JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 3662–3672 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.3662–3672.2005

Use of a Suspension Array for Rapid Identification of the Varieties and Genotypes of the *Cryptococcus neoformans* Species Complex

Mara R. Diaz* and Jack W. Fell

Rosenstiel School of Marine and Atmospheric Science, Division of Marine Biology and Fisheries, University of Miami, Miami, Florida 33149

Received 18 January 2005/Returned for modification 18 February 2005/Accepted 13 April 2005

***Cryptococcus neoformans* is an encapsulated fungal pathogen known to cause severe disease in immunocompromised patients. The disease, cryptococcosis, is mostly acquired by inhalation and can result in a chronic meningoencephalitis, which can be fatal. Here, we describe a molecular method to identify the varieties and genotypic groups within the *C. neoformans* species complex from culture-based assays. The method employs a novel flow cytometer with a dual laser system that allows the simultaneous detection of different target sequences in a multiplex and high-throughput format. The assay uses a liquid suspension hybridization format with specific oligonucleotide probes that are covalently bound to the surface of fluorescent color-coded microspheres. Biotinylated target amplicons, which hybridized to their complementary probe sequences, are quantified by the addition of the conjugate, streptavidin R-phycoerythrin. In this study we developed and validated eight probes derived from sequence analysis of the intergenic spacer region of the rRNA gene region. The assay proved to be specific and sensitive, allowed discrimination of a 1-bp mismatch with no apparent cross-reactivity, and detected 10^1 to 10^3 genome copies. The described protocol, which can be used directly with yeast cells or isolated DNA, can be undertaken in less than 1 h following PCR amplification and permits identification of species in a multiplex format. In addition to a multiplex capability, the assay allows the simultaneous detection of target sequences in a single reaction. The accuracy, speed, flexibility, and sensitivity of this technology are a few of the advantages that will make this assay useful for the diagnosis of human cryptococcal infections and other pathogenic diseases.**

Cryptococcosis is a disease that has become the focus of attention in Europe, America, Africa, and Southeast Asian countries (3, 14, 18, 21, 29, 54, 56, 66, 67, 68, 70). Prior to the availability of highly active antiretroviral treatment, the disease, which is caused by the basidiomycetous yeast *Cryptococcus neoformans*, was considered the fourth major cause of mortality in individuals with AIDS (40). In recent years, the incidence of cryptococcosis in America and Europe has decreased, but nevertheless, it continues to be a serious and fatal disease, with a similar mortality rate as in the past, especially in immunosuppressed human immunodeficiency virus (HIV)-infected individuals and individuals who have limited access to medical care for HIV infection (54). *C. neoformans* has a strong predilection for the meninges and the spinal fluid in AIDS patients; however, cryptococcal pneumonia is common in non-AIDS patients, especially for those who undergo chemotherapy or organ transplantation (34).

The encapsulated yeast *C. neoformans* represents a species complex comprising two species: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and *Cryptococcus gattii* (serotypes B and C). In addition to the four serotypes, there is a hybrid serotype, serotype AD. The species *C. neoformans* appears to affect immunocompromised hosts, whereas *C. gattii* commonly infects patients with healthy immune systems and usually invades the brain parenchyma (8). The species differ in their geographical distributions, ecologies, physiologies, and molecular and morphological characteristics (6, 8, 16, 38). Although *C. neoformans* var. *grubii* rank isolates as the most commonly encountered clinical strains worldwide, *C. neoformans* var. *neoformans* clinical isolates are frequently encountered in Europe (19, 42). *C. gattii* occurs in tropical and subtropical areas, as opposed to the cosmopolitan worldwide distribution of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (4, 8, 24). However, the geographic boundary expanded with the recent outbreak of *C. gattii* in the Vancouver Islands, British Columbia (39, 71).

In addition to classical yeast identification techniques, molecular assays have been used for the identification of *C. neoformans* species complex, some of which include randomly amplified polymorphic DNA analysis (38), amplified fragment length polymorphism (AFLP) analysis (6), karyotyping (7, 59), PCR fingerprinting (12, 23, 38, 53), sequencing (16, 38), and PCR-restriction fragment length polymorphism analysis (19, 43). Even though these techniques have successfully identified *C. neoformans* at the species and the genotypic levels, some of these techniques are not easily adapted for use in routine diagnostic laboratories (43). The present study describes a rapid and reliable molecular bead-based method that allows the simultaneous detection of the varieties and genotypes of the *C. neoformans* species complex. This molecular assay uses specific oligonucleotide probes derived from unique sequence areas of the intergenic spacer (IGS) region of the rRNA gene. Based on sequence divergences in the IGS region, which is a nonconservative, fast-evolving region frequently used as a tool for species identification (16, 17, 25, 62), Diaz et al. (16) showed that *C. neoformans* portrayed five distinct phylogenetic lineages represented by genotype 1, with subgenotypes 1a, 1b,

* Corresponding author. Mailing address: Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149. Phone: (305) 421-4879. Fax: (305) 421-4600. E-mail: mdiaz@rsmas.miami.edu.

and 1c (*Cryptococcus neoformans* var. *grubii*); genotype 2, with subgenotypes 2a, 2b, and 2c (*Cryptococcus neoformans* var. *neoformans*); and genotypes 3, 4, and 5 (*C. gattii*). Recently, a new IGS region genotypic group, which comprised one isolate from Africa and two isolates from India, has been described (14a). Therefore, this new genotypic group within the *C. gattii* complex has been added to our list as genotype 6. The IGS genotype classification correlates with previous AFLP genotypic data described by Boekhout et al. (6). The equivalent types are as follows: IGS genotype 1, AFLP molecular type 1 plus AFLP molecular type 1A; IGS genotype 2, AFLP molecular type 2; IGS genotype 3, AFLP molecular type 6; IGS genotype 4, AFLP molecular type 4; IGS genotype 5, AFLP molecular type 5; and IGS genotype 6, AFLP molecular type 7 (39).

The molecular test developed uses Luminex xMAP technology, a flow cytometer that allows the simultaneous identification of the varieties and their genotypes by mixing different sets of microspheres which contain specific capture probes derived from target sequences. This technology can permit the simultaneous detection of 100 analytes by combining 100 different sets of microspheres in a single reaction. Since each microsphere set is internally dyed with two spectral fluorochromes of different intensities, their unique spectral emissions are recognized by a red laser. Upon hybridization, the biotinylated amplicon bound to the surface of the microsphere is recognized by a green laser that quantifies the fluorescence of the reporter molecule (streptavidin R-phycoerythrin).

The scope of this paper is to report on a rapid, sensitive, and specific molecular assay for the identification of the varieties and genotypic groups of the species complex of *C. neoformans*. The sequence in the nonconservative region of the intergenic spacer region allowed us to develop specific probes that could be used to target the varieties and genotypes.

MATERIALS AND METHODS

Isolates. Clinical and environmental DNA isolates from different geographic areas were analyzed. The sources of isolation, genotypes, and serotypes are described in Table 1. Serotype data were obtained from the Centraalbureau voor Schimmelcultures (CBS) collection, Boekhout et al. (6), or information provided by the depositors of the isolates.

DNA isolation and PCR. PCR amplifications employed either DNA isolated from cultured cells or direct detection from cells. Isolated DNA was obtained from cultured cells as described by Fell et al. (26) by using lysing enzyme and the QIAmp tissue kit (QIAGEN Inc.) or by the cetyltrimethylammonium bromide method (55). Direct detection from cultured cells employed a pinhead-size portion of a colony diluted in 15 μl of sterile, distilled water. The culture was grown for 2 days in glucose-peptone-yeast at 25°C. The microcentrifuge tube was vortexed, after which 4 μl of the cell suspension was transferred into the PCR mixture.

Amplification reactions used the forward primer IG1F (5′-CAGACGACTT GAATGGGAACG), located at positions 3613 to 3633 of the large rRNA region), and the reverse primer IG2R (5′-ATG CAT AGA AAG CTG TTG G), located at position 791 of the IGS1 region. The reverse primer was biotinylated at the 5′ end. The PCR was carried out in microtubes with QIAGEN HotStarTaq Master Mix in a final volume of 50 μl. The master mixture contained 10 ng to 1 pg of genomic DNA; 1.5 mM $MgCl_2$; 0.4 μM of forward and reverse primer pairs; 2.5 units of HotStarTaq polymerase; and deoxynucleoside triphosphates (dNTPs) containing 200 μM each of dGTP, dCTP, dTTP, and dATP. PCR was performed with an MJ Research PTC 100 thermocycler and consisted of an initial activation at 95°C for 15 min, followed by 35 cycles amplification: 30 s of denaturation at 95°C, 30 s of annealing at 50°C, and 30 s of extension at 72°C. A final elongation step was applied at 72°C for 7 min.

