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Identification of Genotypically Diverse *Cryptococcus neoformans* and *Cryptococcus gattii* Isolates by Luminex xMAP Technology▽

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A Luminex suspension array, which had been developed for identification of *Cryptococcus neoformans* and *Cryptococcus gattii* isolates, was tested by genotyping a set of 58 mostly clinical isolates. All genotypes of *C. neoformans* and *C. gattii* were included. In addition, cerebrospinal fluid (CSF) obtained from patients with cryptococcal meningitis was used to investigate the feasibility of the technique for identification of the infecting strain. The suspension array correctly identified haploid isolates in all cases. Furthermore, hybrid isolates possessing two alleles of the Luminex probe region could be identified as hybrids. In CSF specimens, the genotype of the cryptococcal strains responsible for infection could be identified after optimization of the PCR conditions. However, further optimization of the DNA extraction protocol is needed to enhance the usability of the method in clinical practice.

Cryptococcus neoformans and *Cryptococcus gattii* are closely related pathogenic yeasts, as indicated by the previous description of *C. gattii* as a variety of *C. neoformans* (16). Recently, *C. gattii* has been described as a separate species because of differences in ecology, biochemical, and molecular characteristics (17, 18). *C. neoformans* and *C. gattii* both may cause meningoencephalitis, which is fatal unless treated. *C. neoformans* occurs globally and is found primarily in immunocompromised individuals, e.g., human immunodeficiency virus (HIV)-infected patients. Although the incidence of cryptococcosis in AIDS patients has decreased because of the introduction of the highly active antiretroviral treatment, cryptococcosis remains a serious disease, with a mortality rate of 10 to 30% in regions where access to treatment is limited (3, 22), and it continues to be the most important cause of fungal meningitis in immunocompromised patients. In contrast to *C. neoformans*, *C. gattii* mainly infects immunocompetent individuals and was thought to occur only in (sub)tropical areas. However, one of the genotypic groups of *C. gattii* is causing an ongoing outbreak on Vancouver Island (14, 15, 27), which indicates that *C. gattii* may also occur in more temperate areas.

C. neoformans and *C. gattii* differ not only in host range and geographic distribution, but they also differ in clinical manifestation. Although both species infect the central nervous system, *C. gattii* appears to invade the brain parenchyma more commonly than *C. neoformans*. Furthermore, in *C. gattii*-infected patients, pulmonary infections are more likely and pulmonary mass-like lesions occur more commonly than in *C. neoformans*-infected patients (23, 26). Patients infected with *C. gattii* seem to have had their symptoms longer before presentation and therapy is often needed for a longer period of time (23, 26). Because of the differences in clinical manifestations and the outcomes of disease, it is important to accurately identify the species responsible for the infection.

Six haploid genotypic groups within *C. neoformans* and *C. gattii* can be distinguished by several different molecular methods, e.g., by amplified fragment length polymorphism (AFLP) analysis (4), PCR fingerprinting (21), and intergenic spacer (IGS) genotyping (10). The haploid groups within *C. neoformans* correspond to the two varieties *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, while *C. gattii* can be divided into four genotypic groups. Besides these haploid groups, hybrids have been described as well. Hybrids between the two varieties of *C. neoformans* exist, these are the so-called AD hybrids (4, 6, 20, 28), and hybrids between *C. neoformans* var. *neoformans* and *C. gattii* have recently been described (5). The different genotypic groups and the relationship between variety, serotype, and the different genotyping methods are shown in Table 1.

Unfortunately, the diagnostic methods which are currently used do not discriminate between all genotypic groups. As a consequence, the differences in hosts and symptoms between the genotypic groups are not known, which is especially true for the genotypic groups within *C. gattii*. It is likely that more differences in host range and symptoms will be found when the exact genotype of the infecting cryptococcal strain is determined. Another disadvantage of the current diagnostic methods is that they take a considerable amount of time to complete

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TABLE 1. Overview of the varieties, serotypes, and genotypes within *C. neoformans* and *C. gattii*.

Species	Serotype[a,b]	AFLP genotype [b,c]	Molecular genotype[a]	IGS genotype[d]	Luminex probe[e]
C. neoformans					CNNb
C. neoformans var. *grubii*	A	1	VNI/VNII	1A/1B/1C	CNN1b
C. neoformans var. *grubii* × *C. neoformans* var. *neoformans* hybrid	AD	3	VNIII		
C. neoformans var. *neoformans*	D	2	VNIV	2A/2B/2C	CNN2d
C. neoformans var. *neoformans* × *C. gattii* AFLP4 hybrid	BD	8			
C. gattii					CNG
C. gattii	B/C	4	VGI	4A/4B/4C	CNG4c
C. gattii	B/C	5	VGIII	5	CNG5b
C. gattii	B/C	6	VGII	3	CNG3
C. gattii	B/C	7	VGIV	6	CNG6

[a] Meyer et al. (21).
[b] Bovers et al. (5).
[c] Boekhout et al. (4).
[d] Diaz et al. (10).
[e] Diaz and Fell (9).

