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DNA Fingerprinting of *Candida rugosa* via Repetitive Sequence-Based PCR

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A repetitive sequence-based PCR (rep-PCR) technique was developed to characterize the genotypic relatedness among *Candida rugosa* isolates. Two repetitive sequences, viz., Care-2 and Com29 from *Candida albicans*, were used to design primers Ca-21, Ca-22, and Com-21, respectively. When used alone or in combination, these primers generated discriminatory fingerprints by amplifying the adjacent variable regions of the genome. Twenty-three isolates from burn patients, eight from other human sources, and four *C. rugosa* isolates pathogenic in animals were placed into nine fingerprinting groups. Different primers placed these isolates into identical groups, indicating that rep-PCR is a specific and reproducible technique for molecular characterization of *C. rugosa*. Moreover, these primers unequivocally discriminated among other important *Candida* species such as *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, and *C. lusitaniae*. These data confirm the conservation of repetitive sequences in *Candida* species. Because of its ease and sensitivity, rep-PCR offers a relatively rapid and discriminatory method for molecular typing of *C. rugosa* in outbreaks.

The reported incidence of fungal infections has been increasing in hospitalized patients (30). *Candida* species have emerged as major opportunistic pathogens in a variety of settings, including AIDS (25, 27), primary and secondary fungemia (11, 15), bone marrow transplant recipients (26), and burn patients (17). *Candida rugosa* has been associated with hospital-acquired infections in burn patients (10), myelocytic leukemia (25), and catheter-associated infections (18). The mortality rates associated with such infections are extremely high, and their frequency appears to be increasing at an alarming rate (16).

DNA typing methods have advantages over other techniques in studying the genetic relatedness among pathogenic microorganisms that cause outbreaks. Several techniques have been developed for typing of *Candida* species in epidemiologic studies. These include restriction fragment length polymorphism (20, 24), Southern blot hybridizations probed with specific rRNA-encoding gene fragments (3), repetitive sequence Ca3 (8, 22, 23), and repeated sequence 27A (21). Recently, PCR has been applied to fingerprinting of genetically related microorganisms. The small and large rRNA genes have been amplified by PCR and analyzed by either restriction enzyme analysis (13) or hybridization with species-specific oligonucleotides to discriminate among different pathogenic fungi (14, 19). Eukaryotic and prokaryotic repetitive DNA motifs were also employed to generate PCR-based fingerprints to discriminate among *Candida albicans* isolates (28). Random amplification of polymorphic DNA was performed for typing of *C. albicans*, and the patterns were compared with karyotyping by pulsed-field gel electrophoresis and restriction fragment length polymorphisms (1, 2).

Karyotyping by pulsed-field gel electrophoresis and Southern blot hybridization appears to have maximum discriminating power among genetically related *Candida* isolates (1, 2, 23). However, these techniques are labor-intensive and time-consuming and require substantial expertise. Thus, there is a need for developing molecular typing techniques that are specific, rapid, and discriminatory and can be used routinely in hospitals for epidemiologic investigations.

TABLE 1. *C. rugosa* isolates from the Los Angeles County-University of Southern California Medical Center[a]

Isolate	Patient/site	Date of isolation (mo/day/yr)	Assigned type
92-1182	Environmental	03/10/92	A
92-1183	P1/wound	04/28/92	A
92-1184	P1/wound	06/02/92	A
92-1185	P1/blood	06/20/92	A
92-1186	P1/urine	06/30/92	A
92-1187	P2/wound	06/02/92	A
92-1189	P3/wound	06/19/92	A
92-1190	P3/blood	06/23/92	A
95-966	P4/sputum	02/09/95	G
95-967	P5/urine	09/29/94	A
95-969	P6/wound	08/02/94	H
95-970	P6/catheter tip	08/22/94	A
95-972	P7/wound	06/23/94	A
95-973	P5/urethra	10/06/94	A
95-974	P6/wound	08/15/94	A
95-975	P4/wound	09/06/94	A
95-976*	P8/urine	09/16/94	I
95-977**	P9/sputum	12/15/94	G
95-978**	P10/catheter tip	10/06/94	E
95-979	P7/wound	06/10/94	A
95-980	P4/wound	09/09/94	A
95-981	P4/knee	09/16/94	E
95-982	P11/sputum	08/22/94	F
95-984	P5/wound	11/03/94	A
95-985	P5/wound	09/22/94	A
95-986	P12/wound	06/21/94	A

[a] P, patient number; *, neurosurgical intensive care unit; **, medical intensive care unit. All other strains were isolated from burn units.

