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Cryptococcus neoformans-Cryptococcus gattii Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and Epidemiological Cutoff Values for Amphotericin B and Flucytosine

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Clinical breakpoints (CBPs) are not available for the *Cryptococcus neoformans-Cryptococcus gattii* species complex. MIC distributions were constructed for the wild type (WT) to establish epidemiologic cutoff values (ECVs) for *C. neoformans* and *C. gattii* versus amphotericin B and flucytosine. A total of 3,590 amphotericin B and 3,045 flucytosine CLSI MICs for *C. neoformans* (including 1,002 VNI isolates and 8 to 39 VNII, VNIII, and VNIV isolates) and 985 and 853 MICs for *C. gattii*, respectively (including 42 to 259 VGI, VGII, VGIII, and VGIV isolates), were gathered in 9 to 16 (amphotericin B) and 8 to 13 (flucytosine) laboratories (Europe, United States, Australia, Brazil, Canada, India, and South Africa) and aggregated for the analyses. Additionally, 442 amphotericin B and 313 flucytosine MICs measured by using CLSI-YNB medium instead of CLSI-RPMI medium and 237 Etest amphotericin B MICs for *C. neoformans* were evaluated. CLSI-RPMI ECVs for distributions originating in ≥3 laboratories (with the percentages of isolates for which MICs were less than or equal to ECVs given in parentheses) were as follows: for amphotericin B, 0.5 μg/ml for *C. neoformans* VNI (97.2%) and *C. gattii* VGI and VGIIa (99.2 and 97.5%, respectively) and 1 μg/ml for *C. neoformans* (98.5%) and *C. gattii* nontyped (100%) and VGII (99.2%) isolates; for flucytosine, 4 μg/ml for *C. gattii* nontyped (96.4%) and VGI (95.7%) isolates, 8 μg/ml for VNI (96.6%) isolates, and 16 μg/ml for *C. neoformans* nontyped (98.6%) and *C. gattii* VGII (97.1%) isolates. Other molecular types had apparent variations in MIC distributions, but the number of laboratories contributing data was too low to allow us to ascertain that the differences were due to factors other than assay variation. ECVs may aid in the detection of isolates with acquired resistance mechanisms.

Among the non-*Candida* yeasts, members of the *Cryptococcus neoformans-Cryptococcus gattii* complex have been the most common species recovered among clinical isolates (32.9% of 8,717 isolates), as well as the second most common cause of severe fungal infection after *Candida* spp. in certain regions (38, 41). Infections caused by *Cryptococcus neoformans* var. *grubii* (serotype A) are seen worldwide among immunocompromised hosts, followed by *Cryptococcus neoformans* var. *neoformans* (serotype D) (2). On the other hand, *C. gattii* (serotypes B and C) is geographically more restricted and causes infections among immunocompromised as well as nonimmunocompromised patients, and the infections are more difficult to treat (31, 35). Irrespective of the species, cryptococcal infections are associated with high mortality rates (≥12.7%). By use of molecular methodologies, eight major molecular types have been identified among the four serotypes and their hybrids (2, 4, 19, 46). *C. neoformans* comprises molecular types VNI and VNII (both belonging to serotype A), VNIII (an AD hybrid), and VNIV (serotype D), while *C. gattii* comprises VGI (serotype B) and VGII, VGIII, and VGIV (among serotypes B and C). Molecular type VGII has been of particular interest in recent years due to the emergence of the novel strains/subtypes VGIIa, VGIIb, and VGIIc (5, 29). Molecular types VNI to VNIV have also been designated AFLP1 to AFLP3, and types VGI to VGIV have been designated AFLP4 to AFLP7 (19).

