Comparison of DNA-based typing methods to assess genetic diversity and relatedness among *Candida albicans* clinical isolates

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Summary

Three serial isolates of *Candida albicans* were obtained from each of five HIV-infected patients with recurrent oropharyngeal candidiasis from the same geographical area. Isolates from one patient remained susceptible to fluconazole whereas serial isolates from the other four patients showed decreasing susceptibilities to the drug. Strain identity was investigated by pulse-field gel electrophoretic (PFGE) separation of chromosomes, restriction fragment length polymorphism (RFLP) of chromosomal DNA, Southern blot analysis with the moderately repetitive probe Ca3 of the materials present in the RFLP gels after transfer to nylon membranes, and random amplification of polymorphic DNA (RAPD). All techniques were able to group isolates obtained from the same patient. Techniques resulting in more complex banding profiles exhibited increased discriminatory power allowing detection of strain variants. Methods resulting in less complex banding patterns, especially Southern hybridization of *Sfi*I digested chromosomal DNA with the moderately repetitive probe Ca3, were more helpful to determine isogenicity among isolates obtained from the same patient. The combination of results from methods with high discriminatory power (to maximize detection of strain variants) and methods resulting in less complex banding patterns (to allow determination of isogenic isolates) should facilitate the delineation of the epidemiology of *C. albicans* infection.

Key words

Candida albicans, DNA-typing, Azole resistance

Comparación de distintas técnicas de genotipificación para la evaluación de la diversidad genética entre aislamientos clínicos de *Candida albicans*

Resumen

Se obtuvieron tres aislamientos clínicos de *Candida albicans* de episodios consecutivos de candidiasis orofaringea en cinco pacientes infectados con el virus VIH de una misma región geográfica (San Antonio, Texas). En un paciente, los aislamientos permanecieron sensibles al fluconazol, mientras que en los cuatro restantes mostraron sensibilidad decreciente a este fármaco. La tipificación de cepas se llevo a cabo mediante separación de cromosomas bajo electroforesis en campo pulsante (cariotipado), análisis de polimorfismos de longitud de los fragmentos de restricción (RFLP), análisis por Southern blot con la sonda moderadamente repetitiva Ca3, y análisis de polimorfismos del ADN amplificado al azar mediante PCR (RAPD-PCR). Todas las tecnicas fueron capaces de agrupar los aislamientos obtenidos a partir del mismo paciente. Las tecnicas que resultaron en patrones de bandas más complejos exhibieron un poder de discriminación más elevado, permitiendo la detección de sub-cepas y variantes de cepas. Los métodos que resultaron en patrones de bandas más sencillos se mostraron más utiles para determinar la isogenicidad de los aislamientos obtenidos del mismo paciente. La combinación de técnicas con alto y bajo poder de discriminación para la tipificación de cepas facilita la delineacion de la epidemiologia de la candidiasis.

Palabras clave

*Candida albican*s, Tipificación de cepas, Resistencia a azoles

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The epidemiology of *Candida albicans* infection is complex [1-5]. Earlier studies on the epidemiology of candidiasis used a variety of phenotypic properties to assess strain identity, but lacked resolution power [6,7]. With the advent of molecular genetics, new, more powerful DNA-based typing methods have emerged as the "gold standard" for epidemiological studies, and are now widely used for strain delineation of *C. albicans* isolates [6-10]. These include fingerprinting methods such as karyotyping using pulse-field electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA analysis (RAPD), and Southern hybridization with moderately repetitive DNA probes [8,10]. In order to be useful epidemiological tools, these methods should be able to discriminate between unrelated strains, and at the same time to demonstrate the relationship of all organisms isolated from individuals infected through the same source [11]. Methods resulting in more complex banding patterns should presumably provide increased levels of discrimination and allow detection of substrains or strain variants that may evolve from an original strain [11-13]. Time and monetary considerations also play a role in the selection of a method of choice [11].

Several authors have compared different genotyping methods for *C. albicans* [8,13-16]. In general, there is a lack of consensus on the method(-s) of choice as well as in the interpretation of results. The use of a single method may not be optimal and a combination of typing techniques is often required to provide a comprehensive assessment of the epidemiology of candidiasis [6,8,13,15].