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TABLE 2. Other *C. rugosa* isolates[a]

Isolate	Source/site	Date of isolation (mo/day/yr)	Assigned type
92-1188*	P13/right humerus	06/14/92	B
R-922*	P14/blood	03/03/89	F
R-2276*	P15/oral thrush	07/10/93	G
93-662*	P16/blood	04/16/93	E
93-1647*	P17/urine	08/11/93	E
89-31**	Turtle/aspirate	01/20/89	C
89-75**	Penguin/trachea	02/16/89	D
89-134**	Beluga whale/blowhole	03/29/89	B
89-599**	Dolphin/blowhole	12/07/89	E

[a] P, patient number; *, isolates from other human sources; **, isolates from animal sources.

This report describes the development of a repetitive sequence-based PCR (rep-PCR) technique which discriminates among *C. rugosa* isolates from burn patients and other sources. Moreover, this procedure can be applied to other pathogenic *Candida* species.

MATERIALS AND METHODS

Yeast strains. Twenty-six isolates of *C. rugosa* (Table 1) were obtained from the Los Angeles County-University of Southern California Medical Center. Eight isolates (92-1182 to 92-1190) were collected within a span of 4 months in 1992 from the same burn ward (7). One isolate (92-1182) was obtained from an environmental sample from a portable glucose-monitoring device that had been used on multiple patients. The other seven isolates came from wound, blood, and urine samples of three patients. Eighteen isolates (95-966 to 95-986) were collected over a span of 8 months in 1994 and 1995 from nine patients. Of these, three isolates came from neurosurgical and medical intensive care units distant from the burn ward, while the remainder were derived from burn patients. Isolate 92-1188 was obtained from University of Southern California Medical Center at the same time that the initial 1992 isolates were collected from burn patients. It was isolated from an uninfected open humeral fracture of a patient in an orthopedic ward. Four isolates each from human and animal sources were obtained from culture collections of The Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio (Table 2). The clinical isolates were identified to species level by routine microbiological procedures.

The following *Candida* species were obtained from the American Type Culture Collection, Rockville, Md.: *C. rugosa* ATCC 58964, *C. albicans* ATCC 24433, *C. krusei* ATCC 6258, *C. lusitaniae* ATCC 42720, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750, *C. kefyr* ATCC 46764, and *C. parapsilosis* ATCC 22019. All isolates were maintained on Sabouraud agar medium (Difco Laboratories, Detroit, Mich.).

DNA isolation. Cultures were grown in 1.5 ml of YPD broth containing 1% yeast extract, 2% Bacto Peptone, and 2% glucose (Difco Laboratories) with shaking to late log or early stationary phase at 25°C. The DNA was extracted according to the method of Scherer and Stevens (21). After determination of the concentration with the GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Piscataway, N.J.), the DNA was aliquoted and stored at −20°C.

Designing of oligonucleotide primers. Of different repetitive sequences reported in *C. albicans*, Care-2 (GenBank accession number X53050) and RPS1 (GenBank accession number M87288) were selected as targets for the primers to be used in rep-PCR. Two primers from the Care-2 element (12) were designed, Ca-21 (5′-CATCTGTGGTGGAAAGTAAAC-3′) and Ca-22 (5′-ATAATGCT CAAAGGTGGTAAG-3′), and these spanned nucleotide positions 746 to 766 and 247 to 227, respectively. Their directions are opposite from each other so that they did not amplify repetitive regions. Instead, they amplified adjacent variable regions. Com-21 (5′-GCCGTTTTGGCCATAGTTAAG-3′) was derived from the highly repeated Com29 segment present in the RPS1 repetitive sequence (5, 9) and corresponds to 152 to 172 nucleotides. All the primers were checked for compatibility as well as hairpin and dimer formation by the Oligo Ver 4.0 software program (National Biosciences, Inc., Plymouth, Minn.).