Capture probe design and validation. Probe design for *C. neoformans* species complex and their genotypes employed sequence data from the IGS1 region (16). These data, which are available on GenBank, contained over 100 sequences from clinical and environmental strains (16). The sequences were aligned with the Megalign program (DNAStar) to determine unique sequences that could be used for probe development. When possible, probes were designed to be uniform in length (21-mer). However, to avoid potential secondary structures (stem loops) or an unstable delta G structure, some probes underwent length modification. To assess the quality of the probe, the software program Oligo (Molecular Biology Insights Inc.) was employed. The specificity of the prospective probe was screened with GenBank BLAST. The secondary phase of the probe validation was achieved by testing the performance of the probe in a bead-based hybridization assay format. The capture probes, which were complementary in sequence to the biotinylated strand of the target amplicon, were synthesized with a 5′-end amino C-12 modification (Integrated DNA Technologies, Coralville, IA). Each probe was covalently coupled to a different set of 5.6-μm polystyrene carboxylated microspheres by a carbodiimide method (28), with slight modifications (15). Coupling optimization was carried out by adjusting the amount of probe in a range from 0.2 to 0.5 nmol.

Hybridization assay. This bead suspension assay is based upon detection of 5′ biotin-labeled PCR amplicons hybridized to specific capture probes covalently bound to the carboxylated surface of the microspheres. Hybridization was performed in 3 M TMAC (tetramethyl ammonium chloride, 50 mM Tris, pH 8.0, 4 mM EDTA, pH 8.0, 0.1% Sarkosyl) solution. Duplicate samples containing 5 μl of biotinylated amplicon were diluted in 12 μl of 1× TE (Tris-EDTA) buffer (pH 8) and 33 μl of 1.5× TMAC solution containing a bead mixture of ~5,000 microspheres for each set of probes. Prior to hybridization, the reaction mixture was incubated for 5 min at 95°C with a PTC 100 thermocycler (MJ Research). This step was followed by 15 min incubation at 55°C. After hybridization, the beads were centrifuged at 2,250 rpm for 3 min. Once the supernatant was carefully removed, the 96-well plate was incubated for 5 min at 55°C and the hybridized amplicons were labeled for 5 min at 55°C with 300 ng of freshly made streptavidin R-phycoerythrin. The samples were centrifuged and the supernatant was removed. This step was followed by the addition of 75 μl of 1× TMAC. The samples were analyzed on the Luminex 100 analyzer. One hundred microspheres of each set were analyzed, which represents 100 replicate measurements. Median fluorescent intensity (MFI) values were calculated with a digital signal processor and the Luminex 1.7 proprietary software. Each assay was run twice. A blank and a set of positive and negative controls were included in the assay. The signal-to-background ratio represents the MFI signals of positive controls versus the background fluorescence for samples containing all components except the amplicon target. A positive signal corresponds to a signal which is twice the background level after the background has been subtracted.

The sensitivity of the assay was determined with serial dilutions of genomic DNA (10 ng to 1×10^{-3} ng) and amplicons (500 to 1×10^{-3} ng). DNA quantification was determined with a NanoDrop ND-1000 spectrophotometer by using an absorbance of 260 nm. Prior to quantification, amplicons were purified with QIAGEN Quick-spin. Reactions were performed in duplicate, and the experiment was run twice.

To test the detection of multiple targets in a single reaction, amplicons which were generated by a mix of genomic DNA from isolates that represent genotypes 1 to 5 were tested in the hybridization assay format. In order to determine the optimum parameters for multitemplate PCR, several reactions were conducted by using various concentrations of genomic DNA (5 to 10 ng), $MgCl_2$ (1.5 to 2.25 mM), dNTPs (200 to 300 μM), polymerase (2.5 to 3.75 U), and PCR primers (0.4 to 0.8 μM). The PCRs were run with the standard PCR program. Five or 15 μl of amplicon was used in the hybridization assay. The experiments were performed twice.

To test the multiplex capability of the assay, individual sets of probes were pooled into a bead mix and tested in one- and eightplex formats. Each plex assay was tested with amplicons derived from single strains. Reactions were performed in duplicate and the experiment was run twice.

RESULTS

Probe specificity. Eight probes were designed to target the varieties and genotypic groups of the *C. neoformans* species complex. The probes were tested and validated with ~66 clinical and environmental isolates listed in Tables 1 and 2. The probes were designed to have a G+C content greater than 30%, a melting temperature greater than 50°C, and a length of

TABLE 1. Experimental strains used to develop the probes and their sources of isolation, serotypes, and IGS genotypes

Strain[a]	Source of isolation	Serotype	Genotype
C. neoformans var. *grubii*			
AVB12 RDA 4054	AIDS patient, The Netherlands	A	1
CBS 916	Unknown	A	1
Hamdan 214L	AIDS patient, Brazil	A	1
Hamdan MCP2	Pigeon dropping, Brazil	A	1
RV 58146	Wood, Zaire	A	1
RV 59351	Parrot droppping, Belgium	A	1
RV 62210	Cerebrospinal fluid from AIDS patient, Belgium	A	1
WM 148	Human, cerebrospinal fluid, Australia	A	1
WM 553	House dust, Brazil	A	1
WM 554	Dust from pigeon, Brazil	A	1
WM 626	Human cerebrospinal fluid, Australia	A	1
WM 712	Cat paranasal, Australia	A	1
WM 719	AIDS patient, India	A	1
WM 721	Pigeon dropping, India	A	1
WM 723	Environmental isolate, United States	A	1
NIH 192	Desert soil, United States	A	1
NIH 193	Soil, United States	A	1
NIH 443	Soil, United States	A	1
H 99	Patient with Hodgkin's disease, United States	A	1
C. neoformans var. *neoformans*			
AVB6	AIDS patient, The Netherlands	D	2
CBS 132	Institut Pasteur, Paris, France	AD	2
CBS 888	Unknown	D	2
CBS 918	Dead white mouse	D	2
CBS 950	Tumor	AD	2
CBS 5728	Nonmeningitic cellulitis and osteomyelitis, United States	D	2
CBS 6885	Lesion on bone of man, United States	D	2
CBS 6886	Dropping of pigeon	D	2
CBS 6900	Genetic offspring of CBS 6885 × CBS 7000	D	2
CBS 6901	Genetic offspring of CBS 6885 × CBS 7000	D	2
CBS 6995	Cerebrospinal fluid, non-AIDS patient, United States	D	2
CBS 7815	Pigeon droppings, Czechoslovakia	D	2
CBS 7816	Cuckoo dropping, Thailand	D	2
CBS 7824	Unknown	D	2
CBS 7825	Unknown	AD	2
PCC09	Rio de Janeiro, Brazil	D	2
RV 52755	Cerebrospinal fluid, Belgium	D	2
WM 628	Human, cerebrospinal fluid, Australia	AD	2
WM 629	Human, blood, Australia	D	2
J9	AIDS patient, United States	D	2
C. gattii			
CBS 1930	Sick goat, Aruba	B	3
CBS 5758	Unknown	C	5
CBS 6289	Subculture of type strain RV 20186	B	4
CBS 6955	Spinal fluid, of *Filobasidiella bacillispora*, United States	C	5
CBS 6994	Cerebrospinal fluid, United States	C	5
CBS 7523	*Eucalyptus camaldulensis*, Australia	B	4
CBS 7748	Air in hollow, *Eucalyptus camaldulensis*, Australia	B	4
CBS 7749	*Eucalyptus camaldulensis*, Australia	B	4
NIH 139	Patient, United States	C	5
NIH 178	Patient, United States	C	5
IMH 1658	Nest of wasp, Uruguay	B	3
CGBMA6	Pink shower tree, Brazil	B	3
CGBMA15	Pink shower tree, Brazil	B	3
WM 178	Human, lung, Australia	B	3
WM 179	Human, cerebrospinal fluid, Australia	B	4
WM 717	Woody debris of *Eucalyptus terricornis*, United States	B	4
WM 718	Woody debris of *Eucalyptus terricornis*, United States	B	4
WM 726	*Eucalyptus citriodora*, United States	B	5
WM 779	Cheetah, South Africa	C	6
B5742	Human, cerebrospinal fluid, India	C	6
B5748	HIV-infected patient, India	B	6

[a] CBS, Centraalbureau voor Schimmelcultures; RV, Institute of Tropical Medicine; NIH, National Institutes of Health; WM, University of Sydney at Westmead Hospital (Australia). The rest of the isolates were provided by individual researchers from America and Europe.

