures of specimens from other sites that were positive (including urine, wounds, and in one instance, bronchial secretions). Blood was obtained either by vein puncture or by collection through the central venous catheter. Culture of oropharyngeal or stool specimens was not performed.

As shown on Fig. 2, hospitalization of the patients overlapped. Patient B was admitted when patient A was still alive and diagnosed with *Candida* septicemia. Patients B, C, and D were diagnosed with *Candida* septicemia at the same time on 20 and 21 April 1993. Cultures of blood from patient B were positive for *C. albicans* for almost 3 weeks. All patients except patient B were immediately treated with fluconazole (FCZ; 400 mg/day intravenously). All patients died within 4 days (patient D) to 4 weeks (patient A) after the onset of therapy. Deaths occurred from multiple organ failures. Furthermore, patient B had se-

TABLE 1. Characteristics of the patients and comparative results obtained after analysis of the *Candida* isolates by antifungal susceptibility testing and genotyping methods

Subject	Age (yr)	Underlying disease[a]	Burn severity (UBS)[b]	Source of positive culture[c]	Source of isolates studied (no. of isolates)[c]	Species of *Candida*	MIC (μg/ml)[d]				Pattern obtained by the following genotyping method:			
							AMB	FCZ	ITZ	KTZ	CHEF electro-phoresis	*Eco*RI RFLP	*Hin*fI RFLP	AP-PCR
A	90	A	64	B,U	B (1)	*C. albicans*	0.5	≥64	≥64	16	K1	E1	H1	AAA
B	30	E,T	80	B,U,G	B (6)	*C. albicans*	1–2	≥64	≥64	8–32	K2	E1	H2	BBB
C	42	MS,D	40	B,U,G	B (4)	*C. albicans*	0.5–1	≥64	≥64	8–32	K1[e]	E2[e]	H3	CCC
					G (3)	*C. albicans*	0.5–1	≥64	≥64	16–32	K1	E2	H3	CCC
					U (1)	*C. albicans*	1	≥64	≥64	16	K1	E2	H3	C′CC[f]
					B (1)	*C. parapsilosis*	2	1	0.12	0.12	K4	E4	H4	DDD
D	48	A,T	142	B,U,G,L	B (3)	*C. albicans*	1	≥64	≥64	16–32	K2	E1	H2	BBB
					G (1)	*C. parapsilosis*	2	0.5	0.12	0.12	K4	E4	H4	DDD
M				Clothes	(2)	*C. albicans*	0.5–1	≥64	≥64	16–32	K1	E2	H3	CCC
N				Clothes	(6)	*C. albicans*	0.5–1	≥64	≥64	8	K2	E1	H2	B′BB[f]

[a] A, alcoholism; E, epilepsy; T, tabagism; MS, multiple sclerosis; D, diabetes mellitus.
[b] UBS, unit burn standard = percent burned body surface + (3 × third-degree burned surface).
[c] B, blood; U, urine; G, skin graft; L, bronchial secretions.
[d] MICs were determined after 48 h of incubation by a broth microdilution method.
[e] One of the isolates, CH4a, was classified as K3 by CHEF analysis and as E3 by RFLP analysis with *Eco*RI.
[f] Pattern C′ was slightly different from pattern C (as was pattern B′ from pattern B), but there was not enough polymorphism to warrant the definition of a new genotype.

vere neurological dysfunctions for 2 months following a cardiac arrest, and patient D died from acute respiratory distress syndrome related to an esophagotracheal fistula. Nevertheless, cultures of blood and/or urine specimens from patients A and C were still positive for at least 2 weeks after the onset of therapy. The central venous catheters were not removed after diagnosis, nor were they cultured after death.

