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Comparison of Four DNA-Based Methods for Strain Delineation of *Candida lusitaniae*

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Four methods for the accurate delineation of epidemiologically related and unrelated strains of *Candida lusitaniae* were compared. Three pulsed-field electrophoretic methods, including two contour-clamped homogeneous field gel electrophoresis methods (EKP-1 and EKP-2) yielding electrophoretic karyotype patterns of intact chromosomal DNA and a method in which the chromosomal DNA was macrodigested with the endonuclease *Sfi*I prior to pulsed-field electrophoresis (MDP), and a random amplified polymorphic DNA (RAPD) assay were evaluated. A selected panel of 21 well-characterized isolates representing 13 strains of *C. lusitaniae*, including 7 epidemiologically related isolates of one strain (group I-A), 3 epidemiologically related isolates of another strain (group I-B), and 11 epidemiologically unrelated isolates (group II), were tested. All isolates were coded and tested in a blinded manner. All seven group I-A isolates were confirmed to be a single strain by the EKP-1 and MDP methods, and the three group I-B isolates were shown to be a single strain by the EKP-1, EKP-2, MDP, and RAPD methods. Subtle differences were noted with two of the group I-A isolates by the EKP-2 method, whereas three of these isolates were different by the RAPD method. Each group II isolate had distinct patterns by all four methods. These data support the fact that the three pulsed-field electrophoretic methods and the RAPD method can be used to delineate strains of *C. lusitaniae*. The EKP-1, EKP-2, and MDP gave results that correlated with the epidemiologic characteristics of the isolates tested in the study, whereas the RAPD method was perhaps too sensitive in detecting DNA changes for epidemiologic studies.

Candida lusitaniae has emerged as a pathogen in compromised patients, predominantly in granulocytopenic patients undergoing cytoreductive chemotherapy for acute leukemia and in bone marrow transplantation recipients (1, 3–5, 9, 10, 12, 13). Although this species is less virulent than most other *Candida* species, it is important because of its propensity to develop resistance to amphotericin B (3, 4, 10, 11–13).

In order to investigate the colonization with *C. lusitaniae* or the transmission or pathogenesis of infections with *C. lusitaniae*, methods for strain delineation that provide discriminatory power that is as reliable as a fingerprint are necessary. To date, several methods have been developed and tested with *C. lusitaniae*, including isoenzyme profiles (11), restriction enzyme fragment length polymorphisms (14, 15), random amplified polymorphic DNA (RAPD) patterns (8), and karyotype patterns generated by pulsed-field electrophoresis (PFE) (11, 14). Few studies have compared these DNA typing methods.

The purpose of the study described here was to compare three PFE methods and a RAPD method for the accurate delineation of epidemiologically related and unrelated strains of *C. lusitaniae*. Two contour-clamped homogeneous field gel electrophoresis (CHEF) procedures previously described in the literature which generate electrophoretic karyotype (EK) patterns of intact chromosomal molecules were evaluated. In the third method, the chromosomal DNA was macrodigested with a restriction enzyme and the patterns of DNA fragments were visualized following PFE. Finally, a RAPD assay with four different primers was also evaluated.

MATERIALS AND METHODS

Organisms. A panel of 21 isolates was studied. These 21 isolates represented 13 strains of *C. lusitaniae*, including seven epidemiologically related isolates of a single strain (*C. lusitaniae* 1 to 7) designated group I-A as described by King et al. (7), three epidemiologically related isolates (group I-B) recovered from a single patient (*C. lusitaniae* 8 to 10), and 11 epidemiologically unrelated isolates (group II) (*C. lusitaniae* 11 to 21). All isolates were identified by use of the API 20C System (bioMerieux Vitek, Inc., Hazelwood, Mo.) and the Vitek YBC System (bioMerieux Vitek, Inc.). The isolates were coded and tested in a blinded manner. Each laboratory performed its own assay. EKP-2 and MDP were performed in the same laboratory.

CHEF method I (EKP-1). The EK patterns of intact chromosomal DNA molecules were generated by the CHEF method (2) as described previously (11). Briefly, yeast DNA samples were prepared as follows. Cells grown overnight in YEPD broth (yeast extract, 10 g/liter; peptone, 10 g/liter; D-glucose; 20 g/liter) were washed and suspended in low-melting-point agarose. Spheroplasts were produced overnight by incubation of the agarose plugs with Zymolyase 20T (ICN Immunobiologicals, Lisle, Ill.). The spheroplasts were lysed, and the proteins were digested by overnight incubation with *N*-lauroylsarcosine and proteinase K at 45°C. A 25-μl aliquot of the agarose plugs was loaded into dry wells of a gel (1% agarose in 44 mM Tris-borate–2 mM sodium EDTA [pH 8.3]), and CHEF was performed by circulating the same Tris-borate–EDTA buffer (0.5×) at 10°C with 3-min alternating pulses of 120 V for 20 h; this was followed by 6-min alternating pulses of 120 V for another 20 h. Gels were stained with ethidium bromide, and the bands were visualized with UV light (>354 nm). A single isolate was included in each gel, yielding identical patterns each of the 12 times that it was analyzed.

