AAC Journals.ASM.org

Cryptococcus neoformans-Cryptococcus gattii Species Complex: an International Study of Wild-Type Susceptibility Endpoint Distributions and Epidemiological Cutoff Values for Fluconazole, Itraconazole, Posaconazole, and Voriconazole

A. Espinel-Ingroff,[a] A. I. Aller,[b] E. Canton,[c] L. R. Castañón-Olivares,[d] A. Chowdhary,[e] S. Cordoba,[f] M. Cuenca-Estrella,[g] A. Fothergill,[h] J. Fuller,[i] N. Govender,[j] F. Hagen,[k] M. T. Illnait-Zaragozi,[l] E. Johnson,[m] S. Kidd,[n] C. Lass-Flörl,[o] S. R. Lockhart,[p] M. A. Martins,[q] J. F. Meis,[r] M. S. C. Melhem,[q] L. Ostrosky-Zeichner,[s] T. Pelaez,[t] M. A. Pfaller,[u] W. A. Schell,[v] G. St-Germain,[w] L. Trilles,[x] and J. Turnidge[y]

VCU Medical Center, Richmond, Virginia, USA[a]; Hospital Universitario de Valme, Sevilla, Spain[b]; Unidad de Microbiologia Experimental, Hospital Universitario La Fe, Valencia, Spain[c]; Universidad Nacional Autónoma de Mexico, Distrito Federal, México[d]; Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India[e]; Departamento de Micología, Instituto Nacional de Enfermedades Infecciosas, ANLIS "Dr. Carlos G. Malbrán," Buenos Aires, Argentina[f]; Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain[g]; University of Texas Health Science Center, San Antonio, Texas, USA[h]; The University of Alberta, Edmonton, Alberta, Canada[i]; National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa[j]; Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, Netherlands[k]; Institute of Tropical Medicine Pedro Kouri, Havana, Cuba[l]; The HPA Mycology Reference Laboratory, Kingsdown, Bristol, United Kingdom[m]; Women's and Children's Hospital, Adelaide, South Australia, Australia[n]; The Innsbruck Medical University, Innsbruck, Austria[o]; Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[p]; The Adolfo Lutz Institute Public Health Reference Center, São Paulo and Rio Claro, Brazil[q]; Department of Medical Microbiology, Radboud University Medical Centre, Canisius Wilhelmina Hospital, Nijmegen, the Netherlands[r]; University of Texas Health Science Center, Houston, Texas, USA[s]; Hospital General Universitario Gregorio Marañón, Faculty of Medicine-Universidad Complutense, Madrid, Spain[t]; University of Iowa, Iowa City, Iowa, USA[u]; Duke University Medical Center, Durham, North Carolina, USA[v]; Laboratoire de Santé Publique du Québec, Canada[w]; Instituto de Pesquisa Clinica Evandro Chagas-FIOCRUZ, Rio de Janeiro, Brazil[x]; and University of Adelaide, Adelaide, South Australia, Australia[y]

Epidemiological cutoff values (ECVs) for the *Cryptococcus neoformans-Cryptococcus gattii* species complex versus fluconazole, itraconazole, posaconazole, and voriconazole are not available. We established ECVs for these species and agents based on wild-type (WT) MIC distributions. A total of 2,985 to 5,733 CLSI MICs for *C. neoformans* (including isolates of molecular type VNI [MICs for 759 to 1,137 isolates] and VNII, VNIII, and VNIV [MICs for 24 to 57 isolates]) and 705 to 975 MICs for *C. gattii* (including 42 to 260 for VGI, VGII, VGIII, and VGIV isolates) were gathered in 15 to 24 laboratories (Europe, United States, Argentina, Australia, Brazil, Canada, Cuba, India, Mexico, and South Africa) and were aggregated for analysis. Additionally, 220 to 359 MICs measured using CLSI yeast nitrogen base (YNB) medium instead of CLSI RPMI medium for *C. neoformans* were evaluated. CLSI RPMI medium ECVs for distributions originating from at least three laboratories, which included ≥95% of the modeled WT population, were as follows: fluconazole, 8 μg/ml (VNI, *C. gattii* nontyped, VGI, VGIIa, and VGIII), 16 μg/ml (*C. neoformans* nontyped, VNIII, and VGIV), and 32 μg/ml (VGII); itraconazole, 0.25 μg/ml (VNI), 0.5 μg/ml (*C. neoformans* and *C. gattii* nontyped and VGI to VGIII), and 1 μg/ml (VGIV); posaconazole, 0.25 μg/ml (*C. neoformans* nontyped and VNI) and 0.5 μg/ml (*C. gattii* nontyped and VGI); and voriconazole, 0.12 μg/ml (VNIV), 0.25 μg/ml (*C. neoformans* and *C. gattii* nontyped, VNI, VNIII, VGII, and VGIIa,), and 0.5 μg/ml (VGI). The number of laboratories contributing data for other molecular types was too low to ascertain that the differences were due to factors other than assay variation. In the absence of clinical breakpoints, our ECVs may aid in the detection of isolates with acquired resistance mechanisms and should be listed in the revised CLSI M27-A3 and CLSI M27-S3 documents.