On 23 April, after the diagnosis of *Candida* septicemia in three patients (patients B, C, and D), environmental sampling was performed in the ward. This included the clothes of two nurses (nurses N and M, who mostly took care of the patients on the day shift), the matresses, and the infusion pumps. Samples from all sites grew *C. albicans* isolates. Only the isolates recovered from the nurses' gowns were stored. Careful review of the clinical records showed that FCZ was not prescribed prophylactically in this ward. FCZ was prescribed between September and December 1992 for only four patients whose specimens were cultures positive for *Candida* spp. (*C. glabrata* in blood specimens once and *C. albicans* in stool specimens three times). One of these latter patients was still hospitalized when patient A was admitted. These isolates were not stored.

Isolates. Cultures were stored at 4°C on Sabouraud dextrose agar slants until they were used for the present study (Table 1). After two consecutive clonings on Sabouraud dextrose agar, five colonies with identical morphologies were pooled for each sample and were subsequently called isolates (designated by a code starting with the letter assigned to the individual). Identification of the species was checked by chlamydospore formation in rice-Tween agar and by the pattern of sugar assimilation determined by using commercial strips (ID 32C; bioMérieux, Marcy-l'Etoile, France). For one sample, two types of colonies were observed, cloned (CH4a and CH4b), and analyzed separately. All isolates were *C. albicans* except two isolates that were identified as *C. parapsilosis* (Table 1). Reference strains were used as controls for the typing methods (ATCC 32354 or ATCC 28367 for *C. albicans* and ATCC 22019 for *C. parapsilosis*) and for the antifungal susceptibility testing (*Candida kefyr* 706).

Antifungal susceptibility testing. A broth microdilution method was used to determine the MICs (5). Stock solutions of antifungal agents were prepared in sterile distilled water for FCZ (Pfizer Central Research, Sandwich, United Kingdom) and in dimethyl sulfoxide for itraconazole (ITZ; Janssen Pharmaceuticals, Beerse, Belgium), ketoconazole (KTZ; Janssen), and amphotericin B (AmB; Squibb, Princeton, N.J.). Yeast suspensions were adjusted by turbidimetry to 5 × 10^3/ml in RPMI 1640 buffered with 0.165 M morpholinopropanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.) as recommended previously (11). The yeast suspensions (180 μl) and twofold dilutions of the antifungal agents (20 μl; final concentrations ranging from 64 to 0.125 μg/ml for the azoles and from 4 to 0.078 μg/ml for AmB) were added to each well. Growth was recorded at 492 nm (Multiscan Plus; Flow Laboratories, McLean, Va.). The MIC was determined after 48 h of incubation at 28°C as the lowest concentration of the antifungal agent preventing 80% of the growth compared with the growth in the drug-free well. We defined resistance to FCZ by an MIC of ≥16 μg/ml and resistance to KTZ and ITZ by an MIC of ≥4 μg/ml. The following MICs were obtained for the reference strain: 0.25 μg/ml for FCZ, 0.125 μg/ml for ITZ, 0.125 μg/ml for KTZ, and 1 μg/ml for AmB.

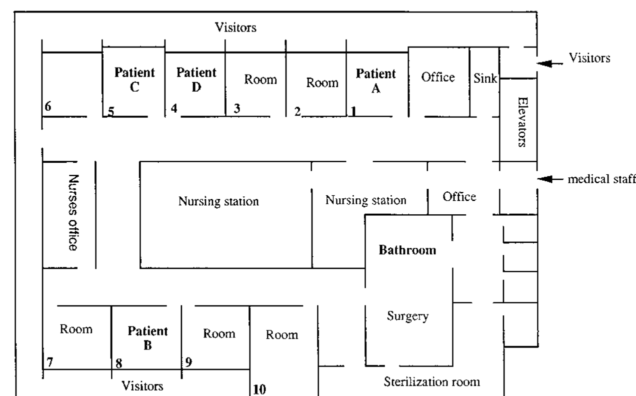

FIG. 1. Schematic plan of the Burn Care Unit. The numbers 1 to 10 indicate patient rooms.