(e.g., culturing) or they can only be used for a limited number of species (e.g., antigen detection). Recently, Luminex xMAP technology has been adapted for the detection of the genotypes within *C. neoformans* and *C. gattii* (9). The xMAP technology is based on uniquely color-coded microspheres, which allows as many as one hundred different species to be detected in a single reaction. This technology has been used for the detection of several species of bacteria and fungi (7, 8, 9, 11, 13, 24, 31). Recently, xMAP technology has been used in several diagnostic kits for the detection of bacterial and viral pathogens.

In our study, we used a set of 48 haploid and 10 hybrid isolates to test a Luminex suspension array, which had been developed for identification of *C. neoformans* and *C. gattii* strains (9). Our set contained isolates obtained from Dutch cryptococcosis patients in the period between 1977 and 2001, as well as *C. gattii* isolates from our own collection. In addition, cerebrospinal fluid (CSF) specimens obtained from patients diagnosed with cryptococcal meningitis were used to investigate the feasibility of this Luminex suspension array for the identification of cryptococci in clinical specimens.

MATERIALS AND METHODS

Genotyping of cryptococcal isolates. Thirty-four isolates obtained from Dutch patients and maintained in the cryptococcal collection of The Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center, Amsterdam, The Netherlands) were used for the genotyping assay. Because almost all of these isolates were *C. neoformans*, we included twenty additional *C. gattii* isolates and four additional hybrid isolates from our own collection. The origins of the haploid and hybrid isolates are shown in Tables 2 and 3, respectively.

DNA was isolated from cultures as described by Bovers et al. (5). Isolates that had not been genotyped before were analyzed by AFLP (4) and *C. neoformans* mating- and serotype-specific PCRs. PCR amplifications were performed in 20-μl volumes containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 8.3), 0.1 mM deoxynucleoside triphosphates, 0.5 U of *Taq* DNA polymerase (Gentaur, Bruxelles, Belgium), 2 to 3 μl of template DNA, and 0.1 μM of both primers. Amplification conditions were as follows: for serotype AD-*MAT*α-specific primer pair JOHE1671/1672 (20), 96°C for 5 min, followed by 25 cycles of 96°C for 30 s, 66°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 5 min; for serotype A-specific primer pair JOHE3241/JOHE2596 (20) and serotype D-specific primer pair JOHE3240/JOHE2596 (20), 96°C for 5 min, followed by 25 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension step of 72°C for 5 min. PCR conditions for the first serotype A-*MAT***a**-specific primer pair JOHE5169/JOHE5170 (20), the second serotype A-*MAT***a**-specific primer pair JOHE7270/JOHE7272 (1), the serotype A-*MAT*α-specific primer pair JOHE7264/JOHE7265 (1), the serotype D-*MAT***a**-specific primer pair JOHE7273/JOHE7275 (1), and the serotype D-*MAT*α-specific primer pair JOHE7267/JOHE7268 (1) were as follows: 96°C for 5 min, followed by 30 cycles of 96°C for 15 s, 66°C for 15 s, and 72°C for 1 min, and a final extension step of 72°C for 5 min.

Flow cytometry and sequencing of hybrid isolates. The diploid nature of all hybrid isolates was confirmed by flow cytometry according to the method of Bovers et al. (5). Furthermore, partial sequences of the IGS1 region of the ribosomal DNA and laccase (*CNLAC1*) gene were determined for all hybrid isolates. The primer sequences were those used by Diaz et al. (12) and Xu et al. (32). The amplicons were cloned into *Escherichia coli* DH5α cells with a TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones were picked randomly, amplified, and purified with a GFX PCR DNA and gel band purification kit (Amersham Biosciences, Piscataway, NY). A BigDye v3.1 Chemistry kit (Applied Biosystems, Foster City, CA) was used for sequencing, and the amplicons were analyzed on an ABI 3700XL DNA analyzer (Applied Biosystems).

Clinical specimens. Clinical specimens were obtained from The Netherlands Reference Laboratory for Bacterial Meningitis in Amsterdam, the University Medical Centre in Utrecht, and the Erasmus Medisch Centrum in Rotterdam, all in The Netherlands, and the University Hospital Gasthuisberg in Leuven, Belgium. CSF from patients with culture-proven cryptococcal meningitis had been stored for up to five years at −80°C. The origins and volumes of the CSF specimens are described in Table 4. After thawing the CSF samples, they were centrifuged for 10 min at 16,000 × *g* and the supernatant was removed. Five hundred microliters of distilled water was added, and the pellet was resuspended to remove human cells that might be present in the CSF. The samples were centrifuged for 10 min at 16,000 × *g* and the supernatant was removed. One milliliter of Novozym 234 (1 mg/ml) (Novo Industri, Bagsvaerd, Denmark) suspended in sorbitol buffer (1 M sorbitol, 0.1 M sodiumcitrate; pH 5.5) was added to the samples. The samples were incubated for one hour at 37°C to generate protoplasts, after which the samples were centrifuged for 5 min at 4,600 × *g* and the supernatant was removed. The tissue protocol of the QIAamp DNA Micro kit (QIAGEN, Venlo, The Netherlands) was used for DNA isolation. The DNA was eluted with 35 μl of AE buffer from the kit.