CHEF method II (EKP-2). Yeasts grown in 10 ml of YEPD broth were packed by centrifugation (1,000 × *g*, 5 min) and washed twice with 50 mM sodium EDTA (pH 8.0) and were suspended in 150 μl of 50 mM sodium EDTA (pH 8.0). The cells were mixed with 80 μl of yeast cell wall-degrading enzymes (Lyticase; L5263, partially purified grade; 1,250 U/ml in 50% [vol/vol] glycerol–0.01 M $NaPO_4$ [pH 7.5]; Sigma Chemical, St. Louis, Mo.), and the mixture was incubated at 37°C for 20 min. Following incubation, 0.56 ml of 1% low-melting-point agarose (Bio-Rad, Richmond, Calif.) was added to each tube at 58°C. A total of 400 μl of the yeast-agarose suspensions was placed in individual molds (Bio-Rad), and the molds were placed at 4°C for 20 min. Individual agarose inserts were incubated at 50°C for 15 h in 1.5 ml of buffer (10 mM Tris-HCl [pH

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TABLE 1. Results of three PFE methods and a RAPD method for delineation of *C. lusitaniae* strains

Epidemiologic group	*C. lusitaniae* isolate	Pattern by the following method[a]:			
		EKP-1	EKP-2	MDP	RAPD
Group I-A					
	1	A	A	A	A
	2	A	B[b]	A	B
	3	A	A	A	C
	4	A	A	A	D
	5	A	A	A	A
	6	A	C[b]	A	A
	7	A	A	A	A
Group I-B					
	8	B	D	B	E
	9	B	D	B	E
	10	B	D	B	E
Group II					
	11	C	E	C	F
	12	D	F	D	G
	13	B[c]	D[c]	B[c]	NT[d]
	14	E	G	E	H
	15	F	H	F	I
	16	G	I	G	J
	17	H	J	H	K
	18	I	K	I	NT
	19	J	L	J	L
	20	K	M	K	NT
	21	L	N	L	M

[a] Each letter represents a different pattern (strain) by the specific method for that column only; comparisons of letters cannot be made among the columns.

[b] Subtle differences were noted with this isolate compared with the other group I-A isolates.

[c] Pattern was identical to that for *C. lusitaniae* 8, 9, and 10. The patient from whom this isolate was obtained was hospitalized in the same institution and at the same time as the patient from whom isolates 8, 9, and 10 were recovered.

[d] NT, not tested by the RAPD method.

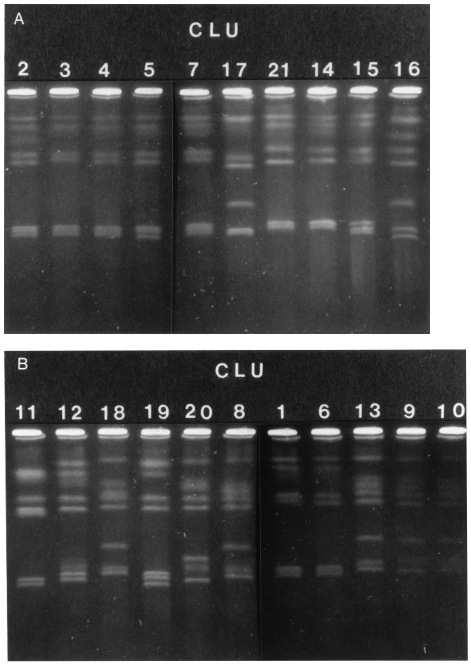

FIG. 1. Electrophoretic karyotype patterns of intact chromosomal DNAs generated by the EKP-1 method. *C. lusitaniae* (CLU) isolate numbers are provided above each lane.