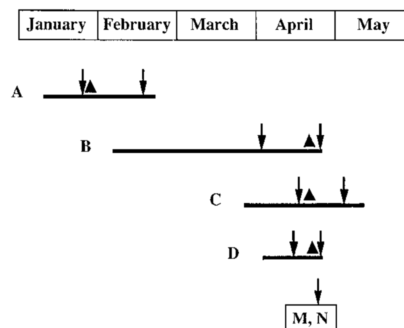

FIG. 2. Schematic representation of the epidemiologic situation. The four patients were hospitalized in 1993 in a burn care unit. The duration of hospitalization is represented by the thick lines. The date of the diagnosis of *Candida* septicemia is indicated by the black triangle. In the time interval bordered by the arrows, several cultures of blood, skin graft, and urine were positive for *C. albicans* (patients A to D) and *C. parapsilosis* (patients C and D). Environmental sampling including the clothes of two nurses (nurses N and M who mostly took care of the patients on the day shift), the matresses, and the infusion pumps, which grew *C. albicans* (arrow).

DNA typing. All experiments were performed on yeasts grown as described above. The origins of the isolates were unknown to the investigators performing and interpreting the arbitrarily primed PCR (AP-PCR) and to one of the investigators interpreting the restriction fragment length polymorphisms (RFLPs) and the karyotyping patterns.

RFLP pattern analysis. Extraction of total DNA, enzymatic digestion, electrophoresis, and staining were performed as described before (2). *Eco*RI-*Hin*dIII digests of bacteriophage λ DNA (Boehringer GmbH, Mannheim, Germany) and the Raoul marker (Appligene, Illkirch, France) were included in each gel as molecular size standards. RFLPs were detected by visual examination of the photographs. Relatedness was based on the number and the positions of the major bands.

Contour-clamped homogeneous electric field (CHEF) electrophoresis. After washing, approximately 10^8 *Candida* cells were resuspended in 150 μl of SCE buffer (1 M sorbitol, 0.1 M sodium citrate, 0.06 M EDTA [pH 7.0]) and mixed with 300 μl of 1% low-melting-point agarose (Bio-Rad, Richmond, Calif.) and 50 μl of SCE containing 6 mM aurintricarboxylic acid (ATA; Sigma) and 2 mg of lyticase (Sigma) per ml as described previously (8). Plugs were incubated for 24 h at 37°C in 1 ml of LET-ATA buffer (0.01 M Tris-HCl, 0.45 M EDTA [pH 8.0], 6 mM ATA) and for 24 h at 50°C in 1 ml of LET containing 2 mg of proteinase K per ml and 1% *N*-lauroylsarcosine. Prior to use, the plugs were incubated for 1 h at room temperature in 0.5× TBE buffer (0.045 M Tris, 0.045 M boric acid, 0.001 M EDTA [pH 8.4]). Electrophoresis in 0.6% agarose (SeaKem GTG agarose; FMC Co., BioProducts, Rockland, Maine) was carried out on the CHEF-DRII apparatus (Bio-Rad) at 70 V with a dually ramped switch time of 120 to 300 s for 30 h and 420 to 900 s for 66 h (26). *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as molecular size standards. For *C. albicans*, variations in chromosomes of less than 2.2 Mb were considered epidemiologically relevant. For variations greater than 2.2 Mb, clones from the same mother cell can exhibit variability in the size or intensity of the bands (2).

PCR-mediated genotyping. The AP-PCR scheme used has been described previously (19). In short, *Candida* DNA was isolated by treatment of fungal cells with zymolyase (Sigma), after which the spheroplasts were lysed and the DNA was purified. For each PCR assay, approximately 50 ng of yeast DNA was used as a template. Thermocycling was performed in a Biomed model 60 machine (Theres, Germany) by using *Thermus thermophilus* DNA polymerase (Sphaero Q, Leiden, The Netherlands). Two types of primers were used. The enterobacterial repetitive intergenic consensus (ERIC1 and ERIC2) primers were applied in combination with annealing at 25°C. The repeat motif primer $(GGA)_7$ was combined with an annealing temperature of 52°C. The banding patterns were interpreted on the basis of visual inspection. Differences between lanes were assessed, and when more than one band was added, disappeared, or shifted position, a new type was defined (capital lettering). When single band differences or changes in ethidium bromide staining intensity were observed, subtypes were indicated by the addition of a prime (B versus B′ and C versus C′; Table 1).

