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KARYOTYPING OF *CANDIDA ALBICANS* AND *CANDIDA GLABRATA* ISOLATES FROM RECURRENT VAGINAL INFECTIONS BY PULSED-FIELD GEL ELECTROPHORESIS

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In the present study, 16 women with recurrent vulvovaginal candidiasis (RVVC) due to *Candida albicans* and *Candida* (*Torulopsis) glabrata* were followed for a period of 4 to 12 months, and 36 vaginal isolates were evaluted by pulsed-field gel electrophoresis (PFGE). Eleven women were infected by *C. albicans* and 5 by *C. glabrata*. Three electrophoretic karyotypes of *C. albicans* and 3 of *C. glabrata* were identified throughout the follow-up. All patients but one was infected with the same karyotype of *C. albicans* or *C. glabrata* during the follow-up period. Two different karyotypes of *C. glabrata* were identified in one patient in the course of 12 months. The results confirmed the diversity of the karyotypes of *C. albicans* and *C. glabrata* causing vulvovaginitis, and demonstrated the persistence of colonization with the same strain over different periods of time despite therapy (15/16 women).

Key words: *Candida albicans, Candida glabrata,* pulsed-field gel electrophoresis (PFGE), karyotyping, recurrent vulvovaginal candidiasis (RVVC)

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Introduction

Vaginal infections in adult women are among the most common situations in clinical practice. *Candida* species are responsible for a major proportion of vaginal infections. Different *Candida* species have been found in the vagina of 5 to 10% of healthy, nonpregnant women, in 18% of nonpregnant women with a vaginal discharge, and in 30% of pregnant women [1]. Recurrent vulvovaginal candidiasis (RVVC) accounts for 20 to 25% of all cases of vaginitis in a variety of clinical settings [2–4]. *C. albicans* is responsible for about 80% of all vulvovaginal yeast infections. Other species of *Candida*, such as *C. glabrata*, account for 3 to 16% of vaginal yeast infections. 20% of women can be shown to be culture-positive for yeast in the vagina within 1 month after clinically successful therapy [2, 3]. The etiology of this recurrent presence of *Candida* species in the vagina of a given patient is still unknown. Theories include frequent vaginal relapse or frequent reinfection due to exogenous acquisition of the strains [5, 6]. Classical biochemical tests are not satisfactory for investigation of the relatedness of different *Candida* species in this respect. The technique of electrophoretic karyotyping by pulsed-field gel electrophoresis (PFGE) makes it possible to separate the large yeast chromosomal DNA on the basis of size [7–9]. The method can be used for a more precise differentiation of different *Candida* species and for karyotyping of the genome of yeasts, including *C. albicans* and *C. glabrata* [10, 11, 12].

Materials and methods

Yeast strains

In the present study, 16 women with RVVC due to *C. albicans* or *C. glabrata* were followed for a period of 4 to 12 months. Thirty-six *Candida/Torulopsis* isolates originating from the vagina of patients with RVVC from the Department of Gynaecology, Faculty of Medicine University of Szeged were involved in this study. Swab samples were obtained from the middle third of the vagina. These samples were spread on CHROMagar Candida (Mast Diagnostica, France) or Sabouraud dextrose agar (Diagnostics Pasteur, France), and then incubated for 48 h at 30 and at 37 °C. The isolated strains were identified by carbohydrate assimilation profile tests (API 20 C AUX BioMerieux, France), and morphologically on rice extract agar (Difco, USA). Identification of *C. albicans* was based on germ tube formation in human serum. The antimycotic susceptibilities of the isolates were tested by E-test (AB BIODISK,

Sweden). Electrophoretic karyotyping analysis was performed by the CHEF-DR II system (BioRad, USA).

Preparation of intact chromosomal DNA

The samples were prepared by a modification of the method of Schwartz, Cantor [7] and Nagy et al. [8]. The *Candida* strains were isolated on Sabouraud agar. Separate yeast colonies were inoculated into 30 ml of YEPD broth (0.25% yeast extract, 0.25% peptone, 1% dextrose, 0.5% malt extract) and incubated for 20 h at 30 °C in a shaking water bath. After centrifugation, the cells were washed twice in a suitable osmotic KCl buffer depending on the species: 1 M KCl for *C. albicans*, and 0.6 M KCl for *C. glabrata* and pelleted. Cells (10^9 cells/ml) were finally resuspended in 1 M KCl containing 0.1% 2-mercaptoethanol, incubated at room temperature for 15 min and centrifuged again (3000 rpm for 10 min).

