JOURNAL OF CLINICAL MICROBIOLOGY, July 2011, p. 2522–2527 Vol. 49, No. 7
0095-1137/11/$12.00 doi:10.1128/JCM.00502-11

Rapid Identification of *Cryptococcus neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* by Use of Rapid Biochemical Tests, Differential Media, and DNA Sequencing▽

Lisa McTaggart,[1] Susan E. Richardson,[1,2,3] Christine Seah,[1] Linda Hoang,[4,5] Annette Fothergill,[6] and Sean X. Zhang[1,2]*

Medical Mycology, Ontario Public Health Laboratories, Ontario Agency for Health Protection and Promotion,[1] Department of Laboratory Medicine and Pathobiology, University of Toronto,[2] and Division of Microbiology, Hospital for Sick Children,[3] Toronto, Ontario, Canada; BCCDC Public Health and Reference Microbiology Laboratory, PHSA,[4] and Department of Pathology and Laboratory Medicine, University of British Columbia,[5] Vancouver, British Columbia, Canada; and Fungus Testing Laboratory, San Antonio, Texas[6]

Received 11 March 2011/Returned for modification 13 April 2011/Accepted 10 May 2011

Rapid identification of *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans*, and *Cryptococcus gattii* is imperative for facilitation of prompt treatment of cryptococcosis and for understanding the epidemiology of the disease. Our purpose was to evaluate a test algorithm incorporating commercial rapid biochemical tests, differential media, and DNA sequence analysis that will allow us to differentiate these taxa rapidly and accurately. We assessed 147 type, reference, and clinical isolates, including 6 other *Cryptococcus* spp. (10 isolates) and 14 other yeast species (24 isolates), using a 4-hour urea broth test (Remel), a 24-hour urea broth test (Becton Dickinson), a 4-hour caffeic acid disk test (Hardy Diagnostics and Remel), 40- to 44-hour growth assessment on L-canavanine glycine bromothymol blue (CGB) agar, and intergenic spacer (IGS) sequence analysis. All 123 *Cryptococcus* isolates hydrolyzed urea, along with 7 isolates of *Rhodotorula* and *Trichosporon*. Eighty-five of 86 *C. neoformans* (99%) and 26 of 27 *C. gattii* (96%) isolates had positive caffeic acid results, unlike the other cryptococci (0/10) and yeast species (0/24). Together, these two tests positively identified virtually all *C. neoformans*/*C. gattii* isolates (98%) within 4 h. CGB agar or IGS sequencing further differentiated these isolates within 48 h. On CGB, 25 of 27 (93%) *C. gattii* strains induced a blue color change, in contrast to 0 of 86 *C. neoformans* isolates. Neighbor-joining cluster analysis of IGS sequences differentiated *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii*. Based on these results, we describe a rapid identification algorithm for use in a microbiology laboratory to distinguish clinically relevant *Cryptococcus* spp.

Cryptococcus neoformans and *Cryptococcus gattii* are closely related species of basidiomycetous yeasts that cause potentially severe pulmonary and central nervous system (CNS) infections. *C. neoformans* primarily infects AIDS patients and other immunocompromised hosts, producing meningoencephalitis and other neurological complications (22). Globally, nearly 1 million persons with HIV develop cryptococcal meningitis each year. Depending on treatment, up to 70% will die within 3 months (26). Historically, human cases of cryptococcosis in temperate climates were primarily attributed to the two subspecies *Cryptococcus neoformans* var. *grubii* and *C. neoformans* var. *neoformans* owing to their worldwide distribution. Conversely, pulmonary and neurological infections of *C. gattii* generally occur at low incidence in immunocompetent hosts and were thought to be confined to tropical and subtropical regions where it is endemic (22). However, since 1999, hundreds of *C. gattii* infections have been identified in British Columbia, Canada, and the Northwestern United States. Characterized as an outbreak, these isolates signify a possible shift in the ecological distribution of *C. gattii* (5). Furthermore, macrophage intracellular proliferation studies and murine inhalation assays suggest that the outbreak strains of *C. gattii* from the Pacific Northwest are more virulent than nonoutbreak strains of *C. gattii* (2, 10, 23). With respect to patient treatment, differences in antifungal susceptibility patterns exist among the molecular subtypes of *C. gattii* and *C. neoformans*, in which molecular genotype VGII isolates, which comprise the majority (95%) of *C. gattii* clinical isolates from the Pacific Northwest of the United States and Canada (2, 17), are significantly less susceptible to fluconazole and other triazoles, as determined by the broth microdilution method, than other *C. gattii* and *C. neoformans* genotype isolates (3, 14, 28).