Rep-PCR and gel electrophoresis. Optimization of amplification conditions was accomplished with the Stratagene Optiprime kit (Stratagene, La Jolla, Calif.). The PCR amplification was performed in a volume of 50 μl containing 200 ng of DNA, 10 mM Tris-HCl (pH 8.8), 3.5 mM $MgCl_2$, 25 mM KCl, 0.2 mM each deoxyribonucleotide triphosphate, 50 pmol of each primer, and 1.25 U of *Taq* polymerase (Perkin-Elmer, Branchburg, N.J.). These conditions were employed when Ca-21 and Ca-22, or Com-21 and Ca-22, were used in combination. However, when Com-21 was used alone, the primer concentration was increased to 100 pmol per reaction, and the pH of 10 mM Tris-HCl was changed to 9.2. The amplification was performed by an initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 5 min in a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer). Negative control reactions without any template DNA were carried out simultaneously. An absence of any band excluded the possibility of cross-contamination of DNA during the PCRs.

PCR products were analyzed by electrophoresis through 2% (wt/vol) Metaphor-XR agarose gel (FMC Bioproducts, Rockland, Maine) containing ethidium bromide (0.5 μg/ml) in 1× TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA). Metaphor-XR agarose was preferred to SeaKem GTG agarose because of its higher resolving power, resulting in sharper banding patterns. A 100-bp DNA ladder (BRL Life Technologies, Inc., Bethesda, Md.) was run concurrently with amplicons for sizing of bands. The rep-PCR profiles of *C. rugosa* isolates were examined in a blind fashion, and the blind was broken after the data sets were collected. The intensities of bands were considered while analyzing the fingerprinting patterns. The PCRs were repeated with different batches of DNA from all isolates to verify the reproducibility of the technique.

RESULTS

Optimization of PCR. The optimization of amplification conditions was accomplished with DNA from isolate 92-1183. A buffer containing 10 mM Tris-HCl (pH 8.8), 3.5 mM $MgCl_2$, and 25 mM KCl was used for Ca-21–Ca-22 and Com-21–Ca-22 primers. However, in Com-21-mediated PCRs, the same buffer adjusted to pH 9.2 yielded optimal fingerprints. The presence of 6 to 10 separate bands of equal intensities was used as the major criterion for determination of optimum conditions. An-

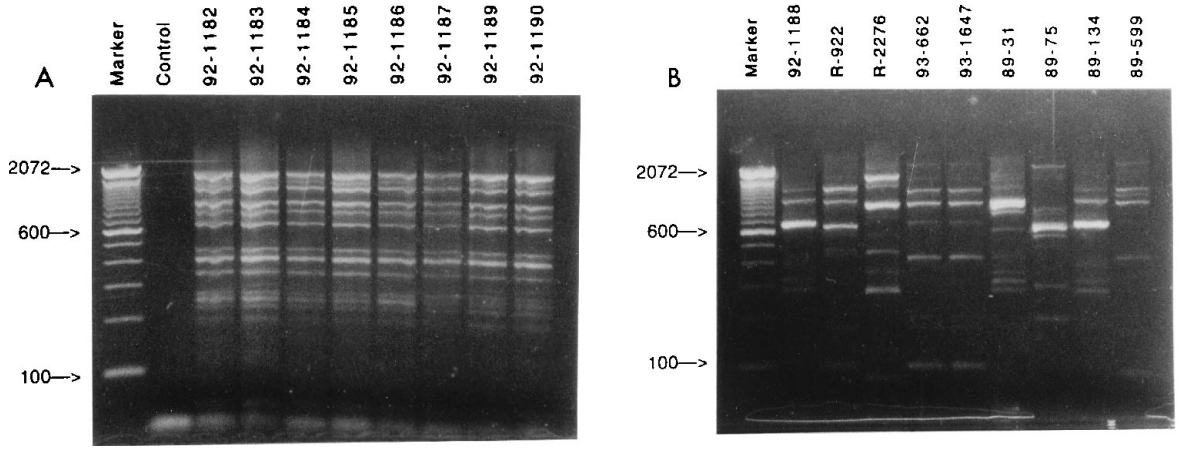

FIG. 1. Rep-PCR fingerprints of *C. rugosa* isolates. Primers Ca-21 and Ca-22 were used in PCRs, and the amplicons were resolved in a 2% Metaphor-XR agarose gel (for details, refer to Materials and Methods). (A) Fingerprints of eight isolates from burn patients; (B) fingerprints of five human and four animal isolates. Control denotes negative control reaction without template DNA, while molecular size markers (100 bp) are in the left lanes.