RESULTS

Antifungal susceptibility testing. The *C. parapsilosis* isolates were all susceptible to the four antifungal agents tested. The *C. albicans* isolates were all susceptible to AmB, and the MICs of FCZ and ITZ (MICs, ≥64 μg/ml) and KTZ (MIC range, 8 to 32 μg/ml) were high (Table 1).

DNA typing. (i) RFLP pattern analysis. When *Eco*RI was used, three types of patterns were seen for the *C. albicans* isolates (Table 1; Fig. 3). Pattern 1 contained isolates from individuals A, B, D, and N, pattern 2 contained isolate CH4a, and pattern 3 contained the other isolates from patient C and the isolates from nurse M. For pattern 3, we later noted small differences between isolates from individual C and isolates from nurse M in the range of 7 to 8 kb. However, these patterns were interpreted blindly as being similar and were thus kept in the same group.

With *Hin*fI, three types of patterns were observed among the *C. albicans* isolates (Fig. 4). The single isolate from patient A was different from the isolates from individuals B, D, and N and from the isolates recovered from nurse M and patient C. Isolate CH4a was identical to the other isolates from patient C. The positions of a triplet of bands seen between 4 and 5 kb were slightly lower for isolate CH2 compared with the positions of the bands for isolates CH4a and M2 (Fig. 4). These small variations were not taken into account when the patterns were interpreted blindly and were therefore disregarded. With both restriction enzymes, the pattern obtained with the reference strain ATCC 28367 was different from those obtained

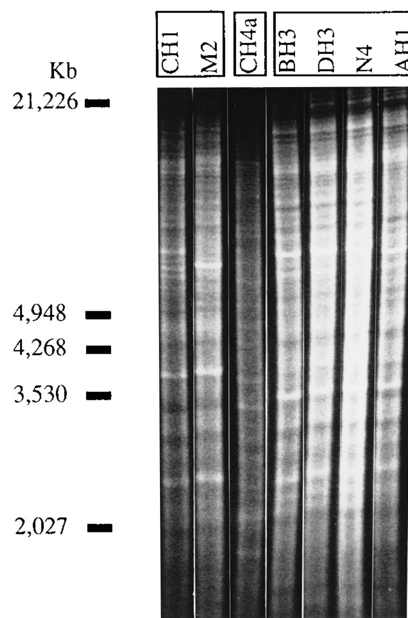

FIG. 3. DNAs from the *C. albicans* isolates were digested with *Eco*RI. For each individual except patient C, one representative isolate is shown; for patient C, two isolates including CH4a were always studied. The origin of the isolate (with the first letter corresponding to the individual) is indicated at the top of each lane. Isolates are grouped (boxed lane headings) according to the similarity of the banding pattern that was obtained during the same run. *Eco*RI-*Hin*dIII-cut phage lambda provided the molecular size standards.

with the clinical isolates. The clinical isolates of *C. parapsilosis* were undistinguishable from the reference strain ATCC 22019 (data not shown).

(ii) Karyotyping analysis. For the individuals from whom multiple isolates were available, the pattern was shown to be identical for all of the isolates except for the isolate from patient C, isolate CH4a, whose pattern was different from those of the other isolates. Three different karyotypes were

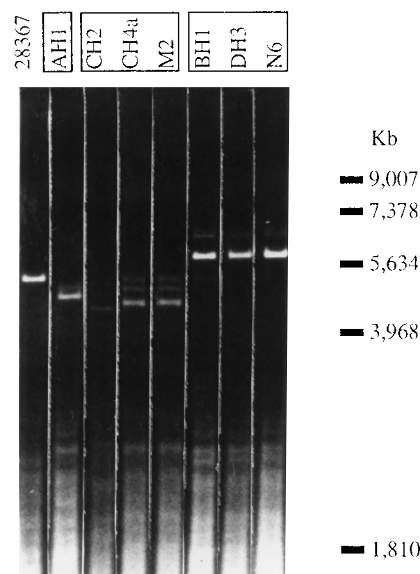

FIG. 4. DNA from *C. albicans* isolates was digested with *Hin*fI. The Raoul marker was used as a molecular size standard.