The *Cryptococcus neoformans-Cryptococcus gattii* complex is the most common species of non-*Candida* yeasts recovered from clinical specimens (32.9% of 8,717 isolates) (41). In addition, cryptococcal disease has been reported as the second most common severe fungal infection in certain regions (41). Infections caused by *C. neoformans* var. *grubii* (serotype A) and, to a lesser degree, by *C. neoformans* var. *neoformans* (serotype D) are seen worldwide among immunocompromised hosts (4). The more geographically restricted *C. gattii* (serotypes B and C) causes infections among immunocompromised as well as nonimmunocompromised patients, and the infections are more difficult to treat (27, 38). Irrespective of the species, cryptococcal disease is associated with high mortality rates (≥12.7%) (15, 17, 38). Using molecular methodologies, eight major molecular types have been identified among the four serotypes and their hybrids (4, 6, 8, 25, 31, 51). *C. neoformans* comprises molecular types VNI and VNII (both serotype A), VNIII (serotype A/D hybrid), and VNIV (serotype D), while *C. gattii* comprises VGI, VGII, and VGIV (all serotype B), VGII, and VGIV (both serotype C) (25). Molecular type VGII has been of particular interest in recent years due to the emergence of the novel subtypes VGIIa, VGIIb, and VGIIc; mo-

Received 29 May 2012 Returned for modification 18 August 2012
Accepted 27 August 2012
Published ahead of print 4 September 2012
Address correspondence to A. Espinel-Ingroff, avingrof@vcu.edu.

doi:10.1128/AAC.01115-12

lecular types VNI to VNIV and VGI to VGIV also have been designated AFLP1 to AFLP3 and AFLP4 to AFLP7, respectively (4, 6, 7). Identification of these different molecular types has been associated with differences in antifungal susceptibility and virulence (4, 6–11, 22, 25, 33, 51).

In addition to the different amphotericin B formulations, fluconazole and itraconazole are recommended as primary alternative induction treatments for infections caused by *C. neoformans* and *C. gattii* and voriconazole and posaconazole as salvage consolidation therapies (35, 38); fluconazole is also the drug of choice for lifelong suppressive (maintenance) therapy or primary therapy in some areas (24). The azoles block the pathway of ergosterol biosynthesis by inhibiting the 14-α-lanosterol demethylase enzyme, which is coded by the *CYP51* gene in *C. neoformans* (Cn*CYP51*; also called *ERG11*) (46). The wide use of fluconazole and other triazoles has led to *in vitro* resistance among *Candida* and *Cryptococcus* isolates, especially to fluconazole more than to the newer triazoles, voriconazole, and posaconazole (38). Two azole resistance mechanisms have been identified in *C. neoformans* (18, 32, 46, 54).

The use of standard testing methods has allowed the recognition of antifungal resistance, as well as the proposal of clinical breakpoints (CBPs) for *Candida* spp. and epidemiological cutoff values (ECVs) for *Aspergillus* spp. by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST) (19–21, 40, 42, 44, 47). More recently, CLSI amphotericin B and flucytosine ECVs were defined for the *Cryptococcus neoformans-Cryptococcus gattii* species complex (22). CBPs are based on MIC distributions, pharmacokinetic and pharmacodynamic (PK/PD) parameters, animal studies, and clinical outcomes to therapy, while the ECV is based mostly on MIC distributions. Numerous surveys indicate that CLSI MICs are ≤16 μg/ml (fluconazole) and ≤0.5 μg/ml (the other three triazoles) for most *C. neoformans* and *C. gattii* isolates (5, 9, 10, 16, 24, 43, 50). In the last few years, azole (mostly fluconazole) antifungal susceptibility differences have been reported for these two species and for their molecular types and serotypes (10, 11, 25, 33, 50, 51). However, CBPs or ECVs based on data from multiple laboratories are not available for either *C. neoformans* or *C. gattii* versus the triazoles. ECVs defined in the present study could help to characterize the susceptibility of these species and to monitor the emergence of strains with mutations that could lead to reduced antifungal susceptibility to fluconazole, itraconazole, posaconazole, and voriconazole.

The purpose of the study was dual: (i) to define wild-type (WT; population of isolates in a species-drug combination with no detectable acquired resistance mechanisms) (14, 52) susceptibility endpoint distributions of each species/molecular type and triazole combination originating from at least 3 laboratories and (ii) to propose ECVs (highest WT susceptibility endpoint) of four triazoles. We aggregated the CLSI RPMI broth microdilution MICs of fluconazole, itraconazole, posaconazole, and voriconazole obtained in 15 to 24 laboratories (2,985 to 5,733 MICs for *C. neoformans* and 705 to 975 MICs for *C. gattii* [species/molecular type and agent/combination dependent]) in Europe, the United States, Argentina, Australia, Canada, Cuba, Brazil, India, Mexico, and South Africa. The 220 to 359 MICs that were obtained using the alternative CLSI yeast nitrogen base (YNB) broth (12, 13) for *C. neoformans* were analyzed separately.