Given the severity of cryptococcosis, the changing ecology and virulence of *C. gattii*, and the differences in antifungal susceptibility, it is imperative that clinical laboratories implement testing algorithms that rapidly detect *C. neoformans* and *C. gattii* and distinguish them from other pathogenic *Cryptococcus* and yeast species. Both urea hydrolysis and detection of melanin production are key characteristics distinguishing *C. neoformans* and *C. gattii* from other yeast and *Cryptococcus*

* Corresponding author. Mailing address: Division of Medical Microbiology, The Johns Hopkins University School of Medicine, 600 North Wolfe St., Meyer B1-193, Baltimore, MD 21287. Phone: (410) 955-5077. Fax: (410) 614-8087. E-mail: szhang28@jhmi.edu.
▽ Published ahead of print on 18 May 2011.

TABLE 1. Study isolates

Yeast group	No. of type, reference, and clinical isolates[a]	Type or reference strain(s)[b]
Cryptococcus		
Cryptococcus albidus	1	ATCC 10666^T
Cryptococcus gattii	27	CBS 6289,[c] ATCC MYA-4561,[c] CBS 6955,[c] ATCC MYA-4563[c]
Cryptococcus laurentii	1	ATCC 18803^T
Cryptococcus luteolus	1	JCM 3689^T
Cryptococcus neoformans var. *grubii*	82	ATCC MYA-4564,[c] ATCC MYA-4565[c]
Cryptococcus neoformans var. *neoformans*	4	ATCC MYA-4567[c]
Cryptococcus magnus	4	CBS 140^T (AF190008.1[d])
Cryptococcus terreus	1	CBS 1895^T
Cryptococcus unigutulatus	2	JCM 3685^T
Non-*Cryptococcus*		
Candida albicans	2	NRRL Y-12983^T
Candida dubliniensis	1	NRRL Y-17841^T
Candida glabrata	3	NRRL Y-65^T
Candida guilliermondii	1	NRRL Y-2075^T
Candida krusei	2	NRRL Y-5396^T
Candida lusitaniae	1	NRRL Y-11827^T
Candida parapsilosis	2	DSM 5784^T
Candida tropicalis	2	DSM 11953^T
Geotrichum candidum	1	JCM 6359^T
Rhodotorula mucilaginosa	2	JCM 8115^T
Saccharomyces cerevisiae	2	JCM 7255^T
Trichosporon asahii	3	JCM 2466^T
Trichosporon asteroides	1	JCM 2937^T
Trichosporon inkin	1	JCM 9195^T

[a] Identification based on >99% ITS and/or >98% IGS sequence identity to a type or reference strain.
[b] Superscript "T" indicates type strain. ATCC, American Type Culture Collection, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; JCM, Japanese Collection of Microorganisms, Saitama, Japan; NRRL, United States Department of Agriculture Agricultural Research Service, Peoria, IL; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
[c] Meyer et al. (16).
[d] GenBank accession number.

spp. However, the biochemical tests developed to detect these characteristics required either lengthy incubation times (2 to 7 days) (18) or tedious preparation of media and reagents (11, 16, 27, 29–31). L-Canavanine glycine bromothymol blue (CGB) agar has been reported to differentiate most *C. gattii* isolates from *C. neoformans* (18). Alternatively, DNA sequence analysis has been suggested to identify *C. neoformans* and *C. gattii*; however, the traditional fungal sequencing targets, such as the internal transcribed spacer (ITS) and the D2 region of the large ribosomal subunit, show >99% similarity between species and/or subspecies (13, 15, 18), suggesting that specialized DNA targets with more discriminatory power, such as the rRNA intergenic spacer (IGS) (1, 6, 7), may be more useful for differentiating isolates at the species and/or subspecies level in a clinical setting.