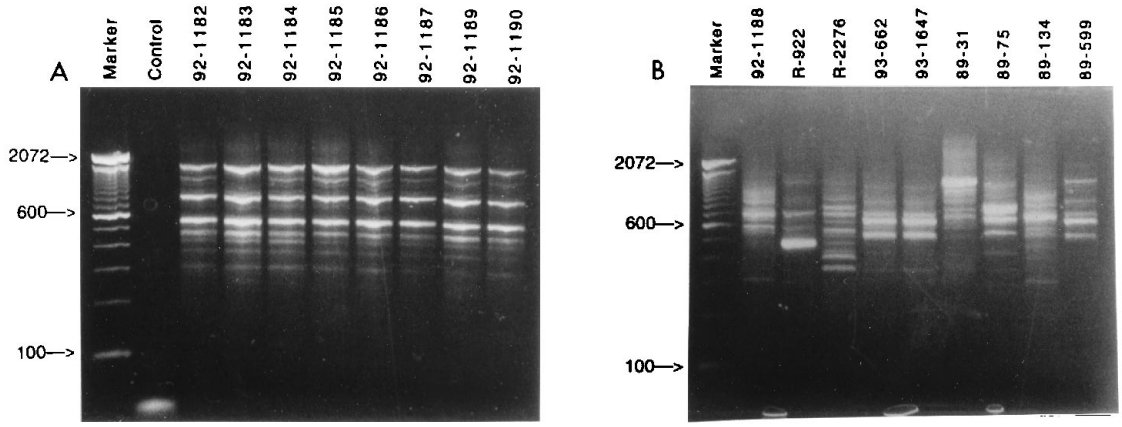

FIG. 2. Rep-PCR fingerprints of *C. rugosa* isolates. Primer Com-21 was used in PCRs. (A) Fingerprints of eight isolates from burn patients; (B) fingerprints of five human and four animal isolates. Control denotes negative control reaction without template DNA, while molecular size markers (100 bp) are in the left lanes.

nealing temperatures ranging from 40 to 50°C were tested, and 45°C was chosen as the optimum annealing temperature for fingerprinting. At temperatures over 45°C, the number of bands remained the same; however, certain bands were preferentially amplified, giving rise to differences in band intensities (data not shown). Additives, such as bovine serum albumin, formamide, glycerol, dimethyl sulfoxide, ammonium sulfate, and Perfect Match DNA polymerase enhancer from the Optiprime kit (Stratagene) were tested. No significant improvements were observed with the use of these additives. All three primers were used alone and in all possible combinations to examine which primer(s) generated acceptable banding patterns and whether certain combinations offered greater discriminatory patterns.

Analysis of rep-PCR results and typing of *C. rugosa* isolates. Three primer combinations (Ca-21 plus Ca-22, Com-21 alone, and Com-21 plus Ca-22) were used to fingerprint 17 isolates of *C. rugosa* from burn patients and other human and animal sources. Eight isolates from burn patients exhibited fingerprinting patterns identical to those of Ca-21 plus Ca-22 (Fig. 1A), Com-21 alone (Fig. 2A), and Com-21 plus Ca-22 (Fig. 3A) primers. Only one strain (92-1183 [Fig. 2A]) did not show the presence of one band of ≈1,300 bp in Com-21-generated fingerprints. However, a single low-intensity band difference was not considered significant, and 92-1183 was classified as analogous to the other isolates.