MATERIALS AND METHODS

Isolates. Each isolate originated from a unique clinical specimen. The MICs of the four triazoles used in the present study for ECV definition were obtained at one of the following medical centers: VCU Medical Center, Richmond, VA; Unidad de Microbiologia Experimental, Hospital Universitario La Fe, Valencia, Spain; Universidad Nacional Autónoma de México, Mexico; Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; Departamento de Micología, INEI, ANLIS "Dr. Carlos G. Malbrán," Buenos Aires, Argentina; Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain; University of Texas Health Science Center, San Antonio, TX; The University of Alberta, Edmonton, Alberta, Canada; National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa; Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands; Institute of Tropical Medicine Pedro Kouri, Havana, Cuba; The HPA Mycology Reference Laboratory, Kingsdown, Bristol, United Kingdom; Women's and Children's Hospital, Adelaide, South Australia, Australia; The Innsbruck Medical University, Innsbruck, Austria; Centers for Disease Control and Prevention, Atlanta, GA; The Adolfo Lutz Institute Public Health Reference Center, São Paulo and Rio Claro, Brazil; Hospital Universitario de Valme, Sevilla, Spain; University of Texas Health Science Center, Houston, TX; Hospital General Universitario Gregorio Marañón, Faculty of Medicine-Universidad Complutense, Madrid, Spain; University of Iowa, Iowa City, IA; Duke University Medical Center, Durham, NC; Institut national de santé publique du Québec, Laboratoire de santé publique du Québec, Canada; and Instituto de Pesquisa Clinica Evandro Chagas-FIOCRUZ, Rio de Janeiro, Brazil. Species and molecular type identification were performed at each medical center using standard methodologies (8, 11, 25, 28, 33, 36, 51). We have aggregated the maximum available CLSI data from each laboratory and agent as follows: 2,985 posaconazole, 4,019 itraconazole, 4,693 voriconazole, and 5,733 fluconazole MICs for *C. neoformans* (including MICs for the VNI to VNIV isolates) and 705 posaconazole, 828 itraconazole, 923 voriconazole, and 975 fluconazole MICs for *C. gattii* (including those for the VGI to VGIV isolates) (8, 11, 24, 25, 26, 33, 36, 51). Sets of 220 (itraconazole) to 359 (fluconazole) MICs obtained using CLSI YNB broth instead of the CLSI RPMI medium (12) for *C. neoformans* were also available and analyzed separately. One or both quality control (QC) isolates (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) were used by the participant laboratories (Table 1) (12, 13).

Antifungal susceptibility testing. In order to include MIC results in the total set of available aggregated CLSI data from the participant laboratories (Tables 2 to 5), triazole 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 that ranged from 0.4×10^3 to 5×10^3 CFU/ml, and 72 h of incubation); MICs were the lowest drug concentrations that produced ≥50% growth inhibition compared to the growth control (12). Testing conditions and interpretation of MICs were otherwise identical for those isolates grown in CLSI YNB broth. MIC data for the two QC reference strains, utilized during the years of testing in each center, were obtained each time that a set of isolates was tested following the CLSI M27-A3 broth microdilution method (12, 13).

Definitions. The ECV, a term previously used in similar fungal reports and also known as the CO_{WT}, is the highest wild-type (WT) cutoff susceptible value (19–22, 44). ECVs are based on MIC distributions where there are two distinct populations: (i) the WT population of isolates/MICs with no detectable acquired or mutational resistance to the drug being evaluated and (ii) the non-WT population or isolates that harbor one or more resistance markers (14, 52). A non-WT organism shows reduced susceptibility to the agent being evaluated compared to the WT population, but it may or may not respond to treatment to the drug being evaluated. When data pertaining to the establishment of clinical breakpoints (CBPs) are not available, ECVs serve as an early indication of emerging

TABLE 1 MICs for QC strains used in 11 to 15 laboratories according to the CLSI broth microdilution method at 48 h[a]

QC isolate	Antifungal agent	QC MIC range (mode) in μg/ml (CLSI document)	% MICs within range (CLSI document)	MIC range in μg/ml (present study)	Modes in μg/ml (present study)[b]
Candida parapsilosis ATCC 22019	Fluconazole	1–4 (2)	98.1	1–4	1, 1, 1, 2, 2, 2, 2, 2, 2, 2, 2, 2, 2, 4, 4, ND
	Itraconazole	0.06–0.5 (0.25)[b]	97.5	0.03–0.5[c]	0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.25, 0.25, ND, ND, ND, ND
	Posaconazole	0.03–0.25 (0.12)	98.8	0.03–0.25	0.06, 0.06, 0.06, 0.06, 0.12, 0.12, 0.12, 0.12, 0.12, 0.12, 0.25, ND, ND, ND, ND, ND, ND, ND
	Voriconazole	0.03–0.25 (0.06)	100	0.01–0.25[c]	0.03, 0.03, 0.03, 0.03, 0.03, 0.03, 0.06, 0.06, 0.06, 0.06, 0.06, 0.06, 0.12, 0.12, 0.12, ND, ND, ND, ND
Candida krusei ATCC 6258	Fluconazole	16–128 (32)	100	2–64[d]	16, 32, 32, 32, 32, 32, 32, 32, 32, 32, 32, 32, 64, ND, ND, ND
	Itraconazole	0.25–1 (0.5)	100	0.03–1[d]	0.12, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.5, 0.5, 0.5, 0.5, ND, ND, ND, ND, ND, ND, ND
	Posaconazole	0.12–1 (0.5)	99.6	0.12–2[d]	0.12, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, ND, ND, ND, ND, ND, ND, ND, ND
	Voriconazole	0.12–1 (0.5)	100	0.12–0.5	0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.25, 0.5, 0.5, 0.5, 0.5, ND, ND, ND, ND

