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Comparison of Molecular Typing Methods for *Candida albicans* Strain Delineation

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Summary

This paper compares the reproducibility and discriminatory power of pulse-field electrophoresis, randomly amplified polymorphic DNA and DNA fingerprinting in C. albicans strain delineation. Seven different karyotypic patterns were identified among 103 C. albicans strains isolated from the oral cavities of healthy carriers and from oral lesions of HIV-seropositive and seronegative patients by pulse-field electrophoresis. The randomly amplified polymorphic DNA technique turned out to be more discriminative than karyotyping, allowing further diversification within strains sharing the same karyotype. However, such a technique did not appear useful when employed as the only DNA typing method, since several strains, characterized by different karyotypes, showed indistinguishable electrophoretic amplification profiles. DNA fingerprinting with the 27A probe (S. Scherer, D. A. Stevens, Proc. Natl. Acad. Sci. USA 85 (1988) 1452–1456) yielded the most resolutive analysis, since about as many polymorphic patterns were obtained as the individual strains typed. Therefore, while C. albicans DNA typing by pulse-field electrophoresis followed by random amplification of polymorphic DNA appears to be adequate for discriminating C. albicans strains into distinct types and subtypes, DNA fingerprinting may be suitable for rapid identification of individual strains in fine epidemiological investigations.

Keywords: Candida albicans; karyotyping, RAPD-RFLP analysis; molecular epidemiology

Introduction

Candida albicans is an opportunistic pathogen existing in human hosts as a commensal yeast, but capable of causing serious and even life-threatening diseases in immunocompromised individuals *(1)*. It is well known that an intact immune response is an essential requirement in the control of *C. albicans* transition from commensal colonizer to pathogen *(2)*. The finding that oral candidiasis occurs in 7–48% of HIV-infected individuals *(3)* and in up to 93% of AIDS patients *(4)* emphasizes the fact that cell-mediated immunity plays a key role against *C. albicans* mucosal infections. Neverthless, several virulence attributes of this organism have been identified, that are differently expressed depending on the infectious strain rather than on the host, which might explain the occurrence of symptomatic mucosal candidiasis in otherwise healthy individuals *(5)*.

Although *C. albicans* has become of increasing medical importance, relatively little is known about the epidemiology of *C. albicans* infection, mostly because available methods for strain delineation are based on phenotypic features which do not allow intraspecific discrimination such as serotyping *(6)*, streak morphology *(7)*, resistance to various chemicals *(8)*, enzyme profiles *(9)*, or susceptibility to toxins *(10)*. To better understand pathogenesis and epidemiology of candidiasis, it is of paramount importance to provide large-scale application DNA typing methods for defining the population genomic structure of this opportunistic yeast and for providing a reliable subdivision of *C. albicans* strains into genetically distinct types.

Karyotyping, random amplification of polymorphic DNA (RAPD) and DNA fingerprinting, with the *C. albicans*-specific moderately repetitive sequence 27A, have been applied to 103 *C. albicans* strains, isolated from the oral cavities of healthy carriers and from oral lesions of HIV-seropositive and seronegative patients. This paper

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compares the three molecular typing methods in order to assess their reliable and reproducible application to *C. albicans* strain delineation.

Materials and Methods

Source of C. albicans isolates

C. albicans strains were obtained from oral lesions of 38 HIV-seropositive individuals, treated as outpatients at the Divisione Malattie Infettive, Ospedale di Cisanello, Pisa, and 41 HIV-seronegative patients, not treated with antimycotics at the sampling time, at the Unità Operativa di Micologia, Dipartimento di Biomedicina Sperimentale, Infettiva e Pubblica, Università degli Studi di Pisa. Samples from control individuals were taken from the buccal epithelium of 24 asymptomatic students, who exhibited no signs of oral thrush. Samples were immediately plated on Sabouraud agar supplemented with gentamycine and chloramphenicol (Difco, Detroit, Michigan, USA). The isolated yeasts were identified as *C. albicans* by demonstration of a typical *C. albicans* pattern of sugar utilization (API, ID 32 C, bioMerieux, Marcy l'Etoile, France) and by the production of germ tubes after 2 h incubation in fetal calf serum (Biological Industries, kibbutz bet haemek, Israel).