Luminex suspension array. The Luminex suspension array, which detects 5′ biotin-labeled PCR amplicons hybridized to specific capture probes, was performed as described by Diaz and Fell (9). Specific oligonucleotide probes for each of the six haploid genotypic groups within the *C. neoformans* species complex as well as oligonucleotide probes targeting either *C. neoformans* or *C. gattii* were used. All probes had been designed based on the IGS1 region of the ribosomal DNA (9). An overview of the targets of each probe is given in Table 1. The probes were synthesized with a 5′-end Amino C-12 modification (Integrated DNA Technologies, Coralville, IA) and covalently coupled to different sets of 5.6-μm polystyrene carboxylated microspheres using a slightly modified

TABLE 2. Origins of haploid isolates and overview of results obtained by AFLP analysis, mating-serotype-specific PCRs, and Luminex suspension array

Isolate[a]	Source of isolation[b]	Location	AFLP genotype	Mating type and serotype	Positive Luminex probes	Luminex identification	Reference
AMC770704	CSF, HIV-negative woman, age 22	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC830410	CSF, HIV-negative woman, age 58	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC860743	CSF, HIV-positive man, age 47	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC880696	Jugular gland, man, age 19	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC900239	CSF, immunocompetent man, age 27	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC900321	Lung, AIDS patient, man, age 43	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC900906	CSF, immunocompetent woman, age 49	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC901081	CSF, HIV-positive man, age 47	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC922148	CSF, AIDS patient, man, age 29	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC931394	CSF, HIV-negative, immunocompromised man, age 52	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC940211	CSF, AIDS suspected, man, age 30	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC940580	CSF, HIV-negative, immunocompromised man, age 73	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC940751	CSF, HIV-positive man, age 44	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC951535	CSF, prednisone usage, HIV-negative, immunocompromised man, age 58	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC981683	CSF, AIDS patient, woman, age 23	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC990558	CSF, encephalopathy, AIDS patient, man, age 37	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC2040734	CSF, man, age 46	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC9402891	CSF, AIDS patient, man, age 45	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
JS9901	CSF, sarcoidosis, man, age 43	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
MN	HIV-positive man, age 30	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
P1953	HIV-negative, immunocompromised man, age 65	The Netherlands	AFLP1	αA	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)	This study
AMC890401	CSF, AIDS patient, man, age 35	The Netherlands	AFLP2	**a**D	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
AMC940038	CSF, AIDS patient, man, age 29	The Netherlands	AFLP2	**a**D	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
AMC941354	CSF, HIV-negative, immunocompromised man, age 69	The Netherlands	AFLP2	αD	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
AMC2010488	CSF, AIDS patient, man, age 50	The Netherlands	AFLP2	αD	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
AMC2031402	CSF, man, age 65	The Netherlands	AFLP2	αD	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
AMC2020797A	CSF, non-Hodgkin lymphoma, woman, age 41	The Netherlands	AFLP2	αD	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	This study
CBS1622	Tumor, man	France	AFLP4	B	CNG, CNG4c	*C. gattii* (AFLP4)	Boekhout et al. (4)
CBS6289	Subculture of type strain of *C. neoformans* var. *gattii* (RV20186: human CSF, Zaire)		AFLP4	B	CNG, CNG4c	*C. gattii* (AFLP4)	Boekhout et al. (4)
CBS7229	Meningitis, type strain of *C. neoformans* var. *shanghaiensis*	China	AFLP4	B	CNG, CNG4c	*C. gattii* (AFLP4)	Boekhout et al. (4)
CBS883	Infected skin, syntype *C. hondurianus*	Honduras	AFLP4	B	CNG, CNG4c	*C. gattii* (AFLP4)	Boekhout et al. (4)
N114	HIV-positive man, age 47	The Netherlands	AFLP4	ND[b]	CNG, CNG4c	*C. gattii* (AFLP4)	This study

Continued on following page

TABLE 2—*Continued*

Isolate[a]	Source of isolation[b]	Location	AFLP genotype	Mating type and serotype	Positive Luminex probes	Luminex identification	Reference
RV54130	Second isolate of *C. neoformans* var. *shanghaiensis*	China	AFLP4	B	CNG, CNG4c	*C. gattii* (AFLP4)	Boekhout et al. (4)
CBS6955	CSF, type strain of *Filobasidiella bacillispora*	California	AFLP5	C	CNG, CNG5b	*C. gattii* (AFLP5)	Boekhout et al. (4)
CBS6993	Human CSF	California	AFLP5	C	CNG, CNG5b	*C. gattii* (AFLP5)	Boekhout et al. (4)
CBS8755	Detritus of almond tree	Colombia	AFLP5	C	CNG, CNG5b	*C. gattii* (AFLP5)	Boekhout et al. (4)
WM726	*Eucalyptus citriodora*	San Diego, CA	AFLP5	B	CNG, CNG5b	*C. gattii* (AFLP5)	Boekhout et al. (4)
113A-5	Air sample from beneath Douglas fir tree	Vancouver Island, Canada	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Kidd et al. (15)
AV54	CSF, HIV-positive man, age 31	Greece	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Velegraki et al. (29)
AV55	Immunocompromised woman, age 26	Greece	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Velegraki et al. (29)
CBS6956	Sputum, immunocompetent human	Seattle, WA	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Boekhout et al. (4)
A1MF3179	Sputum, immunocompetent man	Vancouver, Canada	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Kidd et al. (15)
A1MR265	Bronchial wash, immunocompetent man	Vancouver Island, Canada	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Kidd et al. (15)
ENV133	Douglas fir tree	Vancouver Island, Canada	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Kidd et al. (15)
RB28	Tree stump near alder tree	Vancouver Island, Canada	AFLP6	B	CNG, CNG3	*C. gattii* (AFLP6)	Kidd et al. (15)
B5748	HIV-positive human	India	AFLP7	B	CNG, CNG6	*C. gattii* (AFLP7)	Diaz and Fell (9)
M27055	Clinical specimen	Johannesburg, South Africa	AFLP7	C	CNG, CNG6	*C. gattii* (AFLP7)	Latouche et al. (19)
WM779	Cheetah	Johannesburg, South Africa	AFLP7	C	CNG, CNG6	*C. gattii* (AFLP7)	Kidd et al. (15)

