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Comparison of Restriction Enzyme Analysis versus Pulsed-Field Gradient Gel Electrophoresis as a Typing System for *Torulopsis glabrata* and *Candida* Species Other than *C. albicans*

JOSE A. VAZQUEZ,[1,2,3]* ANN BECKLEY,[2] SUSAN DONABEDIAN,[2] JACK D. SOBEL,[1] AND MARCUS J. ZERVOS[1,2]

Department of Internal Medicine, Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, Michigan 48201[1]; William Beaumont Hospital, Royal Oak, Michigan 48072[2]; and Veterans Administration Medical Center, Allen Park, Michigan 48101[3]

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***Candida* species have recently emerged as important nosocomial pathogens. Because of the lack of a reliable system for detecting differences within the same species, little is known about the epidemiology of infection with *Candida* species. We describe a typing system for *Torulopsis glabrata* and the non-*C. albicans Candida* species that uses contour-clamped homogeneous electric field electrophoresis (CHEF), a version of pulsed-field gradient gel electrophoresis, and compared it with restriction enzyme analysis (REA) of genomic DNA. One hundred seventeen clinical isolates from 40 patients were evaluated. CHEF and REA were performed on each of the isolates, and the results of the two procedures were compared. The REA procedure revealed 8 different types of *Candida lusitaniae*, 20 of *Torulopsis glabrata*, 5 of *Candida tropicalis*, 3 of *Candida parapsilosis*, and 7 of *Candida kefyr*, whereas the CHEF method revealed 14 different types of *C. lusitaniae*, 16 of *T. glabrata*, 10 of *C. tropicalis*, 10 of *C. parapsilosis*, and 7 of *C. kefyr*. The CHEF technique yielded unique patterns of electrophoretic karyotypes that could be used to distinguish intraspecies variations. When compared with REA, CHEF demonstrated greater sensitivity in recognizing subtle strain-to-strain variations in most isolates and will be a useful epidemiologic tool for studying non-*C. albicans Candida* species and *T. glabrata*.**

Candida species have emerged as an important cause of nosocomial infections, particularly when they involve immunocompromised hosts. The rise in nosocomial candidal infections reflects the increase in high-risk patients, including the increase in the number of human immunodeficiency virus-infected individuals. The incidence of disseminated candidiasis has also increased, as proven by large autopsy series (5, 6, 35). *Candida* species are now the fourth most commonly isolated organisms in blood cultures (3). The crude mortality rate associated with *C. albicans* when cultured from blood is postulated to be 70 to 85% (25). For *Torulopsis glabrata* and some of the non-*C. albicans Candida* species, such as *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitaniae*, fungemia has been associated with mortality rates of 90 to 100% (28, 39). The importance of *T. glabrata* and the non-*C. albicans Candida* species is due to their increasing frequency, the myriad of reports demonstrating the emerging tolerance or resistance to the currently available antimycotic agents, and the higher mortality rates associated with infections with these organisms (14, 16, 42, 44, 47, 65, 66).

It has been very difficult to achieve a precise understanding of the epidemiology of *Candida* infections because of the lack of a reliable system of evaluating strain differences or relatedness. Previous methods used in the delineation of *C. albicans* strains have relied upon biotyping (63), enzyme profiles (9), susceptibility to killer toxins (46), streak morphology (45), resistance patterns and biochemical analysis (61), serological agglutination reactions (59), immunoblotting techniques (29), and modifications of older techniques (11, 63). The large number of methods reflects the inadequacies of any one method. With the advent of molecular genetics, newer methodologies have emerged. These methodologies use comparative analysis of chromosomal DNA (30, 36, 48, 56), ribosomal DNA (13, 33), and mitochondrial DNA digested with restriction enzymes (43). This produces numerous restriction fragment length polymorphisms (RFLPs) with unique DNA patterns which are then visually analyzed in a gel after staining with ethidium bromide. This method is known as DNA fingerprinting. The basis for the use of RFLPs is that isolates of the same strain have the same RFLP pattern and unrelated strains have different patterns. These techniques have been used successfully to evaluate the relatedness of various types of bacteria, viruses, and yeasts (17). DNA probes have also been used as a tool for strain delineation (10, 18, 48–50). Restriction enzyme analysis (REA) supplemented with a radioactive probe was initially used by Scherer and Stevens (48, 49), who found that this method, because of its stability and reproducibility, would be useful for large-scale epidemiologic studies of *C. albicans*. A frequent dilemma observed in the initial typing systems has been the problem of clustering, whereby the majority of isolates were sequestered into only a few groups, resulting in a method that lacks the sensitivity to adequately differentiate intrastrain variations. Although these methods have greatly enhanced our knowledge of *C. albicans*, few have tested these methods on *T. glabrata* or the other *Candida* species (2, 13). In 1984, Schwartz and Cantor (51) described the use of pulsed-field gel electrophoresis (PFGE) for the analysis of chromosomes larger than 2 Mb by using *Saccharomyces cerevisiae* as a model. Chu et al. (12), who used the contour-clamped homogeneous electric field electrophoresis (CHEF) technique, a modified version of PFGE, were able to improve the resolution of large chromosomal bands. PFGE is performed on DNA which has previously

* Corresponding author.

