

Comparison of PCR- and *HinfI* Restriction Endonuclease-Based Methods for Typing of *Candida krusei* Isolates

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We compared *HinfI* restriction endonuclease-based analysis of genomic DNA with a PCR-based method for molecular typing of 90 *Candida krusei* isolates from 17 geographically related patients. Strain groupings by these methods were the same for 89 of 90 isolates. Ten of 17 patients were infected with related strains of *C. krusei*.

Candida krusei is an important opportunistic cause of nosocomial candidiasis due to its intrinsic resistance to fluconazole (1, 2, 9). To date, only a few studies have examined the epidemiology, mode of transmission, and pathogenesis of infection for this species (4, 9, 10, 11). Molecular strain typing is a key tool in such investigations. Both *HinfI* restriction endonuclease-based analysis (6, 8, 10) and PCR (7) have been described for this purpose, and we report here a comparison of those approaches for a collection of 90 *C. krusei* isolates from 17 geographically related patients.

A total of 90 *C. krusei* isolates from 17 patients collected from the Fred Hutchinson Cancer Research Center from 1995 to 1998 were studied. *C. krusei* ATCC 6258 (American Type Culture Collection, Rockville, Md.) was also evaluated as a standard strain. The isolates were identified with the API 20C AUX system (bioMérieux Vitek, Inc., Hazelwood, Mo.) according to the manufacturer's instructions.

DNA was extracted as described by Scherer and Stevens with slight modifications (12). A 1.5-ml volume of an overnight growth in YPD medium (1 g of yeast extract, 2 g of Bacto-Peptone, 2 g of glucose per 100 ml of sterile distilled water) was pelleted by centrifugation and washed with 1 M sorbitol. Pellets were resuspended in 1 ml of 1 M sorbitol–50 mM potassium dihydrogen phosphate buffer (pH 7.5) containing 0.2 mg of Zymolase 20T and 0.1% (vol/vol) β -mercaptoethanol per ml and incubated at 30°C for 1 h. After a centrifugation, the resulting spheroplasts were incubated with a lysis buffer (2-mg/ml sodium dodecyl sulfate, 50 mM EDTA at pH 8.5) for 30 min at 65°C. After proteins were precipitated by addition of 5 M potassium acetate, the supernatant was treated with 10-mg/ml RNase (Sigma Chemical Co., St. Louis, Mo.) at 37°C. The DNA was precipitated by addition of 7.5 M ammonium acetate and 100% cold ethanol. Following the centrifugation,

the pelleted DNA was rinsed with 70% cold ethanol, resuspended in TE solution (50 μ l of 10 mM Tris chloride buffer, pH 7.5, 1 mM EDTA), and stored at 4°C.

DNA samples (15 μ l) were subjected to a 3-h digestion at 37°C with *HinfI* (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions and then separated by electrophoresis at 30 V for 18 h in 0.8% (wt/vol) agarose (type II medium EEO; Sigma Chemical Co.) gel in TBE (Tris-borate-EDTA) buffer. A 1-kb DNA ladder (Invitrogen) was used as a molecular marker. The gel was briefly soaked in ethidium bromide (0.5 μ g/ml) and photographed under UV illumination.

PCR was performed with the previously described *C. krusei*-specific primer pair Arno1 and Arno2 (7) designed for the amplification of a specific variable region of *C. krusei* repeated sequence 1 (CKRS-1). The reaction mixture (50 μ l) contained 10 pmol of each primer, PCR Master Mix (Promega), and 1 μ l of template DNA. Cycling conditions consisted of 4 min at 92°C; followed by 32 cycles of 30 s at 55°C, 2 min at 72°C, and 30 s at 92°C; followed by 10 min at 72°C. Amplification products were separated by electrophoresis through 1.0% agarose gel in TBE for 2 h at 100 V, stained with ethidium bromide, and photographed under UV illumination.