[a] AMC, The Netherlands Reference Laboratory for Bacterial Meningitis, Academic Medical Center, Amsterdam, The Netherlands; CBS, CBS—Fungal Biodiversity Centre, Utrecht, The Netherlands; RV, BCCM/IHEM Biomedical Fungi and Yeast Collection, Brussels, Belgium; WM, Wieland Meyer, Molecular Mycology Research Laboratory, Westmead Hospital, Sydney, Australia.

[b] ND, not determined.

carbodiimide method (8). Each microsphere set (MiraiBio, Alameda, CA) contains a unique spectral address by combining different ratios of red and infrared fluorochromes. In a typical reaction, 5×10^6 microspheres were resuspended in 25 μl 0.1 M MES (2[*N*-morpholino]ethanesulfonic acid), pH 4.5, with a determined amount of probe (0.2 to 0.5 nmol). Probe coupling was performed as described by Diaz and Fell (8), and the microspheres were subsequently resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8). A microsphere mixture was made by adding approximately 5,000 microspheres for each of the eight probes to a 1.5× TMAC (3 M tetramethyl ammonium chloride, 50 mM Tris [pH 8], 4 mM EDTA [pH 8], 0.1% Sarkosyl) solution.

To amplify the IGS1 region, forward primer IG1F (5′-CAG ACG ACT TGA ATG GGA ACG-3′) and reverse primer IG2R (5′-ATG CAT AGA AAG CTG TTG G-3′) were used (12). The reverse primer was biotinylated at the 5′ end. The 1× HotStarTaq MasterMix (QIAGEN, Valencia, CA) containing 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, and 2.5 units of HotStarTaq polymerase was used for all PCRs. DNA had been extracted from the cryptococcal isolates prior to amplification, although Diaz and Fell (9) directly used yeast cells for PCR amplification. PCRs were carried out in a total volume of 25 μl. Primers IG1F and IG2R (0.6 μM) and 1.5 μl of template DNA were added to the MasterMix. Amplification conditions were as follows: 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 7 min.

PCR amplification of the first two clinical samples was carried out using the HotStarTaq MasterMix in a 50-μl total volume containing 3 μl of DNA and 0.4 μM of both primer IG1F and primer IG2R. Amplification conditions were as described above.

Optimization of the PCR conditions resulted in the following reaction conditions, which were used for the remaining clinical samples. PCR amplification was carried out with the HotStarTaq MasterMix in a 50-μl total volume containing 0.2% bovine serum albumin, 8 μl of DNA, and 0.6 μM of both primer IG1F and primer IG2R. Amplification conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 69°C for 30 s, and a final extension step of 69°C for 9 min. Amplicons were cleaned with a QIAGEN purification kit (QIAGEN, Valencia, CA) and eluted with elution buffer (10 mM Tris-HCl [pH 8.5]).

To genotype the cryptococcal isolates, 5 μl of biotinylated amplicon was diluted with 12 μl of TE buffer (pH 8), and to genotype the clinical samples, 15 μl of biotinylated amplicon was diluted with 2 μl of TE buffer (pH 8). Thirty-three microliters of the microsphere mixture was added. Each amplicon was tested in duplicate with the Luminex suspension array. The hybridization reaction was performed as described by Diaz and Fell (9).

The hybridized samples were analyzed on the Luminex 100 analyzer (Luminex Corporation, Austin, TX). One hundred microspheres of each set were analyzed, which represents a hundred replicate measurements. Median fluorescence intensity (MFI) values were calculated with a digital signal processor and Luminex 1.7 proprietary software. A positive signal was defined as a signal that is at least twice the background level after subtraction of the background.

Nucleotide sequence accession numbers. All sequences were deposited at GenBank under the accession numbers DQ286656 to DQ286661, DQ286665 to DQ286670, and EF100569 to EF100594.