been embedded in agarose instead of a conventional DNA preparation. This is to avoid excessive handling and, thus, prevent shearing of the large fragments of DNA to be analyzed (53). There are two methods for embedding cells into agarose. In one, the cells are encapsulated into agarose beads; in the second, the cells are immobilized in blocks of agarose called "plugs." Once the intact DNA is inside the agarose, the resolution of large chromosomal DNA is accomplished by producing homogeneous electrical fields. Large DNA molecules are then separated on the basis of their ability to relax and realign themselves through the agarose pores in the alternating electrical fields. These homogeneous electrical fields produce sharper, straighter, and more distinct patterns. The patterns produced allow for the electrophoretic karyotyping of the genomes of various yeasts, including *C. albicans*, *S. cerevisiae*, *Schizosaccharomyces pombe*, and several of the other non-*C. albicans* *Candida* species (8, 26, 27, 32, 34, 38, 40, 52, 54). PFGE has recently been used epidemiologically to analyze strain differentiation in *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, and enterococci (1, 41). A recent study has shown that CHEF is a more sensitive system than REA for the detection of subtle variations in the electrophoretic karyotypes of *C. albicans* strains (58). Several advantages of the CHEF method include the ease of strain differentiation with minimal observer bias and its reproducibility, without the need for radioactivity or hybridization techniques.

In the present study we evaluated the utilities of REA and CHEF as methods for use in assessing strain relatedness in the more commonly isolated non-*C. albicans* *Candida* species and *T. glabrata* and compared the results of the two methods.

MATERIALS AND METHODS

Yeast strains. One hundred seventeen isolates were evaluated. One hundred three isolates were recovered from immunocompromised hospitalized patients in either the Bone Marrow Transplant Unit (BMTU) or the Medical Intensive Care Unit (MICU) of a large tertiary-care hospital. These isolates were isolated from multiple anatomic sites and at separate times over a 1-year period from individual patients as well as from different patients. Six isolates were recovered from the hands of hospital personnel; four isolates were recovered from environmental surfaces in both the BMTU and the MICU. The four control isolates were American Type Culture Collection (ATCC; Rockville, Md.) strains: *C. tropicalis* ATCC 750, *C. lusitaniae* ATCC 34449, *C. parapsilosis* ATCC 22019, and *C. kefyr* ATCC 4135. All specimens were identified by negative germ tube formation, the yeast API 20C method (Sherwood Medical, Plainview, N.Y.), and chlamydospore formation on cornmeal agar and were verified by carbohydrate assimilation and fermentation tests. Yeasts were initially isolated on Sabouraud dextrose agar (Difco, Detroit, Mich.), stored in a 1:1 mixture of brain heart infusion broth (Difco) and glycerol, and frozen at −70°C until analysis.

Preparation of DNA. For the isolation of DNA, yeast cells were grown on Sabouraud agar plates for 24 h at 30°C. Single colonies were then inoculated into 200 ml of YEPD broth (2 g of yeast extract, 2 g of peptone, 2 g of glucose) and incubated in a shaking water bath at 30°C for 36 h. The method used to extract the DNA has been described previously (58). Once the nucleic acids were extracted and purified, the DNA was resuspended in 100 μl of TE buffer and were stored at 4°C until electrophoresis. The DNA yield from this lysis procedure was 0.8 to 1.0 mg/200 ml of yeast cell culture.

Restriction enzyme digest. A single-enzyme digest with 4 μl of restriction endonuclease *Eco*RI or *Msp*I (Bethesda Research Laboratories, Gaithersburg, Md.) and the appropriate reaction buffer were mixed with 40 to 50 μg of DNA following the recommendations of the manufacturer.

Electrophoresis. For the electrophoresis of samples, 20 to 30 μl of DNA per enzyme mixture was used for each REA. This concentration produced the most definitive patterns with the least amount of background haziness. The DNA was run on a 0.7% agarose gel apparatus at 30 V for 18 h. The gel was then stained in a solution of 0.5 μg of ethidium bromide per ml for 30 min and was then washed in distilled water for 60 min. RFLPs were then visualized with short-wave UV light (254 nm) and photographed.

Strain delineation. Differences between isolates were determined by visual comparison of the DNA patterns. The variations in these patterns formed the basis for our groups. Capital letters denote the primary groups when the DNA was digested with *Eco*RI. After digestion with *Msp*I, if any additional groups were revealed, these strains were designated with the original capital letter obtained after digestion with *Eco*RI, and a lowercase letter was added to designate subgroups within the primary group.

Preparation of intact yeast DNA (plugs). After isolation on Sabouraud agar, single colonies of yeasts were inoculated into 40 ml of YEPD broth and were incubated for 36 h at 30°C in a shaking water bath. The DNA plugs were prepared as described previously (58). Briefly, the cell suspension was pelleted at 3,000 × *g* for 5 min, washed in 10 ml of 1 M sodium chloride–0.5 M EDTA (pH 9.0), and pelleted again at 500 × *g* for 10 min. The cells were then suspended in 2.0 ml of the NaCl-EDTA solution and placed in a 37°C water bath. Low-melting-temperature 1% agarose (Sigma, St. Louis, Mo.) was prepared with 2 ml of 125 mM EDTA (pH 7.5) and was placed into the cell suspension at 37°C. Zymolyase 20 T was then added to the suspension at a concentration of 80 U/g of cells. The cell-agarose-Zymolyase suspension was distributed with a Pasteur pipette into a premade mold (Bio-Rad, Richmond, Calif.) and was refrigerated for 60 min at 4°C. The solidified plugs were then placed into equal volumes of 0.5 M EDTA–7.5% β-mercaptoethanol and were incubated overnight in a 37°C water bath with gentle shaking. After 12 h (overnight), the plugs were removed from the solution and washed once with 5 ml of 50 mM EDTA (pH 7.5). The plugs were then transferred to 10-ml tubes, and 3.3 ml of ESP solution (0.5 M EDTA [pH 9.0], 25 ml, 10% Sarkosyl, 2.8 ml; 0.5 mg of pronase per 28 ml) was added. The plugs were incubated overnight in a water bath at 50°C with gentle shaking. After 12 h (overnight), the plugs were refrigerated at 4°C for 1 h and were transferred to microcentrifuge tubes, to which 1 ml of 0.5 M EDTA (pH 9.0) was added. The plugs were then stored at 4°C until electrophoresis.