Our goal was to develop a rapid, accurate, and comprehensive testing algorithm incorporating biochemical tests and/or DNA sequence analysis (i) to distinguish *C. neoformans* and *C. gattii* from other *Cryptococcus* and yeast species and (ii) to differentiate *C. neoformans* and *C. gattii* at the species or subspecies level depending on the required level of discrimination. To this end, we utilized rapid commercial preparations of tests to detect urea hydrolysis (urease test) and melanin production (caffeic acid disk test) in order to positively identify *C. neoformans*/*C. gattii* isolates in 4 h with minimal preparatory work. Within 48 h, isolates were further differentiated at the species level as *C. neoformans* or *C. gattii* by CGB agar. Alternatively, highly discriminatory subspecies identification of *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* was achieved within 48 h using IGS sequence analysis. When implemented in a clinical laboratory, this algorithm provides rapid and accurate species identification to aid both patient diagnosis and epidemiological study.

MATERIALS AND METHODS

Strains. A total of 147 yeast isolates were tested, including 123 *Cryptococcus* strains and 24 non-*Cryptococcus* yeasts. Of the *Cryptococcus* isolates, 47 were isolated from clinical samples submitted to the Mycology Laboratory of the Ontario Public Health Laboratory from 2007 to 2010. Another 49 were obtained from the Fungal Testing Laboratory, Department of Pathology, University of Texas Health Sciences Center, and an additional 15 were from the British Columbia Centre for Disease Control. Twelve type and reference strains (24) were also included in the analysis (Table 1). Of the 24 non-*Cryptococcus* yeast isolates, 10 were clinical isolates from the Ontario Public Health Laboratory and 14 were type strains (Table 1).

Biochemical testing. Urease tests were performed on 24- or 48-hour inhibitory mold agar (IMA; Becton Dickinson [BD], Sparks, MD) or Sabouraud dextrose agar (BD) plus 0.005% chloramphenicol (SAB+C) cultures using Remel rapid urea broth (Remel, Lenexa) or BD BBL urease test broth according to the manufacturer's instructions. Briefly, 3 ml of broth was heavily inoculated with yeast culture and incubated at 37°C. Tubes were inspected at 4 and 24 h (Remel) or 24 and 48 h (BD) for pink color development, indicating positive urea hydrolysis.

Caffeic acid disk tests were performed by inoculating disks from Remel or Hardy Diagnostics (Santa Maria, CA) with cultures previously grown for 72 hours on 1% cornmeal agar plus 0.01% Tween 80 (CMA). (Inoculation of the disks with CMA cultures was recommended by both manufacturers since cultures grown on media containing glucose yield false-negative results.) Disks were incubated at 37°C on CMA medium for 4 h before they were assessed for brown pigment, indicating a positive result.

L-Canavanine glycine bromothymol blue agar (CGB) was prepared as previously described (19). Isolates were grown on CGB at 27°C and examined at 40 to

TABLE 2. Results for the urease broth, caffeic acid disk, and CGB agar biochemical tests

Identification[a]	No. of isolates	No. of isolates with positive reaction/total no. of isolates (%)						
		Urease broth test using:				Caffeic acid disk test		CGB agar
		Remel IMA	Remel SAB+C	BD IMA	BD SAB+C	Remel	Hardy Diagnostics	
C. neoformans	86	86/86 (100)	86/86 (100)	86/86 (100)	85/86 (99)	85/86 (99)	85/86 (99)	0/86 (0)
C. gattii	27	27/27 (100)	27/27 (100)	27/27 (100)	27/27 (100)	26/27 (96)	26/27 (96)	25/27 (93)
Other cryptococci	10	10/10 (100)	9/10 (90)	7/10 (70)	7/10 (70)	0/10 (0)	0/10 (0)	2/10 (20)
Trichosporon	5	5/5 (100)	5/5 (100)	3/5 (60)	3/5 (60)	0/5 (0)	0/5 (0)	5/5 (100)
Rhodotorula	2	2/2 (100)	2/2 (100)	2/2 (100)	2/2 (100)	0/2 (0)	0/2 (0)	0/2 (0)
Geotrichum	1	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Saccharomyces	2	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Candida	14	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	2/14 (14)

[a] Identification based on >99% ITS and/or >98% IGS sequence identity to a type or reference strain.

44 h (most isolates did not grow at 24 h). Development of a bright blue color was indicative of a positive result. If the medium remained yellow-green in color, the result was interpreted as negative. Prolonged incubation of cultures beyond 48 h resulted in false-positive interpretation due to blue color production by non-*C. gattii* cryptococci.