We have been interested in the study of fluconazole resistance in HIV-infected patients with oropharyngeal candidiasis (OPC), including the characterization of the molecular mechanisms responsible for the development of fluconazole resistance in *C. albicans* strains [17,18]. For these studies, the use of DNA-typing techniques is instrumental in inferring strain isogenicity, since determination of molecular mechanisms of resistance requires the use of matched sets of susceptible and resistant isolates [17,19]. Here we report on the comparison between different DNA-typing methods to assess genetic diversity and relatedness among *C. albicans* isolates recovered from successive episodes of OPC from HIV-infected patients in the San Antonio (Texas, USA) area.

MATERIALS AND METHODS

Organisms and culture conditions. Three serial isolates of *C. albicans* were obtained by direct swab or by oral saline rinses from five HIV-infected patients with recurrent OPC enrolled in a longitudinal study to assess significance of fluconazole resistance. Patients were treated initially with fluconazole at 100 mg/day, and increased doses to up to 800 mg/day if necessary for clinical resolution if development of resistance was detected [20,21]. The identity of the clinical isolates as *C. albicans* was confirmed by both biochemical (API 20C, Analytab Products, BioMerieux, France), and microbiological (germ-tube formation in serum-containing medium, color in CHROMagar *Candida* [CHROMagar, France]) procedures. Initial plating of isolates and preliminary assessment of drug susceptibility was performed using a fluconazole-agar dilution method [22,23]. Briefly, dilutions of oral samples are added to plates containing solid medium with and without fluconazole from which individual colonies are recovered. This technique maximizes early detection of resistant isolates. Determination of the fluconazole MICs for the different isolates was performed following the NCCLS broth macrodilution procedure [24]. The different isolates were stored at room temperature as suspensions in sterile deionized water. Table 1 shows the identity of isolates, the patient from which they were recovered, the elapsed time of isolation and the fluconazole MIC values for each isolate.

Table 1. Identity of *Candida albicans* isolates from HIV-infected patients with OPC.

Patient	Isolate Number	Elapsed Time of Isolation (Days)	Fluconazole MIC[a] (µg/ml)
A	1	-	4
A	2	20	0.25
A	3	138	4
B	4	-	0.5
B	5	210	8
B	6	280	>64
C	7	-	4
C	8	7	16
C	9	296	32
D	10	-	0.5
D	11	15	8
D	12	58	16
E	13	-	0.25
E	14	44	8
E	15	100	16

[a]As determined by NCCLS broth macrodilution method.

Strain identification. Strain identity was investigated by pulse-field gel electrophoretic (PFGE) separation of chromosomes (karyotyping), restriction fragment length polymorphism (RFLP) of chromosomal DNA digested with *Eco*RI or *Sfi*I and separated by PFGE, Southern blot analysis with the moderately repetitive probe Ca3 of the materials present in the RFLP gels after transfer to Nylon membranes, and fingerprinting by random amplification of polymorphic DNA (RAPD) with the phage M13 core sequence and $(GACA)_4$ primers.

For electrophoretic karyotyping *C. albicans* isolates were plated on Sabouraud dextrose agar and grown at 30˚C for 48 h. Colonies were suspended in 2 ml of 75 mM NaCl/25 mM EDTA (Ethylenedia-aminetetraacetic acid) to a turbidity of approximately 2.0 McFarland. The suspension was centrifuged at 230 *g* for 10 min and the pellet resuspended in 1 ml of 75 mM NaCl/25 mM EDTA. Plugs were made by mixing together at 37°C: 1) 1 ml of 1.5% low melting point agarose in 125 mM EDTA, pH 7.5, 2) 75 µl of 2000 units/ml zymolase-20T (ICN Biomedicals, Inc., Aurora, Ohio), and 3) 1 ml of the cell suspension. This mixture was distributed into plug molds and refrigerated for 1 h at 4°C. Spheroplasts were made by placing plugs in 4 ml of 0.5 M EDTA, pH 9.0/7.5% ß-mercaptoethanol. Plugs were incubated overnight at 37°C, then rinsed with 5 ml of 50 mM EDTA, pH 7.5. Four ml of ESP (0.5 M EDTA / 10% sarcosyl / 20 µg/ml proteinase K) solution was added to each tube and incubated at 50°C overnight. Plugs were refrigerated at 4°C for 1 h. The chromosomes were resolved on 1.0% low melting agarose gel using Contour-clamped Homogenous Electric Field (CHEF) (CHEF-DR III, Bio Rad, Hercules, Calif.). The conditions used for CHEF electrophoresis were: I) 120 sec 4.5V/cm 21 h; II) 300 sec 4.5V/cm 18 h; III) 300 sec 3.4 V/cm 28 h. After electrophoresis, gels were stained with ethidium bromide, illuminated under ultraviolet light and photographed.