CHEF. One-fifth of the plug was loaded into wells of a 1% agarose gel. The gel was then placed into the electrophoresis chamber of a CHEF DR II (Bio-Rad) apparatus with a running buffer of 0.089 M Tris–0.089 M borate–0.0025 M EDTA diluted in distilled water to a ×0.5 concentration. A constant temperature of 12 to 14°C was provided by using a chiller water bath and a pump. The following parameters were used for the CHEF: (i) a 180-s pulse time for 22 h at 150 V and (ii) a 360-s pulse time for 26 h at 150 V. These parameters were used for the electrophoresis of DNAs from *T. glabrata*, *C. lusitaniae*, *C. parapsilosis*, and *C. kefyr*. The

parameters used for *C. tropicalis* were as follows: (i) a 120-s pulse time for 22 h at 150 V, (ii) a 360-s pulse time for 6 h at 150 V, and (iii) a 360-s pulse time for 24 h at 113 V. The gels were subsequently stained in a solution of 0.5 μg of ethidium bromide per ml for 30 min and were washed in distilled water for 60 min before being photographed.

Differentiation by CHEF was achieved by visual comparison of the more variable electrophoretic karyotypes. The variations in these patterns formed the basis for our groups. Numbers were used to designate CHEF groups, to avoid confusion with the REA group designations.

RESULTS

Restriction enzyme patterns of DNAs from *Candida* species. Distinguishable RFLPs of chromosomal DNA were produced for *T. glabrata* and the non-*C. albicans Candida* species by using the endonuclease *Msp*I or *Eco*RI as a single digest. Each species had characteristic elements in its RFLPs that enabled us to easily differentiate between the species. One hundred seventeen isolates were evaluated by REA. This included 37 *C. lusitaniae* isolates from eight patients and two environmental surfaces and one strain from ATCC; 34 *T. glabrata* isolates from 17 patients; 19 *C. tropicalis* isolates from 7 patients and 1 reference strain from ATCC; 17 *C. parapsilosis* isolates, 2 of which were recovered from environmental surfaces, 6 from the hands of hospital personnel 8 from six patients and 1 strain from ATCC; and 10 *C. kefyr* isolates, 9 from 2 patients and 1 reference strain from ATCC.

Agarose gel electrophoresis of each *Candida* species produced over 100 fragments, with fragment sizes ranging from 2 to 23.1 kb. A summary of the REA results are given in Table 1.

Thirty-seven *C. lusitaniae* isolates were analyzed. Digestion with *Eco*RI produced six groups (types), groups A to F (Table 1). Group A consisted of 30 isolates from four patients. Twenty-two of these isolates were recovered from multiple sites in a single patient over a 6-week period. An additional four isolates were from another patient and were isolated from multiple sites simultaneously. Additionally, there were two patients with one isolate each that belonged to group A, as well as two environmental isolates, one from a cardiac monitor mounted in the central MICU nursing station and the other from an air vent in an open-area room. *Msp*I digestion of the isolates in group A generated three additional subgroups designated Aa, Ab, and Ac. Subgroup Aa contained 28 of the original 30 isolates, 22 from one patient, 4 from an additional patient, and the 2 environmental surface isolates. The patients from whom these isolates were obtained were temporally and geographically related in the MICU, where the environmental isolates were recovered. Subgroups Ab and Ac each consisted of one isolate from two separate patients. Group B consisted of three isolates from two patients who were epidemiologically unrelated. *Msp*I digestion of these isolates did not delineate them any further. Groups C, D, E, and F contained one isolate each, and all isolates in these groups were recovered from different patients except for the isolates in group D. Group F was made up of the ATCC isolate, which was not shared by any patients. The isolates in subgroup Ab and group D were recovered from the rectum of the same patient 4 weeks apart. The RFLPs generated by *Eco*RI were characterized by two intensely staining bands between the 3.5- and 5.0-kb region, with lighter-staining fragments being in the higher molecular-mass region, which varied from strain to strain.

TABLE 1. Comparative analysis of *T. glabrata* and *Candida* species groups on the basis of restriction enzyme patterns

Candida species	*Eco*RI group	No. of isolates				
		Total	In *Msp*I group:			
			a	b	c	d
C. lusitaniae	A	30	28	1	1	
	B	3	3			
	C	1	1			
	D	1	1			
	E	1	1			
	F	1	1			
T. glabrata	A	5	1	2	1	1
	B	3	1	2		
	C	4	1	2	1	
	D	3	3			
	E	2	2			
	F	2	2			
	G	3	3			
	H	1	1			
	I	4	4			
	J	1	1			
	K	1	1			
	L	3	3			
	M	1	1			
	N	1	1			
C. tropicalis	A	13	12	1		
	B	3	3			
	C	2	2			
	D	1	1			
C. parapsilosis	A	13	13			
	B	3	3			
	C	1	1			
C. kefyr	A	3	3			
	B	3	1	1	1	
	C	1	1			
	D	3	2	1		

The RFLPs produced by *Msp*I revealed four to five intensely staining bands, with fragment sizes ranging from 3.0 to 23 kb. Fig. 1, lanes F and G, displays the genomic DNAs of *C. lusitaniae* isolated from two separate patients and digested with *Eco*RI, revealing different patterns. Figure 2, lanes F and G, contains the same DNA shown in Fig. 1, lanes F and G, respectively, digested with *Msp*I, which produced two distinctly different restriction patterns.