RESULTS AND DISCUSSION

AFLP analysis and mating-serotype-specific PCRs performed on the isolates that had not been genotyped before showed that twenty-one isolates belonged to *C. neoformans* var. *grubii MAT*α serotype A (AFLP1), four isolates were *C.*

TABLE 3. Origin of hybrid isolates and overview of results obtained by AFLP analysis, sequence analysis, mating-serotype-specific PCRs, and Luminex suspension array[a]

Isolate[b]	Source of isolation	Location	AFLP genotype	Mating type and serotype	Positive Luminex probes	Luminex identification	IGS1 sequences (no. of alleles)	*CNLAC1* sequences (no. of alleles)	Reference
AMC881205I	CSF, HIV-positive man	The Netherlands	AFLP3	**a**A-αD	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)	2	2	This study
CDC92-26	CDC		AFLP3	**a**A-αD	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)	2	2	This study
Kl#1	Progeny laboratory crossing H99 5-FOAr × JEC171		AFLP3	**a**D-αA	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)	2	2	Lengeler et al. (20)
Kl#45	Progeny laboratory crossing H99 5-FOAr × JEC171		AFLP3	**a**D-αA	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)	2	2	Lengeler et al. (20)
ZG287	Duke Medical Center permanent strain collection		AFLP3	**a**D-αA	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)	2	2	Lengeler et al. (20)
AMC890351	CSF, HIV-positive man, age 44	The Netherlands	AFLP3	**a**D-αA	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	1	2	This study
AMC891529	CSF, AIDS patient, man, age 31	The Netherlands	AFLP3	**a**D-αA	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)	1	2	This study
AMC770616 (=CBS10488)	CSF, brain tumor surgery, man, age 23	The Netherlands	AFLP8	**a**D-αB	CNNb, CNN2d, CNG, CNG4c	Hybrid between *C. neoformans* var. *neoformans* and AFLP4 *C. gattii* (AFLP8)	2	2	Bovers et al. (5)
AMC2010404 (=CBS10489)	CSF, idiopathic intracranial hypertension, man, age 35	The Netherlands	AFLP8	**a**D-αB	CNNb, CNN2d, CNG, CNG4c	Hybrid between *C. neoformans* var. *neoformans* and AFLP4 *C. gattii* (AFLP8)	2	2	Bovers et al. (5)
AMC2011225 (=CBS10490)	CSF, idiopathic intracranial hypertension, man, age 36	The Netherlands	AFLP8	**a**D-αB	CNNb, CNN2d, CNG, CNG4c	Hybrid between *C. neoformans* var. *neoformans* and AFLP4 *C. gattii* (AFLP8)	2	2	Bovers et al. (5)

[a] All isolates were diploid.
[b] AMC, The Netherlands Reference Laboratory for Bacterial Meningitis, Academic Medical Center, Amsterdam, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, GA.

TABLE 4. Origin and volume of CSF for which amplicons could be obtained and the results of Luminex identification

Clinical specimen[a]	Source of isolation	Vol (μl)	Positive Luminex probes	Luminex identification
AMC2031402	CSF, man, age 65	400	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)
AMC2031845	CSF, man, age 73	400	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)
AMC2040592	CSF, AIDS patient, man, age 29	400	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)
AMC2010488 (=JS2002)	CSF, AIDS patient, man, age 50	385	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)
JS9901	CSF, sarcoidosis, man, age 43	185	No positive probes	
AMC2010576 (=JS2003)	CSF, HIV-positive man, age 46	85	No positive probes	
L4	CSF	185	CNNb, CNN2d	*C. neoformans* var. *neoformans* (AFLP2)
L5C (=535615)	CSF, some blood present	1,230	CNNb, CNN1b	*C. neoformans* var. *grubii* (AFLP1)
L5D (=536140)	CSF	2,230	CNNb, CNN1b, CNN2d	Hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (AFLP3)

[a] AMC, The Netherlands Reference Laboratory for Bacterial Meningitis, Academic Medical Center, Amsterdam, The Netherlands; JS and L, University Medical Centre Utrecht, Utrecht, The Netherlands.

neoformans var. *neoformans MAT*α serotype D (AFLP2), and two isolates were *C. neoformans* var. *neoformans MAT***a** serotype D (AFLP2). Finally, one isolate belonged to the *C. gattii* AFLP4 genotype. Some hybrid isolates between the two varieties of *C. neoformans* were detected: two *MAT***a** serotype A-*MAT*α serotype D (AFLP3) and two *MAT***a** serotype D-*MAT*α serotype A (AFLP3) isolates. Results of the AFLP analysis and the mating-serotype-specific PCRs are presented in Tables 2 and 3.

All haploid isolates were identified by two Luminex suspension array probes (Fig. 1). Probes CNNb and CNN1b identified the AFLP1 isolates as *C. neoformans* var. *grubii* (AFLP1), and probes CNNb and CNN2d identified the AFLP2 isolates as *C. neoformans* var. *neoformans* (AFLP2). Probe CNG correctly identified all *C. gattii* isolates as *C. gattii*. In addition, probe CNG4c identified the AFLP4 isolates, probe CNG5b identified the AFLP5 isolates, probe CNG3 identified the AFLP6 isolates, and probe CNG6 identified the AFLP7 isolates. These results show that the Luminex suspension array correctly genotyped all haploid strains (Table 2).