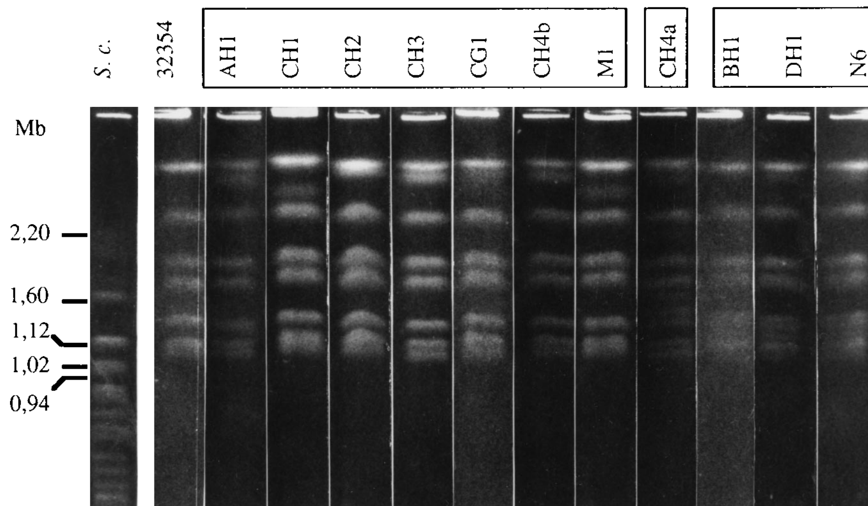

FIG. 5. Karyotypes of clinical *C. albicans* isolates. The origin of the isolate (with the first letter corresponding to the individual) is indicated at the top of each lane. Isolates are grouped (boxed lane headings) according to the similarity of the patterns on the basis of size variability of the chromosomal bands of <2.2 Mb. Molecular size standards were *S. cerevisiae* (*S.c.*) chromosomes.

determined (Table 1; Fig. 5). The first one was specific for isolate CH4a, the second one grouped the isolates from individuals B, D, and N, and the third one was composed of isolates from individuals A and M and the remaining isolates from patient C. The clinical isolates of *C. parapsilosis* shared the same karyotype, which was easily distinguished from that of the reference strain (Fig. 6).

(iii) AP-PCR analysis. Table 1 summarizes the results of the three AP-PCR assays which appeared to present the most comprehensive data [primers $(GGA)_7$, ERIC2, and ERIC1-ERIC2, respectively]. All three assays concordantly identified one genotype for the *C. parapsilosis* isolates and three genotypes for the *C. albicans* isolates. With $(GGA)_7$, patterns B and B′ were slightly different, but they did not present a sufficiently high number of polymorphisms to warrant the definition of a new, additional genotype (Fig. 7). The same was true for C and C′.

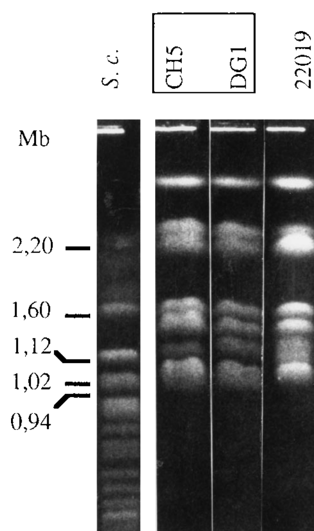

FIG. 6. Karyotypes of *C. parapsilosis* isolates. Total DNA from clinical isolates CH5 and DG1 (from patients C and D, respectively) and from the reference strain ATCC 22019 were compared by CHEF electrophoresis. Molecular size standards were *S. cerevisiae* (*S.c.*) chromosomes.