DNA sequence analysis. As a gold standard, all isolates were identified by DNA sequence analysis of the rRNA internal transcribed spacer (ITS) and the rRNA intergenic spacer (IGS) for *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii*. Products were amplified using Phire polymerase (New England BioLabs, Ipswich, MA) and the primer pairs comprising ITS-1 (5′TC CGTAGGTGAACCTGCGG3′)/ITS4 (5′TCCTCCGCTTATTGATATGC) and IGSF (5′ATCCTTTGCAGACGACTTGA3′)/IGSR (5′GTGATCAGTGCATT GCATGA3′) (24). ITS PCRs were cycled at 98°C for 30 s, followed by 35 cycles at 98°C for 5 s, 56°C for 5 s, and 72°C for 20 s, followed by 72°C for 1 min, while the IGS PCR conditions were 98°C for 30 s, followed by 35 cycles at 98°C for 5 s, 60°C for 5 s, and 72°C for 20 s, followed by 72°C for 1 min. PCR products were sequenced using BigDye v1.1 and a model 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Forward and reverse fragments were aligned and trimmed in BioNumerics v6.0.1 (Applied Maths, Austin, TX). Identities of clinical isolates were assigned based on a >99% match to the ITS sequence of a type strain (Table 1) or a >98% match to the IGS sequence of a *Cryptococcus* reference strain (Table 1). ITS and IGS sequences of all type and reference strains were determined during the course of this study except for the ITS sequence of *Cryptococcus magnus* type strain CBS 140, which was obtained from GenBank (accession no. AF190008.1). Using BioNumerics v6.0.1, IGS sequences were aligned and trimmed to defined start and end positions, yielding fragments of 728 to 761 bp. An unrooted neighbor-joining (NJ) tree was generated in accordance with Kimura-2 parameter (K2P) correction. Bootstrap analysis (500 replicates) was used to access the robustness of the clusters.

RESULTS

When evaluated using Remel rapid urea broth and BD urease test broth, isolates of *Cryptococcus*, *Trichosporon*, and *Rhodotorula* were found to turn the medium bright pink, while other species of yeast produced a negative reaction. The reactions of different isolates within a given genus were more consistent when the isolates were grown on IMA than when they were grown on SAB+C and were found to be more consistent when the isolates were tested using the Remel rapid urea broth than when they were tested using the BD urease test broth (Table 2). While most positive results were obtained in 4 (Remel) or 24 (BD) hours, negative reactions could not be confirmed until 24 (Remel) or 48 (BD) hours, due to a small number of weakly positive isolates (IMA and Remel, 2.3%; IMA and BD, 1%; SAB+C and Remel, 4%; SAB+C and BD, 1%). All but one (85/86 [99%]) of the *C. neoformans* isolates and one (26/27 [99%]) of the *C. gattii* isolates induced a brown color, indicative of a positive reaction, on the caffeic acid disks. All other species of *Cryptococcus* and other yeast species had a negative reaction (Table 2). Nearly all (25/27 [93%]) of the *C. gattii* isolates produced a bright blue color after 40 to 44 h on CGB agar. All 86 of the *C. neoformans* isolates had a negative reaction on CGB. As well, isolates of *Trichosporon asahii* (3/3 [100%]), *Trichosporon asteroides* (1/1 [100%]), *Trichosporon inkin* (1/1 [100%]), *Cryptococcus laurentii* (1/1 [100%]), *Cryptococcus luteolus* (1/1 [100%]), *Candida parapsilosis* (2/2 [100%]), and *Geotrichum candidum* (1/1 [100%]) also produced a positive reaction on CGB agar.

As a gold standard, all clinical isolates were identified by DNA sequence analysis. Except for *C. neoformans* and *C. gattii*, ITS sequence analysis clearly distinguished all yeast species. Clinical isolates demonstrated >99% similarity to type strains, with between-species divergence of >5.8%. With >99% between-species similarity in the ITS region, *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* were distinguished using IGS sequencing. Neighbor-joining cluster analysis clearly differentiated *C. neoformans* var. *grubii* (82 isolates), *C. neoformans* var. *neoformans* (4 isolates), and *C. gattii* (27 isolates), with 100% bootstrap support for each of the three clusters (Fig. 1 and Table 1). Isolates showed >98.9% similarity to a reference strain (24) and >4% divergence between species and subspecies.