TABLE 2. Results of identification of clinical and environmental strains used for the identification of the genotypes and varieties of *Cryptococcus neoformans* species complex

Strain	Source	Serotype	Geographic origin	Species	Genotype
CN 4	CSF[a]	A	Guine (Bissau)	*C. neoformans* var. *grubii*	1
CN 32	Blood	A	Hospital Sta. Maria (Lisbon, Portugal)	*C. neoformans* var. *grubii*	1
CN 38	Blood	AD	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *neoformans*	2
CN 40	CSF	AD	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *neoformans*	2
CN 43	CSF	A	Inst. for Tropical Medicine (Lisbon)	*C. neoformans* var. *grubii*	1
CN 50	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 55	CSF	A	Hospital Sto. Antonio (Oporto, Portugal)	*C. neoformans* var. *grubii*	1
CN 59	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 70	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 74	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 79	CSF	D	Institute Pasteur (Paris, France)	*C. neoformans* var. *neoformans*	2
CN 83	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 92	CSF	A	Hospital Sta. Maria (Lisbon)	*C. neoformans* var. *grubii*	1
CN 95	CSF	A	Prisonal Hosp. (Lisbon)	*C. neoformans* var. *grubii*	1
CN 112	Pigeon droppings	A	Veterinary School (Lisbon)	*C. neoformans* var. *grubii*	1
PYCC 5025	Eucalyptus tree	B	Australia	*C. gattii*	4

[a] CSF, cerebrospinal fluid.

21 bases. Some of the designed probes did not follow these parameters. For example, CNG 5b displays a melting temperature of 48.5°C, and CNN 1b is a 20-mer oligonucleotide. All probes except for CNG 5b were coupled at 0.2 nmol; CNG 5b was used at 0.5 nmol. The probe sequences are depicted in Table 3.

The specificity of each probe was tested against the positive control (perfect match), negative controls (more than three mismatches), and cross-reactive groups (one to three mistmatches). Six probes, represented by CNN 1b (genotype1), CNN 2d (genotype 2), CNG 3 (genotype 3), CNG 4c (genotype 4), CNG 5b (genotype 5), and CNG 6 (genotype 6), were developed to identify the genotypic groups, as described in the IGS region phylogenetic tree of *C. neoformans* species complex (16). In addition, two group-specific probes were designed to identify members of the two main clades, represented by CNN b, which includes strains belonging to *C. neoformans* var. *neoformans*-*C. neoformans* var. *grubii* and CNG, which includes all the genotypic groups (genotypic groups 3 to 6) within *C. gattii.*

Under our capture assay conditions, the results demonstrated that we can discriminate between probe sequences that differ by 1 bp from the target sequence. To illustrate the specificity of our assay, probe CNG 4c, which targets genotype 4 isolates, was challenged against strains belonging to different genotypic groups (Fig. 1). None of the potential cross-reactive strains, represented as isolates displaying 1- to 3-bp differences, were found to cross-react with CNG 4c, indicating the specificity of the assay (Fig. 1).

Figure 2A to H depicts the performance of all eight probes tested against strains representing all six genotypic groups. The probe specificity was accurate, as no cross-reactivity was observed with nontarget isolates. For example, CNG 6 was specific and hybridized only with perfectly matching complementary sequences of strains, e.g., WM 779 and B 5742 (Fig. 2H). No cross-hybridization was documented with nontarget strains (e.g., IMH 1658, CBS 1930, CBS 6289, CBS 7748, CBS 7749, CBS 7523, NHI 139 CBS 5758 CBS 6955, and NHI 178), with two mismatches from the CNG 6 probe sequence, TAA*c*T TCTC*g*CGCCCACTGTG, where the lowercase italic bases indicate the mismatches (Fig. 2H). Overall, the specificity of this bead-based assay was maintained when the base pair difference(s) was centrally located. An exception to this rule was CNG 5b, which maintained specificity when it was tested with genotype 3 isolates (IMH 1658 and CBS 1930) bearing two off-centered base pair differences (indicated by the lowercase italic bases) at positions 5 and 6, respectively, from the 5′ end (AAAA*tg*GGTAAATGTGGTATG) (Fig. 2G).

Some inherent variability in the probe hybridization signal was found among positive control strains when they were challenged with their probe targets (Fig. 2A to H). When CNN b was tested with various genotype 1 isolates, the MFI signals for RV 62210 and CBS 950 were 1,800 to 576 MFI, respectively (Fig. 2A). A similar scenario, where different positive control strains displayed different signal intensities, was observed for the other probes (Fig. 2B to H). Despite the differences in signals among the positive control strains, all isolates displayed

TABLE 3. Probes sequences used for the detection of the varieties and genotypic groups of the *Cryptococcus neoformans* species complex

Probe	Sequence	Target
CNN b	GCTCATTGTGGGTCCAGTCTT	*C. neoformans* var. *grubii*/*C. neoformans* var. *neoformans* (genotypes 1 and 2)
CNN 1b	GGATGGGCAGTAGAATTTTG	*C. neoformans* var. *grubii* (genotype 1)
CNN 2d	ACTGATCACCCAGCTAGAAAG	*C. neoformans* var. *neoformans* (genotype 2)
CNG	TGGTCAAGCAAACGTTTAAGT	*C. gattii* (genotypes 3 to 6)
CNG 3	CTTGCAACTTGTCTGGCCCAC	*C. gattii* (genotype 3)
CNG 4c	GACTCTAATACGCTGGTCAAG	*C. gattii* (genotype 4)
CNG 5b	AAAACAGGTAAATGTGGTATG	*C. gattii* (genotype 5)
CNG 6	TAAGTTCTCTCGCCCACTGTG	*C. gattii* (genotype 6)

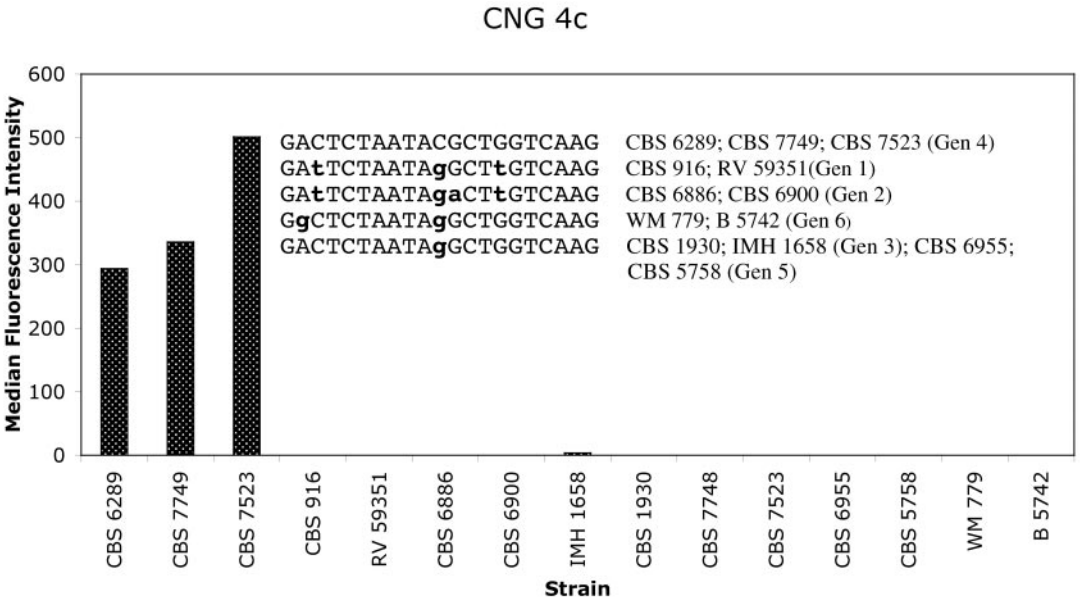

FIG. 1. CNG 4c complex probe tested with strains representing all genotypic groups within the *C. neoformans* species complex. Nucleotide variations from the probe sequence are depicted in boldface and lowercase letters.

MFI values of sufficient strength to allow differentiation of positive from negative samples. In addition to the differential hybridization response among strains with complementary target sequences, we found that the fluorescence intensities among the probes varied considerably. For example, CNG 5b and CNG 4c displayed fluorescent signals ranging ~250 to 500 MFI, whereas others, CNN b, CNN 2d, CNG, and CNG 6, displayed MFI values of over 1,000.