Although several newer antifungal agents are available, the conventional deoxycholate formulation of amphotericin B (especially in resource-limited settings) and its lipid formulations remain important therapeutic choices for the systemic treatment of cryptococcal infections caused by *C. neoformans* and *C. gattii* (32, 35). Among the three lipid amphotericin B formulations, liposomal amphotericin B has been licensed for the treatment of cryp-

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tococcal meningitis among AIDS patients (32, 35). The polyenes bind to ergosterol in the cell membrane, leading to pore formation and eventually to cell death (49). In contrast, flucytosine inhibits DNA replication and protein synthesis. In polyene-resistant *Candida* and *Cryptococcus* isolates, ergosterol content has been lower than in susceptible isolates (28). Several enzymes (purine-cytosine permease, cytosine deaminase, uracil phosphoribosyltransferase) are involved in the flucytosine mode of action as well as fungal resistance to this agent (14, 50, 51). The combination of flucytosine and amphotericin B is one of the treatments recommended for cryptococcal infections (35). However, relapses are frequent.

The availability of reference methodologies has enabled the recognition of resistant isolates as well as the proposal of clinical breakpoints (CBPs) and epidemiologic cutoff values (ECVs) for *Candida* spp. and *Aspergillus* spp. with regard to most available antifungal agents by both the Clinical and Laboratory Standards Institute (CLSI) and the Subcommittee on Antifungal Susceptibility Testing of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) (15–17, 37, 39, 40, 42, 43). CBPs are based on MIC distributions, pharmacokinetic and pharmacodynamic (PK/PD) parameters, animal studies, and clinical outcomes of therapy, while ECVs are based mostly on MIC distributions. CLSI MICs are ≤1 μg/ml (amphotericin B) and ≤8 μg/ml (flucytosine) for most *C. neoformans* and *C. gattii* isolates (3, 13, 18, 23, 36, 45), but acquired resistance is frequent during flucytosine monotherapy (1, 22). In the past few years, antifungal susceptibility differences have been reported between these two species, as well as among the molecular types and serotypes (6–8, 19, 24, 45, 47). However, neither CBPs nor ECVs are available for either *C. neoformans* or *C. gattii* versus amphotericin B or flucytosine. In the absence of CBPs, ECVs could help to characterize the susceptibilities of these species to amphotericin B, its lipid formulations, and flucytosine and to monitor the emergence of strains with mutations that could lead to reduced susceptibility to these antifungal agents.

We are proposing ECVs (see "Definitions" below) for amphotericin B and flucytosine against *C. neoformans* (VNI and nontyped isolates) and *C. gattii* (VGI, VGII, and nontyped isolates). The purposes of the study were (i) to define the wild-type (WT) (population of isolates in a species-drug combination with no detectable acquired resistance mechanisms [11, 48]) susceptibility endpoint distributions (see "Definitions" below) of each species/molecular type and agent combination by using aggregated CLSI-RPMI broth MICs of amphotericin B and flucytosine gathered in 8 to 16 laboratories (3,590 to 3,045 MICs for *C. neoformans* and 985 to 853 MICs for *C. gattii*, species/molecular type and agent/combination dependent) in Europe, the United States, Australia, Brazil, Canada, India, and South Africa and (ii) to propose ECVs on the basis of data originating from at least 3 of these laboratories. The 442 amphotericin B and 313 flucytosine MICs that were obtained using the alternative CLSI-yeast nitrogen base (CLSI-YNB) broth (9, 10) and the 237 amphotericin B Etest MICs for *C. neoformans* were analyzed separately.