RFLP patterns were generated by digestion of genomic DNA with *Sfi*I or *Eco*RI (Boehringer-Mannheim, Indianapolis, Ind.) and subsequent separation of DNA fragments by PFGE. Briefly, plugs prepared as described before were incubated in the presence of the corresponding restriction endonuclease overnight at 37°C (for *Eco*RI) or 50°C (for *Sfi*I). After digestion, the plugs were

loaded in wells of a 0.8% agarose gel. This gel was placed in a CHEF gel chamber (DR III, BioRad) with electrophoresis performed using the following parameters: For *Sfi*I pulse times were ramped from 5 sec to 35 sec for 24 h at 6 V/cm. For *Eco*RI pulse times were ramped from 5 sec to 35 sec for 18 hours at 4.5 V/cm. After the run gels were stained with ethidium bromide and photographed.

For Southern-hybridization with the moderately repetitive probe Ca3 the materials present in the RFLP gels were transferred to nylon membranes (Nytran, Schleicher and Schuell, Keene, N.H.) overnight using the Turboblotter apparatus (Schleicher and Schuell) and 20x SSC (3 M NaCl, 0.3 M Na citrate, pH 7.0) buffer. Subsequently, materials present in the Nylon membranes were hybridized with a Ca3 probe [25-27] radioactively labeled by random priming (Random DNA Labeling System, GibcoBRL, Gaithersburg, Md.). Prehybridization and hybridization were performed using Rapid-hyb buffer (Amersham Life Science Inc., Arlington Heights, Ill.) following manufacturer's instructions. The membranes were then washed and exposed to autoradiography film (Du Pont, Wilmington, Del.).

For RAPD analysis, genomic DNA from the different isolates was extracted using YeaStar Genomic DNA (Zymo Research, Orange, Calif.). The primers used in the PCR reactions were the core sequence of phage M13: (5'-3') (GAGGGTGGCGGTTCT) and the simple repeat sequence $(GACA)_4$. PCR reactions were performed in volumes of 50 µl containing 10 ng *C. albicans* DNA; 1 µM of each single primer; 0.6 U of Taq DNA (Life Technologies Gibco-BRL, Gaithersburg, Md.); 200 µM each of dATP, dCTP, dGTP and dTTP; 5 µl of 10x Taq-polymerase buffer (Gibco BRL); 3 mM $MgCl_2$ and sterile water to 50 µl. The following program was used: Initial denaturation for 1 min at 95°C; folowed by 35 cycles of denaturation (30 sec at 95°C), primer annealing (40 sec at 50°C) and extension (30 sec at 75°C). The amplification ended with a 5 min extension at 75°C, finally cooling down to 4°C. After amplification 5 µl of gel loading buffer was added to each reaction tube, and the amplification products separated in 1% agarose gels. After the run, gels were stained with ethidium bromide and photographed.

Documentation. Pictures of the gels or films were scanned using a Kodak Digital Camera and Image Analysis System (EDAS 120, Eastman Kodak, Rochester, N.Y.). For preparation of figures, digital images were processed using the Adobe Photo Shop program (Adobe Systems Inc., Mountain View, Calif.).

Visual analysis of band patterns. The fingerprints obtained were compared for similarity by visual inspection of band patterns. Sizes of DNA fragments amplified by PCR were determined by direct comparison with the DNA marker (100 bp ladder, Gibco-BRL). Fingerprints were considered highly similar when all visible bands obtained had the same migration distance for each isolate. Variations in intensity and shape of bands among isolates were not considered differences. The presence or absence of one distinct band was considered a difference.

Computer-assisted analysis of fingerprinting patterns. All fingerprints were analyzed with the Molecular Analyst Fingerprinting Software (Bio-Rad Laboratories, Hercules, Calif.). Bands were automatically identified by the resident software, verified and edited manually. Dendrograms were generated by the hierarchic unweighted pair-group method with arithmetic averages (UPGMA) cluster algorithm. Gel normalization, standardization, fingerprint analysis and the methods and algorithms used in this study were performed according to manufacturer's instructions.