The Luminex suspension array was also used to genotype ten hybrid isolates. Five out of seven serotype AD (AFLP3) hybrid isolates, namely AMC881205I, CDC92-26, Kl#1, Kl#45, and ZG287, were identified as hybrids between the two varieties of *C. neoformans* by probes CNNb, CNN1b, and CNN2d (Fig. 2a). However, two serotype AD (AFLP3) hybrid isolates, namely AMC890351 and AMC891529, were identified as *C. neoformans* var. *neoformans* by probes CNNb and CNN2d (Fig. 2b). The three serotype BD (AFLP8) hybrid isolates were identified as hybrids between *C. neoformans* var. *neoformans* and *C. gattii* AFLP4 by probes CNG, CNG4c, CNNb, and CNN2d (Fig. 3). Interestingly, when the suspension array identified a hybrid isolate, low signal intensities, namely 16% to 53% of an average positive signal, were obtained for probes which identified one of the parental genotypes. The results of the suspension array correlated with the number of clones that were found for each allele. For example, more AFLP4 than AFLP2 IGS1 clones were obtained for the serotype BD hybrid isolates. The Luminex probes gave a similar outcome: the signals for probes CNG and CNG4c, which identify the *C. gattii* AFLP4 genotype, had normal intensities, i.e., 546 to 940 MFI. The probes which identify *C. neoformans* var. *neoformans* (AFLP2), namely CNNb and CNN2d, were positive with MFI values ranging from 119 to 311, but the MFI values that were obtained were only 16% to 19% of an average positive signal (Fig. 3).

Flow cytometry confirmed that all hybrid isolates were diploid or close to diploid (data not shown). In addition, when a part of the *CNLAC1* region was cloned and sequenced, two alleles could be obtained for all hybrid isolates. However, when the IGS1 region was used for cloning and sequencing, two alleles were obtained for five serotype AD (AFLP3) hybrid isolates, namely AMC881205I, CDC92-26, Kl#1, Kl#45, and ZG287, but only one IGS1 allele was found in two serotype AD (AFLP3) hybrid isolates, namely AMC890351 and AMC891529, even though thirty clones were sequenced. All three serotype BD (AFLP8) hybrid isolates possessed two IGS1 alleles. Our results show that all hybrid isolates were diploid or close to diploid, they possessed two *CNLAC1* alleles, and most hybrid isolates possessed two IGS1 alleles. All hybrid isolates that possessed two IGS1 alleles, the region on which the Luminex probes are based, could be identified as hybrids. In order to further improve the identification of cryptococcal hybrids, a probe derived from another gene could be included. In a multigene study of thirty-one serotype AD hybrid isolates (AFLP3), five isolates possessed two IGS1 alleles, but twenty-seven isolates had two *TEF1*α alleles, twenty-six isolates possessed two *RPB1* alleles, and twenty-three isolates had two *CNLAC1* alleles (M. Bovers, unpublished data). This indicates that *CNLAC1*, *RPB1*, and *TEF1*α are potential regions that could be used to improve the identification of cryptococcal hybrids.

The detection limit of the suspension array was calculated to vary from 4×10^1 to 2×10^3 cells for the different probes (9). In clinical practice, the concentration of cryptococcal cells ranges from 1,000 to 10,000,000 cells per ml of CSF (25). Therefore, the Luminex technology could be a powerful tool for the detection and identification of cryptococcal

