Identification by Random Amplification of Polymorphic DNA of a Common Molecular Type of Cryptococcus neoformans var. neoformans in Patients with AIDS or Other Immunosuppressive Conditions

Sharon C. A. Chen, Alan G. Brownlee, Tania C. Sorrell, Patricia Ruma, David H. Ellis, Tania Pfeiffer, Bryan R. Speed, and Graeme Nimmo

Centre for Infectious Diseases and Microbiology, University of Sydney, Westmead Hospital, Westmead; CSIRO, Division of Animal Production, Blacktown; Women’s and Children’s Hospital, Adelaide; Fairfield Hospital, Fairfield; and Princess Alexandra Hospital, Wollongabba, Australia

Sixty clinical isolates of Cryptococcus neoformans var. neoformans were analyzed by random amplification of polymorphic DNA (RAPD) using 12- to 22-mer primers in pairs. Five major profiles, which clearly distinguished between serotypes A (profiles I–III), AD (profile IV), and D (profile V), were identified. Forty-two of 58 serotype A isolates were assigned to profile I, 13 to profile II, and 3 to profile III. Profile I comprised 5 subtypes (profiles la–Ie), with 37 of 42 isolates in profile la. Twenty-seven of 28 isolates from patients with AIDS belonged to profile la (P < .001), as did 7 of 10 isolates from otherwise immunocompromised patients. Isolates from immunocompetent hosts were broadly distributed (profile I, 8 isolates; profile II, 10 isolates; profile III, 2 isolates). RAPD profiles were independent of body site and geographic origin of isolates. Isolate pairs from 3 patients produced identical profiles. A predominant genetic profile among serotype A strains from AIDS patients has not been reported previously.

Cryptococcus neoformans is an important opportunistic pathogen in patients with AIDS [1, 2]. In addition, a significant proportion of infections occurs in immunocompetent hosts [3]. Two varieties of C. neoformans are recognized: C. neoformans var. neoformans (serotypes A, D, or AD) and C. neoformans var. gattii (serotypes B or C). These varieties differ in their ecology and epidemiology; the former is found in nature in avian excreta, whereas the latter has been associated with eucalyptus trees in Australia [2]. Immunocompromised persons are nearly always infected with C. n. var. neoformans [1, 2]. In contrast, C. n. var. gattii has a propensity to cause disease in immunocompetent hosts [3].

Genetic differences among strains of cryptococci have been demonstrated by several methods, including electrophoretic karyotyping [4] and Southern blot hybridization with DNA probes based on repetitive DNA sequences from C. neoformans [5]. These techniques are time-consuming and not readily adaptable in the clinical laboratory for epidemiologic purposes. Recently, a method was developed to discriminate between strains of the same microorganism by amplifying polymorphic DNA with the polymerase chain reaction (PCR) using arbitrarily chosen oligonucleotide primers (RAPD) [6]. Use of this technique to type C. neoformans has demonstrated marked heterogeneity among strains [7–9]. However, in most cases, a single primer or a small number of primers was used, and experience with RAPD analysis of cryptococcal strains remains limited. We have shown previously that a modification of the RAPD technique using 20- to 22-mer primers in pairs produces stable DNA patterns that clearly distinguish between the 2 varieties of C. neoformans [10].

Although most cryptococcal disease is caused by C. n. var. neoformans serotype A, there is evidence that strains vary in their pathogenicity [11]. Furthermore, infection occurs in both immunocompromised and immunocompetent hosts. There is in general a paucity of information relating strain variation in cryptococci to host factors; one analysis of C. neoformans serotype D isolates suggested an association between strain characteristics and individual risk factors for human immunodeficiency virus (HIV) infection [12]. We describe the analysis by RAPD of 60 clinical isolates of C. n. var. neoformans and relate the RAPD profile to the epidemiology of the infection and to underlying host disease.

Materials and Methods

Cryptococcal strains. Clinical isolates of C. n. var. neoformans were obtained from Westmead Hospital, Women’s and Children’s Hospital, Princess Alexandra Hospital, and Fairfield Hospital. Two North American clinical isolates were a gift from S. Rosenthal (New York Medical Center, New York City). All isolates were identified and biotyped by standard techniques [2]. Serotyping was done using the Crypto Check agglutination test (Latron Laboratories, Tokyo). For each isolate, a single colony was picked and
subcultured twice on Sabouraud’s dextrose agar. Cultures were maintained at 30°C in air for 72 h before DNA extraction.