DISCUSSION

As a severe fungal infection of the CNS leading to meningoencephalitis and neurological complications, successful treatment of cryptococcosis depends on rapid and accurate identification of the causative agents, *C. neoformans* and *C. gattii*. Although cryptococcosis infections in temperate regions were previously attributed to *C. neoformans*, the recent outbreak of *C. gattii* in the Pacific Northwest region of North America suggests a recent ecological expansion of *C. gattii* from tropical and subtropical regions where *C. gattii* was previously endemic into temperate climates (17). Additional differences in host preference (22), virulence (2, 10), and antifungal drug susceptibility patterns (3, 14, 28) necessitate that clinical laboratories implement testing algorithms that differentiate these species in order to aid patient treatment and advance the understanding of the disease and its epidemiology. Here, we describe a testing algorithm (Fig. 2) using two rapid

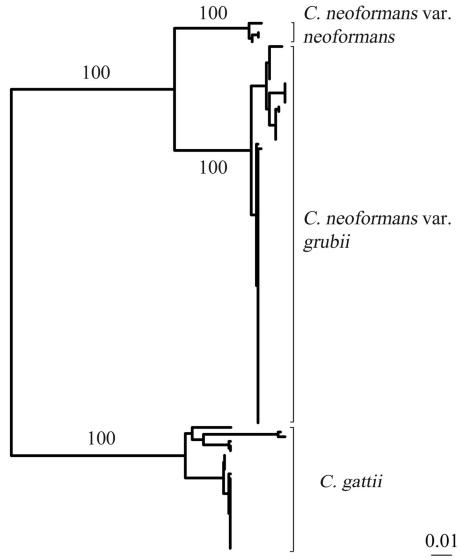

FIG. 1. NJ tree constructed using IGS sequences (bp) from 82 isolates of *C neoformans* var. *grubii*, 4 isolates of *C. neoformans* var. *neoformans*, and 27 isolates of *C. gattii*. Bootstrap values (500 replicates) are indicated. Bar, 1% nucleotide substitutions.

commercial biochemical tests that distinguishes the majority (98%) of *C. neoformans*/*C. gattii* isolates from other *Cryptococcus* and yeast species in 4 h. Growth on CGB selective medium and/or IGS sequencing further differentiates *C. neoformans*/*C. gattii* isolates at the species or subspecies level within 48 h. This testing algorithm represents a rapid and accurate method for identifying clinically relevant isolates of *Cryptococcus*.

Using various test broth formulations, studies have shown that urea hydrolysis with medium alkalinization is a pivotal, accurate biochemical test in the dichotomous classification of yeasts, with positive results distinguishing the genera *Cryptococcus*, *Rhodotorula*, and *Trichosporon* (18, 27, 31). When coupled with the detection of melanin production through brown pigmentation of caffeic acid agar medium (11, 16, 29) or caffeic acid-ferric citrate-infused paper disks (30), *C. neoformans*/*C. gattii* are easily distinguished from other *Cryptococcus* and yeast species. In this study, we utilized rapid commercial forms of these two biochemical tests, which are advantageous over formulations that require >24 h of incubation for results or require tedious preliminary preparation of media and reagents. All *Cryptococcus*, *Rhodotorula*, and *Trichosporon* isolates were differentiated from other yeasts by use of the two commercial urea broth tests. However, growth on IMA yielded the most-consistent results, and the Remel rapid urea broth provided more-accurate and -rapid results (4 h) than the BD urease test broth (24 h). With the use of the caffeic acid disk test, all but 2 of the *C. neoformans*/*C. gattii* isolates (98%) were differentiated from other *Cryptococcus* spp., *Rhodotorula*, and *Trichosporon*. Although the performances of the caffeic acid disks from Remel and Hardy Diagnostics were identical, the disks from Hardy Diagnostics were significantly less expensive ($1.70/disk) than those from Remel ($3.00/disk) based on the list price indicated on the website. With the use of the Remel rapid urease test and the caffeic acid disk test as outlined in Fig. 2, most *C. neoformans*/*C. gattii* isolates were identified in 4 h. Since the caffeic acid disk test produced two false negatives (1.8% [2/113]), also noted by Klein et al. (18), the testing algorithm was designed to refer these isolates (positive for the urease test and negative for the caffeic acid disk test) for ITS sequencing (Fig. 2). ITS sequencing clearly distinguished these false negatives as *C. neoformans*/*C. gattii* and identified other *Cryptococcus*, *Trichosporon*, and *Rhodotorula* species as expected (4).