RESULTS

All fifteen *C. albicans* isolates in this study, three from each of five HIV-infected patients with OPC (Table 1), were included in the analysis by each of the methods described above. Fingerprinting patterns obtained by each of the different typing methods are shown in the top panel (panel A) of Figures 1 through 6. The dendrograms generated from banding patterns obtained by each technique are shown in the bottom panels (panel B) of the corresponding figure.

All PFGE-based techniques, including karyotyping (Figure 1), RFLP analysis with *Sfi*I (Figure 2) and Ca3 hybridizations with *Sfi*I and *Eco*RI digests (Figures 3 and 4 respectively) were able to group isolates obtained from the same patient, and discriminate from those obtained from all other patients. Ca3 probe hybridization of *Eco*RI digested chromosomal DNA (Figure 4) resulted in the most complex banding patterns and exhibited increased discriminatory power allowing detection of differences among isolates obtained from the same patient. These differences may be indicative of the presence of sub-strains or strain variants with sequential episodes of OPC. However, without the need of blotting and hybridization, direct staining of RFLP gels for *Sfi*I-digested chromosomal DNA separated by PFGE (Figure 2) exhibited discriminatory power similar to the Ca3 probe hybridization of *Eco*RI digests, and was also able to detect subtle differences among serial isolates from the same patient. Ca3 hybridization fingerprints of *Sfi*I digested chromosomal DNA (Figure 3) yielded simple and easy to interpret patterns that allowed determination of isogenic isolates for each patient. Because of the increased resolution power obtained by using PFGE, this technique was also able to distinguish between strains recovered from the different patients. Despite the fact that *C. albicans* exhibits unstable karyotypes, the karyotyping patterns obtained (Figure 1) also indicated that serial isolates obtained from each patient were isogenic, but differed among patients. Of interest, dendograms obtained from karyotyping, RFLP with *Sfi*I and Ca3 hybridization fingerprints of *Sfi*I-digested DNA consistently assessed a higher degree of relatedness between isolates obtained from patients 1 (isolates 1-3), 4 (isolates 10-12) and 5 (isolates (13-15) compared to isolates from patients 2 (isolates 4-6) and 3 (isolates 7-9) that clustered in a different branch of the dendogram for all three typing methods.

For RAPD analysis (Figures 5 and 6) each primer generated between 1 and 10 major bands for an individual isolate. RAPD analysis grouped most, but not all related isolates. Some discordance was also detected according to the individual primer used for RAPD analysis.

DISCUSSION

DNA-typing techniques have emerged as powerful epidemiological tools for the investigation of strain identity in a number of infections [11]. These techniques are used to determine the origins of infection, the routes of acquisition and transmission of strains, their persistence and the micro-evolution within a single strain [11]. A number of DNA-based typing techniques have been described to aid the strain delineation during *C. albicans* infections. These include karyotyping, RFLP, hybridiza-

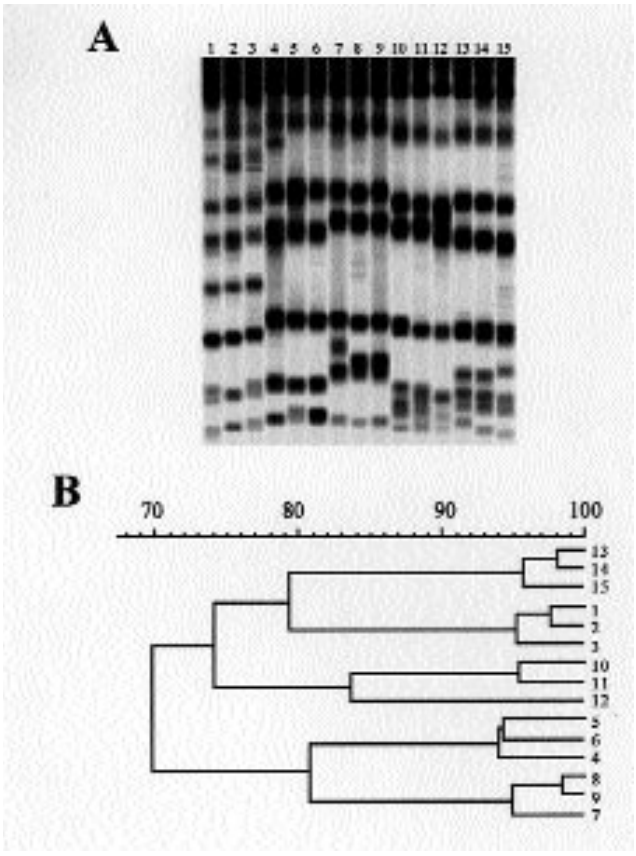

Figure 1. Electrophoretic karyotypes of *Candida albicans* clinical isolates recovered from HIV-infected patients (Panel A). The dendrogram generated from this analysis is shown in Panel B. See Table 1 for identity of isolates.