Probe multiplexing. Experiments were designed to test the multiplex capability of the assay, which employed multiple probes in a single reaction. After the probes were pooled, they were challenged with a single amplicon target per well. The results showed that all probes performed similarly when they were tested in the uniplex and the eightplex formats. For example, when tested in the uniplex and the eightplex formats, the signal intensities of probes CNG 4c, CNG, and CNN 1b differed by only 8, 2.7, and 12%, respectively (data not shown).

Probe validation with blind test isolates derived from clinical and environmental sources. Probe validation was undertaken with a blind collection of isolated DNA from 16 clinical and environmental strains. Fourteen samples were clinical isolates from HIV-positive individuals recovered from various hospitals in Portugal; CN 79 originated from the Institute Pasteur in Paris, France. Two strains, PYCC 5025 and CN 112, were recovered from environmental sources. Table 2 describes the sources of isolation, serotypes, and origins for each of the isolates, which were disclosed after the blind testing was conducted. Through employment of the multiplex assay format, we determined without ambiguity the varietal status and the genotypic classification for each of the strains (Table 2). The varietal classification was in agreement with the identification submitted by the donors, who used an array of morphological, biochemical, and PCR molecular techniques to identify the isolates (I. Spencer-Martins, personal communication). Among the strains studied, all 12 serotype A isolates belonged to *C. neoformans* var. *grubii* genotype 1 (CN4, CN 32, CN 43, CN 50, CN55, CN 59, CN 70, CN 95, CN83, CN 112, CN 92, and CN 74), followed by 3 strains (serotype AD strains CN 38 and CN 40 and serotype D strain CN 79) identified as *C. neoformans* var. *neoformans* genotype 2 (Table 2). The remaining isolate (serotype B strain PYCC 5025) belonged to *C. gattii* genotype 4 (Table 2).

Multitarget detection. In order to determine the feasibility of the Luminex xMAP assay to identify multiple strains in a single sample, a multitemplate PCR was carried out with the genomic DNAs from the following isolates: WM 554 (genotype 1), CBS 132 (genotype 2), CGBMA6 (genotype 3), CBS 7523 (genotype 4), and CBS 6955 (genotype 5). The amplifications used 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 units of polymerase, and equimolar concentrations (0.6 μM) of the primer set IG1F and IG2R. PCRs were tested with 5 and 10 ng of genomic DNA from each of the strains. The generated multitarget amplicon was hybridized with the probes in a multiplex format. Our results show that 5 or 10 ng of genomic template in the PCR enabled the detection of the isolates. Overall, the sensitivity of the multiple-genome PCR was lower than that of the single-genome PCRs (Fig. 3). However, the fluorescent signal could be improved by increasing the amount of amplicon in the hybridization reaction (Fig. 3). When 15 μl of the amplicon target was used, the hybridization signals were comparable to those results obtained with single target amplicons (Fig. 3).

Genomic and amplicon detection limits. To determine the minimum amount of detectable genomic DNA in the PCRs, serial dilutions of genomic DNA, which ranged from 10 to 10^{-3} ng, were performed with CNN b, CNN 1b, CNN 2d, CNG, CNG 3, and CNG 4c. The lowest limit of detection was 1 pg (CNN 2d), followed by 10 pg (CNN b and CNG). Other probes, CNN 1b, CNG 4c, and CNG 3, showed detection limits of ~50 pg (Fig. 4). Below 10-pg levels, the signal was barely detectable for all probes except CNN 2d, which showed detec-

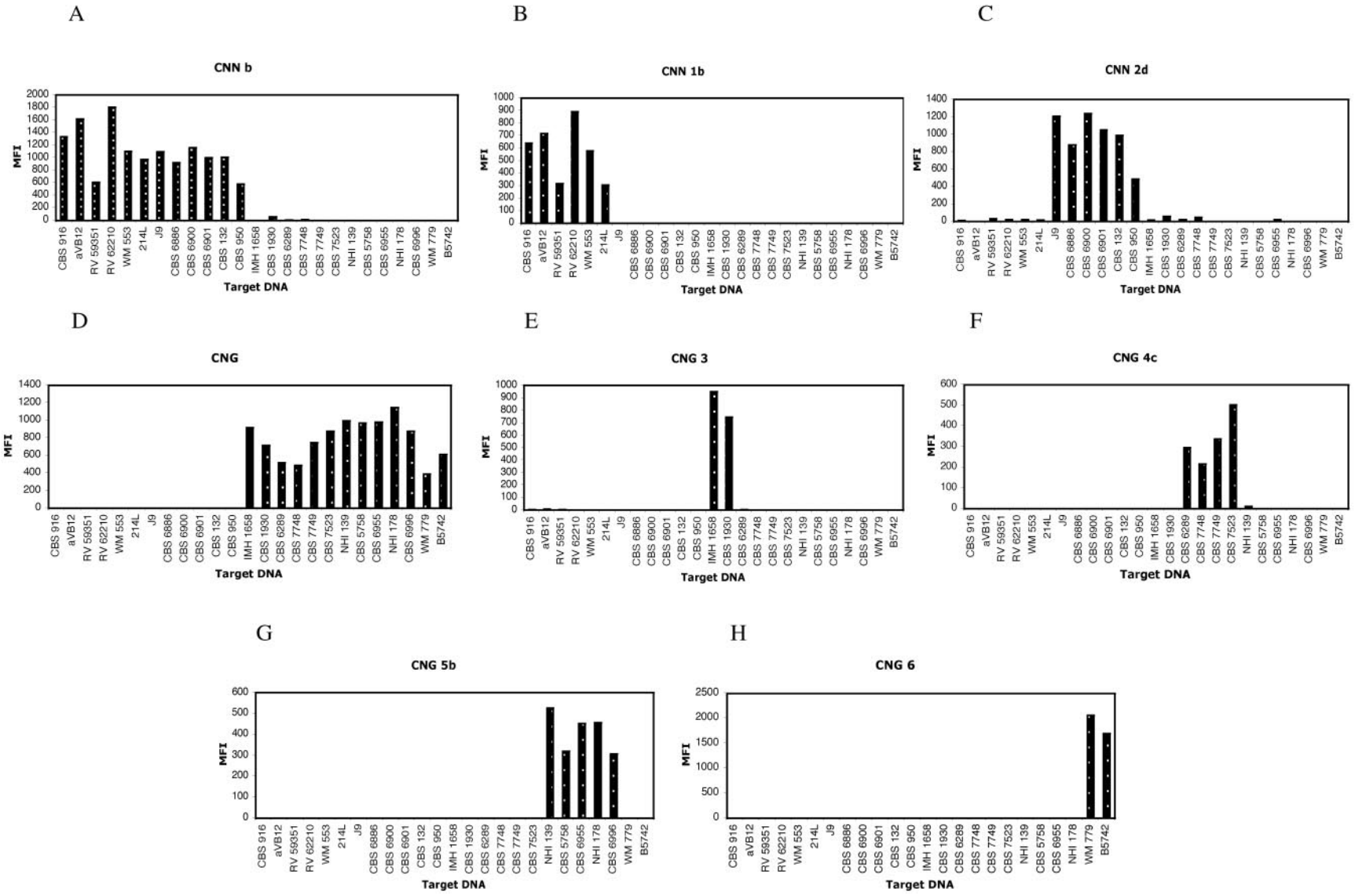

FIG. 2. Probe responses with target and nontarget DNA. Hybridization was performed at 55°C with amplicons biotinylated at the 5′ end. All probes were tested in a multiplex format (eightplex assay). The background signal was subtracted. (A) CNNb (genotypes 1 and 2); (B) CNN 1b (genotype 1); (C) CNN 2d (genotype 2); (D) CNG (genotypes 3, 4, 5, and 6); (E) CNG 3 (genotype 3); (F) CNG 4c (genotype 4); (G) CNG 5c (genotype 5); (H) CNG 6 (genotype 6).