7.5], 450 mM EDTA [pH 8.0], 1% *N*-lauroylsarcosine) containing proteinase K (1 mg/ml; protease type XVIII; 20 U/mg; Sigma), washed three times with 50 mM sodium EDTA (pH 8.0), incubated overnight at 25°C, washed three more times, and stored in 50 mM EDTA (pH 8.0) at 4°C. The inserts were loaded into wells of a 0.8% agarose gel in 0.5× TBE (0.090 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]), and electrophoresis was carried out in the same TBE buffer at 13°C by using a CHEF-DR II apparatus (Bio-Rad) with 120-s pulses of 150 V for 24 h; this was followed by 240-s alternating pulses at 150 V for 36 h. The gels were stained with ethidium bromide. DNA extracted from *Saccharomyces cerevisiae* was run as a size marker. Reproducibility by this method was ≥95% on the basis of replicate determinations.

CHEF method III (MDP). CHEF method III (MDP) was used for generating patterns of macrodigested chromosomal DNA molecules. Agarose plugs containing chromosome-sized DNA were prepared as described above for the EKP-2 method and were placed into 100 mM Tris-HCl buffer (pH 8.0) containing 5 mM magnesium chloride. After three washes, the agarose inserts were placed in 100 μl of buffer containing 20 U of the low-frequency-cutting restriction endonuclease *Sfi*I (Gibco BRL Life Technologies Inc., Gaithersburg, Md.). Digestion was performed overnight as directed by the endonuclease manufacturer. CHEF PFE was performed at 13°C for 24 h at 200 V in a 1% agarose gel (Bio-Rad). Pulse times were ramped from 5 s to 35 s throughout the 24-h period. Bacteriophage lambda ladder DNA (Bio-Rad) was used as a standard molecular weight marker on each gel. Ethidium bromide-stained agarose gels were inspected visually. Reproducibility by this method was ≥95% on the basis of replicate determinations.

RAPD assay. *C. lusitaniae* strains were subcultured onto Sabouraud dextrose agar (Difco, Detroit, Mich.) plates, and the plates were incubated for 18 to 24 h at 37°C in 5% CO_2 in air. DNA extraction was carried out as described by Woods et al. (16), with slight modification. A heavy suspension (greater than a McFarland no. 5 standard) was prepared in 1 ml of sterile saline in a 1.5-ml microcentrifuge tube, the tubes were centrifuged at 16,000 × *g* for 2 min, and the pellet was resuspended in 0.2 ml of 0.25 M Tris buffer containing 1.5% sodium dodecyl sulfate and 0.1 mM EDTA. The cap was secured with a cap lock (PGC Scientifics, Gaithersburg, Md.), and the suspension was boiled for 30 min, chilled on ice, extracted twice with phenol-chloroform (4:1) and once with chloroform, and then precipitated with ethanol. The recovered DNA was dissolved in water and quantitated by measuring the A_{260}, and purity was assessed by determining the A_{260}/A_{280} ratio. PCR was performed with 200 ng of DNA as a template and one of the four primers under the following conditions: Stoffel buffer (Cetus, Emeryville, Calif.); 3 mM $MgCl_2$; 400 nM primer (primer 1247, 1253, 1281, or 1283 as described by Kersulyte et al. [6]); 200 μM (each) dATP, dTTP, dCTP, and dGTP; and 5 U of Stoffel fragment *Taq* DNA polymerase (Cetus) in a final volume of 50 μl with a sterile light mineral oil overlay. Samples were denatured at 94°C for 3 min with a Perkin-Elmer 480 thermocycler; this was followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, ending with 7 min at 72°C. The reaction products (25%) were analyzed by electrophoresis in 2% Seakem-NuSieve GTG agarose (3:1) in 1× TBE buffer at 8 to 10 V/cm, with visualization of the bands performed following staining with ethidium bromide.

Analysis of DNA banding patterns. The DNA banding patterns obtained by the EKP-1, EKP-2, and MDP methods were inspected visually. Differences of one or more discrete bands among the isolates was used as the criterion sufficient to designate different strains. By the RAPD method, all four patterns for the isolates needed to be identical in order to designate the isolates as being a single strain; any difference in DNA pattern obtained with any one of the four primers was sufficient to designate different strains.

RESULTS

Patterns from many gels were analyzed. Gels illustrating the results obtained by the four methods are presented in Fig. 1 to 4. The results of all assays are collated in Table 1.