Preparation of protoplasts

For protoplast formation, pretreated yeast cells were resuspended in 20 ml of 1 M KCl containing 0.125% lysing enzyme (from *Trichoderma harzianum* Sigma, Germany) and 0.1% 2-mercaptoethanol. This mixture was incubated for up to 4 h at 30 °C in a shaking water bath. The protoplasts were collected by centrifugation (2000 rpm for 5 min) and washed twice in the appropriate KCl solution (1 M or 0.6 M KCl). Approximatly 4×10^8 protoplasts/ml were mixed with an equal volume of 1.4% low gelling temperature agarose (Type VII agarose, Sigma, Germany). The agarose blocks were cast and allowed to solidify at 4 °C for 5 min. The plugs were transferred into incubation buffer (0.5 M EDTA, 10 mM TRIS-HCl, 1% sodium-lauryl-sarcosine, 1 mg/ml Proteinase K, pH 9.5) and incubated for 2 days at 52 °C in a water bath. Finally, they were washed three times with 50 mM EDTA (pH 8.0) and stored at 4 °C until further use.

Conditions of PFGE

The plugs were loaded into 0.8% (w/v) chromosomal-grade agarose (BioRad) gels and placed into the electrophoresis chamber of a contour-clamped homogeneous electric field electrophoresis (CHEF) apparatus (CHEF-DR II, BioRad) with 0.5×TBE buffer (45 mM TRIS, 45 mM borate, 50 mM EDTA). The DNA molecules were separated at a constant temperature of 10 °C. The separations were carried out at 125 V for 72 h with 450 s switching time, followed by 125 V for 24 h with 250 s switching time.

After electrophoresis, the gel was stained for 15 min with ethidium bromide (0.5 μg/ml), then destained for 30 min in distilled water. DNA bands were visualized with 254 nm UV light and photographed.

DNA from *Saccharomyces cerevisiae* YNN 295, *Schizosaccharomyces pombe* ATCC 2411, *Candida albicans* ATCC 14053 and *Candida* (*Torulopsis*) *glabrata* ATCC 39316 served as DNA size markers.

Table I

Electrophoretic karyotypes of C. albicans *and* C. glabrata *duration of follow-up study of RVVC*

Patient no.	No. of isolates	Duration of follow-up (months)	Total no. of bands	Electrophoretic karyotype
C. albicans:				
1	3	12	6	A
2	2	6	6	A
3	2	5	6	A
4	2	8	8	B
5	3	12	6	A
6	2	10	6	A
7	2	8	6	A
8	2	4	6	A
9	2	6	6	A
1	3	12	5	C
11	2	4	6	A
C. glabrata:				
12	2	12	10, 6	I, II
13	3	9	10	I
14	2	8	10	I
15	2	6	10	I
16	2	10	11	III

Table II

Estimated chromosomal DNA sizes (kb) of three electrophoretic karyotypes of C. albicans *and* C. glabrata

C. albicans			*C. glabrata*		
A	B	C	I	II	III
3300*	3300	3300	2600	1600	2800
2800	2800*	1800	2200	1120	1500
2200	2200	1600*	1480	1020	1400
1800	1800	1200*	1400	945*	1200
1400	1400	700*	1200	700	1120
1200*	1300		1120*		1020
	1180*		770		945*
	1070*		700		770
			620		700
					620

Sizes of the detected chromosomal DNA molecules (kb) have been calculated in comparision with those of *Sch. pombe* and *S. cerevisiae*. Possible chromosomal comigrations indicated by asterisks.

Results

Sixteen women with RVVC due to *C. albicans* (11 patients) or *C. glabrata* (5 patients) were followed for 4 to 12 months. At frequent intervals during this period, the patients received multiple antimycotic therapy: first a short course of local topical therapy (nystatin or clotrimazole) later a single-dose fluconazole therapy. Altogether, 36 *Candida* isolates were obtained from the vagina of these patients. The fluconazole susceptibilities of *Candida* isolates were checked before and after therapy, the MIC values for *C. albicans* were found to be in the range of 0.125 to 4 mg/l (susceptible), *C. glabrata* isolates were resistant to fluconazole (≥64 mg/l).

Table I lists data relating to the electrophoretic karyotypes of *C. albicans*. We identified 3 different electrophoretic karyotypes from 25 *C. albicans* isolates. In these strains the number of chromosomal mobility groups varied from 5 to 8. The molecular size (kb) of the major bands varied between 3.3 and 2.8 Mb, 2.2 and 1.4 Mb, and 1.2 and 0.7 Mb. Each isolate produced single or double bands in these molecular ranges. The most frequent electrophoretic karyotype (20 out of 25 isolates) was A, with a total of 6 bands. The 5 other isolates involved karyotype B (8 bands) or karyotype C (5 bands).