**DNA preparation.** Cells were disrupted by boiling for 20 min in extraction buffer (0.2 M sodium hydroxide, 0.4% N-lauroylsarcosine, 20 mM EDTA) in microcentrifuge tubes. Samples were then centrifuged for 5 min at 14,000 g. Supernatants were transferred to a clean microcentrifuge tube containing 0.5 vol of 7.5 M ammonium acetate, mixed, and centrifuged as before. The supernatant was transferred to a clean tube, and DNA was precipitated with 0.6 vol of isopropanol at room temperature. Centrifuged pellets were suspended in 1 mL of 10 mM TRIS with 1 mM EDTA, pH 8.0 (TE), and extracted twice with phenolchloroformisoamyl alcohol (25:24:1) [13]. DNA was precipitated using 1/10 vol of 3 M sodium acetate, pH 5.2, and 1 vol of isopropanol. The centrifuged pellet was redissolved in 0.5 mL of 0.05 M TE [13] and 0.28 M sodium chloride, pH 6.0, precipitated with 0.1% cetyltrimethyl ammonium bromide, washed with 70% ethanol, and resuspended in 50 μL of TE. DNA concentrations were determined spectrophotometrically.

**RAPD analysis.** Three 20- to 22-mer primers were chosen arbitrarily from laboratory stocks; their designations and sequences are (5’ to 3’): CN1 (TACCCCCGCCCATATTTCCAT), MYC1 (GAGGAAAGGTGGGATGACGT), and 5SOR (ATGGGAAATCGACGTCTGTAG). They were used in three combinations: CN1/MYC1, MYC1/5SOR, and 5SOR/CN1. In addition, 3 12-mer primers: FPKI-01 (ACACGAGCTCA), FPKI-05 (ACTTGGCGCCCT), and FPKI-07 (ACCTGGCTCATC) (Bresatec, Thebarton, Australia) were used in two combinations: FPKI-01/FPKI-05 and FPKI-05/FPKI-07. RAPD analysis was thus done with five primer pairs. PCR was done in a thermal cycler (PTC-100; MJ Research, Bresatec) using 25-μL reaction volumes, each containing ~10 ng of genomic DNA, 10 pmol of each of the two primers, 6 mM MgCl₂, 200 μM each dNTP, and 0.75 U of Taq DNA polymerase (Promega, Sydney) under the recommended buffer conditions. Reactions were cycled 10 times at 93°C for 1 min, 35°C for 1 min, and 72°C for 1 min followed by 20 cycles at 93°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min. Where the primer pair SSOR/CN1 was used, the annealing temperature was changed to 40°C for 1 min at low-stringency conditions.

Products of amplification were electrophoresed in a 7% polyacrylamide gel and visualized by silver staining (BioRad Laboratories, North Ryde, Australia). Band sizes were estimated from comigrating 100-bp DNA molecular size standards (Life Technologies GIBCO BRL, Five Dock, Australia).

DNA preparations made from 2 separate cultures of each isolate were run in parallel in RAPD analysis. RAPD assays of each DNA preparation were repeated at least once. Reproducibility of the method was confirmed by analyzing further DNA preparations from selected isolates on different occasions and by repeating experiments on stored DNA preparations over a 2-year period and in two different laboratories under identical test conditions. In all instances, identical profiles were obtained. Variations were sometimes observed in the intensities of amplicons from separate DNA preparations of the same organism despite standardization of DNA template concentrations. RAPD profiles were thus scored according to the number and reproductibility of bands independent of their intensity. A major profile was defined as a pattern that was reproducible and distinct from the others by at least four of the five primer combinations. Subtypes were defined by a consistent difference in profile associated with the use of one primer pair. Assignment of patterns to major and minor profiles was validated by an independent observer. Isolates that were not serotyped (or untypeable) were assigned to a serotype on the basis of their RAPD profile. Statistical analysis was done using the χ² test or Fisher’s exact test.