Table 1 also illustrates the concordance between AP-PCR and *Hin*fI RFLP results. The results of *Eco*RI RFLP were different with respect to strain grouping, because isolates with AP-PCR codes AAA and BBB could not be discriminated and isolate CH4a differed from the other isolates from patient C. Karyotyping also identified isolate CH4a from patient C as a novel type.

DISCUSSION

Since *Candida* septicemia is not unusual in burn patients, we attempted to determine whether the isolates responsible for candidemias in a burn care unit were genetically related. All *C. albicans* isolates were considered resistant to the azoles tested by a method that has been shown to correlate with treatment efficacy in AIDS patients with oropharyngeal candidiasis (5). Although the broth microdilution method is known to give higher values than the broth macrodilution method (15), the levels reached were high enough to be considered indicative of resistance. Since three patients treated with FCZ died after the onset of therapy, the question arises whether in vitro resistance was associated with therapeutic failure. The fact that cultures were still positive might be supporting evidence in this respect. In an experimental model of hematogenous candidiasis in mice, results of in vitro susceptibility testing are predictive of clinical outcome (1), but clinical investigators rarely rely on them (15). Clearly, ascertainment of the clinical relevance of the antifungal susceptibility testing methods in the setting of systemic infection requires additional studies. Usually, selected resistance to azoles, especially FCZ, is the result of prolonged or repeated treatments (4), and natural resistance has rarely been described (6). Because none of the *C. albicans* isolates recovered prior to the described episode in the ward was available, the genuine origins of the resistant isolates are not clear.

Genotyping methods can demonstrate the relatedness of fungal isolates. Molecular typing methods are not always reproducible, and results can be difficult to interpret because of the complexity of the patterns (13, 21). Misinterpretation and

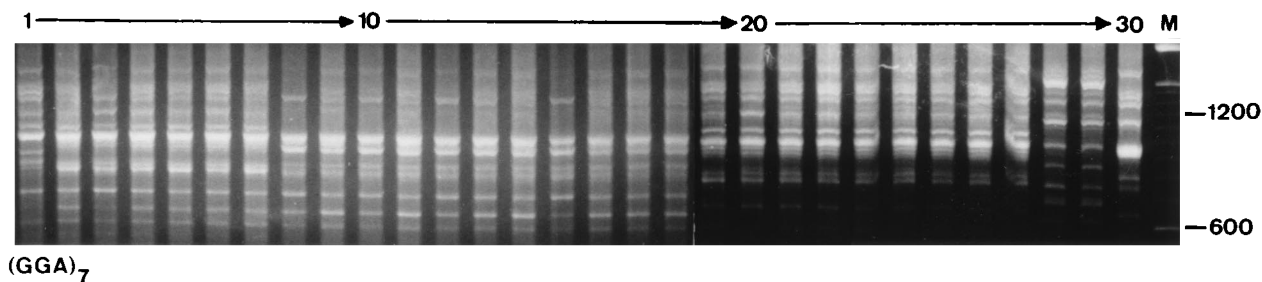

FIG. 7. AP-PCR patterns obtained with primer $(GGA)_7$ for *C. albicans* and *C. parapsilosis* clinical isolates. Isolates of *C. albicans* from patients A (lane 1), B (lanes 2 to 7), C (lanes 10 to 18) and D (lanes 19 to 21) and from nurses M (lanes 8 and 9) and N (lanes 22 to 27) as well as *C. parapsilosis* isolates from patient C (lane 28) and D (lane 29) and the reference strain *C. albicans* B311 (lane 30) were analyzed. Molecular size standards (in base pairs) are given in lane M (100-bp ladder from Pharmacia).

