The identification of pathogenic yeast strains by electrophoretic analysis of their chromosomes

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Summary. Epidemiological studies require characterisation of pathogenic yeasts at and below the species level. The chromosomes of 130 strains of four pathogenic species of the genus *Candida*, isolated from clinical material, were separated by pulsed field electrophoresis with the clamped homogeneous electric field (CHEF) technique. Each species was characterised by a distinct electrophoretic karyotype (EK). Furthermore, smaller variations of the EK amongst strains belonging to the same species appeared to offer a useful means of strain differentiation. A karyotyping system is proposed for *C. albicans*. The EKs were assigned to a code of four numbers which designated the number of bands that could be resolved in each of four sets of chromosomes. Morphotypes of the colonies of *C. albicans* on malt agar plates, which did not correlate with the EK, could provide a complementary means of strain characterisation in epidemiological studies.

Introduction

Infections with opportunistic yeasts of the genus *Candida* are becoming more frequent. The species determination is achieved routinely by the results of sugar fermentation tests, although *C. albicans*, which is most frequently isolated from clinical specimens, can be identified selectively by germ-tube formation in human serum.¹ An epidemiological approach requires characterisation below the species level. Previous studies reporting strain identification and differentiation, generally concerned with *C. albicans*, included morphotyping,^{2,3} biotyping by a series of plate tests,^{4,5} resistogram typing,^{6–8} serotyping,⁹ differential susceptibility to killer toxins,¹⁰ immunoblotting,¹¹ and DNA fingerprinting.¹² Recently, *C. albicans* was reported to have a variable karyotype by orthogonal field agarose gel electrophoretic (OFAGE) analysis performed with clinical isolates.¹³

Resolution of DNA fragments larger than 50 kb cannot be achieved by conventional agarose gel electrophoresis. Pulsed field gel electrophoresis (PFGE) was introduced for resolving DNA of chromosomal size in yeasts^{14,15} and protozoa.¹⁶ Contour clamped homogeneous electric field (CHEF) gel electrophoresis, the most recent devel-

opment of the basic PFGE, allows good resolution, with preservation of sharp bands, straight lanes and reproducible separation of DNA molecules greater than 5000 kb.¹⁷ Preliminary work has shown that this technique can be used in routine work for screening *Candida* strains. Therefore, the electrophoretic karyotype (EK) was investigated in 130 strains of four pathogenic species of the genus *Candida*, isolated from clinical material.

Materials and methods

Yeast isolates

The test strains—100 isolates of *C. albicans* and 10 each of *C. parapsilosis*, *C. glabrata* (formerly *Torulopsis glabrata*) and *C. tropicalis*—were isolated from 130 different patients at the Centre Hospitalier Universitaire Vaudois. All strains were grown on Sabouraud Agar and maintained on agar slants at 4°C.

Isolates were identified as *C. albicans* by germ tube production in human serum.¹ Strains that did not produce germ tubes were identified on the basis of sugar fermentation tests (API system).

Sample preparation for electrophoresis

Cells were grown overnight in Sabouraud Broth to late logarithmic phase at 30°C. They were then harvested and washed with 50 mM EDTA, pH 8, and 100 µl of the final

cell pellet resuspended in 150 μ l of 50 mM EDTA, pH 8, were treated at 37°C for 20 min with 80 μ l of lyticase solution containing (/ml) 2 mg of enzyme (Sigma 5263) in 10 mM sodium phosphate, pH 8, and glycerol 50%; 300 μ l of the cell-enzyme suspension were mixed with 1 ml of low gelling-temperature agarose 1% w/v prepared in 125 mM EDTA, pH 7.5 and cooled to 50°C. The mixture was poured into mould chambers and allowed to cool to 4°C for 20 min. The blocks of treated cells in gellified agarose were recovered and incubated overnight at 37°C in LETbuffer (0.5 M EDTA, pH 8.0, 0.01 M Tris, pH 7.5, 2-mercaptoethanol 7.5%). They were subsequently incubated for 24 h at 50°C with proteinase K (Boehringer 1.000.144), 1 mg/ml in NDS buffer (0.5 M EDTA, pH 8.0, 0.01 M Tris, pH 7.5, sodium laurylsarcosinate 1%). Finally, the agarose plugs were washed three times for 24 h with 50 mM EDTA, pH 8.0, at room temperature, and stored at 4°C in the same solution in a test tube.