MATERIALS AND METHODS

Isolates. Each isolate from unique clinical specimens was tested at each medical center: the University of Texas Health Science Center, San Antonio, TX; the University of Iowa College of Medicine, Iowa City, IA; the University of Texas Health Science Center, Houston, TX; the Centers for Disease Control and Prevention, Atlanta, GA; Duke University Medical Center, Durham, NC; VCU Medical Center, Richmond, VA; Canisius Wilhelmina Hospital, Department of Medical Microbiology and Infectious Diseases, Nijmegen, The Netherlands; the HPA Mycology Reference Laboratory, Kingsdown, Bristol, United Kingdom; the Innsbruck Medical University, Innsbruck, Austria; the Hospital General Universitario Gregorio Marañón, Madrid, Spain; Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo, Spain; Facultat de Medicina, IISPV, URV, Reus, Spain; the University of Alberta, Edmonton, Alberta, Canada; the Adolfo Lutz Institut, São Paulo State, Brazil; Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; the Instituto de Pesquisa Clinica Evandro Chagas-FIOCRUZ, Rio de Janeiro, Brazil; and the Women's and Children's Hospital, North Adelaide, Australia. Species and molecular types were identified at each medical center using standard methodologies (8, 19, 20, 24, 33, 47). We have aggregated the maximum available CLSI data from each laboratory and agent (a total of 3,590 to 3,045 MICs of amphotericin B and flucytosine MICs for *C. neoformans* [including those for 1,002 VNI isolates] and 985 and 853 MICs for *C. gattii* [including those for 42 VGIIc to 259 VGI isolates]) (8, 19, 20, 24, 33, 47). A set of 313 flucytosine and 442 amphotericin B MICs obtained using CLSI-YNB broth instead of the CLSI-RPMI medium (9), as well as 237 Etest amphotericin B MICs for *C. neoformans*, were also available; both of these sets were analyzed separately. One or both of the quality control (QC) isolates (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) were used by the participating laboratories (9, 10) (see Table 1).

Antifungal susceptibility testing. In order to include MIC results in the total set of available aggregated CLSI data from the participant laboratories (see Table 2), amphotericin B and flucytosine MICs were obtained at each center by following the CLSI M27-A3 broth microdilution method (standard RPMI-1640 broth [0.2% dextrose]; final inoculum concentrations ranging from 0.4×10^3 to 5×10^3 CFU/ml; 72 h of incubation); MICs were the lowest drug concentrations that produced either ≥50% (flucytosine) or 100% (amphotericin B) inhibition of growth compared to that of the control (9). For isolates grown in YNB broth, testing conditions and interpretation of MICs were otherwise identical. The set of amphotericin B Etest MICs was obtained by following the manufacturer's instructions, and endpoints were rounded up to the nearest 2-fold dilution. MIC data for the two QC reference strains, utilized during the years of testing in each center, were obtained by following the CLSI M27-A3 broth microdilution method (9, 10).

Definitions. The WT population is that population of isolates/MICs in a species-drug combination with no detectable acquired resistance mechanisms (11, 48). The highest WT susceptibility endpoint has been defined as either the WT cutoff value (CO_{WT}) or the ECV, which is the critical drug concentration that may identify those strains with decreased susceptibility or serve as an early warning of emerging changes in the patterns of susceptibility of organisms to the agent being evaluated (11, 48). The latter term has been used previously in similar fungal reports (15–17, 37, 39, 40, 42, 43).

Data analysis. Several amphotericin B and flucytosine MIC distributions were obtained for *C. neoformans*: (i) aggregated CLSI-RPMI data from 13 and 10 laboratories, respectively, for isolates that were not molecularly typed (referred to below as "nontyped isolates") and (ii) from 2 to 4 laboratories for molecular types VNI to VNIV (referred to below as "typed isolates"), (iii) single-laboratory CLSI-YNB data, and (iv) single-laboratory amphotericin B Etest data (see Table 2). The final WT distributions for *C. gattii* were (i) aggregated CLSI-RPMI amphotericin B and flucytosine data from 4 laboratories for nontyped isolates and (ii) data from 2 to 5 laboratories for VGI to VGIV (also referred to below as "typed isolates") (Table 2). The aggregated MIC distributions obtained in at least three laboratories were used to define ECVs by the statistical method (48), where the modeled population is based on fitting a normal distribution at the lower end of the MIC range, working out the mean and standard deviation of that normal distribution, and using those parameters to calculate the MICs that capture at least 95%, 97.5%, and 99% of the modeled