Thirty-four *T. glabrata* isolates were evaluated. *Eco*RI digestion produced 14 groups, groups A to N. Group A was the largest and contained five isolates from three patients. Two patients had two isolates each. Subsequent digestion of these five isolates with *Msp*I produced a total four subgroups, subgroups Aa, Ab, Ac, and Ad. One patient had two isolates that were previously in group A; these two isolates were subdivided into two groups, Aa and Ac, and were collected from the perianal area 10 weeks apart. For the other patient with two group A isolates, the isolates retained the same strain type; these two isolates, on the other hand, were isolated simultaneously from different body sites. Group C contained four isolates from two patients; three of these isolates were recovered from one patient simultaneously and from three different body sites. Upon digestion of these isolates by *Msp*I, three subgroups were delineated

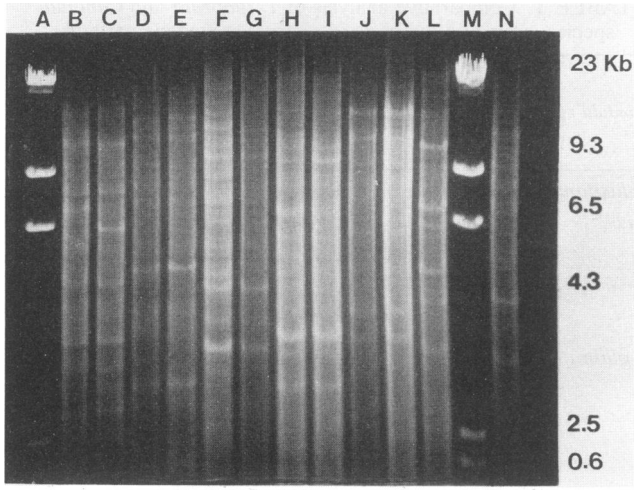

FIG. 1. Chromosomal DNAs from clinical *Candida* and *Torulopsis* species. Lanes A and M, bacteriophage lambda DNA digested with *Hin*dIII; lanes B and C, *C. albicans*; lanes D and E, *T. glabrata*; lanes F and G, *C. lusitaniae*; lanes H and I, *C. tropicalis*; lanes J and K, *C. parapsilosis*; lanes L and N, *C. kefyr*. All isolates were digested with *Eco*RI.

as Ca, Cb, and Cc. Subgroups Ca, with one isolate, and Cb, with two isolates, were recovered at the same time from the same patient at two separate sites. The subgroup Cc strain was isolated from a different patient and had a distinctly different RFLP. Group I also contained four isolates obtained from different body sites of four separate patients at various times in the study. Digestion with *Msp*I did not differentiate these isolates any further. Groups B, D, G, and L each contained three isolates when digested with *Eco*RI. The three group B isolates were obtained from two different body sites of the same patient 1 week apart. Digestion with *Msp*I further subdivided them into two subgroups; subgroup Ba comprised one isolate from the urinary tract, and subgroup Bb comprised two isolates, one from the pharynx,

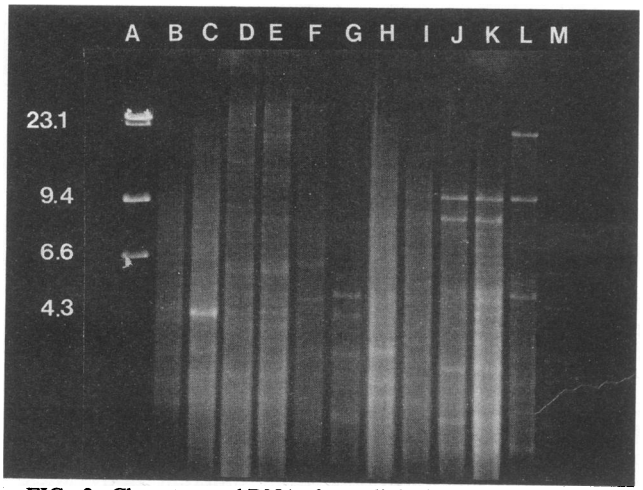

FIG. 2. Chromosomal DNAs from clinical *Candida* and *Torulopsis* species digested with *Msp*I. Lane A, bacteriophage lambda DNA digested with *Hin*dIII. Lanes B and C, *C. albicans*; lanes D and E, *T. glabrata*; lanes F and G, *C. lusitaniae*; lanes H and I, *C. tropicalis*; lanes J and K, *C. parapsilosis*; lanes L and M, *C. kefyr*.

which was recovered simultaneously with strain Ba, and one from the urinary tract, which was recovered 1 week after the other two isolates were recovered. The three group D isolates were recovered at the same time from three different body sites (perianal region, vagina, and urine) of one patient. The isolates in groups G and L were also isolated from individual patients at the same time and from three body sites: perianal region, vagina, and pharynx for group G isolates and perianal region, urine, and pharynx for group L isolates. All isolates from groups D, G, and L were submitted to *Msp*I digestion; no further differentiation was accomplished. Groups E and F contained two isolates apiece. The isolates from group E were recovered from two sites of the same patient at the same time, as were the group F isolates. Groups H, J, K, M, and N contained one isolate each. Four patients were infected with isolates of the same subgroup, subgroup Ia; there was no epidemiologic association, and they were recovered at separate times over a 3-week period. One patient was found to be infected with strains, of three subgroups, subgroups Aa, Ac, and Ia. All were isolated from the perianal region at different times. One patient harbored two different strains, Ac and Ia; these were recovered from different sites (urine and perianal region) 1 week apart. Figure 1, lanes D and E, shows the chromosomal DNA of *T. glabrata* isolated from separate patients and digested with *Eco*RI. It reveals identical patterns, with restriction fragment lengths ranging in size from 23 kb to less than 4 kb. Fig. 2, lanes D and E, shows the same clinical isolates shown in Fig. 1, lanes D and E, respectively, digested with *Msp*I; these isolates demonstrated different patterns with different fragment lengths. Both isolates were identical in the mid to small range of fragment lengths below 6.6 kb, with much greater variability in the higher-molecular-mass range of 9.4 to 23 kb.