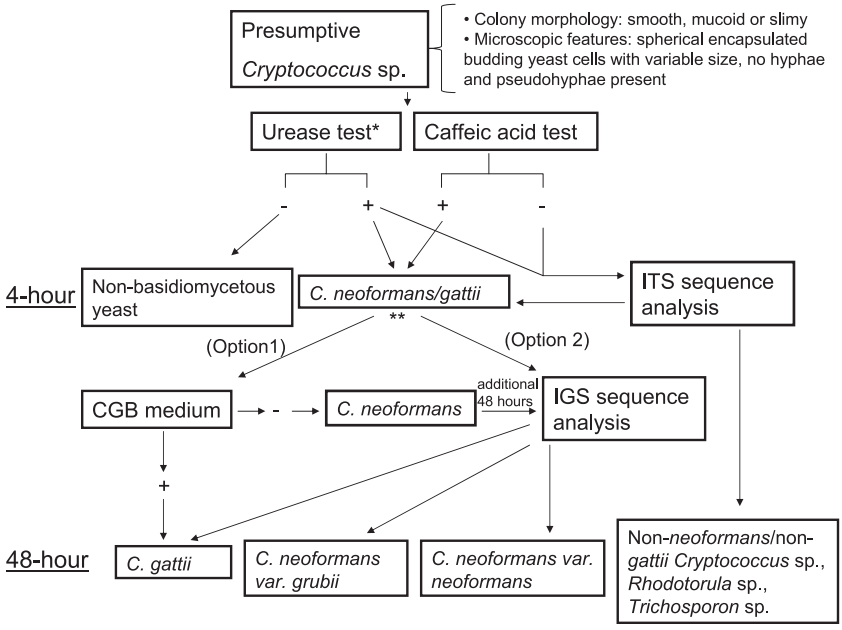

FIG. 2. Clinical laboratory testing algorithm for rapidly identifying *Cryptococcus* spp. *, urease test. Optimal test performance is achieved by testing culture isolates grown on IMA agar and using Remel rapid urea broth. **, *C. neoformans*/*C. gattii* isolates can be further differentiated using either CGB medium (option 1) or IGS sequence analysis (option 2).

The test algorithm presented in Fig. 2 provides two options for further differentiating the *C. neoformans*/*C. gattii* isolates identified using the urease and caffeic acid disk tests. As option 1, CGB selective medium was used to distinguish *C. gattii* from *C. neoformans* based on the former's ability to grow in the presence of L-canavanine and metabolize glycine in the process, thereby raising the pH and causing the bromothymol blue indicator to turn the agar blue (25). Similar to results reported by Klein et al. (18), none of the *C. neoformans* isolates in this study grew on CGB agar; however, two of the *C. gattii* isolates (7%) failed to produce a positive reaction (Table 2). Thus, the use of GCB medium is an inexpensive and feasible way to distinguish *C. neoformans* from *C. gattii* in 48 h. CGB-negative isolates can be referred for IGS sequence analysis to identify the small number of CGB-nonreactive *C. gattii* isolates in an additional 48 h (Fig. 2). Besides *C. gattii*, seven other yeast species (10 isolates) of *Trichosporon* spp., *Geotrichum* spp., and *C. parapsilosis* turned the CGB medium blue, also noted by Klein et al. (18). However, these species are distinguishable from *C. gattii* based on microscopic examination and biochemical analysis. In addition to the fact that these species are negative for the urease (except for *Trichosporon* sp.) and caffeic acid disk tests, the presence of arthroconidia distinguishes *Trichosporon* and *Geotrichum* from *Cryptococcus* while well-developed pseudohyphae differentiate *C. parapsilosis* from *Cryptococcus* (21). Thus, microscopic examination and the urease and caffeic acid disk tests are crucial in conjunction with CGB medium in order to positively identify *C. gattii* (Fig. 2).