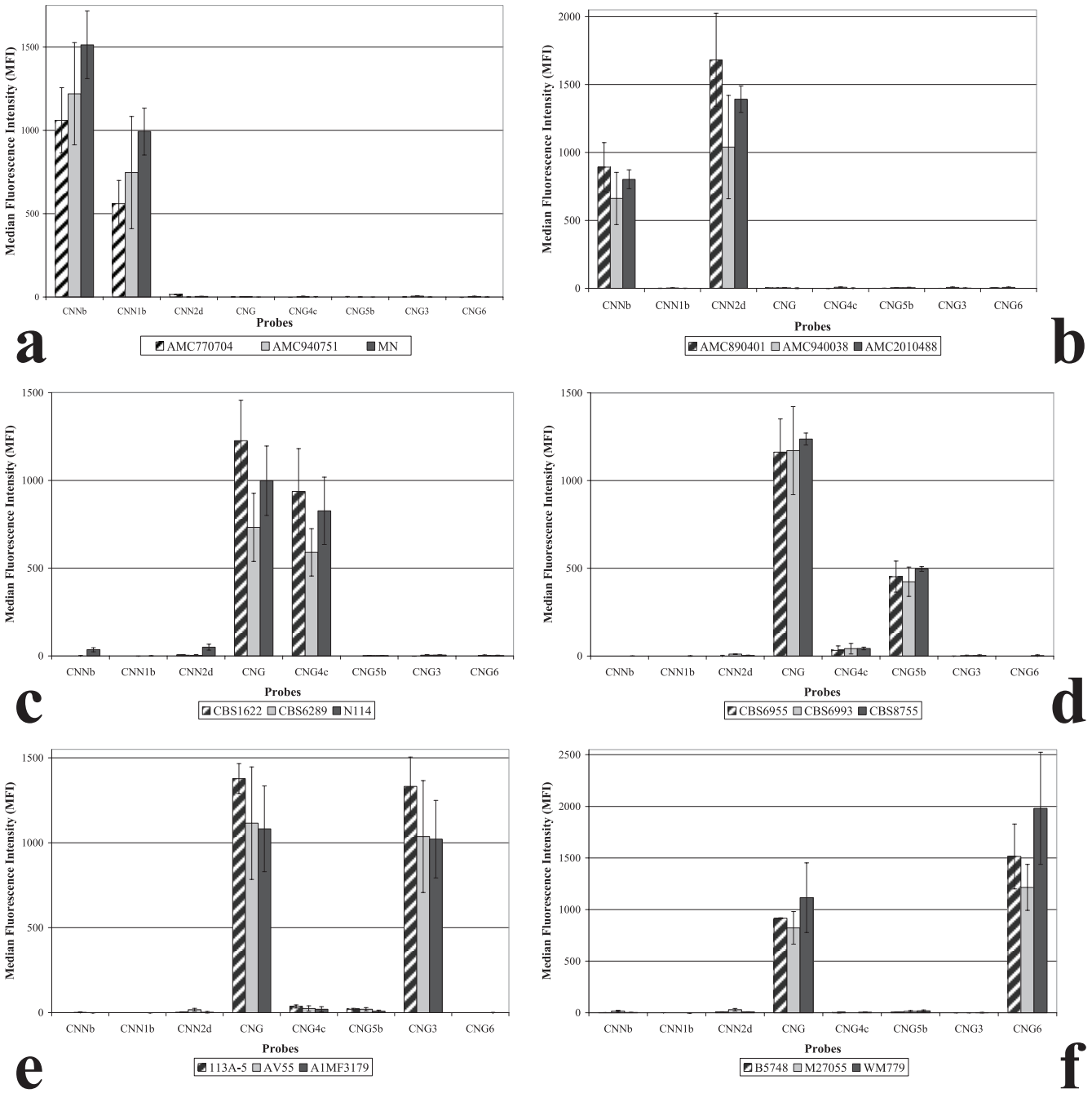

FIG. 1. Results obtained with the Luminex suspension array for all six haploid groups within *C. neoformans* and *C. gattii*. Examples of the results obtained with the AFLP1, -2, -4, -5, -6, and -7 genotypic groups are depicted in panels a, b, c, d, e, and f, respectively.

cells in CSF. CSF specimens from various Dutch and Belgian hospitals that had been stored for up to five years at −80°C were used to test the Luminex suspension array on clinical specimens. Only nine out of twenty CSF specimens obtained from patients with culture-proven cryptococcal meningitis gave an amplicon of the targeted IGS locus. Unfortunately, we do not have additional information, i.e., about the number of (undamaged) cells or about the presence of interfering substances (30) in the samples, to explain why an amplicon could not be obtained. Amplicons of the PCR-positive samples were subsequently used for genotypic identification by the suspension array (Fig. 4), and the results are shown in Table 4. The first two samples that were analyzed, namely JS9901 and AMC2010576, had probe signals that were too low to be considered positive. Optimization of the PCR conditions, i.e., adding 0.2% bovine serum albumin and increasing the amount of DNA and primers in the reaction mix as well as decreasing the temperature of the elongation step to 69°C, improved detection. As a consequence, the infecting agent of the remaining seven samples

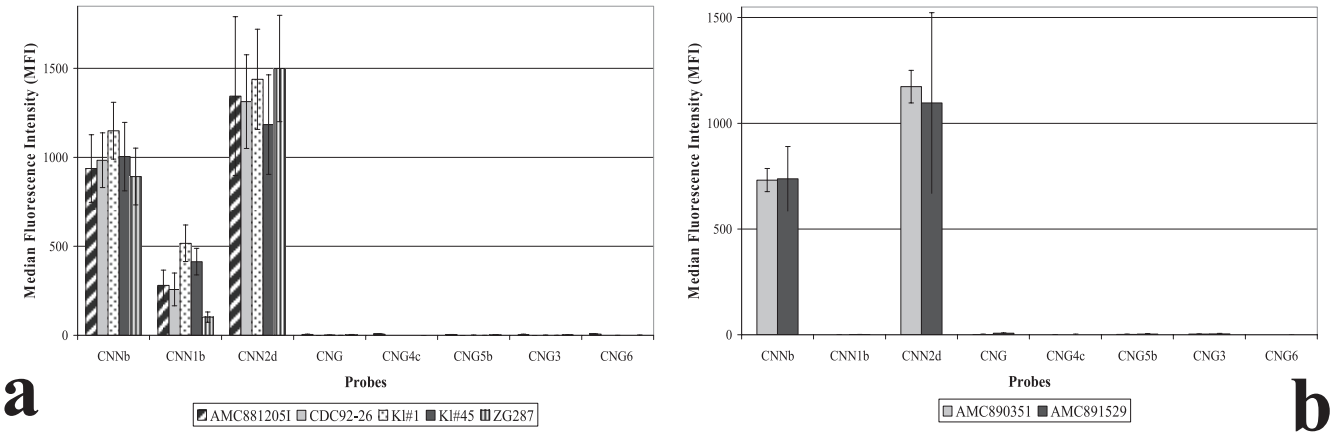

FIG. 2. Results obtained with the Luminex suspension array for all of the serotype AD (AFLP3) hybrids of *C. neoformans*. Panel a shows five serotype AD hybrids that were identified by three probes, and panel b shows two serotype AD hybrids that were identified by two probes.