Nineteen isolates of *C. tropicalis* were evaluated. Digestion with *Eco*RI produced four groups, groups A to D. Group A contained 13 isolates from four patients and the ATCC strain. After digestion with *Msp*I, only one isolate could be differentiated further; this isolate was placed into subgroup Ab. The difference between this isolate in subgroup Ab and the remaining 11 isolates in subgroup Aa was that the subgroup Ab strain was isolated 4 weeks after the other isolates were recovered. The remaining 12 isolates in subgroup Aa were isolated from four patients who were unrelated and were isolated from various body sites over a 10-week period. Group B contained three isolates recovered from the same patient at the same time. Digestion with *Msp*I did not delineate the isolates any further. Group C had two isolates; both were from the same patient and different body sites and were obtained 1 week apart. Group D consisted of one isolate from a different patient who was temporally associated with the isolates from group C. The RFLPs produced by *Eco*RI revealed two to three brightly staining bands ranging in size from 3 to 10 kb. The use of *Msp*I on the same isolates yielded patterns with 12 to 15 bands ranging in size from 3 to 24 kb. Figure 1, lanes H and I, demonstrates the chromosomal DNAs isolated from *C. tropicalis* and digested with *Eco*RI, revealing an identical banding pattern. Figure 2, lanes H and I, are the same isolates shown in Fig. 1, lanes H and I, respectively, digested with *Msp*I; this comparison disclosed different banding patterns.

Seventeen isolates of *C. parapsilosis* were studied. Digestion with *Eco*RI produced three groups, groups A to C. Group A consisted of 13 isolates from five patients, 4 isolates from the hands of four hospital staff (1 from the MICU, three from the BMTU) and 2 isolates from environmental surfaces

from two separate patient rooms, one from a countertop and one from an air vent. Digestion with *Msp*I did not differentiate the isolates any further. Group B contained three isolates; two isolates were from the hands of MICU staff and were isolated at the same time and one was the ATCC isolate. This group did not contain any patient isolates. *Msp*I digestion did not help in further differentiation. Group C contained one patient isolate which was temporally unrelated to any of the other isolates. Both enzymes produced few intensely staining bands, usually three to five, ranging in size from 2 to 25 kb. Figure 1, lanes J and K, demonstrates the chromosomal DNA isolated from *C. parapsilosis* and digested with *Eco*RI; the isolates were identical, even though they were isolated from different patients. Figure 2, lanes J and K, are the same clinical isolates shown in Fig. 1, lanes J and K, respectively, digested with *Msp*I, and again, they revealed an identical banding pattern.

Ten isolates of *C. kefyr* from two patients and one ATCC strain were evaluated. Digestion with *Eco*RI produced four different groups, groups A to D. Group A consisted of three isolates, all of which were recovered from the same patient simultaneously, but from different body sites. Digestion with *Msp*I did not differentiate the groups any further. Group B consisted of three isolates from the same patient that were isolated simultaneously from different body sites. *Msp*I digestion further delineated these isolates into three subgroups, subgroups Ba, Bb, and Bc. The isolate of subgroup Ba was recovered from the perianal region, whereas the isolates of subgroups Bb and Bc were recovered from the patient's urine. Group C consisted of one isolate, which was the ATCC strain. Group D contained three isolates, all of which were recovered from the perianal region of the patient who also carried a group B strain. Digestion with *Msp*I differentiated the three isolates into two subgroups, subgroup Da, with two isolates, and subgroup Db, with one isolate. The two isolates in subgroups Da were recovered 1 week before the isolates found in group B were recovered, and the isolate in subgroup Db was recovered at the same time as the isolates in group B were recovered. Five different strains of *C. kefyr* were isolated from one of the patients who we evaluated. Four of the isolates were recovered simultaneously; two were from the urine and two were from the perianal region. The RFLPs produced by *Eco*RI digestion demonstrated patterns with eight intensely staining bands. The RFLPs produced by *Msp*I revealed five intensely staining bands ranging in size from 2.5 to 23 kb. Figure 1, lanes L and N, demonstrates the chromosomal DNAs from *C. kefyr* digested with *Eco*RI; both lanes have similar patterns in the lower-molecular-size range, but with most of the differences being in the higher-molecular-size region. The isolates were recovered from different patients. Figure 2, lanes L and M, consists of the same DNAs shown in Fig. 1, lanes L and N, respectively, digested with *Msp*I; the isolates revealed different patterns.

Reproducibility was observed with repeat testing in triplicate. All isolates retained the same restriction patterns with repeat testing. The stabilities of the patterns were determined by repeated evaluation of the isolates after passage through multiple generations.