[a] MICs were determined as described by the CLSI M27-A3 document using RPMI 1640 broth (12).
[b] Modes were not listed for 9 of the 25 laboratories because only 24-h (from 4 to 5 laboratories) or a single (from two to four laboratories) MIC(s) for QC isolates (*C. parapsilosis* and *C. krusei*) were reported for small subsets of isolates (8 to 84 isolates). However, MICs were in range (95 to 100%) and available 24-h modes were within 1 to 2 dilutions. ND, not determined or obtained using the CLSI YNB broth.
[c] MICs were outside the range in one (itraconazole [4.5%]) and two (voriconazole [2 to 3%]) laboratories for QC isolate *C. parapsilosis* ATCC 22019.
[d] MICs were outside the range in two (fluconazole [0.3 to 5%] and posaconazole [1.5 to 2%]) to four (itraconazole [0.8 to 5%]) laboratories for QC isolate *C. krusei* ATCC 6258.

changes in the patterns of susceptibility of organisms to the agent being evaluated.

Data analysis. MIC distributions from each laboratory (laboratories were coded) for each available combination of triazole and species or molecular type were listed in Excel spreadsheets before they were aggregated for the analysis. The MIC distributions of each of the four triazoles for *C. neoformans* (Tables 2 to 5) were as follows: (i) CLSI RPMI medium aggregated data from 11 to 18 laboratories for nonmolecular typed isolates (here referred to as nontyped isolates), (ii) CLSI RPMI medium aggregated data from 2 to 6 laboratories for VNI to VNIV molecular types (here referred to as typed isolates), and (iii) single-laboratory CLSI YNB data. The final WT distributions for *C. gattii* (Tables 2 to 5) were as follows: (i) CLSI RPMI medium aggregated data of each of the four triazoles from 4 to 6 laboratories for nontyped isolates and (ii) CLSI RPMI medium aggregated data from 1 to 7 laboratories for VGI to VGIV (also referred to as typed isolates). The aggregated MIC distributions obtained in at least

TABLE 2 WT fluconazole MIC distributions for the *Cryptococcus neoformans-Cryptococcus gattii* species complex[a]

Species or molecular type	Medium[b]	Genotyped[c]	No. of labs[d]	Total no. of isolates	No. of isolates for which the MIC (μg/ml) was:[e]									
					≤0.12	0.25	0.5	1	2	4	8	16	32	≥64
C. neoformans	RPMI	N	18	4,446	92	133	258	543	1,225	1,372	569	180	52	22
VNI		Y	6	1,137	4	12	40	127	376	456	89	20	10	3
VNII		Y	4	42	1	1	1	7	13	12		5	2	
VNIII		Y	4	54		1	3	9	9	22	8	1	1	
VNIV		Y	4	54		2	17	19	6	6	2		2	
All isolates		Both	24	5,733	97	149	319	705	1,629	1,868	668	206	67	25
C. neoformans	YNB	N	1	359	1	5	13	23	73	84	126	26	8	
C. gattii	RPMI	N	6	137			1	8	19	71	25	6	4	3
VGI		Y	7	260		1	18	39	69	101	29	3		
VGII[f]		Y	4	101				3	3	26	32	17	13	7
VGIIa		Y	3	200			2	1	16	93	80	6	2	
VGIIb		Y	2	106				3	9	31	54	9		
VGIIc		Y	2	42						1	2	21	17	1
All VGII isolates		Y	6	449			2	7	28	151	168	53	32	8
VGIII		Y	3	43		1	1	6	12	18	4	1		
VGIV		Y	3	86			2	3	21	19	32	5	4	
All isolates		Both	10	975		2	24	63	149	360	258	68	40	11

[a] Fluconazole MICs were determined by the CLSI broth microdilution method (12).
[b] RPMI and YNB, RPMI 1640 and yeast nitrogen base, respectively, as described by the CLSI M27-A3 document (12).
[c] Y, yes; N, no.
[d] Number of laboratories contributing data to each MIC distribution.
[e] The modal MIC for each distribution is underlined.
[f] Isolates identified as belonging to the VGII molecular type and not being one of the VGIIa, VGIIb, or VGIIc subtypes examined as a separate group.

Table 3 WT itraconazole MIC distributions for *Cryptococcus neoformans-Cryptococcus gattii* species complex[a]

Species or molecular type	Medium[b]	Genotyped[c]	No. of labs[d]	Total no. of isolates	No. of isolates for which the MIC (μg/ml) was:[e]									
					≤0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	≥4
C. neoformans	RPMI	N	11	2,731		106	174	377	949	865	222	32	4	2
VNI		Y	6	1,145	25	54	147	315	447	144	13			
VNII		Y	4	32	1	3	3	15	7	2	1			
VNIII		Y	4	54		1	8	20	14	4	7			
VNIV		Y	4	57		17	15	9	6	6	4			
All isolates		Both	20	4,019	26	181	347	736	1,423	1,021	247	32	4	2
C. neoformans	YNB	N	1	220	1		5	22	38	91	61	1		1
C. gattii	RPMI	N	5	70		4	9	16	25	12	4			
VGI		Y	6	257	3		11	42	77	103	20	1		
VGII[f]		Y	3	47			2	13	15	10	6	1		
VGIIa		Y	2	176			7	15	44	63	41	6		
VGIIb		Y	2	106	4			5	34	44	14	4	1	
VGIIc		Y	2	42					1	9	24	7	1	
All VGII isolates		Y	4	371	4		9	33	94	126	85	18	2	
VGIII		Y	3	44		1	1	3	14	17	6	2		
VGIV		Y	3	86	1		1	4	22	24	29	3	2	
All isolates		Both	9	828	8	5	31	98	232	282	144	24	4	