TABLE 1 MICs for quality control strains used in 13 (flucytosine) and 16 (amphotericin B) laboratories according to the CLSI broth microdilution method[a]

QC isolate and antifungal agent[b]	QC MIC (μg/ml) range (mode)	% of CLSI MICs within range	MIC (μg/ml) obtained in 13 to 16 laboratories	
			Range	Modes
Candida parapsilosis ATCC 22019				
AMB	0.5–4 (2)	91.7	0.12–2[c]	0.5, 0.5, 0.5, 0.5, 0.5, 1, 1, 1, 1, 1, 1, 1, 2, 2, ND,[d] ND
FCT	0.12–0.5 (0.25)	97.9	0.12–0.5	0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.25, 0.25, 0.25, ND, ND
Candida krusei ATCC 6258				
AMB	1–4 (2)	100	1–2	1, 1, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 2, ND, ND
FCT	8–32 (16)	99.6	8–32	8, 8, 8, 8, 8, 16, 16, ND, ND, ND

[a] MICs were determined as described by CLSI document M27–A3 using RPMI–1640 broth (9, 10).
[b] AMB, amphotericin B; FCT, flucytosine.
[c] MICs were outside the range in one laboratory for QC isolate *C. parapsilosis* ATCC 22019 2% of the time (1 of 49 times).
[d] ND, not determined or value obtained using the CLSI-YNB medium broth or Etest.

WT population. The MIC value that captured ≥95% of the observed population ("visual" method) was calculated as in previous studies (15–17, 37, 39, 40, 42).

RESULTS AND DISCUSSION

For susceptibility testing to be clinically relevant, the test result should predict with some reliability the clinical outcome for an infected patient treated with the specific agent evaluated; this test result is the CBP (11, 26). CBPs are established on the basis of clinical trial data (in particular, clinical and microbiological outcomes versus the MIC for the infecting pathogen), global susceptibility surveillance, resistance mechanisms, and PK/PD parameters from model systems. On the other hand, the ECV is based on MIC distributions and is the highest MIC that belongs to the WT (isolates with no detectable acquired resistance mechanisms) distribution. While the CBP predicts the clinical outcome of therapy, the ECV could monitor the emergence of strains with reduced susceptibility (due to mutations) to the agent being evaluated, e.g., the reduced susceptibility of *C. neoformans* or *C. gattii* to either flucytosine or amphotericin B. No CBPs for any antifungal agent against either *C. neoformans* or *C. gattii* are available, owing to the paucity of data on PK/PD and clinical outcomes compared to MICs. In addition, attempts to correlate *in vitro* results with clinical outcome have not been successful (12, 30). The amphotericin B and flucytosine ECVs established in the present study for *C. neoformans* and *C. gattii* may aid in the evaluation of clinical isolates by identifying those strains that may have acquired resistance mechanisms and may serve as an early warning of emerging changes in the susceptibility patterns of these organisms. Even though the frequencies of cryptococcal meningitis and other infections have decreased with the use of antiretroviral therapies in developed countries, these infections are still a major problem among immunosuppressed patients and in certain geographical areas. Amphotericin B and flucytosine continue to be the primary therapeutic agents for cryptococcal infections (32, 35).

Variability is expected when MICs from different laboratories are compared, despite standardization efforts (9, 10). Table 1 shows the MIC data obtained in participant laboratories for one or both QC isolates (overall range and individual modes), *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, when clinical isolates were tested. Although most ranges were within the CLSI established limits (98 to 100%) for the two QC strains (10), a certain degree of mode variability was observed (mostly ±2-fold dilution). Intralaboratory reproducibility was also excellent (10 and 20 replicate tests) in the two laboratories using either the CLSI-YNB medium or Etest methodology (data not shown in Table 1). The modal variability among the participant laboratories for QC strains reflected the modal MIC differences for the clinical *Cryptococcus* sp. isolates. Similar modal variability has been observed in previous ECV studies for *Aspergillus* spp. with several antifungal agents (15–17) and may indicate individual interpretations of MIC endpoints or may result from the use of different lots of antifungal powders, or both.