CHEF patterns of DNAs from *Candida* species. All 117 clinical isolates evaluated by REA were also subjected to PFGE by the CHEF method. Comparison of the groups obtained by REA and CHEF are shown in Table 2. CHEF yielded definitive patterns for all of the *Torulopsis* and *Candida* species. As with REA, each species tended to have characteristic bands which were generally species specific and which did not vary a great deal within species. The electrophoretic karyotypes generally demonstrated 6 to 11 bands (chromosomes) ranging in size from 245 to >2,200 kb; this corresponded to the electrophoretic karyotypes of the various *Candida* species and *T. glabrata*.

Thirty-seven isolates of *C. lusitaniae* were evaluated by CHEF. This method yielded 14 electrophoretic karyotypes, which was 6 more than the number yielded by REA (Table 2). The three isolates of group 1 all belonged to a single patient and were isolated simultaneously from three separate body sites. Group 2 contained 17 of the isolates, also from the same patient, all of which were recovered over a 6-week period from three different sites (urine, vagina, and perianal region). Some of the isolates were recovered at the same time from different body sites, and some were recovered at weekly intervals. The environmental isolates of subgroup Aa, as determined by REA, had very similar but distinctly different patterns by CHEF, as did the other four isolates from patients that were in subgroup Aa by REA. Group B determined by REA, which consisted of three isolates, was further differentiated into two separate groups by CHEF; two identical isolates were from one patient and the other isolate belonged to a patient who was epidemiologically unrelated to the other patient. Otherwise, the remaining CHEF groups, groups 5, 9, 10, and 11, contained only one isolate in each group, and each isolate belonged to individual patients. It is important to point out that CHEF groups 1, 2, 4, 5, 6, 13, and 14 were classified as subgroup Aa by REA. Figure 3, lanes C and D, shows the electrophoretic karyotype of *C. lusitaniae*; each isolate contained seven to eight bands. The patterns observed revealed only minor variations in band sizes. In general, all isolates had five bands of between 1,600 and 2,200 kb and three bands in the lower-molecular-size region (1,000 to 800 kb).

The 34 *T. glabrata* isolates were also evaluated by CHEF; 16 electrophoretic karyotypes were observed, 4 fewer than were observed by REA (Table 2). Generally, there was agreement between REA and CHEF except for subgroups Ca, Cb, and Cc by REA; CHEF was not able to distinguish the differences in these isolates that had been recovered from the same patient. The CHEF patterns revealed either 10 or 11 bands that ranged in size from 400 kb to greater than 2,200 kb. The CHEF patterns revealed three stable bands in the region of 700 to 1,000 kb, four to five bands between 2,000 and 1,600 kb, and one or two bands in the high-molecular-size region of 2,200 kb and greater. The variability that produced the different patterns was generally observed in the regions above 2,000 kb. The karyotypes are shown in Fig. 3; lanes A and B of Fig. 3 reveal the different patterns of two separate patient isolates.

Nineteen *C. tropicalis* isolates were evaluated by CHEF. Ten electrophoretic karyotypes were observed (Table 2). Subgroup Aa by REA, which contained 12 isolates from four patients and one ATCC strain, was further differentiated into seven groups by CHEF. Isolates from three of the four patients had their own electrophoretic karyotypes; for one patient with five isolates who previously carried one strain by REA, had three strains were detected by CHEF. Each of the strains detected by CHEF was isolated at different times. The ATCC isolate also had its own pattern by CHEF; previously it was subgroup Aa by REA. Groups B, C, and D each maintained the same groups by REA and CHEF. The electrophoretic karyotype of *C. tropicalis* revealed six to seven chromosomal bands, with stable bands in the middle molecular sizes. One band was at 1.1 Mb and another band was at the 1.4-Mb region; both bands were consistently

TABLE 2. Comparative analysis of REA and CHEF for typing of *T. glabrata* and *Candida* species

Species and REA subgroup	No. of isolates	No. of isolates with the following CHEF groups:															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
C. lusitaniae																	
Aa	28	3	17		1	2	2						1	1	1		
Ab	1			1						1							
Ac	1			1													
Ba	3							1	2								
Ca	1					1											
Da	1									1							
Ea	1										1						
Fa	1											1					
T. glabrata																	
Aa	1														1		
Ab	2		2														
Ac	1							1									
Ad	1																1
Ba	1											1					
Bb	2												2				
Ca	1													1			
Cb	2													2			
Cc	1										1						
Da	3															3	
Ea	2							2									
Fa	2									2							
Ga	3					3											
Ha	1						1										
Ia	4					1	1	1			1						
Ja	1								1								
Ka	1	1															
La	3		3														
Ma	1			1													
Na	1				1												
C. tropicalis																	
Aa	12		1	2	1	2	1		4	1							
Ab	1					1											
Ba	3							3									
Ca	2										2						
Da	1	1															
C. parapsilosis																	
Aa	13		3	2	2		3	1	1		1						
Ba	3				1	1				1							
Ca	1											1					
C. kefyr																	
Aa	3			1	2												
Ba	1					1											
Bb	1			1													
Bc	1							1									
Ca	1								1								
Da	2						2										
Db	1			1													

present in all of our isolates. Variations used to differentiate groups were usually noted in the bands located between 1.6 and 2.2 Mb. The band at the approximately 2.2-Mb region was also consistent in all of our groups and was thicker and stained more intensely than the other bands. Figure 3, lanes E and F, reveals the electrophoretic karyotype of *C. tropicalis*, with the isolates in both lanes having identical patterns.