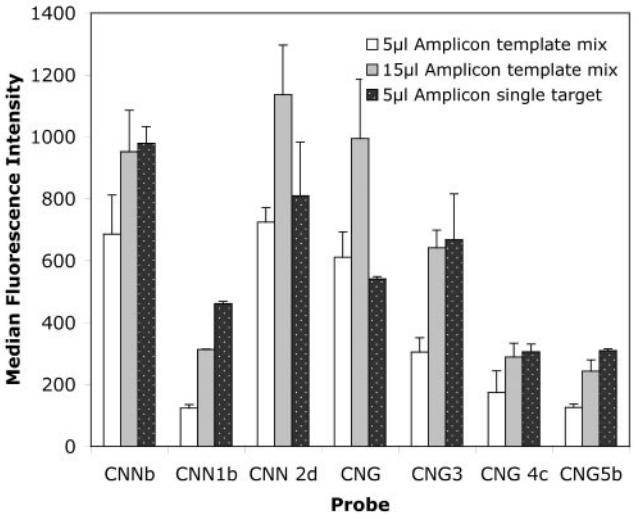

FIG. 3. Effects of various amounts of the amplicon template mixture on hybridization intensities. The amplicon products were derived from the simultaneous PCR amplification of five different DNA targets: WM 554, CBS 7523, CGBMA6, CBS 6955, and CBS 132. The PCR mixture used 5 ng of each of the targets described above. A comparison of the signals between the single-target PCR (one strain) and the multitarget PCR (five strains) is provided.

tion limits as low as 1 pg of DNA with a signal intensity ~50 MFI (Fig. 4).

The detection limits of the amplicon targets were determined with cleaned PCR products serially diluted from 500 to 10^{-3} ng. The amplicon detection limits determined with CNG and CNN 1b demonstrated that this assay can detect 0.5 ng with signal intensities over 50 MFI (data not shown).

Direct detection from cultures. Direct yeast cell amplification, which was performed with a pinhead-size portion of a colony diluted in 15 μl of sterilized water, demonstrated that 4 μl of the cell suspension is sufficient to generate an amplicon that can be used for the identification of the isolates without DNA extraction. For this particular experiment, we used a set of reference strains (Table 1) that had been typed by PCR fingerprinting and *URA5* restriction fragment length polymorphism analysis (52).

As shown in Fig. 5 we identified all six strains at the variety and genotypic levels by direct detection, with the fluorescence signals (MFI values) ranging from 210 to 867. The identities of the strains at the genotypic level were as follows: WM 628 and WM 629, genotype 2; WM 626, genotype 1; WM 178, genotype 3; WM 179, genotype 4; and WM 779, genotype 6. For some probes, e.g., CNG and CNG 6, the MFI values obtained from direct amplification (i.e., WM 779) were reduced by ~42 to 52% compared to those obtained with extracted DNA material (data not shown). Nevertheless, the signal intensities of the probes displayed with nonextracted cells ranged from ~10- to 25-fold above the background levels. The reduction in the signal is probably due to the differential amplification efficiencies of both techniques, which resulted in different concentrations of PCR product. For instance, the PCR product concentration obtained by direct amplification (i.e., WM 779) averaged ~33 ng/μl, whereas it was ~50 ng/μl when the DNA was extracted. By increasing the amount of amplicon in the hybridization assay to 15 μl, the probe signals from cells from which DNA was not extracted were enhanced by nearly 50% and were similar to those from cells from which DNA was extracted (data not shown).

DISCUSSION

Molecular biology-based PCR methods, e.g., reverse cross blot hybridization (63), nested real-time PCR (5), and multiplex PCR (9, 50) have successfully been applied to the identification of *C. neoformans* in clinical specimens. However, none of these methods identified the species at the variety or genotypic level. In the present study we successfully adapted the Luminex xMAP technology to differentiate between the varieties and genotypes of one of the most important fungal pathogens, *C. neoformans*. Differences in the nonconservative region

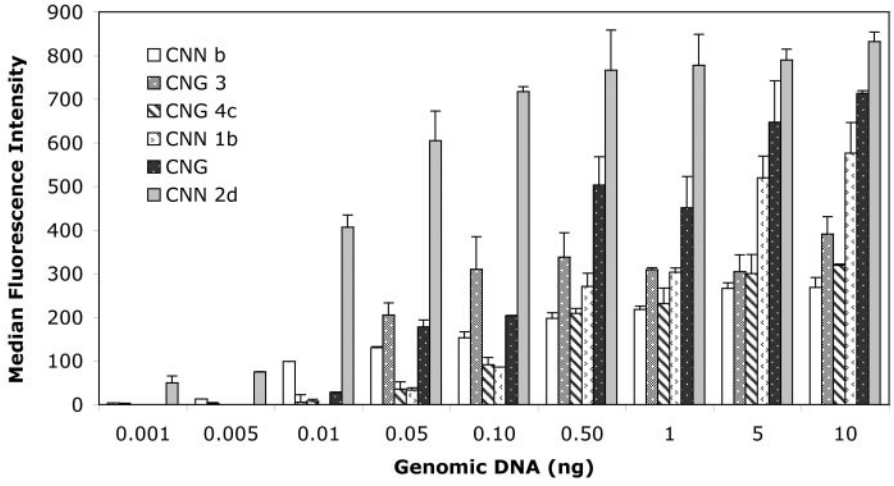

FIG. 4. Detection levels of genomic DNA with different amounts of genomic DNA in the PCR mixture. Following the amplification, 5 μl of amplicon was used in the hybridization reaction. The probes (strains) tested were as follows: CNN b (214L), CNG 3 (IMH 1658), CNG 4c (CBS 6289), CNN 1b (WM 554), CNG (CBS 6955), and CNN 2d (CBS 132).

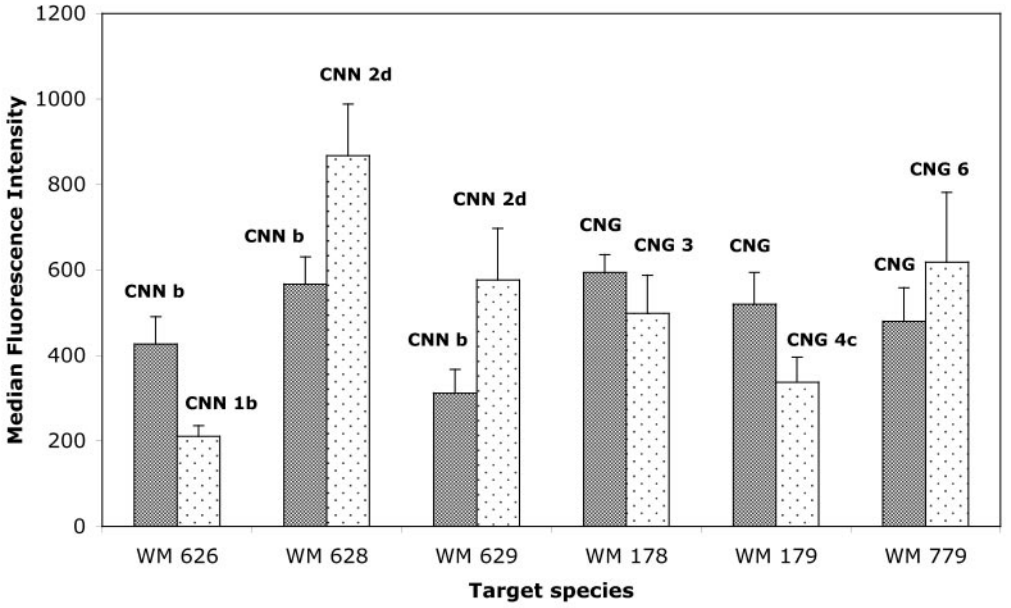

FIG. 5. Direct amplification and detection of DNA targets. After hybridization, 5 μl of the PCR product was tested with its complementary probe sequence. The hybridization assay was performed in an eightplex assay. Samples were run in duplicate, and the experiment was run twice.

of the rRNA gene, the IGS region, allowed us to develop and validate eight different probes that can target the varieties and the different molecular genotypes of the species. This technique, which incorporates flow cytometry and a bead-based capture hybridization assay, was a reliable method for the detection of members of the *C. neoformans* species complex. The assay has been successfully employed for the identification of all species within the genus *Trichosporon* (15).