Discriminatory banding patterns were observed when Ca-21 plus Ca-22 (Fig. 1B), Com-21 alone (Fig. 2B), and Com-21 plus Ca-22 (Fig. 3B) were used as primers for fingerprinting *C. rugosa* isolates from humans and animals. Isolates 92-1188 and 89-134 exhibited identical fingerprints. Three other isolates (93-662, 93-1647, and 89-599) displayed identical patterns, except for the presence of an additional band of ≈1,000 bp in 89-599 (Fig. 1B, last lane). However, the fingerprints of these three isolates were indistinguishable when run on 2% SeaKem GTG agarose (data not shown). It is possible that the additional band visible in Fig. 2A may be due to the better resolving power of Metaphor-XR agarose. The remaining four isolates yielded fingerprints entirely different from each other.

Eighteen additional *C. rugosa* isolates were fingerprinted to test the discriminatory power of this technique. One primer pair (Ca-21 plus Ca-22) was selected on the basis of previous results and used in fingerprinting these isolates. Eleven of these isolates displayed identical fingerprints (Fig. 4), which were the same as those previously isolated from burn patients. Three isolates (95-976, 95-977, and 95-978) collected during the same time from patients in intensive care units distant from the burn ward were used as controls.

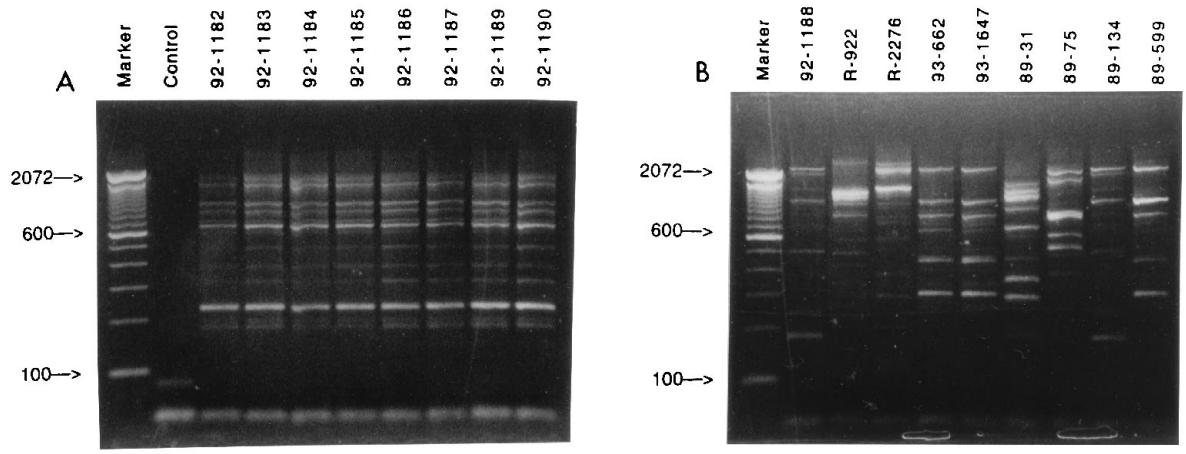

FIG. 3. Rep-PCR fingerprints of *C. rugosa* isolates. Primers Com-21 and Ca-22 were used in PCRs, as described above. (A) Fingerprints of eight isolates from burn patients; (B) fingerprints of five human and four animal isolates. Control denotes negative control reaction without template DNA, while molecular size markers (100 bp) are in the left lanes.