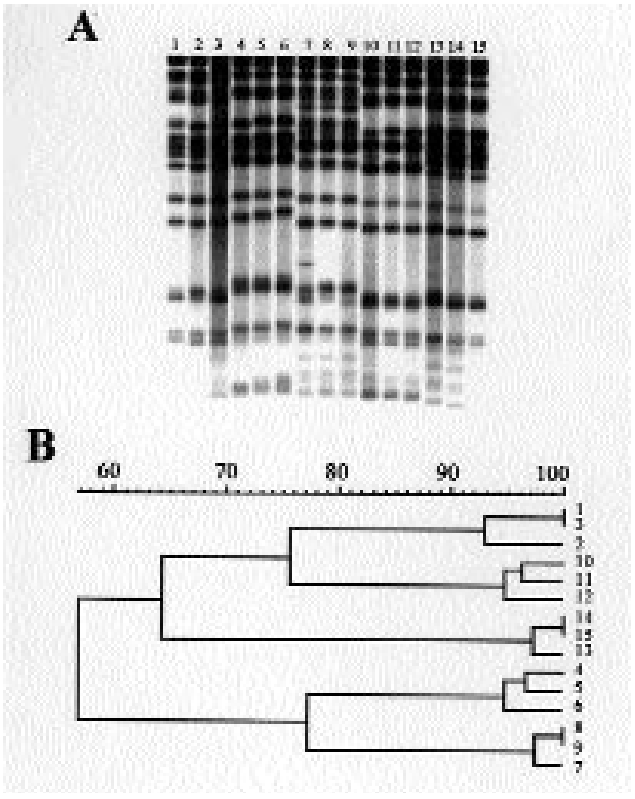

Figure 2. RFLP patterns generated by digestion with *Sfi*I of genomic DNA of *Candida albicans* clinical isolates recovered from HIV-infected patients (Panel A). The corresponding dendrogram is shown in Panel B. See Table 1 for identity of isolates.

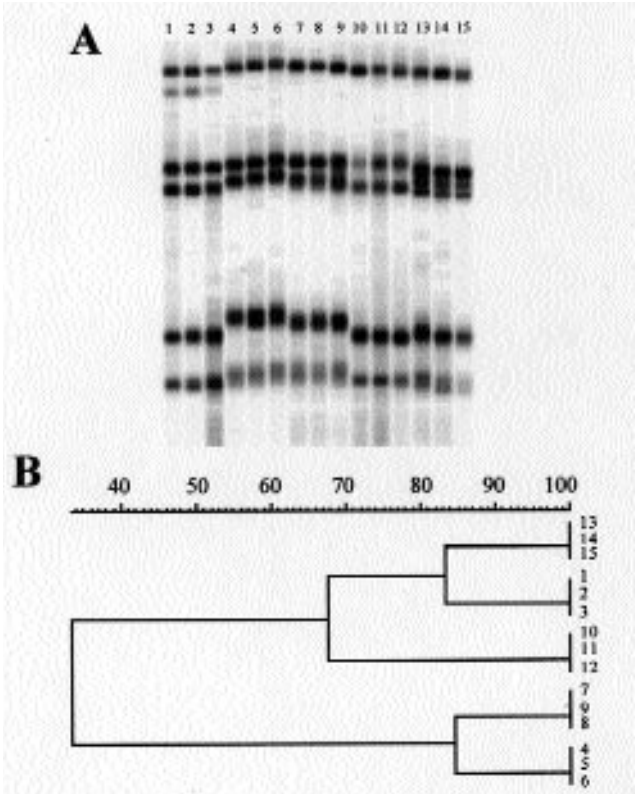

Figure 3. Hybridization profiles obtained with the moderately repetitive probe Ca3 of genomic DNA of *Candida albicans* clinical isolates recovered from HIV-infected patients digested with *Sfi*I (Panel A). The corresponding dendrogram is shown in Panel B. See Table 1 for identity of isolates.