could successfully be identified at species and genotypic levels, but because no CSF of JS9901 and AMC2010576 remained, the genotype of the infecting agent could not be determined for those two samples. Probes CNNb and CNN1b, with MFI signals ranging from 260 to 1,402, identified the infecting agent in L5c, AMC2040592, and AMC2031845 as *C. neoformans* var. *grubii*. Probes CNNb and CNN2d identified the source of infection of AMC2010488, L4, and AMC2031402 as *C. neoformans* var. *neoformans*, with MFI signals ranging from 112 to 1,780. Probes CNNb, CNN1b, and CNN2d, with MFI signals ranging from 199 to 1,446, identified the cryptococcal strain responsible for infection of the patient of specimen L5d as a serotype AD (AFLP3) strain of *C. neoformans*. Interestingly, CSF specimens L5c and L5d were obtained from the same patient, thus suggesting a dual infection by a haploid *C. neoformans* var. *neoformans* and a serotype AD (AFLP3) hybrid strain. Our results show that the suspension array is highly specific, as both varieties of *C. neoformans* (*C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*) and a hybrid could be identified in CSF specimens. The clinical applicability of the Luminex suspension array might be improved by optimization of the DNA isolation protocol, as this is one of the critical steps in any molecular detection system (2). In addition, to determine the robustness of the method, fresh clinical specimens for which the amount of cryptococcal cells and the antigen titers are known should be tested and follow-up studies should be performed on samples with false-negative results.

In summary, the Luminex suspension array has the potential to become an efficient diagnostic method with high specificity that not only identifies cryptococcal isolates at the species and genotype levels but that also allows identification of hybrid isolates that possess two IGS1 alleles. Furthermore, our results show that the Luminex suspension array is able to identify cryptococci in CSF specimens. Identification in CSF occurs at the species, genotype, and hybrid levels, but optimization of DNA extraction methods is

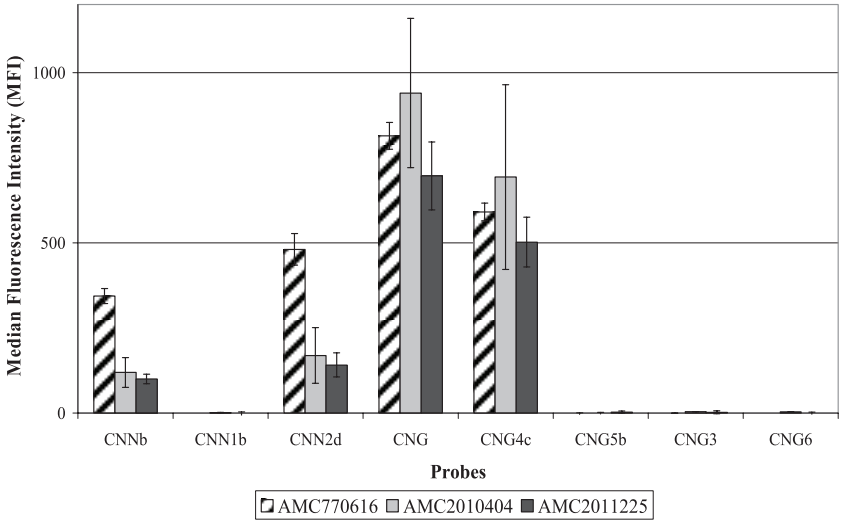

FIG. 3. Results obtained with the Luminex suspension array for the serotype BD (AFLP8) hybrid isolates.

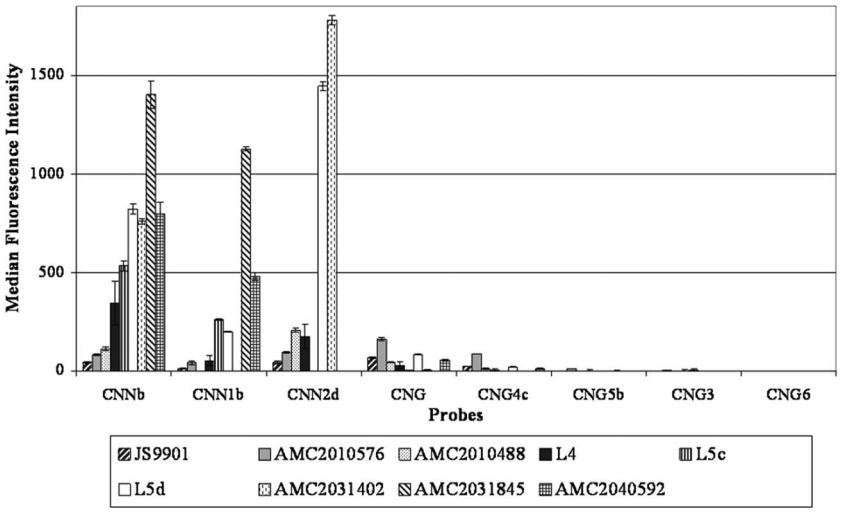

FIG. 4. Luminex suspension array results for the amplicons obtained from CSF specimens.

needed before the method is suited for routine use in clinical laboratories.

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