Determination of C. albicans electrophoretic karyotypes

Preparation of *C. albicans* DNA for electrophoretic karyotyping and conditions for the resolution of chromosome-sized DNA molecules, by pulse field electrophoresis on agarose gel with a CHEF-DR II system (Bio-Rad, Richmond, Calif.), were performed as previously described *(11)*.

C. albicans DNA preparation for RAPD analysis and Southern blot hybridization

Yeast cells were grown on Sabouraud plates. One colony for each strain was picked up and inoculated in 50 mL of 15 g/L Malt Extract Broth (Difco, Detroit, Michigan, USA). At least five individual colonies of each *C. albicans* strain were used in separate experiments. The cultures were incubated at 37 °C for 18 h and the cells, harvested at the early stationary phase, were washed with 1.0 M sorbitol buffered with 4.0 mM potassium phosphate (pH = 7.0). The pellet was resuspended in 1.25 mL of the same solution containing 5 mg/mL lyticase (Sigma-Chemical Co, Milan, Italy). The cell suspension was incubated at 37 °C for 30–60 min until an average of 80% spheroplasts were observed. The spheroplasts were spun down and lysed by incubation at 60 °C for 30 min in lysis buffer containing 0.2 M NaCl, 0.1 M EDTA, 5% sodium dodecyl sulfate, 50 mM Tris-HCl (pH = 8.5) and 20 μg/mL proteinase K. Nucleic acids were extracted with phenol-chloroform-isoamyl alcohol (25:24:1); the aqueous phase was recovered and treated with chloroform-isoamyl alcohol (24:1). Purified DNA was precipitated with 2 volumes of absolute ethanol and 0.1 volume of 3.0 M sodium acetate. The precipitated DNA was washed with 70% ethanol, dried for 30 min at 37 °C and suspended in 10 mM Tris containing 1 mM EDTA (pH = 8.3) (TE buffer). Following an incubation for 30 min at 30 °C with RNAse (20 μg/mL; Sigma-Chemical Co., Milan, Italy), DNA was precipitated once more and stored at 4 °C in TE buffer.

C. albicans DNA analysis by RAPD fingerprinting

RAPD fingerprinting *(12)* of DNA was performed with the primer HLWL 85 (5′ ACAACTGCTC 3′). Polymerase chain reactions were carried out in 0.5 mL microcentrifuge tubes in a final reaction mixture of 50 μL, containing 1μM HLWL 85, 200 μM of each dNTP (dATP, dGTP, dCTP, dTTP), 5 μL $MgCl_2$-reaction buffer (10 mM Tris-HCl, pH = 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 2.5 U_s of *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden) and 0.1 μg *C. albicans* genomic DNA. Amplification reactions were performed in a DNA 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, USA) set at the following conditions: 94 °C, 5 min; 36 °C, 5 min, and 72 °C, 5 min, for four cycles; 94 °C, 1 min; 36 °C, 1 min, and 72 °C, 2 min for 30 cycles; a final period at 72 °C for 10 min. The amplified DNA products were separated by electrophoresis in 2% agarose gel, containing ethidium bromide (0.5 μg/mL). TAE (0.04 M Tris acetate, 0.001 M EDTA, pH = 8) was used as the electrophoresis buffer and marker III (λ DNA-*Eco*RI-*Hin*dIII, Boehringer Mannheim Italia SpA, Monza, Italy) as molecular size standard.

Southern blot hybridization

DNA of each strain was digested with *Eco*RI (Boehringer Mannheim Italia SpA, Monza, Italy), and the DNA fragments obtained were separated in 1.0% agarose gel, containing ethidium bromide (0.5 μg/mL). *Eco*RI-digested DNA of *C. tropicalis* was included in every Southern blot of *C. albicans* strain to assess the species-specificity of the 27A probe, a 6 kbp dispersed, repeated DNA segment *(13)*. DNA was transferred to a nylon membrane by capillary blotting and fixed by drying for 40 min at 80 °C in a conventional oven. The membrane was incubated for 2 h at 65 °C in prehybridization solution (0.2 mL/cm^2), containing SSC (6X), SDS (0.5%), Denhardt's solution (5X), salmon sperm DNA (10 μg/mL) and hybridized with digoxigenin-labeled 27A probe (DIG Easy Hyb; Boehringer Mannheim Italia SpA, Monza, Italy) at 65 °C for 18 h. The labelling of the 27A sequence with digoxigenin-11-dUTP was perfomed by the random priming method (DIG-High Prime Labelling and Detection Starter Kit I; Boehringer Mannheim Italia SpA, Monza, Italy). The hybridization products were detected by the colorimetric reagents NBT and BCIP (DIG-High Prime Labelling and Detection Starter Kit I; Boehringer Mannheim Italia SpA, Monza, Italy).