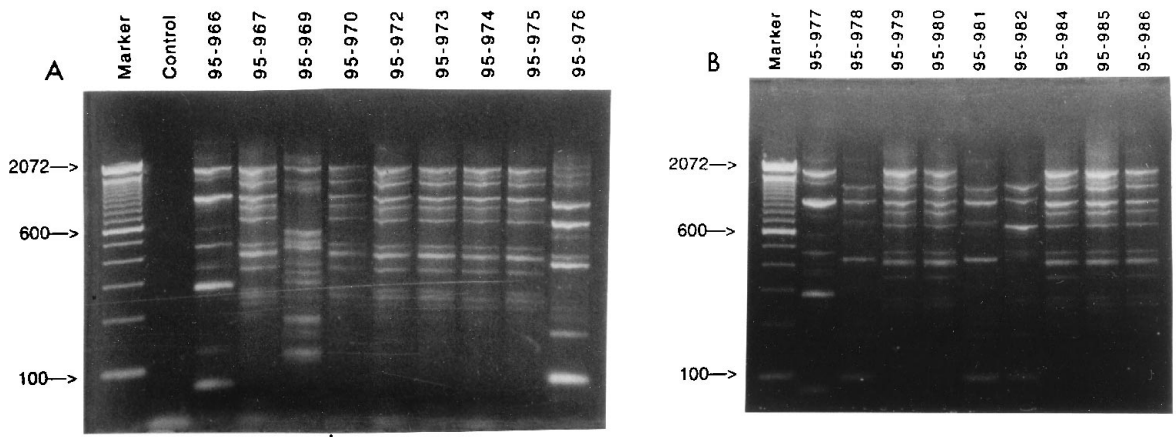

FIG. 4. Rep-PCR fingerprints of additional *C. rugosa* isolates. Primers Ca-21 and Ca-22 were employed in these PCRs, which are described in Materials and Methods. (A) Fingerprints of nine isolates; (B) fingerprints of remaining nine isolates. Control lane represents negative control reaction without template DNA, while molecular size markers (100 bp) are in the left lanes.

Twenty-three isolates of *C. rugosa* from burn patients, eight from other humans, and four isolates of animal origin were typed on the basis of fingerprinting patterns. These isolates were placed in nine distinct groups, viz., A (19), B (2), C (1), D (1), E (5), F (2), G (3), H (1), and I (1). Representative molecular fingerprints from each group are displayed in Fig. 5. The group to which individual isolates belonged is indicated in Tables 1 and 2. The majority of isolates from burn patients belonged to group A. Two other significant groups (E and G) contained burn and human pathogens, whereas the rest of the groups appeared to be unique. The animal isolates were different from burn and human isolates with the exception of 89-599. The other primer pair (Com-21 plus Ca-22) was also used on additional *C. rugosa* isolates. The resulting patterns placed these isolates in the same fingerprinting groups (data not shown).

Fingerprinting of different *Candida* species. Various strains of *Candida* species were employed to demonstrate the application of this technique. The fingerprinting patterns of different ATCC strains are displayed in Fig. 6. All the strains exhibited different patterns, indicating that this procedure can have a wide range of applications. It also indicated conservation of Care-2 and Com29 repetitive sequences in *Candida* species.

DISCUSSION

The objective of this study was to develop a rep-PCR-based fingerprinting technique to discriminate among different *C. rugosa* isolates from burn patients and apply it to other *Candida* species. The requisites for such a technique are that it should be simple, relatively rapid, highly discriminatory, cost-effective, and not labor-intensive and should allow analysis of large numbers of samples. Moreover, the procedures should be technically less complex (not requiring sophisticated skills) and capable of being performed by hospital technologists.

Rep-PCR has been used in fingerprinting bacterial strains in epidemiologic investigations (6, 29). This strategy utilizes primers that are derived from seemingly randomly distributed repetitive sequences on the genome to amplify adjacent variable regions. The moderately repeated Care-2 element and highly repeated Com29 domain were used to select primers for *C. rugosa* from several repetitive sequences reported in *C. albicans.*

The fingerprinting patterns of *C. rugosa* isolates were unambiguous, reproducible, and discriminatory. The differences in patterns are an indication of distances between repetitive tar-

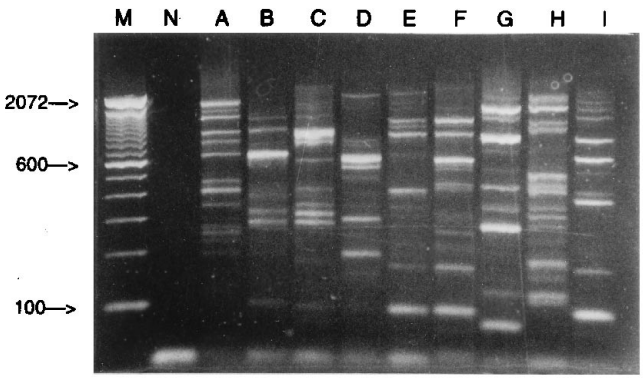

FIG. 5. Rep-PCR fingerprints of *C. rugosa* isolates representing nine groups. Primers Ca-21 and Ca-22 were utilized in PCRs. Thirty-five strains from burn, human, and animal sources were placed in nine distinct groups (refer to Results for details). Lane M represents molecular size markers (100 bp), while lane N denotes negative control reaction without template DNA.