[a] Itraconazole MICs were determined by the CLSI broth microdilution method (12).
[b] RPMI and YNB, RPMI 1640 and yeast nitrogen base, respectively, as described by the CLSI M27-A3 document (12).
[c] Y, yes; N, no.
[d] Number of laboratories contributing data to each MIC distribution.
[e] The modal MIC for each distribution is underlined.
[f] Isolates identified as belonging to the VGII molecular type and not being one of the VGIIa, VGIIb, or VGIIc subtypes examined as a separate group.

three laboratories were used to calculate ECVs by the statistical method (52), 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 MIC that captures at least 95%, 97.5%, and 99% of the modeled WT population (19–22). A search for outlier laboratories in each distribution was also performed, and only one outlier set of voriconazole MICs was excluded from the analysis. In addition, ECVs were not esti-

Table 4 WT posaconazole MIC distributions for *Cryptococcus neoformans-Cryptococcus gattii* species complex[a]

Species or molecular type	Medium[b]	Genotyped[c]	Total no. of labs[d]	No. of isolates	No. of isolates for which the MIC (μg/ml) was:[e]									
					≤0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	≥4
C. neoformans	RPMI	N	13	2,120		52	152	418	938	440	92	23	5	
VNI		Y	3	759	21	49	108	334	203	42	2			
VNII		Y	2	24		1	15	5	2	1				
VNIII		Y	2	35		2	19	12	2					
VNIV		Y	2	47		18	11	11	4	3				
All isolates		Both	15	2,985	21	122	305	780	1,149	486	94	23	5	
C. neoformans	YNB	ND		ND										
C. gattii	RPMI	N	4	68	1	8	8	17	20	12	1	1		
VGI		Y	3	182	3		6	37	64	58	13	1		
VGII[f]		Y	1	5			1	1	1	1	1			
VGIIa		Y	2	176			7	18	51	62	35	3		
VGIIb		Y	2	106	5			6	60	31	4			
VGIIc		Y	2	42		1			3	6	21	11		
All VGII isolates		Y	2	329	5	1	8	25	115	100	61	14		
VGIII		Y	2	42			2	14	7	8	10	1		
VGIV		Y	2	84			2		17	25	28	12		
All isolates		Both	6	705	9	9	26	93	223	203	113	29		

[a] Posaconazole MICs were determined by the CLSI broth microdilution method (12).
[b] RPMI and YNB, RPMI 1640 and yeast nitrogen base, respectively, as described by the CLSI M27-A3 document (12).
[c] Y, yes; N, no.
[d] Number of laboratories contributing data to each MIC distribution.
[e] The modal MIC for each distribution is underlined.
[f] Isolates identified as belonging to the VGII molecular type and not being one of the VGIIa, VGIIb, or VGIIc subtypes examined as a separate group.

Table 5 WT voriconazole MIC distributions for *Cryptococcus neoformans-Cryptococcus gattii* species complex[a]

Species or molecular type	Medium[b]	Genotyped[c]	Total no. of labs[d]	No. of isolates	No. of isolates for which the MIC (μg/ml) was:[e]									
					≤0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	≥4
C. neoformans	RPMI	N	12	3,473	34	412	814	1,090	748	252	92	20	6	5
VNI		Y	5	1,089	35	48	100	385	376	119	19	5	1	1
VNII		Y	3	27		5	6	9	3	4				
VNIII		Y	3	51		9	7	19	12	3	1			
VNIV		Y	3	53		13	19	12	6	2	1			
All isolates		Both	22	4,693	69	487	946	1,515	1,145	380	113	25	7	6
C. neoformans	YNB	N	1	281			35	78	105	53	10			
C. gattii	RPMI	N	5	98	2	7	13	37	23	11	5			
VGI		Y	6	258	3	13	38	69	90	45				
VGII[f]		Y	3	96		1	1	29	38	23	4			
VGIIa		Y	3	197		6	3	47	113	23	3	2		
VGIIb		Y	2	106	3		2	6	46	47	2			
VGIIc		Y	2	42			1	2	7	19	11	2		
All VGII isolates		Y	5	441	3	7	7	84	204	112	20	4		
VGIII		Y	2	42		1	3	12	21	5				
VGIV		Y	2	84			8	28	29	16	3			
All isolates		Both	9	923	8	28	69	230	367	189	28	4		

[a] Voriconazole MICs were determined by the CLSI broth microdilution method (12).
[b] RPMI and YNB, RPMI 1640 and yeast nitrogen base, respectively, as described by the CLSI M27-A3 (12).
[c] Y, yes; N, no.
[d] Number of laboratories contributing data to each MIC distribution.
[e] The modal MIC for each distribution is underlined.
[f] Isolates identified as belonging to the VGII molecular type and not being one of the VGIIa, VGIIb, or VGIIc subtypes examined as a separate group.

mated when (i) the distribution was grossly skewed, which precluded statistical fitting, or (ii) there were data from less than 3 laboratories or the total number of isolates in a molecular type was less than 50.