Two independent observers analyzed the restriction endonuclease-based analysis of genomic DNA (REAG) profiles by visual grouping of the patterns without knowledge of isolate-patient relationships. Discrepancies were resolved by consensus and, in some cases, by preparing a new gel. All of the REAG profiles had to match exactly in order to classify the isolates as identical. Depending on the strain, *HinfI* yielded 9 to 13 bands of variable sizes in the range of 6.2 to 2 kb and both the numbers and sizes of the fragments varied greatly among the strains. When isolate-patient relationships were considered, it was immediately apparent that single-band differences could be seen within the isolates from any given patient (Fig. 1A). (Note that two isolates from patient B appear identical, whereas the pattern differs among the isolates from patient A by one band from left to right.) Similar to the results seen by others when single-band differences are discounted (13), the

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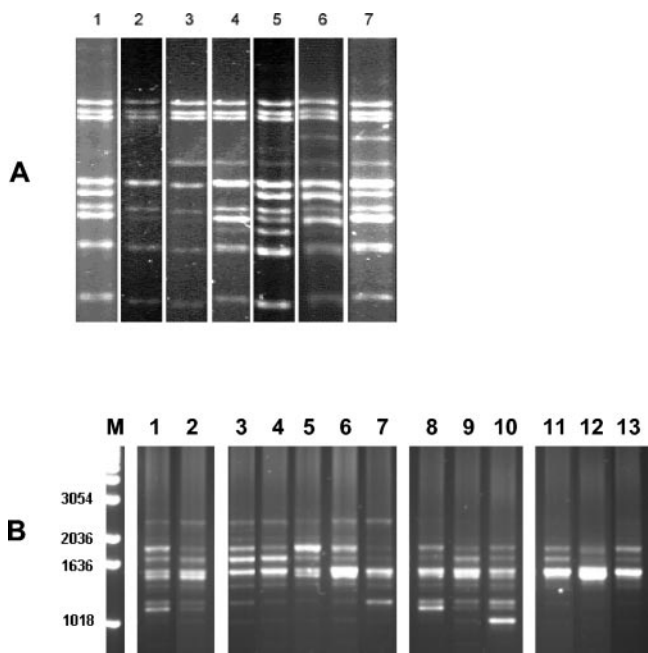


FIG. 1. (A) Agarose gel electrophoresis of REAG patterns of *Hinf*I-digested DNA. Patterns for different isolates *C. krusei* from two different patients in lanes 1 to 5 (patient A) and 6 and 7 (patient B) are shown. (B) Agarose gel electrophoresis of PCR-amplified DNA products generated with *C. krusei*-specific primers Arno1 and Arno2. Minor banding variations among isolates of *C. krusei* are seen for the four individual patients (lanes 1 and 2, 3 to 7, 8 to 10, and 11 to 13). Lane M is a molecular weight marker (1-kb DNA ladder).

net effect of single-band differences is that some pairs (e.g., lanes 1 and 5) differ by two bands. However, between the patients, the patterns revealed several band differences. Therefore, isolates were accepted as similar when no more than a one-band difference was seen (10). Based on this rule, seven different patterns (A, B, G, K, L, M, and R) were identified from 17 patients. Using this rule, only one type of *C. krusei* was detected from 14 subjects, whereas 3 patients demonstrated two types. The PCR-based banding patterns were analyzed in the same manner. Band sizes ranged from 1 to 2 kb, and, as with REAG-based analysis, single-band differences could be seen within the patterns of multiple isolates from the same patients (Fig. 1B). When grouped, the isolates showing differences of one band or less, which showed six distinct patterns (a, b, g, k, m, and r), were observed. As for the REAG-based analysis, 14 subjects showed only one strain whereas the same 3 subjects showed evidence for infection with two strains. Dendrograms of similarities between *C. krusei* isolates using REAG typing and PCR typing results are given in Fig. 2. For this analysis, each different band seen in the pool of all isolates was given a unique number and then each isolate was coded to show whether its banding pattern included each possible band. Hierarchical cluster analysis (SPSS for Windows version 11.0.0; SPSS, Inc., Chicago, Ill.) with “between-groups linkage” and the Dice binary similarity coefficient was used to cluster the isolates. Horizontal line lengths are proportional to arbitrary distance units. The initial letter for each unique isolate type corresponds to the patterns discussed in the text. The multiple