CHEF electrophoresis

The chromosomes of each strain were separated by pulse field electrophoresis on agarose gels by the CHEF technique (contour clamped homogeneous electric field), in a CHEF-DR II system (Bio-Rad, USA). Pieces of 10 $\times$ 2 $\times$ 1 mm cut from sample preparation plugs were used to load the wells of the gels. The resolution of chromosomal DNA was performed in a 10 cm $\times$ 10 cm agarose 0.9% gel, run at 150 V at a 120-s switch for 24 h, followed by a 180-s switch for 16 h.

Saccharomyces cerevisiae chromosomal DNA size standards, expressed in kilobases (kb) were obtained from Biorad, USA.

Gels were stained with ethidium bromide (0.5 μ g/ml) for 20 min and washed with distilled water for 5 min. DNA bands were identified under UV light (254 nm). Photographs were taken with Polaroid type 667 film through a red filter (Kodak wratten 22) in a MP6 Polaroid camera.

Morphotype

Strains were streaked on malt extract agar (Oxoid) 6% as described by Hunter *et al.*, 1989 and Phongpaichit *et al.*, 1987.^{2,3} After incubation for 10 days at room temperature, each strain was examined and assigned a morphotype code.

Results

The EK of the strains of *Candida* showed profiles which were characteristic for each of the four species (figs. 1, 2, 3). Variations of the band pattern were due to differences both in the number of bands and in their migration distance. Differences observed allowed differentiation not only at the species level, but also between strains belonging to the same species (figs. 1 and 2). The EK of *C. tropicalis* appeared to be less variable than that of the three other species, *C. albicans*, *C. parapsilosis* and *C. glabrata*.

Fig. 1. Variation of the EK of 12 strains of *C. albicans*. Chromosomes of the yeast *S. cerevisiae* (arrow) were used as DNA size standards (indicated in kb on the left of the figure). The strains of *C. albicans* are assigned to a code of four numbers which designate the number of bands that can be resolved in four sets of chromosomes. The four sets are shown on the right of the figure.

Fig. 2. Variation of the EK of 6 strains each of *C. parapsilosis* (lines A3–A8), *C. glabrata* (lines B3–B8), and *C. tropicalis* (lines C3–C8). The chromosomes of *S. cerevisiae* (lines A1, B1, C1) are used as DNA size standards. The EK of six strains of the three yeast species is compared to the EK of a strain of *C. albicans* (no. 76 in our collection, EK code 0212) used as a reference (lines A2, B2, C2).

When the CHEF electrophoresis patterns of 100 clinical isolates of *C. albicans* were studied, bands which were single in some strains appeared to be double in others. Eleven bands have previously been resolved in *C. albicans* and four double bands

turned out to be homologous chromosomes,¹⁸ which results in seven genetically independent chromosomes. In the numbering system of Magee *et al.*,¹⁸ the largest chromosome was called no. 1 and the remainder were numbered successively in order of

Fig. 3. Characteristic band patterns of *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. tropicalis*. For *C. albicans* and *C. tropicalis*, bands which were single in some strains appeared to be double in others. The size of the bands is indicated (in kb) when it was constant in 90–100% of the strains. The size of the other bands varied between the strains. The chromosomes larger than 2000 kb are represented by a square. Bands occasionally present are not drawn on this diagram.

increasing distance of migration. For practical purposes we assigned the EK of the strains of *C. albicans* to a code of four numbers (fig. 1). These numbers designate the number of bands that could be resolved in four sets of chromosomes under the electrophoresis conditions used. Variations between strains occurred mainly in the chromosomes with smaller sizes. Consequently, the number of bands in the set of chromosomes migrating furthest was written in the first position. The first number designates the bands of less than 850 kb. DNA of this size was found in only eight strains which have a chromosome that apparently corresponded to none of the seven previously identified.¹⁸ This eighth chromosome appeared to be diploid as attested by a double band observed in the EK of one strain (fig. 1, class 2212). The second and third numbers refer to bands between 850 and 1125 kb, Magee's chromosomes nos. 6 and 7, and between 1125 and 1600 kb, Magee's chromosome no. 5, respectively.¹⁸ The latter three chromosomes are

Fig. 4. Electrophoretic karyotypes of nine strains of *C. albicans* belonging to class 0212.