technical artifacts should be monitored closely by performing independent, duplicate assays. The procedures described in this report gave results which were identical on a day-to-day basis. Artifactual genetic relatedness can also result from a lack of discriminatory power of the enzyme used in RFLP studies. It has been demonstrated that for *C. albicans*, *Hin*fI provides better discrimination than *Eco*RI (9). Furthermore, *Hin*fI is much more comfortable to use because of the small number of fragments produced. Changes in some band positions are sometimes the result of epidemiologically nonrelevant genetic variability, as documented by CHEF electrophoresis in which size changes in the three large chromosome bands can be seen for clones isolated from the same mother cell (2). With AP-PCR also, minor variations observed for some isolates obtained on culture of specimens from the same patient were disregarded. No established rule for the interpretation of the AP-PCR data has been defined as yet. In a recent study on the reproducibility of AP-PCR assays for the typing of *Staphylococcus aureus*, it has been suggested that optimal typing is achieved by a combination of the results gathered by multiple assays (18). This implies that the restriction applied in the present study (ignore one band or staining intensity differences) is valid, but additional studies are certainly required. Finally, the performance of a given *C. albicans* typing scheme cannot be extrapolated to non-*C. albicans* species, as reported before for RFLP (20) and as shown here. The *C. parapsilosis* isolates were indistinguishable from the reference strain by RFLP analysis, whereas the CHEF electrophoresis was more discriminatory.

In accordance with previous studies (13, 21, 24), it is shown that some of the typing techniques should not be used alone and without using caution in the interpretation of the results (Table 1). In our study, two genotyping methods (CHEF electrophoresis and RFLP with *Eco*RI) and morphology singled out one isolate, CH4a, as being unique, whereas the results obtained by the other methods (RFLP with *Hin*fI and AP-PCR) demonstrated that CH4a was related to the other isolates from patient C. Whether patient C was infected with a single or multiple *C. albicans* isolates will remain unclear, but it has limited clinical implication. For the isolate from patient A also, interpretation of the results led to three different classifications, depending on the technique used (RFLP with *Eco*RI, CHEF analysis, or RFLP with *Hin*fI and AP-PCR). Although two techniques (RFLP with *Hin*fI and AP-PCR) gave similar results for all of the isolates tested after blind interpretation of the results, we cannot be sure that they are more reliable than the other techniques because of the limited number of patients and isolates studied. There are as yet no standard rules for the interpretation of differences between DNA fingerprints of any kind. We thus will recommend any combination of two different reproducible techniques (such as those used in the present study) when *C. albicans* typing is concerned.

Apparently, the cluster of cases of *Candida* septicemia observed in the Burn Care Unit was not due to a single isolate. Three different strains of *C. albicans* and one of *C. parapsilosis* were involved. Patients B and D were cross-infected by the same strain of *C. albicans*, and patients C and D were cross-infected (most probably) by the same strain of *C. parapsilosis*. It was also demonstrated that nurses carried on their clothes the isolate that was found to be responsible for the infection of at least one patient. The isolate from nurse N was identical to the isolates cultured from patients B and D. Patient C and nurse M also shared identical isolates. These results demonstrate at least the transmission of isolates from patients to nurses and suggest that the gowns provided a reservoir for the transmission of isolates from one patient to another. This indicates that the program of prevention should be improved in this ward. Besides the known vehicles of transmission like infusions, hands, or furniture (16, 17, 23, 25), the clothes of the health care workers could be involved.

The epidemiology of nosocomial *C. albicans* infection is complex, and a retrospective analysis of the factors contributing to the spread of isolates is probably of limited value. The mechanism by which our patients acquired their strains remains unclear, as is often the case, even during prospective studies (22). The present study also suggests that more frequent testing of in vitro susceptibility to azoles of the *C. albicans* isolates recovered in intensive care units could be useful for guiding antifungal therapy and for elucidating the question of natural resistance among isolates. Finally, since not all available *C. albicans* typing methods were used and since a limited number of isolates were studied, the perfect typing method has yet to be established. A reliable typing scheme should thus include a combination of two different typing techniques.

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