RESULTS AND DISCUSSION

The ultimate goal of susceptibility testing is to predict with some reliability the clinical outcome when an infected patient is treated with the specific agent evaluated and is referred to as the CBP (14, 30). CBPs are based on the correlation of *in vitro* data, or the MIC for the infecting pathogen, with clinical and microbiological outcomes in order to differentiate an organism as treatable or nontreatable (14, 30). On the other hand, and as previously defined, ECVs are based on MIC distributions that comprise the WT and non-WT populations; the ECV is the highest MIC that belongs to the WT population. While CBPs predict clinical outcome of therapy, the role of the ECV is to detect emerging resistance or those non-WT strains with reduced susceptibility (due to mutations) to the agent being evaluated. Attempts to correlate *in vitro* fluconazole results with clinical outcome have not been successful (15, 55) and, to our knowledge, have not been reported for the other three triazoles. Therefore, CBPs for either *C. neoformans* or *C. gatti* infections versus any antifungal agent are not available due to the paucity of data on PK/PD and clinical outcomes compared to MICs. However, the ECVs of the four triazoles established in the present study for *C. neoformans* and *C. gattii* may identify the non-WT clinical isolates and serve as an early indication of emerging changes in the susceptibility patterns of these organisms. Even though cryptococcal meningitis and other infections 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. The triazoles, especially fluconazole, are primary or salvage therapeutic agents for cryptococcal infections (2, 38).

Despite standardization efforts, variability is common during interlaboratory comparisons of MIC results (12, 13). Table 1 depicts 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. As in previous CLSI ECV studies (19–22), the majority of MIC ranges were within the CLSI established limits for the two QC strains (≥98%), with a certain degree of interlaboratory modal variability (mostly ±2-fold dilution); inconsistencies were more frequent when testing itraconazole, where MICs were outside the range of 0.8 to 5% for *C. parapsilosis* ATCC 22019 (1 laboratory) and *C. krusei* ATCC 6258 (4 laboratories). Intralaboratory reproducibility was excellent in the laboratory using the CLSI YNB medium (data not shown). Similar modal variability was also observed among the laboratories for the clinical *Cryptococcus* spp. isolates in this and previous multicenter studies establishing ECVs of several antifungal agents for *Aspergillus* spp. and *Cryptococcus* spp. (19–22). The most probable reason is the individual interpretations of MIC endpoints, and/or it may be due to the use of different lots of antifungal powders.

The aggregated MIC distributions of the four triazoles for *C. neoformans* and *C. gattii* (for nontyped and typed isolates) are depicted in Tables 2 to 5, which also list single-laboratory distributions for *C. neoformans* (CLSI YNB data). Fluconazole modal MICs measured in RPMI medium were 1 to 4 μg/ml for *C. neoformans* typed and nontyped isolates, with the lowest mode for VNIV. Modes were consistently higher among the *C. gattii* groups (4 to 16 μg/ml), with the highest for VGIIc (Table 2). Fluconazole modes from individual contributing laboratories were either 2 or

TABLE 6 ECVs and percentages of isolates of the *Cryptococcus neoformans-Cryptococcus gattii* species complex above each triazole WT distribution obtained in 3 to 18 laboratories by the CLSI M27-A3 broth microdilution method

Species	Genotype[a]	Antifungal Agent	Mode (μg/ml)[b]	ECV (μg/ml) (% of observations above each statistical ECV or non-WT)[c]		
				Statistical ECV >95%	Statistical ECV >97.5%	Statistical ECV >99%
C. neoformans	Nontyped isolates	Fluconazole	4	16 (1.7)	16 (1.7)	32 (0.5)
		Itraconazole	0.12	0.5 (1.1)	1 (0.2)	1 (0.2)
		Posaconazole	0.12	0.25 (5.7)	0.5 (1.3)	0.5 (1.3)
		Voriconazole	0.06	0.25 (3.5)	0.25 (3.5)	0.5 (0.9)
	VNI	Fluconazole	4	8 (2.9)	8 (2.9)	16 (1.1)
		Itraconazole	0.12	0.25 (1.1)	0.5 (0)	0.5 (0)
		Posaconazole	0.06	0.25 (0.3)	0.25 (0.3)	0.25 (0.3)
		Voriconazole	0.06	0.25 (2.4)	0.25 (2.4)	0.5 (0.6)
	VNIII	Fluconazole	4	16 (1.9)	16 (1.9)	32 (0)
		Voriconazole	0.06	0.25 (2)	0.25 (2)	0.5 (0)
	VNIV	Voriconazole	0.03	0.12 (5.7)	0.12 (5.7)	0.12 (5.7)
C. gattii	Nontyped isolates	Fluconazole	4	8 (9.5)	8 (9.5)	16 (5.1)
		Itraconazole	0.12	0.5 (0)	0.5 (0)	1 (0)
		Posaconazole	0.12	0.5 (1.5)	0.5 (1.5)	1 (0)
		Voriconazole	0.06	0.25 (5.1)	0.5 (0)	0.5 (0)
	VGI	Fluconazole	4	8 (1.2)	16 (0)	16 (0)
		Itraconazole	0.25	0.5 (0.4)	0.5 (0.4)	1 (0)
		Posaconazole	0.12	0.5 (0.5)	0.5 (0.5)	1 (0)
		Voriconazole	0.12	0.5 (0)	0.5 (0)	1 (0)
	VGII	Fluconazole	8	32 (6.9)	32 (6.9)	64 (1)
		Itraconazole	0.12	0.5 (2.1)	0.5 (2.1)	1 (0)
		Voriconazole	0.12	0.25 (4.1)	0.5 (0)	0.5 (0)
	VGIIa	Fluconazole	4	8 (4)	16 (1)	16 (1)
		Voriconazole	0.12	0.25 (2.5)	0.25 (2.5)	0.25 (2.5)
	VGIII	Fluconazole	4	8 (2.3)	8 (2.3)	16 (0)
		Itraconazole	0.25	0.5 (4.5)	1 (0)	1 (0)
	VGIV	Fluconazole	8	16 (4.7)	32 (0)	32 (0)
		Itraconazole	0.5	1 (2.3)	1 (2.3)	2 (0)