**Results**

Sixty-three isolates of C. n. var. neoformans from 60 patients were studied. Concurrent isolates were available from two body sites (cerebrospinal fluid [CSF] and blood) in each of 2 patients and from CSF obtained initially and 6 months later from 1 patient; in each instance the first isolate obtained was selected as the representative strain for the study. A total of 60 isolates was included in scoring the RAPD profiles.

Five major RAPD profiles were identified using the 20- to 22-mer primer combinations (figure 1A, B). Amplification with the primer pair CN1/MYC1 (figure 1A, table 1) showed that for serotype A isolates, 42 (72%) of 58 fell into profile I. Profile II comprised 13 (22%) and profile III comprised 3 (5%) isolates. Profiles IV and V consisted of 1 isolate each of serotype AD and D, respectively. The primer combination SSOR/CN1 revealed 5 subtypes within profile I (figure 1B, C). Thirty-seven of the 42 (88%) isolates were classed as profile Ia. Profiles Ib, Ic, and Id comprised 1 isolate each, while 2 cryptococcal strains were assigned to profile le. The 12-mer primer pairs distinguished only 4 major profiles.

There was a good correlation between the RAPD profile and serotype of C. n. var. neoformans. Forty-one isolates were serotyped; 38 were serotype A, 1 was serotype D, 1 was serotype AD, and 1 was untypeable. The 19 isolates that were not serotyped and the strain that could not be serotyped had profiles typical of serotype A.

**RAPD profile and host status.** Two isolates were obtained from HIV-infected persons in New York City. Of 58 Australian isolates, 28 were from patients with AIDS, 20 from immunocompetent hosts, and 10 from patients with immunosuppression due to malignancy, connective tissue disorder, or immunosuppressive therapy. The relationship between RAPD profile and underlying host conditions is shown in table 1. Twenty-seven (96%) of 28 serotype A isolates from HIV-infected patients were classified as profile Ia, compared with 10 (33%) of 30 isolates from patients without HIV infection (odds ratio [OR], 54; 95% confidence interval [CI], 5.1–1220; P < .001). The patterns of the 2 American isolates were identical to those of the Australasian strains. Heterogeneity was observed in isolates recovered from immunocompetent hosts, with 8 assigned to profile I (40%), compared with 96% of isolates obtained from HIV patients; OR, 40.5; CI, 4.2–972; P < .001), 10 (50%) to profile II, and 2 (10%) to profile III. Moreover, 5 of the 8 isolates in profile I were distinguishable from profile Ia and from each other by amplification with the primer pair...
**Figure 1.** RAPD profiles of *C. neoformans* amplified with primer pairs CN1/MYC1 (A) and 5S0R/CN1 (B, C) illustrating major types and subtypes. M, molecular size reference markers (100-bp ladder); other lanes consist of amplified DNA fragments from duplicate DNA preparations of individual strains. Bands were included in analysis regardless of intensity. RAPD profiles or subprofiles are shown at top.

SSOR/CN1 (figure 1B, C). Ten (77%) of 13 strains classed as profile II were isolates from immunocompetent patients. Most isolates (70%) from patients with immunosuppression not due to HIV were assigned to profile Ia.

**RAPD profile and body site of recovery.** Isolates were recovered from CSF (40), lung (8), blood (5), skin or soft tissue (4), and bone (1). Thirty-one (78%) of 40 CSF isolates were assigned to profile I, 5 to profile II, 2 to profile III, and 1 each to profiles IV and V. Six (75%) of 8 lung isolates were assigned to profile II (OR, 17.6; 95% CI, 2.4–162; *P* = .001); 5 of the 6 were recovered from immunocompetent patients, as were 77% of isolates assigned to this profile. After allowing for host status, there was no apparent link between RAPD profile and the clinical source of isolates. Of the remaining 10 isolates, 8 were assigned to profile I and 2 to profile II. Isolate pairs from 3 patients produced identical patterns.

**Table 1.** Association between RAPD profile and underlying host condition, as determined by amplification of isolates with primers CN1/MYC1.

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<th>Underlying host condition</th>
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<th>Other condition</th>
<th>Immunocompetent</th>
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**NOTE.** Data are no. of isolates. HIV, human immunodeficiency virus. * Not all strains were serotyped; however, RAPD profiles indicate serotype A.