For the second option, IGS sequence analysis was used to distinguish *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* as previously reported (1, 6, 7). NJ cluster analysis of IGS sequences clearly differentiated *C. gattii*, *C. neoformans* var. *grubii*, and *C. neoformans* var. *neoformans* with 100% bootstrap support (Fig. 1). Although more labor-intensive and costly, DNA sequencing is rapidly becoming a common procedure in most clinical laboratories because is has greater discriminatory power than that generally provided by differential media and biochemical tests. In laboratories where DNA sequencing is routinely available, IGS sequence analysis represents the optimal method for identification of *Cryptococcus* because it identifies all *C. gattii* isolates, including CGB-nonreactive isolates, and differentiates *C. neoformans* at the subspecies level. While there are no clinical data suggesting any treatment or outcome differences for *C. neoformans* var. *grubii* versus *C. neoformans* var. *neoformans* infections, there are epidemiological differences that suggest that it is prudent for clinical reference laboratories to track the species distribution. For instance, although the majority of clinical infections worldwide are caused by *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* infections are more prevalent in certain geographic locations (India, France, Italy, and Denmark) (8, 12, 20) and are associated with infections in the elderly, the presence of skin lesions, and corticosteroid use (9).

C. neoformans and *C. gattii* cannot be differentiated by routine methods used in clinical laboratories for yeast identification, such as API 20C AUX (bioMérieux, Durham, NC), Vitek (bioMérieux), and MicroScan (Siemens, West Sacramento, CA). Serotyping distinguishes *C. neoformans* (serotypes A and D) from *C. gattii* (serotypes B and C); however, the only commercial kit for serotyping cryptococci (Crypto-Check kit; Iatron, Inc., Tokyo, Japan) was discontinued. Multilocus sequence analysis identifies *C. neoformans* and *C. gattii* and delineates these species into eight molecular types (24); however, this method is costly and time-consuming, and this level of discrimination is usually unnecessary in a clinical laboratory. DNA sequencing of the D2 region of the 28S large ribosomal subunit also distinguishes *C. neoformans* and *C. gattii* (18) but may not be able to reliably distinguish *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (13). Similarly, we found that DNA sequencing of the ITS region differentiates *C. gattii* from the other two groups but has poor discrimination (≥99.5% similarity) between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*.

The testing algorithm presented here (Fig. 2) provides an easy, rapid, and accurate method of identifying *Cryptococcus* species from among other yeasts in a clinical laboratory. *C. neoformans*/*C. gattii* are distinguished from other species in 4 h and then further differentiated at the species or subspecies level in 48 h. Depending on the required level of discrimination, cost constraints, workflow, and expertise of personnel, the testing algorithm provides laboratories with the option of CGB medium for biochemical differentiation of *C. gattii* and *C. neoformans* or IGS sequence-based distinction of *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii*. Rapid and accurate identification will aid patient treatment and epidemiological understanding of cryptococcosis.