Table. Distribution of 100 *C. albicans* strains isolated from various sites

EK code	Number of strains isolated from				Total
	genito-urinary tract (24)	oral cavity (32)	skin (26)	deep infections (18)	
0212	5	17	15	6	43
0222	10	6	5	5	26
0312	6	1	4	5	16
0322	0	3	2	2	7
1212	1	1	...	...	2
1213	...	1	...	...	1
1222	1	1	...	...	2
1223	...	1	...	...	1
1312	...	1	...	...	1
2212	1	...	...	...	1

diploid and can show pairs of homologous bands. The fourth number is generally 2, and corresponds to the chromosomes nos. 3 and 4 which are haploid.¹⁸ A third band was observed in some strains. Chromosomes larger than 2000 kb were not considered in our analysis. Using this code the strains of *C. albicans* could be separated into 10 classes (table). Their corresponding EK pattern is shown in fig. 1. Classes 0212 (43%) and 0222 (26%) were the most frequently found. The EK of the strains in the same class were not identical, with variations occurring in the exact position of the bands in the gel (fig. 4). Co-migration in adjacent wells of the chromosomal DNA of two such strains, or co-migration with the chromosomal DNA of a strain chosen as standard, may be necessary to achieve further strain differentiation.

Many different morphotypes were observed when strains of *C. albicans* were streaked on malt extract

Fig. 5. Morphotype variation of nine strains of *C. albicans* with EK of the class 0212. The EK of each strain, numbered 1–9, is shown in fig. 4.

agar.^{2,3} After incubation for 10 days at room temperature, yeasts with an EK belonging to the classes 0212, 0222 and 0312 exhibited colonies with or without a fringe, and smooth, rough, cratered or wrinkled surface. Morphotype and EK appeared to be independent characters as illustrated by comparing figs. 4 and 5. The other classes, containing a small number of specimens, were not examined for correlations between morphotype and EK.

Discussion

Conditions of electrophoresis were chosen for the resolution of bands between 500 and 3000 kb, which allows a separation of the chromosomes of *Candida* thought to be optimal for strain identification. Different chromosome preparations of the same strain gave identical band patterns under the same conditions of electrophoresis. Five transfers on conventional Sabouraud medium did not alter the EK of two strains each of *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. tropicalis* which were tested as a control. CHEF electrophoresis appears to be a simple and reproducible method of differentiation at and below the species level. The interpretation of the results is unequivocal and does not depend on the reader. Interestingly, in this study, strains of *C. albicans* with a small chromosome (400–650 kb) supplementary to the seven previously reported by Magee *et al.*¹⁸ were found.

These strains were identified as *C. albicans* by germ tube production in human serum, and the identity was confirmed by sugar fermentation.

Strains of *C. albicans* were also differentiated on the basis of morphology of colonies streaked on malt agar.^{2,3} Morphotyping appeared to be independent of the EK. These methods appear to be complementary and, used in conjunction, could yield a finer strain characterisation in epidemiological studies.

No clear association between a given EK and the anatomical source of the isolates was found (table) although a striking finding was the low and high proportion of EK of classes 0212 and 0222 respectively in isolates from the genitourinary tract. Further studies are needed to confirm or invalidate this unexpected result for which no obvious explanation is evident.

Interestingly there are data that indicate that strains showing identical EK can be isolated from different body sites in the same patient.¹³ Currently, the origin of the yeasts involved in systemic candidiasis occurring in immunocompromised patients, and in chronic mucocutaneous infections, is being investigated, and electrophoresis with the CHEF technique should permit a more precise approach.

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