The aggregated amphotericin B and flucytosine MIC distributions for *C. neoformans* and *C. gattii* (for nontyped and typed isolates) are shown in Table 2, which also lists the single-laboratory distributions for *C. neoformans* (YNB and Etest data). The highest amphotericin B modal MICs (0.5 μg/ml) measured in RPMI medium were for *C. gattii* nontyped and VGII isolates; the modal MIC for all other distributions was 0.25 μg/ml. Amphotericin B modes observed in the data from most individual contributing laboratories were similar (0.25 or 0.5 μg/ml) for both species. The exceptions were the mode (0.12 μg/ml) from one laboratory for nontyped *C. neoformans* isolates and for VNIII and VNIV typed isolates, but the number of isolates was low for the latter two sets. Overall, our values reflect amphotericin B MIC_{50}s or geometric means (0.25 and 0.5 μg/ml) obtained in previous studies for similar sets of nontyped and typed isolates (7, 12, 23, 36, 45); results have been higher (>0.5 μg/ml) in other studies (3, 13). For *C. neoformans*, amphotericin B MICs measured in YNB appeared to be higher (mode, 1 μg/ml) than those measured in RPMI medium. In contrast, amphotericin B MICs by the Etest were lower (mode, 0.12 μg/ml) (Table 2). We are not sure why the YNB and Etest data are different from those obtained using RPMI broth, because each set was from a single laboratory, which precluded comparisons. However, both modes were different from those observed in 12 of the 13 laboratories using RPMI medium for the similar set of nontyped isolates. In prior reports, amphotericin B MIC_{50}s have been either 0.12 μg/ml (12) or 1 μg/ml (3) by Etest and 0.25 μg/ml by use of YNB (7). These results underline the variability of susceptibility test results using different methodologies as well as the need to evaluate MIC distributions before ECV definition.

Flucytosine modes were more dependent on the species, molecular type, and medium (Tables 2 and 3). The modes for *C. neoformans* nontyped and VNI isolates were the same (4 μg/ml),

TABLE 2 Wild-type MIC distributions of amphotericin B and flucytosine for the *Cryptococcus neoformans-Cryptococcus gattii* species complex[a]

Species or molecular type	Antifungal agent[b]	Medium[c]	Genotyped[d]	No. of labs[e]	Total no. of isolates	No. of isolates for which the MIC (μg/ml)[f] was:											
						≤0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	≥64
C. neoformans	AMB	RPMI	N	13	2,526	4	68	159	985	918	355	34	3				
VNI			Y	4	1,002	2	12	229	511	220	26	2					
VNII			Y	2	8			1	6		1						
VNIII			Y	2	15		2	7	4	1	1						
VNIV			Y	2	39		1	20	15	2	1						
All isolates			Both		3,590	6	83	416	1,521	1,141	375	36	3				
C. neoformans	AMB	YNB	N	1	442			5	27	132	271	7					
		Etest	N	1	237	13	8	116	94	5	1						
C. neoformans	FCT	RPMI	N	10	1,981			50	75	138	260	497	527	342	65	6	21
VNI			Y	4	1,002		2	4	11	28	98	286	422	117	10	3	21
VNII			Y	2	8					3	2	1	2				
VNIII			Y	2	15							4	8	3			
VNIV			Y	2	39					1	1	4	19	9	2	2	1
All isolates			Both		3,045		2	54	86	170	361	792	978	471	77	11	43
C. neoformans	FCT	YNB	N	1	313				2	5	16	86	77	104	14	3	6
C. gattii	AMB	RMPI	N	4	130		1	22	45	52	8						
VGI			Y	5	259	1	4	49	164	39	2						
VGII[g]			Y	4	122			14	35	58	14	1					
VGIIa			Y	3	200		2	29	111	53	5						
VGIIb			Y	2	106		1	11	53	38	3						
VGIIc			Y	2	42			8	23	11							
VGIII			Y	2	42	1	1	15	19	6							
VGIV			Y	2	84			17	57	10							
All isolates			Both		985	2	9	165	507	267	32	1					
C. gattii	FCT	RPMI	N	4	55				3	12	19	11	8	2			
VGI			Y	4	256			1	2	11	90	126	15	4			7
VGII[g]			Y	4	68				1	2	11	20	18	12	2	1	1
VGIIa			Y	2	200						3	31	61	81	24		
VGIIb			Y	2	106					1	3	17	40	26	16	2	1
VGIIc			Y	2	42						1	3	7	20	9	2	
VGIII			Y	2	42					2	7	14	12	5	1		1
VGIV			Y	2	84					2	15	31	24	11	1		
All isolates			Both		853			1	6	30	149	253	185	161	53	5	10