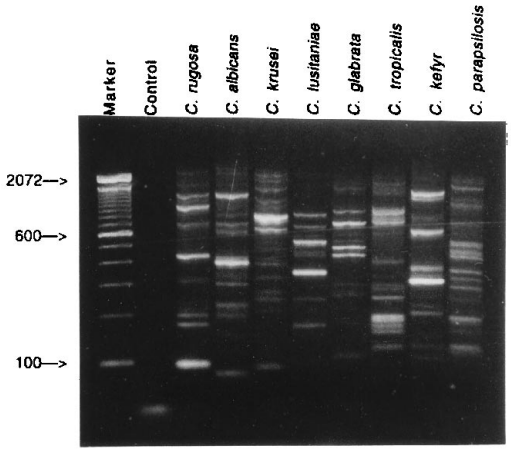

FIG. 6. DNA fingerprints of different *Candida* species. Primers Ca-21 and Ca-22 were utilized in PCRs, and the amplicons were analyzed as described above. Control denotes negative control reaction without template DNA, while molecular size markers (100 bp) are in the left lane.

get sequences along the chromosomes. It is significant that, regardless of the primer(s) used, the isolates were placed in similar fingerprinting groups. This indicates that the rep-PCR strategy adopted in the present study displays a high degree of specificity. Similarly, the same fingerprinting patterns were observed when repeated on different batches of DNA, suggesting the reproducibility of the technique. One of the isolates (92-1182) obtained from a portable glucose-monitoring device displayed fingerprints identical to those of the majority of isolates from burn patients (group A), indicating genetic relatedness. It is noteworthy that this device was used on multiple patients (7) and may have contributed to the outbreak. Moreover, these burn isolates have exhibited similar susceptibility patterns for various antifungal antibiotics, including the unusual characteristic of nystatin resistance coupled with amphotericin B susceptibility (7), which corroborates their fingerprinting patterns. This supports the general notion that most of the *C. rugosa* infections are acquired in hospitals (7, 10). It is also interesting to note that the early burn ward isolates (1992) were identical but that the later ones (1994) were more heterogeneous, suggesting the introduction of new strains. Another significant observation was that the pattern of 95-978, an isolate from a location physically distant from the burn unit, was identical to those of one isolate (95-981) from the burn ward and two other isolates from humans from distant geographic sites (93-662, 93-1647).

A total of nine distinct banding patterns were identified in *C. rugosa* isolates. This catalog of bands is by no means complete, and additional groups could be identified on the continuation of this work. Isolates belonging to group A appear to be predominant in burn patients analyzed in this study. This is consistent with the theory that pathogenic *C. rugosa* isolates have emerged and persisted because of extensive use of topical nystatin (7), wherein a single strain could be selected over a period of time.

The data indicate conservation of at least two repetitive sequences in *Candida* species whose biological function is not known. Thus fingerprinting by rep-PCR has potential for use in typing and differentiation in *Candida* species. This is important, as it appeared to be impossible to discriminate between *C. albicans* and *C. tropicalis* when the cytochrome P-450–L_1A_1 gene from four major human pathogenic *Candida* species was analyzed by PCR (4). In yet another report, a combination of techniques such as amplification of small rRNA gene followed by reamplification of variable sequences by nested PCR and probing of these amplicons by species-specific oligonucleotides was used to discriminate between *C. albicans* and *C. tropicalis* (14). The method described in this report could provide an alternative to labor-intensive typing methods in *Candida* outbreaks.

In conclusion, the rep-PCR technique could be used routinely for typing or discrimination of *C. rugosa* isolates in epidemiologic investigations because of its ease, sensitivity, and reproducibility. This methodology also portends application to other species of this genus.

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