In conventional hybridization assays, discrimination between duplexes with perfect matches and single-base-pair mismatches is generally achieved by controlling the temperature or ionic strength, including formamide, or adding stringent wash steps with low salt concentrations (51). Another strategy is to analyze the melting profiles of individual probes spotted on a chip surface (47). Under the present hybridization assay format, which involved a short incubation at 55°C, we were able to meet the stringent conditions necessary to discriminate among sequences with 1-bp mismatches by the inclusion of 3 M TMAC. This quarternary alkylammonium salt eliminates the preferential melting of AT versus GC base pairs and allows multiple probes with different base pair compositions to be employed under similar hybridization conditions (73). An example of the specificity of the assay is illustrated in Fig. 1, where no cross-reactivity was observed among isolates bearing one mismatch from the probe sequence. A similar specificity was attained in our previous study, in which we employed a similar hybridization assay for the detection of *Trichosporon* spp. (15). As observed, the specificity of the probe was maintained if the mismatches were located at positions 9 through 11 from the 5′ or 3′ end (Fig. 1). However, if a probe sequence had two consecutive mismatches that were off centered at positions 5 to 6 from the 5′ end, it was possible to retain the specificity. For instance, none of the strains (genotype 3) bearing two consecutive mismatches from the probe sequence of CNG 5b cross-reacted with that particular oligonucleotide. According to the kinetics of dissociation, the maximum destabilizing effect of a mismatch is achieved when the mismatches are in the center of the sequence (30) and when the mismatches involve A-A, T-T, C-T, and C-A (35). Double consecutive mismatches after the last three end positions are known to produce unstable duplexes, especially if one of the mutations like those portrayed in CNG 5b involves a C-T, which is considered a significant destabilizing mismatch (35, 45). Mismatches involving C-T can lead to a significant distortion in the helical structure due to the small size of the pyrimidine-pyrimidine base pair, which results in an unstable duplex (35).

In the current study, some heterogeneity in hybridization signals was observed among strains belonging to the same genotypic groups. This effect has been reported by others and is manly due to differential yields in PCR products or PCR labeling efficiencies (49), which can be associated with the quality and/or concentration of the genomic template. Similarly, different probes exhibited different signal intensities after they hybridized with their perfectly matched target. This wide range of fluorescence signals, which in our case ranged from ~250 to 2,000 MFI values above background levels, has been attributed to base composition, base stacking interaction, steric hindrance, the position of the probe binding site, the secondary structures of the single-stranded target molecule, hairpin structures in the probe sequence, and kinetics (31, 36, 58, 69). Although all of these factors can have a profound effect on the duplex yield and the fluorescence intensity associated with the probe-target match, the complex interaction of the mechanisms described above remains a puzzle. In trying to elucidate this dilemma, some investigators have developed secondary structural maps of domains D1 and D2 of rRNA or the 23 rRNA gene to evaluate the accessibility of fluorescent probes based on the secondary structural conformations of the differ-

ent domains, but the results have been inconclusive (27, 31, 36).

The sensitivity of the assay, as determined by the amount of genomic DNA in the PCR, indicated that under our assay conditions we detected between 10 and 50 pg of genomic DNA. However, for probe CNN 2d, we detected as little as 1 pg. These detection levels can be improved by increasing the amount of amplicon in the assay, as we demonstrated in a similar assay format for the detection of the pathogenic yeast *Trichosporon* (15). Our detection levels are more sensitive than those from studies based on PCR-enzyme immunoassay (PCR-EIA) and molecular beacon probes, which have reported detection limits of 1 ng and 100 pg for the detection of clinically important fungi (22, 57). Sensitivities equal to or slightly higher than those documented in this study have been reported for the detection of *Candida* spp., *C. neoformans*, *Aspergillus* spp., and other yeast-like fungal pathogens in studies that used such PCR-based methods as real-time cycler PCR, PCR-EIA, and panfungal PCR-multiplex liquid hybridization (1, 32, 33, 46, 72). Other studies that have employed nested PCR reported sensitivities at femtogram levels for the identification of fungal pathogens such as *Candida* and *Aspergillus* (37) and as few as 10 cryptococcal cells (65). Nested PCR, which uses multiple PCR amplifications, is known to increase the sensitivity of the assay, but it can lead to false-positive results due to carryover contamination (2, 37, 61). Considering the genome size of *C. neoformans* (24 megabases), we estimated that 1, 10, and 50 pg of genomic DNA template correspond to detection limits of ~38, 380, and 1,900 genome copies, respectively. When these values are converted to cell numbers, the detection limits for *C. neoformans* species complex ranged from 4×10^1 to 2×10^3 cells. When it is considered that the numbers of pathogenic yeast cells in positive blood cultures normally exceed 10^5 CFU/ml (10) and that the quantity of yeasts in cerebrospinal fluid specimens ranges from 10^3 to 10^7 CFU/ml (60), our detection levels appear to be sensitive for the detection and identification of this pathogen in clinical specimens.

The detection limits of the amplicon products, which were assessed with dilution series of the amplification products, showed that the smallest amount of product for both CNG and CNN 1b was 0.5 ng, which represents 5.81 fmol and 1.65 fmol, respectively. These detection levels are identical to those reported by Chen et al. (11), who employed the same technology for the identification of single-nucleotide polymorphisms. Diaz and Fell (15) reported slightly less sensitive values, ranging from 1 to 5 ng, for the identification of *Trichosporon* spp. A sensitivity of 1 ng was reported for the identification of *Candida* species by the use of PCR-EIA (22). After correction for amplicon length and copy numbers, this sensitivity is equivalent to 10^6 amplicon copies for CNN 1b and 10^7 amplicon copies for CNG. These amplicon detection levels are concordant with those reported by Dunbar et al. (20), who used the same technology for the identification of bacterial pathogens.

Direct amplification from cultures demonstrated the feasibility of the assay to be undertaken without DNA extraction. This 2-day culture procedure, which was used to standardize the assay conditions, can be applied to cultures of any age to provide a rapid identification. The successful amplification of intact cells was probably due to factors associated with the sufficient content of template in the cell suspension and the high numbers of copies of the target region rRNA gene, which in fungi are present in hundreds of copies (41, 64). The high copy number can act as a preamplification step, enabling an increase in amplicon yield (46, 48).

Multitemplate PCRs, which were carried out with five strains representing five different genotypic groups, demonstrated that we can detect and correctly identify multiple strains in a single sample by the hybridization assay format described here. However, to accommodate all five strains in a single PCR and to minimize preferential amplification of target sequences, certain modifications involving an increase in primer concentration, DNA template, and amplicon amount were necessary to achieve successful amplification and identification. The simultaneous screening of pathogenic strains is a practical way to identify multiple species or IGS region genotypes that coexist in a single host or environmental source. For instance, Lazera et al. (44) reported the occurrence of *C. neoformans* var. *neoformans* and *C. gatti* in the same environmental habitat. Even though multiple infections with strains with different IGS region molecular types can be a rare occurrence in specimens from single patients or a single environmental source, the fact that we can identify *C. neoformans* at the species, variety, or IGS region genotypic level in a single sample illustrates the potential and capability of the assay, which could be easily adapted for the simultaneous identification of other fungal pathogenic species.

In conclusion, we adapted this high-throughput technology to the identification of the species complex *C. neoformans* from culture-based material. The assay described in this study proved to be specific, sensitive, and flexible and allowed a complete array of different target species to be identified in a multiplex format by pooling probes of interest. The assay can be executed in less than an hour after the amplification step. Although most of our experiments used extracted DNA, we demonstrated that this step could be omitted, as biotinylated amplicons can be generated directly from intact yeast cells. These options decrease the time for sample preparation, the amounts of reagents and samples required, and the cost of the assay. Once the probes are developed, the cost of operation is relatively low, as the microspheres are inexpensive and the assay format requires small reagent volumes. All these aspects make this assay useful for applications in clinical settings and epidemiological studies where there is a demand for a high-throughput system that allows the creation of multiple testing platforms for routine testing. Further studies involving clinical cultures and clinical specimens are under way.

ACKNOWLEDGMENTS

We are indebted to Adele Statzell-Tallman for her assistance with cryptococcal isolates and to Sherry Dunbar for critical comments on the manuscript. Our special gratitude goes to B. Theeleen, M. Bovers, and M. da Luz Martins for conducting DNA isolation and to T. Boekhout, I. Spencer-Martin, and W. Meyer for providing isolates.

This research was funded by National Institutes of Health grant 1-UO1 AI53879-01.

REFERENCES

1. **Aguirre, L., S. F. Hurst, J. S. Choi, J. H. Shin, H. P. Hinrikson, and C. J. Morrison.** 2004. Rapid differentiation of *Aspergillus* species from other medically important opportunistic molds and yeasts by PCR-enzyme immunoassay. J. Clin. Microbiol. **42:**3495–3504.
2. **Atkins, S. D., and I. A. M. Clark.** 2004. Fungal molecular diagnostics: a mini-review. J. Appl. Genet. **45:**3–15.