REFERENCES

1. **Bovers, M., et al.** 2007. Identification of genotypically diverse *Cryptococcus neoformans* and *Cryptococcus gattii* isolates by Luminex xMAP technology. J. Clin. Microbiol. **45:**1874–1883.
2. **Byrnes, E. J., III, et al.** 2010. Emergence and pathogenicity of highly virulent *Cryptococcus gattii* genotypes in the northwest United States. PLoS Pathog. **6:**e1000850.
3. **Chong, H. S., R. Dagg, R. Malik, S. Chen, and D. Carter.** 2010. In vitro susceptibility of the yeast pathogen *Cryptococcus* to fluconazole and other azoles varies with molecular genotype. J. Clin. Microbiol. **48:**4115–4120.
4. **Ciardo, D. E., G. Schar, E. C. Bottger, M. Altwegg, and P. P. Bosshard.** 2006. Internal transcribed spacer sequencing versus biochemical profiling for identification of medically important yeasts. J. Clin. Microbiol. **44:**77–84.
5. **Datta, K., et al.** 2009. Spread of *Cryptococcus gattii* into Pacific Northwest region of the United States. Emerg. Infect. Dis. **15:**1185–1191.
6. **Diaz, M. R., T. Boekhout, T. Kiesling, and J. W. Fell.** 2005. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast *Cryptococcus neoformans*. FEMS Yeast Res. **5:**1129–1140.
7. **Diaz, M. R., and J. W. Fell.** 2005. Use of a suspension array for rapid identification of the varieties and genotypes of the *Cryptococcus neoformans* species complex. J. Clin. Microbiol. **43:**3662–3672.
8. **Dromer, F., S. Mathoulin, B. Dupont, and A. Laporte.** 1996. Epidemiology of cryptococcosis in France: a 9-year survey (1985–1993). French Cryptococcosis Study Group. Clin. Infect. Dis. **23:**82–90.
9. **Dromer, F., S. Mathoulin, B. Dupont, L. Letenneur, and O. Ronin.** 1996. Individual and environmental factors associated with infection due to Cryptococcus neoformans serotype D. French Cryptococcosis Study Group. Clin. Infect. Dis. **23:**91–96.
10. **Fraser, J. A., et al.** 2005. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature **437:**1360–1364.
11. **Gokulshankar, S., K. Babu, S. Valli, A. J. Ranjitsingh, and M. S. Ranjith.** Cowitch seed agar medium—a simple new medium for identification and melanin production of *Cryptococcus neoformans.* Mycoses, in press.
12. **Gokulshankar, S., S. Ranganathan, M. S. Ranjith, and A. J. Ranjithsingh.** 2004. Prevalence, serotypes and mating patterns of *Cryptococcus neoformans* in the pellets of different avifauna in Madras, India. Mycoses **47:**310–314.
13. **Gueho, E., L. Improvisi, R. Christen, and G. S. de Hoog.** 1993. Phylogenetic relationships of *Cryptococcus neoformans* and some related basidiomycetous yeasts determined from partial large subunit rRNA sequences. Antonie Van Leeuwenhoek **63:**175–189.
14. **Iqbal, N., et al.** 2010. Correlation of genotype and in vitro susceptibilities of *Cryptococcus gattii* strains from the Pacific Northwest of the United States. J. Clin. Microbiol. **48:**539–544.
15. **Katsu, M., et al.** 2004. The internal transcribed spacers and 5.8S rRNA gene

show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. FEMS Yeast Res. **4:**377–388.
16. **Kaufmann, C. S., and W. G. Merz.** 1982. Two rapid pigmentation tests for identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **15:**339–341.
17. **Kidd, S. E., et al.** 2007. Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. Appl. Environ. Microbiol. **73:**1433–1443.
18. **Klein, K. R., et al.** 2009. Identification of *Cryptococcus gattii* by use of L-canavanine glycine bromothymol blue medium and DNA sequencing. J. Clin. Microbiol. **47:**3669–3672.
19. **Kwon-Chung, J., I. Polacheck, and J. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
20. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
21. **Larone, D. H.** 1995. Medically important fungi: a guide to identification. ASM Press, Washington, DC.
22. **Lin, X., and J. Heitman.** 2006. The biology of the *Cryptococcus neoformans* species complex. Annu. Rev. Microbiol. **60:**69–105.
23. **Ma, H., et al.** 2009. The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. Proc. Natl. Acad. Sci. U. S. A. **106:**12980–12985.
24. **Meyer, W., et al.** 2009. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med. Mycol. **47:**561–570.
25. **Min, K. H., and K. J. Kwon-Chung.** 1986. The biochemical basis for the distinction between the two *Cryptococcus neoformans* varieties with CGB medium. Zentralbl. Bakteriol. Mikrobiol. Hyg. A **261:**471–480.
26. **Park, B. J., et al.** 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS **23:**525–530.
27. **Roberts, G. D., C. D. Horstmeier, G. A. Land, and J. H. Foxworth.** 1978. Rapid urea broth test for yeasts. J. Clin. Microbiol. **7:**584–588.
28. **Trilles, L., B. Fernandez-Torres, S. Lazera Mdos, B. Wanke, and J. Guarro.** 2004. In vitro antifungal susceptibility of *Cryptococcus gattii*. J. Clin. Microbiol. **42:**4815–4817.
29. **Vidotto, V., et al.** 2004. A new caffeic acid minimal synthetic medium for the rapid identification of *Cryptococcus neoformans* isolates. Rev. Iberoam. Micol. **21:**87–89.
30. **Wang, H. S., R. T. Zeimis, and G. D. Roberts.** 1977. Evaluation of a caffeic acid-ferric citrate test for rapid identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **6:**445–449.
31. **Zimmer, B. L., and G. D. Roberts.** 1979. Rapid selective urease test for presumptive identification of *Cryptococcus neoformans*. J. Clin. Microbiol. **10:**380–381.