[a] Data from laboratories using the CLSI RPMI broth (12).
[b] Mode, MIC most frequently obtained for each distribution.
[c] Calculated ECVs comprising >95%, >97.5%, or >99% of the statistically modeled population for which MIC distributions originated in at least three laboratories.

4 μg/ml for *C. neoformans* nontyped and most typed isolates; the exception was the mode (1 μg/ml) from two of the four laboratories that contributed data for VNIV, but the number of isolates was small. Overall, our values reflect previous fluconazole MIC_{90}s and modes for similar sets of typed or nontyped *C. neoformans* and *C. gattii* isolates and the fact that MICs are usually higher (MIC_{90}s and modes, 2 to 8 μg/ml versus 8 to 32 μg/ml) for *C. gattii* independent of testing conditions (10, 16, 23, 50). However, the MIC_{90} has also been higher (16 μg/ml) for *C. neoformans* nontyped isolates (5, 39), but some of those results were obtained by the EUCAST method (39). The modal MIC for *C. neoformans* measured in CLSI YNB medium was higher (mode, 8 μg/ml) than those measured in CLSI RPMI medium; this discrepancy has been reported previously (10, 55). These results further underline the variability of susceptibility test results using different methodologies.

Modes for the other three triazoles were more species-, molecular type- and medium-dependent (Tables 3 to 5). In agreement with previous data (10, 16, 23, 50), the MICs of itraconazole, posaconazole, and voriconazole for *C. gattii* also tended to be higher (modes, 0.06 to 0.5 μg/ml, typed and nontyped isolates) than those for *C. neoformans* (modes, 0.016 to 0.12 μg/ml, typed and nontyped isolates), with the highest modes for VGIIb and VGIIc (voriconazole mode, 0.25 μg/ml) and VGIIc and VGIV (itraconazole and posaconazole mode, 0.5 μg/ml) (Tables 3 to 5). Reflecting fluconazole distributions, modes from individual participant laboratories were within one dilution of each other (modes, 0.12 to 0.25 μg/ml and 0.06 to 0.12 μg/ml for itraconazole and both posaconazole and voriconazole, respectively). Based on these data and the wide geographical range over which the MICs have been collected in the present study, we surmise that we are presenting valid data.

Table 6 depicts the proposed fluconazole, itraconazole, posaconazole, and voriconazole ECVs for the aggregated distributions of *C. neoformans* and *C. gattii* (typed or nontyped isolates). ECVs were proposed when the data originated from at least three laboratories using the statistical methodology that comprised ≥95% of the modeled population instead of the observed population; the values that comprised ≥97.5 and ≥99% of the population were also obtained. Some of the proposed ECVs were based on small numbers of isolates (Tables 2 to 5, $n < 100$), and those could be considered tentative values. Although three other distributions (fluconazole versus VNIV and itraconazole versus VNIII and VNIV) originated in at least three laboratories, statistical fitting was not possible and ECVs were not proposed. The CLSI

fluconazole ECVs for the different subsets of *C. neoformans* isolates were either 8 μg/ml (VNI) or 16 μg/ml (nontyped and VNIII isolates). Fluconazole ECVs for *C. gattii* ranged from 8 μg/ml (nontyped, VGI, VGIIa, and VGIII isolates) to 32 μg/ml (VGII). It is interesting that a fluconazole MIC of ≥16 μg/ml is anecdotally believed to be the resistance cutoff, but this value can be perceived here as a non-WT value for some of the subsets of the two species. So far mutations have not been observed when the fluconazole MIC is ≤16 μg/ml (49). ECVs of the three other triazoles were also species-, agent-, and molecular type-dependent as follows: (i) itraconazole ECVs of 0.25 μg/ml (VNI), 0.5 μg/ml (nontyped isolates of both species and VGI to VGIII), and 1 μg/ml (VGIV); (ii) posaconazole ECVs of 0.25 μg/ml (*C. neoformans* nontyped and VNI) and 0.5 μg/ml (*C. gattii* nontyped and VGI); and (iii) voriconazole ECVs of 0.12 μg/ml (VNIV), 0.25 μg/ml (nontyped isolates of both species, VNI, VNIII, VGII, and VGIIa), and 0.5 μg/ml (VGI). The overall trend was that ECVs for *C. neoformans* molecular types were lower than those for *C. gattii*. Previous MIC_{90}s and modes of these three triazoles have been similar (9, 10, 23, 44, 50). Because the distributions in CLSI YNB originated from a single laboratory, ECVs were not proposed for these distributions (Tables 2, 3, and 5). Tentative values of 16 μg/ml for fluconazole (encompassing 97.8% of the isolates), 2 μg/ml for itraconazole (encompassing 99.5% of the isolates), and 0.5 μg/ml for voriconazole (encompassing 100% of the isolates) can be suggested for CLSI YNB MICs (data not shown). Additional data from other laboratories using either CLSI YNB or CLSI RPMI medium (at least three laboratory subsets), or for subsets comprising at least 100 isolates, should corroborate/extend our tentative values. ECVs encompassing both ≥97.5% and ≥99% of the modeled population were mostly the same or one dilution higher (Table 6); the same applied when all isolates of each species were pooled together, e.g., all VGIIs (data not shown). Although the proposed ECVs in the present study are not predictors of clinical outcome, these critical drug concentrations may aid in identifying those cryptococcal strains with decreased susceptibility to the triazole being evaluated.