3. **Bava, A. J., R. Negroni, and M. Bianchi.** 1993. Cryptococcosis by a urease negative strain of *Cryptococcus neoformans*. J. Med. Vet. Mycol. **31:**87–89.
4. **Bennett, J. E., K. J. Kwon-Chung, and D. Howard.** 1977. Epidemiologic differences among serotypes of *Cryptococcus neoformans*. Am. J. Epidemiol. **105:**582–586.
5. **Bialek, R., M. Weiss, K. Bekure-Nemariam, L. K. Najvar, M. B. Alberdi, J. R. Graybill, and U. Reischl.** 2002. *Cryptococcus neoformans* DNA in tissue samples by nested and real-time PCR assay. Clin. Diagn. Lab. Immunol. **9:**461–469.
6. **Boekhout, T., B. Theelen, M. Diaz, J. W. Fell, C. J. Hop, E. C. A. Abeln, F. Drommer, and W. Meyer.** 2001. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology **147:**891–907.
7. **Calvo, B. M., A. L. Combo, O. Fischman, A. S. Santiago, L. Thompson, M. Lazera, F. Telles, K. Fukushima, K. Nishimura, R. Tanaka, M. Myajy, and L. Morettii-Branchini.** 2001. Antifungal susceptibilities, varieties, and electrophoretic karyotipes of clinical isolates of *Cryptococcus neoformans* from Brazil, Chile, and Venezuela. J. Clin. Microbiol. **30:**2348–2350.
8. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans*, p. 1–541. ASM Press, Washington, D.C.
9. **Chang, H. C., S. N. Leaw, A. H. Huang, T. L. Wu, and T. C. Chang.** 2001. Rapid identification of yeasts in positive blood cultures by a multiplex PCR method. J. Clin. Microbiol. **39:**3466–3471.
10. **Chang, H. C., J. J. Chang, A. H. Huang, and T. C. Chang.** 2000. Evaluation of a capacitance method for direct antifungal susceptibility testing of yeasts in positive cultures. J. Clin. Microbiol. **38:**971–976.
11. **Chen, J., M. A. Iannone, M. S. Li, J. D. Taylor, P. Rivers, A. J. Nelsen, K. A. Slentz-Kesler, A. Roses, and M. P. Weiner.** 2000. A microsphere-based assay for multiplexed single nucleotide polymorphism analysis using single base chain extension. Genome Res. **10:**549–556.
12. **Cogliati, M., M. Allaria, A. M. Tortorano, and M. A. Viviani.** 2000. Genotyping *Cryptococcus neoformans* var. *neoformans* with specific primers designed from PCR-fingerprinting bands sequenced using a modified PCR-based strategy. Med. Mycol. **38:**97–103.
13. **Corrêa, M. P. S. C., E. C. Oliveira, R. R. B. S. Duarte, P. P. O. Pardal, F. M. Oliveira, and L. C. Severo.** 1999. Criptococose em crianças no Estado do Pará, Brasil. Rev. Soc. Bras. Med. Trop. **32:**505–550.
14. **Darzé, C., R. Lucena, I. Gomes, and A. Melo.** 2000. Características clínicas laboratoriais de 104 casos de meningoencefalite criptocócica. Rev. Soc. Bras. Med. Trop. **33:**21–26.

14a. **Diaz, M. R., T. Boekhout, T. Kiesling, and J. W. Fell.** Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic fungus *Cryptococcus neoformans*. FEMS Yeast Res., in press.

15. **Diaz, M. R., and J. W. Fell.** 2004. High-throughput detection of pathogenic yeasts in the genus *Trichosporon*. J. Clin. Microbiol. **42:**3696–3706.
16. **Diaz, M. R., T. Boekhout, B. Theelen, and J. W. Fell.** 2000. Molecular sequence analyses of the intergenic spacer (IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. Syst. Appl. Microbiol. **23:**535–545.
17. **Diaz, M. R., and J. W. Fell.** 2000. Molecular analyses of the IGS and ITS regions rDNA of the psychrophilic yeasts in the genus *Mrakia*. Antonie Leeuwenhoek **77:**7–12.
18. **Dromer, F., S. Mathoulin-Pelissier, A. Fontanet, O. Ronin, B. Dupont, O. Lortholary, and the French Cryptococcosis Study Group.** 2004. Epidemiology of HIV-associated cryptococcosis in France (1985–2001): comparison of the pre and post-HAART eras. AIDS **18:**555–562.
19. **Dromer, F., A. Varma, O. Ronin, S. Mathhoulin, and B. Dupont.** 1994. Molecular typing of *Cryptococcus neoformans* var. *neoformans* serotype D clinical isolates. J. Clin. Microbiol. **32:**2364–2371.
20. **Dunbar, S. A., C. A. Vander Zee, K. G. Oliver, K. L. Karem, and J. W. Jacobson.** 2003. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMap system. J. Microbiol. Methods **53:**245–252.
21. **Dupont, B., H. H. Crewe Brown, K. Westermann, M. D. Martins, J. H. Rex, O. Lortholary, and C. A. Kauffmann.** 2000. Mycoses in AIDS. Med. Mycol. **38:**259–267.
22. **Elie, C. M., T. J. Lott, E. Reiss, and C. J. Morrison.** 1998. Rapid identification of *Candid*a species with species-specific probes. J. Clin. Microbiol. **36:**3260–3265.
23. **Ellis, D., D. Marriott, R. A. Haijjeh, D. Warnock, W. Meyer, and R. Barton.** 2000. Epidemiology: surveillance of fungal infections. Med. Mycol. **38:**173–182.
24. **Ellis, D. H., and T. J. Pfeiffer.** 1990. Natural habitat of *Cryptococcus neoformans* var. *gatti*. J. Clin. Microbiol. **28:**1642–1644.
25. **Fell, J., and G. Blatt.** 1999. Separation of strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis. J. Ind. Microbiol. Biotechnol. **23:**677–681.
26. **Fell, J. W., T. Boekhout, A. Fonseca, G. Scorzetti, and A. Statzell-Tallman.** 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Bacteriol. **50:**1351–1371.
27. **Fuchs, B. E., K. Syutsubo, L. Wolfgang, and R. Amann.** 2001. In situ accessibility of *Escherichia coli* to 23S rRNA to fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **67:**961–968.
28. **Fulton, R., R. McDade, P. Smith, L. Kienker, and J. Kettman.** 1997. Advanced multiplexed analysis with the FlowMetrix system. Clin. Chem. **43**: 1749–1756.
29. **Goldman, D. L., H. Khine, J. Abadi, D. J. Lindenberg, L. Pirofski, R. Niang, and A. Casadevall.** 2001. Serologic evidence for *Cryptococcus neoformans* Infection in early childhood. Pediatrics **107:**66.
30. **Gotoh, M., Y. Hasegawa, Y. Shinohara, M. Schimizu, and M. Tosu.** 1995. A new approach to determine the effect of mismatches on kinetic parameters in DNA hybridization using an optical biosensor. DNA Res. **2:**285–293.
31. **Graves, J. D.** 1999. Powerful tools for genetic analysis come to age. Trends Biochem. Technol. **17:**127–134.
32. **Hendolin, P. H., L. Paulin, P. Koukila-Kahkola, V. J. Anttila, H. Malmberg, M. Richardson, and J. Ylikoski.** 2000. Panfungal PCR and multiplex liquid hybridization for detection of fungi in tissue specimens. J. Clin. Microbiol. **38:**4186–4192.
33. **Hsu, M. C., K. W. Chen, H. J. Lo, Y. C. Chen, M. H. Liao, Y. H. Lin, and S. Y. Li.** 2003. Species identification of medically important fungi by use of real time light cycler PCR. J. Med. Microbiol. **52:**1071–1076.
34. **Husain, S., M. Wagener, and A. Singh.** 2001. Cryptococcus infection in organ transplant recipients: variables influencing clinical characteristics and outcome. Emerg. Infect. Dis. **7:**375–381.
35. **Ikuta, S., K. Takagi, R. B. Wallace, and K. Ikura.** 1987. Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing single mismatched base pairs. Nucleic Acids Res. **15:**797–811.
36. **Inacio, J., S. Behrens, B. M. Fuchs, A. Fonseca, I. Spencer-Martins, and R. Amann.** 2003. In situ accessibility of *Saccharomyces cerevisiae* 26S rRNA to Cy3-labeled oligonucleotide probes comprising the D1 and D2 domains. Appl. Environ. Microbiol. **69:**2899–2905.
37. **Jaeger, E. E. M., N. M. Carrol, S. Choudhury, A. A. S. Dunlop, H. M. A. Towler, M. M. Matheson, P. Adamson, N. Okhravi, and S. Lightman.** 2000. Rapid detection and identification of *Candida*, *Aspergillus*, and *Fusarium* species in ocular samples using nested PCR. J. Clin. Microbiol. **38:**2902–2908.
38. **Katsu, M., S. Kidd, A. Ando, M. L. Morettii-Branchini, Y. Mikami, K. Nishimura, and W. Meyer.** 2004. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of *Cryptococcus neoformans* species complex. FEMS Yeast Res. **4:**377–388.
39. **Kidd, S. E., F. Hagen, R. L. Tscharke, M. Huynh, K. H. Barlett, M. Fyfe, L. Macdougall, T. Boekhout, K. J. Kwong-Chung, and W. Meyer.** 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. USA **101:** 17258–17263.
40. **Kovacs, J. A., A. A. Kovacs, M. Polis, W. C. Wright, V. J. Gill, C. U. Tuazon, E. P. Gelmann, H. C. Lane, R. Longfield, G. Overturf, A. M. Macher, A. S. Fauci, J. E. Parrillo, J. E. Bennett, and H. Masur.** 1985. Cryptococcosis in the acquired immunodeficiency syndrome. Ann. Intern. Med. **103:**533–538.
41. **Kovayashi, T., D. J. Heck, M. Nomura, and T. Horiuchi.** 1998. Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. Genes Dev. **12:**3821–3830.
42. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
43. **Latouche, G. N., M. Huynh, T. C. Sorrell, and W. Meyer.** 2003. PCR-restriction fragment polymorphism analysis of the phospholipase B (*PLB1*) gene for subtyping of *Cryptococcus neoformans* isolates. Appl. Environ. Microbiol. **69:**2080–2085.
44. **Lazera, M. S., M. A. Salmito Cavalcanti, A. T. Londero, L. Trilles, M. M. Nishikawa, and B. Wanke.** 2000. Possible primary ecological niche of *Cryptococcus neoformans*. Med. Mycol. **38:**379–383.
45. **Lee, I., A. A. Dombkowski, and B. D. Athey.** 2004. Guidelines for incorporating non perfectly matched oligonucleotides into target-specific hybridization probes for DNA microarray. Nucleic Acids Res. **32:**681–690.
46. **Lindsley, M. D., S. F. Hurst, N. J. Iqbal, and C. J. Morrison.** 2001. Rapid identification of dimorphic and yeast like fungal pathogens using specific probes. J. Clin. Microbiol. **39:**3505–3511.
47. **Liu, W. T., A. D. Mirzabekov, and D. A. Stahl.** 2001. Optimization of an oligonucleotide microchip for microbial identification studies: a non equilibrium dissociation approach. Environ. Microbiol. **3:**619–629.
48. **Lott, T. J., R. J. Kuykendall, and E. Reiss.** 1993. Nucleotide sequence analysis of the 5.8S rDNA and adjacent ITS2 region of *Candida albicans* and related species. Yeast **9:**1199–1206.
49. **Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K. H. Schleifer, and M. Wagner.** 2002. Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. **68**:5064–5081.
50. **Luo, G., and T. G. Mitchell.** 2002. Rapid identification of pathogenic fungi directly from cultures by using multiplex PCR. J. Clin. Microbiol. **40:**2860–2865.

51. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Chem. **138:**267–284.
52. **Meyer, W., A. Castañeda, S. Jackson, M. Huynh, and E. Castañeda.** 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg. Infect. Dis. **2:**189–195.
53. **Meyer, W., K. Marszewska, M. Amirmostofian, R. P. Igreja, C. Hardtke, K. Methling, M. A. Viviani, A. Chindamporn, S. Sukroongreung, M. A. John, D. H. Ellis, and T. C. Sorrell.** 1999. Molecular typing of global isolates of *Cryptococcus neoformans* by a polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA: a pilot study to standardize techniques on which to base a detailed epidemiological survey. Electrophoresis **20:**1790–1799.
54. **Mirza, S. A., M. Phelan, D. Rimland, E. Graviss, R. Hamill, M. E. Brandt, T. Gardner, M. Sattah, G. P. de Leon, W. Baughman, and R. A. Hajjeh.** 2003. The changing epidemiology of cryptococcosis: an update from population-based active surveillance in 2 large metropolitan areas, 1992–2000. Clin. Infect. Dis. **36:**789–794.
55. **O'Donnell, E. Cigelnik, N. S. Weber, and J. M. Trappe.** 1997. Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia **89:**48–65.
56. **Pappalardo, M. C., and M. S. Melhem.** 2003. Cryptococcosis: a review of the Brazilian experience for the disease. Rev. Inst. Med. Trop. Sao Paulo **45:** 299–305.
57. **Park, S., M. Wong, A. Salvatore, E. Marras, E. W. Cross, T. E. Kiehn, V. Chaturvedi, S. Tyagi, and D. S. Perlin.** 2000. Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. J. Clin. Microbiol. **38:**2829–2836.
58. **Peplies, J., F. O. Glockner, and R. Amann.** 2003. Optimization strategies for DNA microarray-based detection of bacteria with 16s RNA-targeting oligonucleotide probes. Appl. Environ. Microbiol. **69:**1397–1407.
59. **Perfect, J. R., N. Ketabchi, G. M. Cox, C. W. Ingram, and C. L. Beiser.** 1993. Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J. Clin. Microbiol. **31:**3305–3309.
60. **Perfect, J. R., D. T. Durack, and H. A. Gallis.** 1983. Cryptococcemia. Medicine **62:**98–109.
61. **Picken, M. M., R. N. Picken, D. Han, Y. Cheng, and F. Strle.** 1996. Single-tube nested polymerase chain reaction assay based on flagellin gene sequences for detection of *Borrelia burgdorferi* sensu lato. Eur. J. Clin. Microbiol. Infect. Dis. **15:**489–498.
62. **Polanco, C., and M. Pérez de la Vega.** 1994. The structure of the rDNA intergenic spacer of Avena sativa L.: a comparative study. Plant Mol. Biol. **25**:751–756.
63. **Posteraro, B., M. Sanguinetti, L. Masucci, L. Romano, G. Morace, and G. Fadda.** 2000. Reverse cross blot hybridization assay for rapid detection of PCR amplified from *Candida* species, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* in clinical samples. J. Clin. Microbiol. **38:**1609–1614.
64. **Prokopowich, C. D., T. R. Gregory, and T. J. Crease.** 2003. The correlation between rDNA copy numbers and genome size in eukaryotes. Genome **46:**48–50.
65. **Rappelli, P., R. Are, G. Casu, P. L. Fiori, P. Cappuccinelli, and A. Aceti.** 1998. Development of a nested PCR for detection of *Cryptococcus neoformans* in cerebrospinal fluid. J. Clin. Microbiol. **36:**3438–3440.
66. **Saag, M. S., G. A. Cloud, J. R. Graybill, J. D. Sobel, C. U. Tuazon, P. C. Johnson, W. J. Fessel, B. L. Moskovitz, B. Wiesinger, D. Cosmatos, L. Riser, C. Thomas, R. Hafner, W. E. Dismukes, et al.** 1999. A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated cryptococcal meningitis. Clin. Infect. Dis. **30:**291–296.
67. **Sar, B., D. Monchy, M. Vann, C. Keo, J. L. Sarthou, and Y. Buisson.** 2004. Increasing in vitro resistance to fluconazole in *Cryptococcus neoformans* Cambodian. J. Antimicrob. Chemother. **54:**563–565.
68. **Singh, N., S. Husain, M. De Vera, T. Gayowski, and T. V. Cacciarelli.** 2004. *Cryptococcus neoformans* infection in patients with cirrhosis, including liver transplant. Medicine **83:**188–192.
69. **Southern, E., K. Mir, and M. Shchepinov.** 1999. Molecular interactions on microarrays. Nat. Genet. **21**(Suppl.)**:**5–9.
70. **Steenbergen, J. N., and A. Casadevall.** 2000. Prevalence of *Cryptococcus neoformans* var. *neoformans* (serotype D) and *Cryptococcus neoformans* var. *grubii* (serotype A) isolates in New York City. J. Clin. Microbiol. **38:**1974–1976.
71. **Stephen, C., S. Lester, W. Black, M. Fyfe, and S. Raverty.** 2002. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. Can. Vet. J. **43:**792–794.
72. **Tanaka, K. I., T. Miyazaki, S. Maesaki, K. Mitsutake, H. Kakeya, Y. Yamamoto, K. Yanagihara, M. A. Hossain, T. Tashiro, and S. Kohno.** 1996. Detection of *Cryptococcus neoformans* in patients with pulmonary cryptococcosis. J. Clin. Microbiol. **34:**3438–3440.
73. **Wood, W., J. Gitschier, L. A. Lasky, and R. M. Lawn.** 1985. Base pair composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82:**1585–1588.