Results

C. albicans karyotypes

Resolution of chromosome-sized DNA molecules into electrophoretic karyotypes was carried out on 103 *C. albicans* strains, isolated from healthy carriers and from HIV-seropositive and seronegative patients, suffering from symptomatic oral candidiasis. Variability of *C. albicans* karyotypes was found to be restricted to only

seven different karyotypes, designed as types a, b, c, d, x, y and z, which showed electrophoretic profiles indistinguishable from those already described in a limited number of *C. albicans* strains, isolated from the oral cavities of human hosts in the same geographical locale *(11)*. Most karyotypes, with the exception of types x and y, were isolated as commensal strains from healthy carriers, and all of them were associated with oral candidiasis of HIV-infected and non-infected individuals (data not shown). *C. albicans* strains exhibiting karyotypes b and c offered the most common commensal strains as well as the most common infectious agents, giving roughly 70% of shared karyotypes b and c in asymptomatic healthy individuals and in symptomatic HIV-infected and non-infected patients. However, appreciable differences were observed in the frequency of isolation of such karyotypes from healthy carriers (42.3% type b and 34.6% type c), HIV-seropositive (38.3% type b and 29.4% type c) and HIV-seronegative individuals (16.3% type b and 51.1% type c) (Fig. 1).

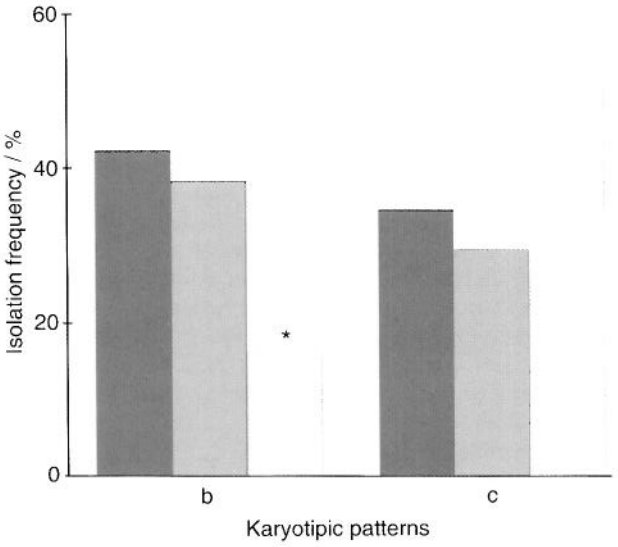

Fig. 1. Isolation frequency of *C. albicans* karyotypes b and c from oral lesions of HIV-seropositive (▓) and seronegative (▒) patients, and from the oral cavities of healthy carriers (▒). * Differences in isolation frequency of karyotype b between HIV-seronegative patients and HIV-seropositive patients as well as healthy carriers were statistically significant ($p<0.05$).

C. albicans RAPD profiles

HLWL 85 primer used in DNA amplification reactions gave a great variety of RAPD profiles, the reproducibility of which was assayed by testing the same clinical strains of *C. albicans* in triplicate in separate experiments. The number, disposition and band thickness of DNA products obtained by RAPD enabled recognition of 22 different patterns, some of which are presented in Fig 2. In all RAPD profiles obtained, some DNA products were constantly observed, with comparable intensity, at an electrophoretic migration corresponding to 1.9, 1.1, 0.8 and 0.2 kbp. Although RAPD analysis was more discriminating than karyotyping, some *C. albicans* strains exhibiting different karyotypes were undistinguishable when RAPD analysis was used as the only typing method. For example, RAPD profile numbered as 3 in Fig. 3 was obtained from different *C. albicans* strains exhibiting either karyotype a, b, c, or d. Neverthless, *C. albicans* DNA typing by RAPD analysis was suitable to further discriminate strains exhibiting the same karyotype. For example, four different subtypes, designed as b1, b2, b3 and b4, were resolved within type b karyotype (Fig. 3).