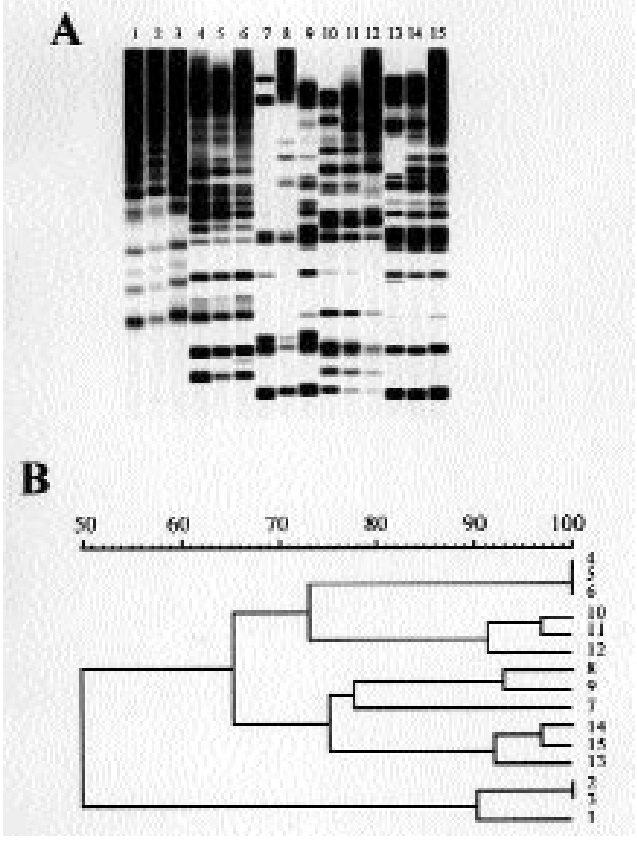

Figure 4. Hybridization profiles obtained with the moderately repetitive probe Ca3 of genomic DNA of *Candida albicans* clinical isolates recovered from HIV-infected patients digested with *Eco*RI (Panel A). The corresponding dendrogram is shown in Panel B. See Table 1 for identity of isolates.

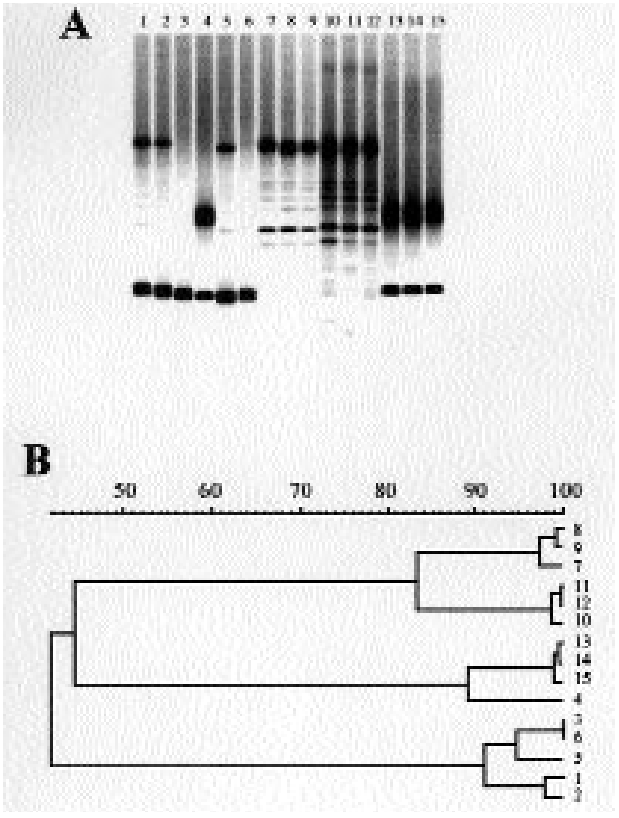

Figure 5. RAPD fingerprinting patterns obtained with the M13 primer for *Candida albicans* clinical isolates recovered from HIV-infected patients. (Panel A). The corresponding dendrogram is shown in Panel B. See Table 1 for identity of isolates. The figure is a composite of two different gels run under the same electrophoretic conditions, standardized and aligned by the fingerprinting software.