Twelve DNA patterns (Fig. 1A and B) that were interpreted as indicating distinct strains were detected among the 21 isolates by the EKP-1 method. All seven group I-A-related isolates were identical. The minor difference noted between *C. lusitaniae* 2 and 7 was not apparent when the isolates were run on the same gel. All three group I-B related isolates were also identical. Ten of the 11 isolates unrelated to group II isolates were distinct, although *C. lusitaniae* 14 and 21 were quite similar. *C. lusitaniae* 13 in group II had a pattern identical to those of the group I-B isolates. In retrospect, it was found that the patient from whom *C. lusitaniae* 13 was recovered was hospitalized at the same institution and at the same time as the

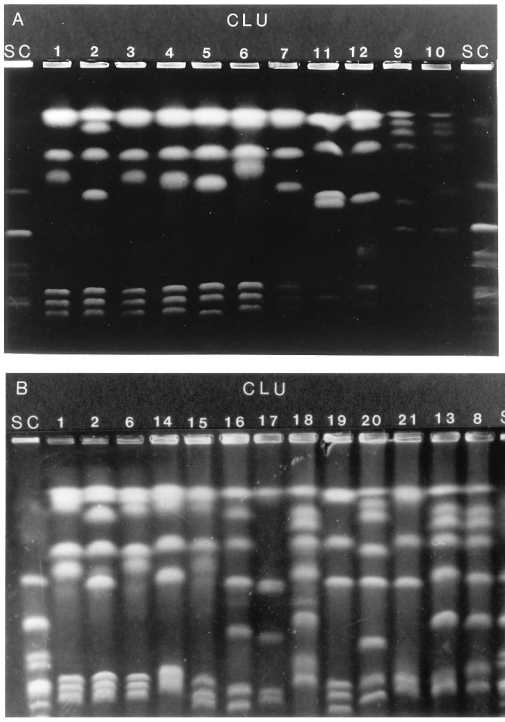

FIG. 2. Electrophoretic karyotype patterns of intact chromosomal DNAs generated by the EKP-2 method. *C. lusitaniae* (CLU) isolate numbers are provided above each lane; SC, *S. cerevisiae.*

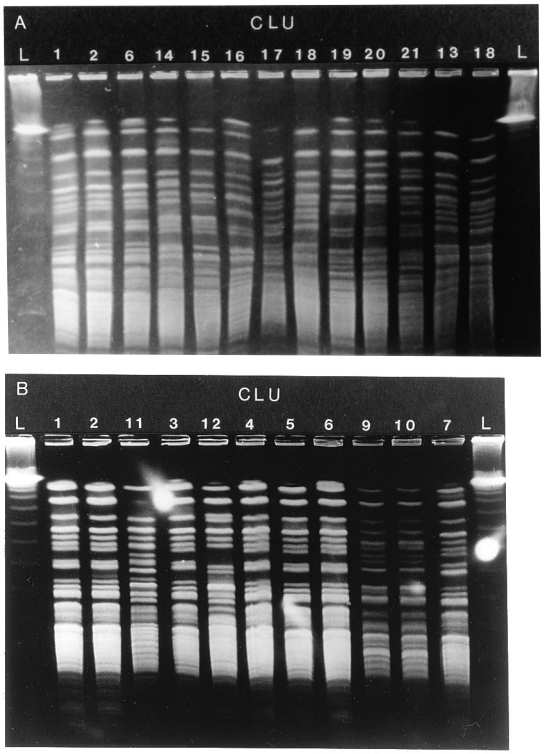

FIG. 3. Patterns of DNA fragments generated by the MDP method. *C. lusitaniae* (CLU) isolate numbers are provided above each lane; L, a bacteriophage lambda DNA ladder as size markers.

patient from whom group I-B isolates (*C. lusitaniae* 8 to 10) were recovered.

Fourteen DNA patterns (strains) were noted among the 21 isolates by the EKP-2 method (Fig. 2A and B). Three patterns were noted within the seven group I-A-related strains. However, two isolates (*C. lusitaniae* 2 and 6) had minor differences. All three group I-B isolates and *C. lusitaniae* 13 had identical patterns, whereas all of the unrelated isolates in group II had distinct patterns.

The MDP method delineated 12 strains among the 21 isolates tested, since 12 DNA patterns were detected (Fig. 3A and B). All related isolates in group I-A had the same pattern. The group I-B isolates and *C. lusitaniae* 13 had identical patterns. The remaining 10 unrelated group II isolates each had a distinct pattern.

The RAPD method delineated 14 strains among the 18 isolates tested. The delineation was based on any difference in a DNA pattern with any of the four primers tested (Fig. 4A to D). Four strains were delineated among the seven related isolates in group I-A, although one isolate (*C. lusitaniae* 3) had a pattern identical to those of four other isolates with one set of primers, primer set 1281. The three related isolates in group I-B had identical patterns, and all unrelated isolates in group II had distinct patterns.

DISCUSSION

All four methods tested in the present study can be used to distinguish epidemiologically related from unrelated strains of *C. lusitaniae*. The results confirm previous studies with this yeast species and others which showed that an individual patient is usually colonized and/or infected with a single strain (13).