Seventeen *C. parapsilosis* isolates were evaluated by CHEF, yielding 10 electrophoretic karyotypes, 7 more than by REA, representing groups 1 through 10 (Table 2). Group A by REA, which contained 13 isolates, was further differentiated by CHEF into six electrophoretic karyotypes. The strains from two patients still shared one electrophoretic karyotype, and the strains from one patient shared the same electrophoretic karyotypes with the environmental isolates. Overall, however, the strains from each patient maintained individual electrophoretic karyotype patterns by CHEF. Group B determined by REA, which contained three isolates, was differentiated by CHEF into three distinct groups. The ATCC isolate was also differentiated by CHEF from group B by REA into its own group by CHEF. The electro-

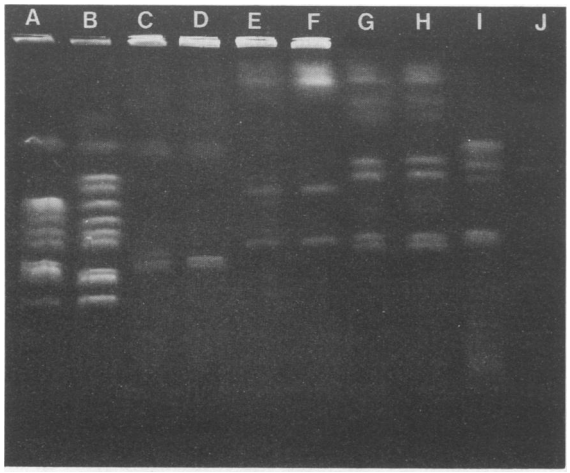

FIG. 3. CHEF electrophoresis of genomic DNAs from clinical *Candida* and *Torulopsis* species. Lanes A and B, *T. glabrata*; lanes C and D, *C. lusitaniae*; lanes E and F, *C. tropicalis*; lanes G and H, *C. parapsilosis*; lanes I and J, *C. kefyr*.

phoretic karyotype of *C. parapsilosis* revealed 10 to 11 bands, with similarities among all of our isolates in bands 1, 2, 3, 4, 6, 7, 10, and 11. Figure 3, lanes G and H, demonstrates the electrophoretic karyotype of *C. parapsilosis*. The bands ranged in size from 945 to greater than 2,200 kb, with the majority of variations in bands 5, 8, and 9.

By using the CHEF method on the 10 isolates of *C. kefyr*, seven electrophoretic karyotypes were detected, the same number that was detected by REA (Table 2). There was no difference in strain delineation by either of the methods. All isolates retained the same group, with no further delineation. The electrophoretic karyotype of *C. kefyr* revealed six to seven chromosomal bands ranging in size from 800 to greater than 2,200 kb, with similarities being in the lower-molecular-size bands and the major differences being in the higher-molecular-size regions. Figure 3, lanes I and J, demonstrates the electrophoretic karyotype of *C. kefyr*; both isolates revealed very different karyotypes.

The reproducibility of the CHEF method was established by repeat testing, which showed identical patterns. Stability was also demonstrated by showing identical patterns when the strains were passed through multiple generations.

Comparison of isolates recovered from multiple sites and from individual patients over a period of time generally revealed that they were the same strain by REA and CHEF. Separate isolates from individual patients who were epidemiologically unrelated generally had different patterns.

Evaluation of the longevity of the yeast plugs revealed that for isolates evaluated 2 and 4 months apart, there were no differences between the results obtained with newer and the older yeast plugs.

DISCUSSION

Modern medical therapy of neoplastic diseases, together with advances in surgical procedures and burn therapy, has not only increased survival but at the same time has contributed to an increasing rate of nosocomial infection (7, 55). Among the nosocomial infections, fungal infections, especially those produced by *Candida* species, have emerged as an increasingly important cause of morbidity and mortality. Although *C. albicans* remains the most common cause of fungemia (3, 5, 28), other species such as *T. glabrata* (24, 28) and *Candida* species such as *C. tropicalis* (19, 64), *C. parapsilosis* (25, 28), *C. krusei* (39, 65), and *C. lusitaniae* (4, 21, 22, 37) have also occurred with an increased frequency. Wey et al. (62) recently reported the enormous impact of systemic candidiasis on hospitalized patients. The crude mortality rates in patients with candidemia versus that in controls was reported to be 57 and 19%, respectively, producing an attributable mortality rate of 38%. The same investigators also found that the mean length of stay for a patient with candidemia was significantly greater (70 days versus 40 days for controls), thus having an attributable excess length of stay of 30 days.

C. lusitaniae is of special interest because of its reported intrinsic resistance to either polyene or azole antifungal agents and its ability to develop resistance during therapy (22, 39). *T. glabrata* and several of the other *Candida* species have also been reported to develop resistance during therapy or to carry intrinsic resistance to the antifungal agents currently in use (15, 16, 42, 44, 60, 66).