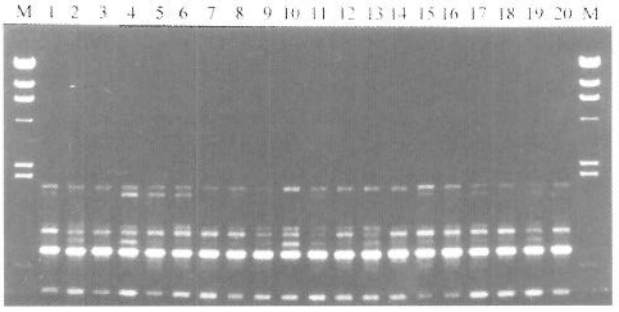

Fig. 2. Separation of DNA products obtained by RAPD from *C. albicans* strains with HLWL-85 as primer. Lanes: 1–4, karyotype a; 5–9, karyotype b; 10–13, karyotype c; 14–18, karyotype d; 19, karyotype y; 20, karyotype z; M, marker λDNA-*Eco*RI/*Hind*III.

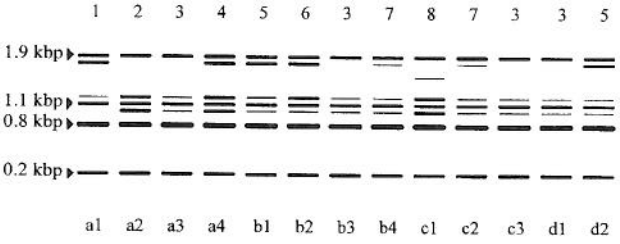

Fig. 3. Schematic representation of RAPD profiles of *C. albicans* strains with HLWL-85 as primer. Letters indicate karyotypes of *C. albicans* isolates and contiguous numbers indicate RAPD profiles within the same karyotype. Thin and thick lines correspond to band thickness and intensities of ethidium bromide staining. Numbers at the top of the figure indicate different RAPD profiles.

C. albicans DNA fingerprinting and hybridization with C. albicans-specific probe

Hybridization-based fingerprinting, performed with the *C. albicans*-specific repetitive sequence 27A, was applied to 49 clinical strains of *C. albicans*. The different patterns obtained were almost as many as the strains typed. A representative example of hybridization profile obtained from clinical strains of *C. albicans* with the probe 27A is shown in Fig. 4, together with the specificity of the probe, tested with *C. tropicalis* (line 21 of Fig. 4). The huge variability in the hybridization profiles did not appear to be due to the method itself, since a given strain was resolved into the same hybridization profile in separate experiments. Therefore, *C. albicans* DNA fingerprinting followed by hybridization with the specific probe 27A showed a discriminatory power higher than

karyotyping and RAPD analysis, being potentially suitable for discriminating individual strains.

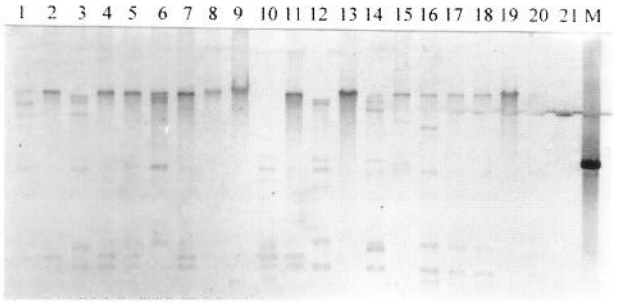

Fig. 4. Hybridization of *Eco*RI-digested genomic DNA from oral isolates of *C. albicans* with the *C. albicans*-specific sequence 27A probe. Lanes: 1–18, oral isolates of *C. albicans*; 19–20, two colonies of the same *C. albicans* strain; 21, *C. tropicalis*; M, 27A probe. Digoxigenin-labeled 27A probe was used and hybridization products were detected by colorimetric reagents.

Discussion

Several genome typing methods are being developed for discriminating clinical strains of *C. albicans* with the aim of answering questions such as whether a new episode of the disease is caused by reinfection or relapse, whether commensal strains are the source of infection, and whether there are certain strains adapted to particular body niches or geographical locales.

None of the DNA typing methods used have been proven to ensure accurate intraspecific discrimination of *C. albicans* strains, and combinations of several methods have been tried out in order to clearly identify individual strains *(14–17)*.