[a] Amphotericin B and flucytosine MICs were determined by the CLSI broth microdilution method (9); amphotericin B Etest MICs were determined according to the manufacturer's instructions.
[b] AMB, amphotericin B; FCT, flucytosine.
[c] RPMI and YNB, RPMI-1640 and yeast nitrogen base, respectively, as described by CLSI document M27-A3 (9).
[d] Y, yes; N, no.
[e] Number of laboratories contributing data to each MIC distribution.
[f] The modal MIC for each distribution is underlined.
[g] Isolates identified as belonging to the VGII molecular type but not to one of the VGIIa, VGIIb, or VGIIc subtypes, which are examined as separate groups.

but more variability was observed for *C. gattii*, with the lowest mode for the small set of nontyped isolates (1 μg/ml) and the highest (8 μg/ml) for two of the three VGII subtypes (VGIIa and VGIIc). Our mode data for flucytosine susceptibility reflect differences found between *C. neoformans* (MIC_{50}, 4 μg/ml) and *C. gattii* (MIC_{50}, 2 μg/ml), as well as differences among genotypes of the latter species (MIC_{50}s, 1 to 4 μg/ml) (7, 45), where the highest value was that for VGII. YNB yielded a higher mode (8 μg/ml) than that for the similar set of nontyped *C. neoformans* isolates measured in RPMI medium (Table 2). Since this value was based on results from a single laboratory, it is not clear if this discrepancy is medium or reader related. However, modes in each participant laboratory for *C. neoformans* (both nontyped and typed isolates) were either 2 or 4 μg/ml (data not shown in Tables 2 and 3). Based on these data and the wide geographical range over which MICs were collected in the present study, we surmise that the data are valid.

Table 3 shows the proposed amphotericin B and flucytosine ECVs for the aggregated distributions of *C. neoformans* and *C. gattii* (typed or nontyped isolates) where the data originated in three or more laboratories (using the methodology that comprised ≥95%, ≥97.5%, and ≥99% of the modeled population). The CLSI amphotericin B ECV was 1 μg/ml for distributions of *C. neoformans* and *C. gattii* nontyped and VGII isolates (encompassing 98.5, 100, and 99.2% of the isolates, respectively) and 0.5 μg/ml for VNI, VGI, and VGIIa isolates (encompassing 97.25%,

TABLE 3 ECVs for amphotericin B and flucytosine against the *Cryptococcus neoformans-Cryptococcus gattii* species complex obtained in 3 to 16 laboratories by the CLSI M27-A3 broth microdilution method