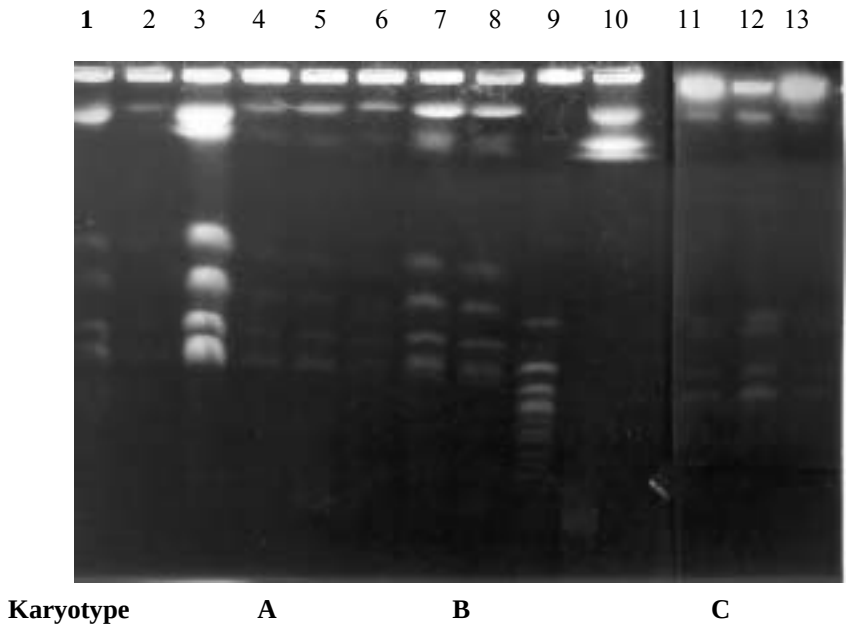

Fig. 1. Different electrophoretic karyotypes of genomic DNA from *C. albicans* isolated from patients 1, 2, 4 and 10. Identical strains from patient 2 (lanes 1–2), genomic DNA isolated from *C. albicans* ATCC 14053 (lane 3), identical strains from patient 1 (lanes 4–6), identical strains from patient 4 (lanes 7–8), *S. cerevisiae* YNN 295 and *Sch. pombe* ATCC 2411 as DNA size markers (lanes 9–10), identical strains from patient 10 (lanes 11–13).

Figure 1 shows the 3 different electrophoretic karyotypes of *C. albicans* revealed by CHEF. Karyotype A was identified in the isolates from patients 2 and 1, obtained during 12 and 6 months, respectively. Lanes 7 and 8 reveal karyotype B in the isolates from patient 4; the duration of follow-up in this case was 8 months. The 3 isolates obtained from patient 10 during 12 months proved to be karyotype C. In these 11 women, despite the multiple courses of numerous types of antimycotics, only 1 strain type was seen per patient. Five women were infected with *C. glabrata* (11 isolates). Three different karyotypes of *C. glabrata* were identified in this study (Table I). The total numbers of bands and the approximate molecular sizes are detailed in Table II. Karyotype I with 3 molecular size ranges (9 bands) was the most frequent (8 out of 11 isolates). Only in one case (patient 12) were two different karyotypes (I and II) obtained. The second isolate from patient 12 involved karyotype II (1 out of 11 isolates). Only 2 of 11 isolates (patient 16) exhibited karyotype III.