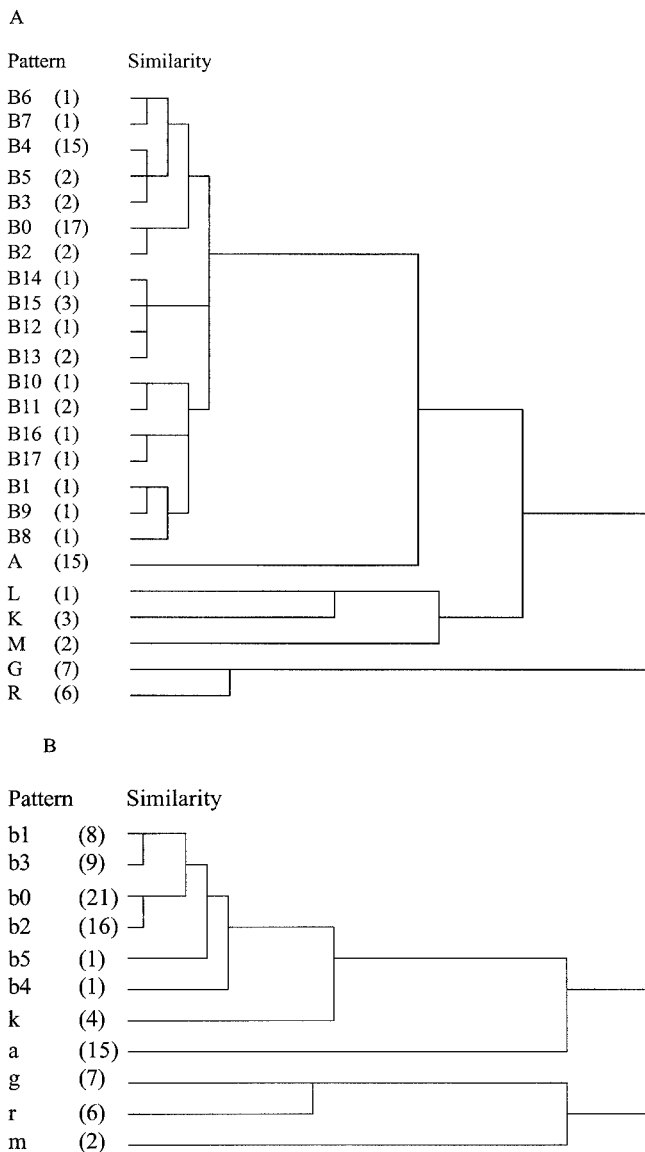


FIG. 2. Dendrograms of similarities between *C. krusei* isolates obtained with (A) REAG typing and (B) PCR typing results. The parenthetical number following each pattern's name is the number of isolates with that banding pattern.

variations seen for the B pattern are indicated by an additional number.

In 89 of 90 isolates, the subtypes detected by both PCR- and REAG-based typing were found to be the same. A, B, G, R, M, and K subtypes of REAG were all typed as a, b, g, r, m, and k by PCR, respectively. Only one isolate from one patient was assigned to a different strain group by the two methods (L by REAG and k by PCR).

The B or b pattern determined by REAG or PCR, respectively, was isolated in 10 of the 17 patients. When the isolates of these 10 patients were examined regarding any possible temporal relationship, it was observed that the dates of culture were distributed equally between June 1995 and January 1998.

C. krusei has recently emerged as an important opportunistic

pathogen (9, 11, 13). Genetic discrimination among *C. krusei* isolates may offer some important clues to understanding transmission and pathogenesis. We compared PCR- and REAG-based methods and found near-perfect correlation for the two methods, with only 1 of 90 isolates grouped differently by the two methods.

As a practical problem, single-band differences were common among isolates from the same patient. A similar tolerance for minor variations has been required in previous studies of *Candida albicans*, *Candida glabrata*, and *C. krusei* (3, 4, 5, 10). When many isolates are available from the same patient, the net effect can be that some pairs of isolates show two band differences (Fig. 1A). This effect has been noted before (10). Despite this, a common pattern that permits accurate isolate typing can be discerned. However, between patients, the differences amounted to several bands.

In summary, both PCR- and REAG-based techniques are accurate for the typing of *C. krusei* isolates to clarify the epidemiology of nosocomial infections. The greater simplicity of the PCR method should make it the first choice. Single-band differences should be ignored when typing *C. krusei* isolates by these methods.

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