Species[a]	Antifungal agent[b]	MIC (μg/ml)		Calculated ECV (μg/ml) comprising the following proportion of the statistically modeled population for each MIC distribution[d]:		
		Range	Mode[c]	≥95%	≥97.5%	≥99%
C neoformans						
Nontyped	AMB	≤0.03–4	0.25	1	1	2
VNI		≤0.03–2	0.25	0.5	1	1
C neoformans						
Nontyped	FCT	0.125–≥64	4	16	16	32
VNI		0.06–≥64	4	8	8	16
C. gattii (nontyped)	AMB	0.06–1	0.5	1	1	2
VGI		0.03–1	0.25	0.5	0.5	0.5
VGII		0.125–2	0.5	1	1	2
VGIIa		0.06–1	0.25	0.5	1	1
C. gattii (nontyped)	FCT	0.25–8	1	4	8	8
VGI		0.125–>64	2	4	4	4
VGII		0.25–≥64	2	16	16	16

[a] ECVs were defined only for distributions from at least three laboratories (see Table 2 for other molecular type distributions) using RPMI-1640 as described in CLSI document M27-A3 (9).
[b] AMB, amphotericin B; FCT, flucytosine.
[c] MIC most frequently obtained for each distribution.
[d] ECVs defined for all isolates of each species (Table 2) were similar.

TABLE 4 Percentages of isolates of the *Cryptococcus neoformans-Cryptococcus gattii* species complex above the amphotericin B and flucytosine wild-type distribution obtained in 3 to 16 laboratories by the CLSI M27-A3 broth microdilution method

Species and genotype[a]	Antifungal agent	ECV (μg/ml) comprising the following proportion of the statistically modeled population (% of observations above each statistical ECV):		
		≥95%	≥97.5%	≥99%
C. neoformans				
Nontyped	AMB	1 (1.5)	1 (1.5)	2 (0.1)
VNI		0.5 (2.8)	1 (0.2)	1 (0.2)
C. neoformans				
Nontyped	FCT	16 (1.4)	16 (1.2)	32 (1.1)
VNI		8 (3.4)	8 (3.4)	16 (2.4)
C. gattii	AMB	1 (0)	1 (0)	2 (0)
VGI		0.5 (0.8)	0.5 (0.8)	0.5 (0.8)
VGII		1 (0.8)	1 (0.8)	2 (0)
VGIIa		0.5 (2.5)	1 (0)	1 (0)
C. gattii	FCT	4 (3.6)	8 (0)	8 (0)
VGI		4 (4.3)	4 (4.3)	4 (4.3)
VGII		16 (2.9)	16 (2.9)	16 (2.9)

[a] Data from laboratories using CLSI-RPMI broth.

99.25%, and 97.5% of the isolates, respectively). It is interesting that an amphotericin B MIC of 2 μg/ml is anecdotally believed to be the breakpoint for resistance yet can be perceived as a WT value here. The flucytosine ECVs for *C. neoformans* nontyped and VNI distributions were 16 and 8 μg/ml (encompassing 98.65% and 96.6% of the isolates, respectively). For *C. gattii*, flucytosine ECVs were either 4 μg/ml (nontyped and VGI isolates) or 16 μg/ml (VGII isolates), encompassing 96.45%, 95.75%, and 97.1% of the isolates. Because only two laboratories contributed data for the other molecularly typed isolates, and both amphotericin B and flucytosine distributions in YNB were obtained in a single laboratory, ECVs were not proposed for these distributions (Table 1). Although tentative values of 1 μg/ml (amphotericin B; encompassing 98.4% of the isolates) and 16 μg/ml (flucytosine; encompassing 97.1% of the isolates) can be suggested for YNB MICs (data not shown in Table 3), additional data from other laboratories using this medium are needed to corroborate these tentative values. Visual ECVs were similar to those obtained by using ≥95% of the modeled population, and some higher ECVs were observed using >95% of the modeled populations (48). Although our newly defined amphotericin B and flucytosine ECVs are not predictors of clinical outcome, these susceptibility cutoff concentrations may aid in identifying those cryptococcal strains with decreased susceptibility to these two agents.