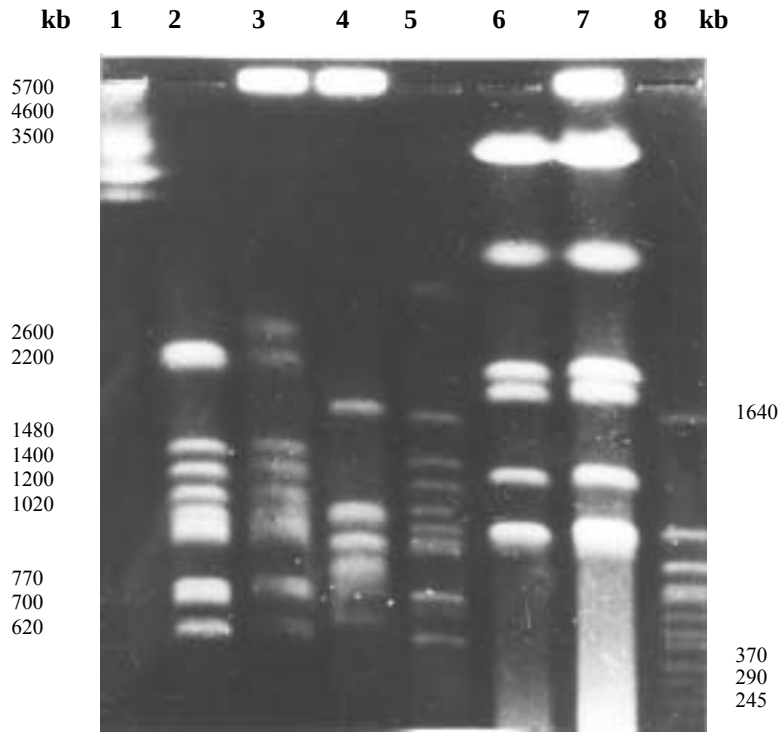

Fig. 2. Different electrophoretic karyotypes of genomic DNA from *C. glabrata* isolated from patients 12 and 16. Genomic DNA isolated from *Sch. pombe* ATCC 2411 (lane 1), and from *C. glabrata* ATCC 39316 (lane 2), two different isolates from patient 12 (lanes 3–4), one isolate from patient 16 (lane 5), *C. albicans* isolates from patient 6 (lanes 6–7), genomic DNA from *S. cerevisiae* YNN 295 as DNA size marker (lane 8).

Figure 2 showes to the 3 different karyotypes of *C. glabrata*. Lanes 3 and 4 illustrate 2 different isolates from patient 12 during 12 months. Lane 5 depicts karyotype III in one isolate from patient 16. Lanes 6 and 7 demonstrate the chromosomal DNA of *C. albicans* isolated from patient 6, and lanes 1 and 8 showe the chromosomal DNA from *Sch. pombe* ATCC 2411 and *S. cerevisiea* YNN 295, respectively, which served as DNA size markers, while lane 2 relates to the genomic DNA isolated from *C. glabrata* ATCC 39316, which served as control for the preparation of intact DNA.

All isolates were typeable by PFGE by three criteria: typeability, reproducibility and discrimination. The isolates were classified (karyotyped) on the basis of

chromosomal DNA molecular size, the molecular size of each band being determined by comparison with known standards.

In 15 patients, despite the long antimycotic therapy, only one electrophoretic karyotype was seen per patient. The results suggest the persistence of colonization with the same strain over a prolonged period of therapy. CHEF typing confirmed the diversity of the types of clinical isolates of *C. albicans* and *C. glabrata*, and demonstrated the persistence of colonization with the same strain (15 patients) or the appearance of a new *Candida* infection (1 woman).

Discussion

The incidence of vulvovaginal candidiasis is elevated in pregnancy and during the use of oral contraceptives. Other predisposing factors are poorly controlled diabetes mellitus [4], steroid therapy, treatment with broad-spectrum antibiotics, acquired immunodeficiency [14] syndrome, and the wearing of tight-fitting nylon underclothing, which increases perineal moisture and temperature [13]. Chemical contact, local allergy and a hypersensitivity reaction may also predispose to symptomatic vaginitis. There is no evidence that an iron deficiency predisposes to *Candida* infection [13].

The pathogenesis of the recurrent presence of *Candida* species in the vagina of a given patient remains unknown. Theories include frequent vaginal relapse or frequent reinfection due to the exogenous acquisition of the strains, either from a persistent gastrointestinal source or by sexual transmission [5, 6, 15]. After systemic and topical antimycotic therapy, negative vaginal cultures regain positivity for *Candida* within 30 days in 20 to 25% of women, which strongly supports the hypothesis that vaginal relapse is responsible for RVVC infection [4, 5]. Another theory is that, after successful therapy that eliminates clinical signs and symptoms, small amounts of yeast persist in the vagina despite the negativity of the vaginal cultures and increase after the cessation of antimycotic therapy [14].

For many years, isolates from women with RVVC were tested by the biotyping methods used by O'Connor and Sobel [15]. The results with this typing system indicated a large number of phenotypically distinct strains of *C. albicans*. However, classical biochemical tests are not satisfactory for investigation of the relatedness of different *Candida* species in this respect. The molecular typing methods, such as genomic DNA analysis, and electrophoretic karyotyping by PFGE, allow the separation of the large yeast chromosomal DNA on the basis of size [6, 7, 11]. The method can be used for a more precise differentiation of different *Candida* species and for karyotyping

of the genome of yeasts, including *C. albicans* and *C. glabrata*, for subspecies determination.

Our results revealed differences between the banding patterns of 3 different karyotypes each of *C. albicans* and *C. glabrata*. The karyotype similarity of the strains in a given patient during follow-up (month after month) supports the concept of chronic colonization and vaginal relapse. The persistence of the individual strains over a period of 4 to12 months, despite different periods of therapy, demonstrated the genetic stability of the Candida isolates, though reinfection with the identical strain cannot be excluded. The diversity of the karyotype of *C. glabrata* in one patient after a long period (12 months) was indicative of the appearance of a new infection.

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