Discussion

Our analysis of 60 clinical isolates of *C. n. var. neoformans* confirms previous observations that genetic polymorphisms are present in *C. neoformans* [5–9, 14]. We have also shown that RAPD analysis can discriminate effectively between strains of *C. n. var. neoformans* and that our method is suitable for large-scale epidemiologic studies.

This study is the first to demonstrate a distinct molecular profile of *C. n. var. neoformans* serotype A in patients with AIDS and includes isolates from different continents. In contrast, molecular typing methods applied previously to *C. n. var. neoformans* from AIDS patients have indicated substantial genetic diversity [7, 12, 14]. Dromer et al. [12] noted that selected DNA patterns may be associated with different risk factors for acquisition of HIV, though the number of patients in each risk group was small. The majority of HIV patients in our study acquired their infection via homosexual activity.

The explanation of a common molecular profile in cryptococcal isolates from HIV-infected patients is uncertain. Possibilities include a point source of infection, person-to-person spread, and the selection of a particular molecular type of *C. neoformans* in the presence of immunodeficiency. The first two possibilities cannot readily explain the occurrence of the same molecular type from two continents and many sites across Australasia, although demographic data and travel patterns of the patients are not available. Within the Australian isolates, we noted that RAPD profile Ia occurred frequently in patients...
immunocompromised by conditions other than HIV as well as those with AIDS in the absence of common environmental exposure. Person-to-person spread of infection has been documented only once, during corneal transplantation from a patient with disseminated cryptococcosis [15]. There may be a predominant environmental strain in Australia; however, the broad spectrum of RAPD patterns in immunocompetent patients from similar locations suggests this is unlikely. Analysis of environmental isolates is underway.

RAPD profiles in our study were predictive of serotype in C. n. var. neoformans, as has been observed with alternative methods of differentiating DNA patterns [5, 9]. However, Crampin et al. [7] reported that RAPD analysis of 12 isolates of C. neoformans yielded a unique pattern for each, without correlation with serotype. The discrepancy may be due to their use of a single short primer for DNA amplification. In our hands, the 20–22-mer primers produced reproducible and more informative profiles than did 12-mer primers, although the latter were useful in confirming major profiles. We chose to use coupled primers, as variable results were noted with the use of single primers. Others recommend the use of at least two primers together to detect a higher number of polymorphisms and to maximize reproducibility [16].

Isolate pairs from the same person taken at different times or from different body sites at the same time produced identical profiles, suggesting that relapse of infection occurred with the same molecular type of C. neoformans and that, in disseminated infection, the population of cryptococci is homogenous. This confirms the findings of previous studies using alternative typing methods [4, 5, 17]. In contrast, one group used RAPD analysis and DNA restriction fragment length polymorphism (RFLP) analysis to demonstrate genetically distinct cryptococcal strains in 5 patients with AIDS [8]. These results were evident with only 1 of 3 primers used in the RAPD and were confirmed by 1 of 5 probes used in the RFLP analysis. Since the probes and primers were directed at microsatellite sequences in C. neoformans, the Southern blot hybridization results were not independent of the RAPD analyses. Though these results raise the possibility of coinfection with different cryptococcal strains and of reinfection with a unique strain, they require confirmation.

RAPD profile II predominated among the small numbers of isolates recovered from lung in our study; however, lung isolates were primarily obtained from immunocompetent patients. In this group, the majority of isolates were also assigned to profile II. Dromer et al. [12], using two alternative molecular techniques, observed that serotype D isolates obtained from the lungs of HIV-infected patients in France were associated with a predominant identifying pattern. Analysis of more cryptococcal isolates from different clinical sources is required to determine if strains differ in their tropism for a given organ or if variation is dependent on the host’s ability to control infection. In our study, RAPD profiles of isolates recovered from immunocompetent patients can, in most circumstances, be distinguished from those obtained from patients with an immunodeficiency. It is possible, but untested, that cell- or complement-mediated immune mechanisms or cofactors associated with HIV infection or immunosuppressive therapy contribute to selection of a predominant cryptococcal genotype and that the amplicons obtained by RAPD represent sequences linked to cryptococcal virulence. RAPD analysis of serotype A isolates obtained from additional AIDS patients from different countries is required to confirm the epidemiologic association.

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References