Overall, there was agreement among the four methods evaluated. The three PFE methods (EKP-1, EKP-2, and MDP) yielded identical results (100% agreement) with 12 strains among the 21 isolates, if minor differences were considered insignificant. Minor differences probably indicate an epidemiologic relatedness but not identity. If, however, these differences were considered significant, then the EKP-1 and the MDP methods still agreed 100%, whereas the EKP-2 method showed a 90.5% agreement with the EKP-1 method (19 of 21 isolates). Therefore, these three assays were of equal value for assessing epidemiologically related and unrelated strains of *C. lusitaniae.*

The RAPD method, evaluated in the present study by using four primers capable of amplifying multiple DNA fragments of various sizes, had more discriminatory power than the PFE methods. The RAPD assay distinguished 14 strains, whereas the PFE methods distinguished 13 strains, among the 18 isolates evaluated by all four methods. Four strains were distinguished among the seven epidemiologically related group I-A isolates; one strain was represented four times and the other three strains were represented once each. Minor DNA changes possibly owing to a single-base change can be detected by the RAPD assay, especially when using four primers, whereas major changes must be present before the PFE methods can detect differences. These seven related isolates were collected over a period of time, and DNA changes may have occurred; however, there is strong evidence that they are closely related. Data to support the fact that they are closely related, if not identical, are as follows: (i) all seven strains were recovered from a single hospital unit over 2 months; (ii) all seven isolates had a second marker and all were highly resistant to amphotericin B; and (iii) all seven strains had a third marker and all were resistant to flucytosine. Minor alterations in PCR condi-

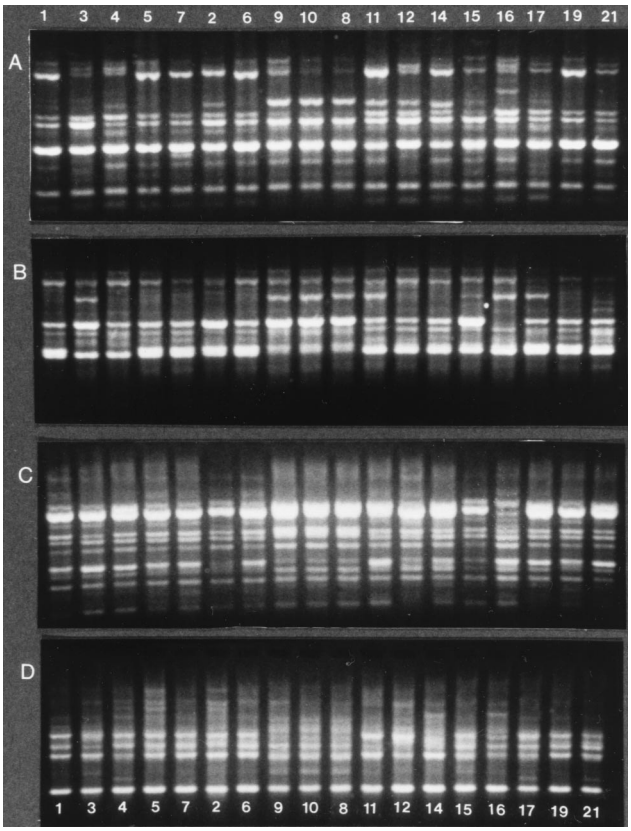

FIG. 4. Patterns of DNA fragments generated by the RAPD method. *C. lusitaniae* isolate numbers are provided above each lane. A, DNA patterns obtained with primer 1247; B, DNA patterns obtained with primer 1253; C, DNA patterns obtained with primer 1281; D, DNA patterns obtained with primer 1283.

tions can result in significant alterations in RAPD patterns. Thus, problems with reproducibility were considered and eliminated. A major limitation of any assay, and especially the three PFE methods, is that large numbers of organisms cannot be run on a single gel. This limitation should be recognized, and several gels should be run to compare individual isolates directly. Overall, it seems reasonable to conclude that the RAPD assay is more discriminatory than the PFE methods; however, it may be overly sensitive because it identifies differences among epidemiologically related as well as epidemiologically unrelated isolates.

In summary, all four methods described here could be used to distinguish strains of *C. lusitaniae*. The RAPD method was more discriminatory than the three PFE methods and might be a powerful tool for detecting minor changes in a population. Results of the PFE methods correlated better with the epidemiologic relationships than did the results of the RAPD method. Application of these methods will help to expand our knowledge about this interesting opportunistic fungal pathogen, and these methods can now be used to investigate potential outbreaks. Currently, since extensive equipment is necessary, these techniques probably should be relegated to reference laboratories until simpler methods can be developed.

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