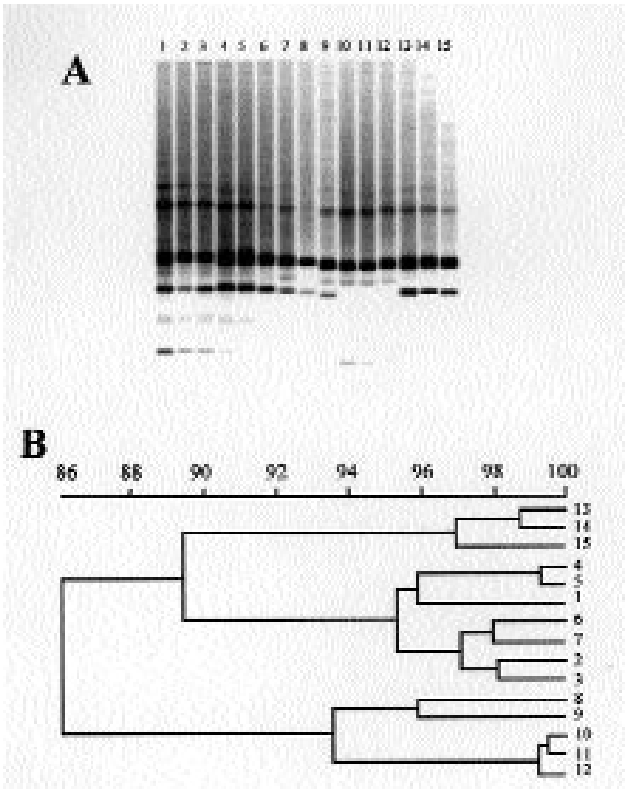

Figure 6. RAPD fingerprinting patterns obtained with the $(GACA)_4$ primer for *Candida albicans* clinical isolates recovered from HIV-infected patients. (Panel A). The corresponding dendrogram is shown in Panel B. See Table 1 for identity of isolates. The figure is a composite of two different gels run under the same electrophoretic conditions, standardized and aligned by the fingerprinting software.

tion with fingerprinting probes, and RAPD [6,8-10,12,14,15,28]. Each of these techniques has proven to be useful in the study of the epidemiology of *Candida*. Factors to be taken into account when choosing a particular typing method are the ease with which the resulting patterns can be interpreted, and the technical difficulty, time and cost of the analysis [6,11].

In general, the DNA-typing methods used in the present study exhibited the ability to group isolates recovered from the same patient and also differentiate from those obtained from different patients, both visually and with the aid of computer-assisted procedures. Some of the techniques were also able to discriminate differences in highly related isolates (serial isolates from the same patient). In general, PFGE-based techniques demonstrated superior clustering and discriminatory power compared to PCR-based techniques. All PFGE-based techniques demonstrated good clustering capabilities. RFLP analysis with *Sfi*I and Ca3 hybridization of *Eco*RI digests resulted in the most complex banding patterns and provided increased levels of discrimination and comparison. This is particularly important when searching for presence of substrains or strain variants generated from an initial strain [29]. On the other hand, Southern hybridization of *Sfi*I digested chromosomal DNA with the moderately repetitive probe Ca3 was the method resulting in fewer polymorphic bands. However, due to the increased resolution achieved by PFGE, this technique was still able to discriminate between strains infecting the different patients. The resulting patterns were easier to interpret (both visually and with computer-assistance) and more helpful to unequivocally determine isogenicity among isolates obtained from the same patient, which could be important in the determination of matched sets of susceptible and resistant isolates required for meaningful analysis of resistance mechanisms [17,19]. Overall, there was a very good congruence in clustering results between karyotype, RFLP with *Sfi*I and Ca3 hybridization of *Sfi*I digests, and the combination of these three techniques provided a good assessment of both genetic diversity and relatedness between the different isolates.

Results from RAPD analysis displayed lower grouping capabilities compared to PFGE techniques. However, it has to be noted that RAPD analysis has compared favorably with PFGE-based methods in studies by other authors [13-15,28]. The differences with the present study may be attributed to the different set of primers and conditions used. In general RAPD assays lack the reproducibility and standardization characteristic of PFGE-based methods [11]. However, development of efficient, high-discriminatory, easy to use RAPD-techniques for strain delineation in *C. albicans* is certainly a possibility. RAPD analysis may provide a less expensive and time-consuming alternative for typing, but sometimes at the expense of discriminatory power.

Overall results from the present study support those by others indicating that a combination typing techniques may be required for strain delineation in *C. albicans* infections [6,8,12,15,30]. Ideally, the combination of results from methods with high discriminatory

power, (to maximize detection of strain variants) and lower discriminatory, but simpler to interpret methods (to allow determination of isogenic isolates) should facilitate the delineation of the epidemiology of *C. albicans* infection.

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