In the present study, clinical strains of *C. albicans*, isolated from the oral cavities of asymptomatic healthy carriers and from oral lesions of symptomatic HIV-infected and non-infected individuals, have been typed by three molecular typing methods such as: (1) karyotyping by pulse-field electrophoresis; (2) analysis of restriction fragment length polymorphism after hybridization with the DNA probe 27A; (3) random amplification of polymorphic DNA with the arbitrary primer HLWL 85.

The application of karyotyping to 103 oral isolates of *C. albicans* produced only seven different karyotypes, designed as types a, b, c, d, x, y and z, as already observed for a more limited number of *C. albicans* strains isolated from the oral cavities of human hosts in the same geographical locale *(11)*. Moreover, only two karyotypes, types b and c, were isolated as the most common infectious agents of oral candidiasis, as well as the most frequent commensal strains of the same body niche in healthy carriers (Fig. 1). Although determination of karyotypic patterns was found to be highly reproducible, the observation that only a limited number of distinct karyotypes could be recognized within oral isolates of *C. albicans* suggested that this typing method could not be used as the only DNA typing approach to track *C. albicans* strains within a host or between hosts, or to associate an individual strain to reinfection or relapse episodes. Random amplification of polymorphic DNA with HLWL 85 as a primer showed higher resolving power than karyotyping (Fig. 2). As many as 22 different RAPD profiles were reproducibly obtained, thus allowing discrimination of *C. albicans* strains sharing the same karyotype into genetically distinct subtypes (Fig. 3). However, the RAPD technique yielded similar electrophoretic patterns from genomic DNA of *C. albicans* strains, characterized by different karyotypes (Fig. 3). Genotypic characterization of *C. albicans* strains by DNA fingerprinting with the repeated element 27A as a probe was found to be the most discriminative molecular approach since about as many polymorphic patterns were obtained as the individual strains typed (Fig. 4).

Consequently, while karyotyping and RAPD profile analysis allowed reliable clustering of *C. albicans* strains into distinct types and subtypes proving to be applicable to epidemiological studies, 27A DNA fingerprinting seemed to be more adequate for fine epidemiological studies, since it enables the identification of the same strain isolated at different times during the course of infection, and the discrimination of different strains at a single site of infection.

Conclusions

Comparison of molecular typing methods in *Candida albicans* strain delineation showed that: (1) karyotyping by pulse field electrophoresis is a highly reproducible method for discriminating *C. albicans* strains into distinct types; (2) analysis of RAPD profiles is more discriminative than karyotyping, allowing further diversification of strains showing the same karyotype into distinct subtypes; (3) DNA fingerprinting with the 27A probe shows excellent discriminatory power, being potentially suitable for discriminating individual *C. albicans* strains for fine epidemiological investigations.

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Usporedba postupaka molekularne tipizacije za označavanje soja *Candida albicans*

Sažetak

U radu su uspoređeni reproducibilnost i sposobnost razlikovanja postupka elektroforeze u pulsirajućem polju i postupka »fingerprinting« nasumce amplificiranih polimorfnih DNA i RNA radi označavanja soja C. albicans. Između 103 soja C. albicans, izoliranih iz usne šupljine, zdravih ispitanika i oralnih lezija HIV-seropozitivnih i seronegativnih pacijenata, elektroforezom u pulsirajućem polju utvrđeno je 7 različitih kariotipova. Postupkom s nasumce amplificiranom DNA bilo je moguće daljnje razlikovanje unutar sojeva koji imaju isti kariotip. Međutim, postupak »fingerprinting« nedovoljan je ako se koristi kao jedini način za tipizaciju DNA zbog toga što su mnogi sojevi, okarakterizirani različitim kariotipovima, pokazivali elektroforetske amplifikacijske profile koji se nisu međusobno razlikovali. Postupak »fingerprinting« za DNA s 27A sondom (S. Scherer, D. A. Stevens, Proc. Natl. Acad. Sci. USA, 85 (1988) 1452-1456) davao je najbolje rezultate razlikovanja, jer se dobilo toliko polimorfnih uzoraka koliko je bilo pojedinačnih tipova sojeva. Stoga, dok tipizacija soja C. albicans, elektroforezom u pulsirajućem polju i naknadnom amplifikacijom polimorfne DNA, daje adekvatne rezultate u razlikovanju sojeva C. albicans u određene tipove i podtipove, postupak DNA »fingerprinting« pogodan je za brzu identifikaciju pojedinih sojeva u preciznim epidemiološkim istraživanjima.