The frequency of amphotericin B and flucytosine MICs above the ECV (non-WT) differed according to the distribution analyzed (Table 4). The rate of non-WT MICs was lower for amphotericin B distributions of both species (0% to 2.8%) than for flucytosine (1.4% to 4.3%) (ECVs encompassing ≥95% of the calculated populations). The rate of non-WT flucytosine MICs was lower among all *C. neoformans* isolates (4.3%) than among all *C. gattii* isolates (10.8%), but the rates of non-WT amphotericin B MICs were almost the same for the two species (4.3% and 4.1%, respectively). Although cryptococcal infections by these two species are clinically similar, infections caused by *C. gattii* show a delayed treatment response, and other complications, relative to infections due to *C. neoformans* (21, 35). *In vitro* resistance to amphotericin B (0.6% to 5.3%) and flucytosine (2.2% to 46%) has been reported among a smaller number of *C. neoformans* isolates than those in our study; although the susceptibility cutoff has been consistent for amphotericin B (≥2 μg/ml), it has been variable for flucytosine (≥8 and ≥32 μg/ml), in the different studies (3, 13, 36, 45). All these results underscore the utility of antifungal susceptibility testing and WT cutoffs as a practical tool with which to detect amphotericin B and flucytosine resistance among *Cryptococcus* isolates; species identification is clinically useful. These results also indicated that ECVs should be species specific and, for this fungal group, molecular type specific.

Although the mechanisms for flucytosine resistance in *C. neoformans* have been elucidated, those for resistance to amphotericin B are poorly understood despite the usage of this drug since the 1960s (14, 22, 27, 44, 50, 51). In addition, the link between genetic mutations in *Cryptococcus* spp. and high MICs of these two agents has not been determined. Susceptibility testing by M27-A3 methodologies demonstrated that high MICs for *C. neoformans* were predictive of clinical failure of the azoles and, to a lesser degree, of both polyenes and flucytosine (34). Amphotericin B MICs of >2 μg/ml have been associated with clinical failure, a defect in the target enzyme sterol delta 8→7 isomerase, other mutations in the sterol biosynthesis pathway (e.g., 5,6-desaturase), or an increase in the efflux of the drug from the fungal cell (25, 28). Primary and acquired resistance of *C. neoformans* was frequently reported when the use of flucytosine began in the 1970s, and high flucytosine MICs correlated with failure (as high as 57%) among patients receiving this agent alone (1, 22, 50). Resistance to this agent oc-

curs by mutations within the pyrimidine pathway (either cytosine deaminase or uracil phosphoribosyltransferase) specifically linked in haploid *C. neoformans* cells to a single mutation at either the *FCY1* or the *FCY2* gene (50). Poor correlation between amphotericin B and flucytosine CLSI MICs and clinical outcome has been reported (12), but the number of patients was low (4 to 19 in each set of therapy success or failure groups), geometric mean MICs were similar (0.22 to 0.39 μg/ml for amphotericin B and 2.24 to 2.87 μg/ml for flucytosine), and other factors could have influenced the outcome (some patients were infected with HIV). It is noteworthy that the combination of these two agents is synergistic *in vitro* despite resistance to flucytosine (44). Therefore, much needs to be examined and determined regarding the relationship between non-WT strains and resistance mechanisms.

In conclusion, the ECVs of amphotericin B (0.5 to 1 μg/ml) and flucytosine (4 to16 μg/ml) proposed in this study for the *C. neoformans-C. gattii* species complex were species and molecular type specific. Further investigation should determine the relationship between the molecular mechanisms of amphotericin B and flucytosine resistance and our proposed non-WT values. Some of the distributions were small (especially for various *C. neoformans* molecular types), and continuing surveillance should either corroborate or extend the information provided in the present study. In the absence of CBPs, these ECVs may aid in detecting non-WT isolates that have reduced susceptibility to amphotericin B and that harbor flucytosine resistance mechanisms. ECVs should be included in the revised version of the CLSI M27-A3 document.

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