The lack of a reliable and simple system for the delineation of *Torulopsis* and *Candida* species has hampered the investigative efforts of most researchers in understanding the evolution of nosocomial *Candida* infections, the avenues of their dissemination, and the strains most likely to cause invasive disease as opposed to colonization. Numerous typing systems for *C. albicans* have existed for the past decade. Few typing systems have evaluated *T. glabrata* or the non-*C. albicans Candida* species. Most of these studies have lacked the epidemiologic information necessary to evaluate the relatedness of isolates. To further evaluate the sensitivity and specificity of REA in the present study, we comparatively evaluated REA and CHEF techniques using 117 isolates from separate patients in a large tertiary-care center. Using a single-enzyme digest with *Eco*RI or *Msp*I, we were able to distinguish distinct patterns for *T. glabrata*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*, and *C. kefyr*. For most of our isolates, the endonuclease *Msp*I was the more useful enzyme for demonstrating variation when compared with *Eco*RI. The restriction patterns observed with *Msp*I were also sharper, more distinct, and easier to evaluate, thus providing a greater ability for differentiating between clinical isolates. In many circumstances, *Eco*RI added no further sensitivity to what was found with *Msp*I alone. This effect could possibly be because *Eco*RI is a high-frequency-cleavage restriction endonuclease, producing large numbers of fragments that are more closely related in size, thereby making it more difficult to distinguish definite restriction patterns. *Msp*I also appears to have fewer recognition sites in *Candida* species, which results in fewer bands and thus sharper, more distinct restriction patterns. As in prior studies (30, 48, 49), we were also able to distinguish intensely staining bands in our restriction patterns. These intense bands are postulated to be repetitive sequences of DNA. Another possibility is that some of the more intensely staining bands are mitochondrial fragments. We found that *T. glabrata* could also be differentiated by using the larger-molecular-size fragments produced by REA. This variation found only in *T. glabrata* formed the basis for our strain types in this species and enabled the use of REA for detecting greater variation than CHEF. The variations in the restriction patterns are possibly due to the heterozygosity of the rDNA genome (30).

Unfortunately, as in prior studies which used REA of whole-cell DNA (30, 48), more than 50% of our patient isolates were clustered into one group. This phenomenon

was seen with *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* in our study; it was not observed, however, in *T. glabrata* or *C. kefyr*. Thus, further differentiation of strains was required. The need for greater differentiation among *Candida* species and *T. glabrata* prompted us to postulate the use of PFGE, specifically, the CHEF technique, as a method for analyzing the variations in the electrophoretic karyotypes of *Candida* species. PFGE has previously been used in the karyotyping of various other yeasts, including *C. albicans*, *T. glabrata*, *S. cerevisiae*, and *S. pombe*, and in the analysis of human DNA and a number of unicellular organisms such as plasmodium, trypanosomas, and leishmania, all with good results (20, 23, 27, 38, 57, 58).

The results of the present study demonstrate that the CHEF method is generally more sensitive than REA for the differentiation of strains. CHEF was most useful for three of the five species tested, specifically, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*, all of which revealed group clustering by REA. CHEF was as sensitive as REA when analyzing *C. kefyr*, but it was not as sensitive as REA when analyzing *T. glabrata* isolates.

In general, the correlation between patients and the patterns observed by REA and CHEF, taking into account all species that we studied, tend to agree with the correlation obtained by previous investigators (50). That is, in most patients, one strain tends to be isolated from multiple body sites simultaneously and, in many instances, even over a prolonged period of time. In several of our patients, however, we found multiple strains from different body areas and greater variability if the isolates were obtained over a period of time. On occasion, several patients and environmental surfaces shared the same strain, leading us to believe that there is a possible role of the environment, which acts as an "innocent bystander" for the nosocomial acquisition of *Candida* species. This was especially important with *C. lusitaniae* and *C. parapsilosis*, the two *Candida* species that were recovered from environmental surfaces and on the hands of hospital personnel.

Overall, the isolates tested had very distinct electrophoretic karyotypes from species to species, despite the innate variations that distinguished each species. The isolates of *C. tropicalis* proved to be the most difficult to resolve for two reasons. First, they required 6 more h of electrophoresis than the other isolates did, and three different parameters were required to achieve separation of their electrophoretic karyotypes. Second, the bands in the high-molecular-mass region proved very difficult to separate, despite numerous attempts with different parameters. These high-molecular-mass bands are possibly homologous chromosomes that we were not able to separate effectively. As observed in prior studies on *C. albicans* by Vazquez et al. (58), Merz et al. (38), and Magee et al. (31), one of the major assets of the CHEF procedure is the ease of strain differentiation in most *Candida* species, but the method has several other important features. The procedure is reproducible and is much simpler and easier to perform than REA. Reproducibility was demonstrated by repeat testing of the same isolates after multiple generations, revealing identical patterns. Another advantage of the CHEF method is the durability of the yeast plugs, which retain the same patterns and clarity after 4 to 6 months of storage. This enables comparison of clinical isolates obtained several months apart without having to repeat the plug preparation, thus saving time, effort, and resources. CHEF is much easier than REA for strain differentiation because of the fewer numbers of bands, which helps to eliminate observer bias, a drawback of REA. An additional advantage of CHEF is that radioactive probes are not required, thus making it easier to use and more accessible in the clinical laboratory. The major disadvantages of CHEF when compared with REA and radioactive probes are the initial investment cost of the equipment, the inability to run more than 20 samples at one time, and the amount of time required to carry out the entire procedure, usually 3 days for the plug preparation and 2 to 3 days for the electrophoresis, depending on the size of the genome evaluated. Several epidemiologic studies by Vazquez et al. (58) and Kaufman and Merz (27) have demonstrated the efficacy of CHEF as a means of strain delineation in *C. albicans* and *T. glabrata*.

CHEF is also a promising method of electrophoretically monitoring the entire genome for minor chromosomal variations and karyotyping of yeast genomes. It also provides a convenient and reproducible method of assigning genes to chromosomes and investigating the epidemiology of *C. albicans* and the non-*C. albicans Candida* species.

The present study demonstrated that the CHEF method is an effective tool for the strain delineation of the non-*C. albicans Candida* species. The CHEF method appears to be superior to REA as a marker of strain differentiation for clinical *Candida* isolates. However, for the *T. glabrata* isolates, the REA methodology proved to be superior to the CHEF technology in differentiating subtle differences between the isolates. Because of the numerous typing methods currently being evaluated and used, further epidemiologic studies will be necessary to determine the most appropriate epidemiologic and clinical settings for its use.

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