The frequency of MICs above the ECV (non-WT) varied according to the distribution analyzed (Table 6). The rate of fluconazole non-WT MICs was higher (1.7 to 9.5%) than those of the other three triazoles (0 to 5.7%). Between the two species, the fluconazole and itraconazole rates of non-WT MICs were usually lower for all *C. neoformans* isolates (1.1 to 2.9%) than for all *C. gatti* isolates (0 to 9.5%) and almost the same for voriconazole. In contrast, the rate was lower for posaconazole (0.3 to 5.7% versus 0.5 to 1.5%). However, *C. gattii* MIC distributions were small (Tables 2 to 5). Although cryptococcal infections caused by both species are clinically similar, there is more of a delayed treatment response and other complications in infections caused by *C. gattii* than those due to *C. neoformans* (38). *In vitro* resistance to fluconazole (3.1% to 46%) and itraconazole (0.5% to 17.4%) has been reported for *C. neoformans* isolates; although the itraconazole cutoff was ≥1 μg/ml, it was variable for fluconazole (≥16 μg/ml and ≥64 μg/ml) (5, 16, 39). Based on arbitrary voriconazole cutoffs of ≥2 μg/ml for both *Cryptococcus* spp., *in vitro* resistance to this agent has been low or absent (0% to 0.9%) (23, 24, 43, 50); the same applies to posaconazole (23, 24, 43). Species identification and antifungal susceptibility testing emphasize the utility of WT cutoffs as a practical tool to detect triazole resistance among *Cryptococcus* isolates. Our results also point out that ECVs should be species-specific and, for this fungal group, molecular type-specific. This information is important; it has been reported that 20% of cryptococcal infections are caused by multiple strains or molecular types, but generally only one isolate per infection is tested (17).

During the 1990s, the early years of fluconazole therapy, relapses were not associated with *in vitro* resistance due to the higher initial fluconazole doses (38). Since then, relapses have been attributed to the excessive use of this agent as primary and prophylactic therapies, as well as the use of suboptimal doses for long periods of time, especially among AIDS patients. As relapses began to be associated with high fluconazole MICs and/or a 4-fold MIC increase (1, 24, 37, 38), several aspects of sterol metabolism in these isolates were investigated to determine azole resistance mechanisms in *C. neoformans*. A point mutation in the *ERG11* (Cn*CYP51*) gene resulting in a G484S substitution in the target enzyme has been observed in *C. neoformans* strains with high fluconazole MICs (≥32 μg/ml); this mutation alters the affinity of the drug for the target enzyme (32, 48, 54). The overexpression of the gene Cn*AFR1* that encodes the membrane efflux pumps has been found to reduce cell drug (fluconazole) content in *C. neoformans* (29, 45). Azole cross-resistance among cryptococcal strains is rare; the lack of cross-resistance between itraconazole and fluconazole has been attributed to the dual itraconazole target in *C. neoformans*, which appears to block both the lanosterol 14-α-demethylase and the NADH-dependent 3-ketosteroid reductase (37). This was recently confirmed in a *C. neoformans* isolate, where cross-resistance was evident between fluconazole (MICs > 64 μg/ml) and voriconazole (MICs ≥ 2 μg/ml) but not with itraconazole. In contrast, increased itraconazole susceptibility (lower MIC) and no change in the posaconazole MIC were observed (49). A missense mutation (Y145F substitution) in the *ERG11* gene (*P450Dm*, *ERG11*) led to fluconazole-voriconazole cross-resistance. In addition, a high level of heteroresistance to fluconazole, more commonly observed in *C. gattii* than in *C. neoformans* isolates, was observed in the same isolate (49, 53). Some of these findings explain the different rates of non-WT isolates found in the present study.

Based on small numbers of patients/isolates and the use of different methodologies, correlations between a variety of fluconazole MICs and clinical outcomes (1, 3, 34) have been reported: more rapid CSF sterilization and infection eradication has been associated with MICs of 4 to 8 μg/ml and clinical failure or recurrence with MICs of ≥16 μg/ml. In addition, either a lack of correlation (15) or a relationship that was dependent on the combination of high MICs with other factors have been documented (55). A more useful MIC cutoff that would predict azole failure in treatment of cryptococcal infections has also been hampered by other factors that influence outcome to therapy in addition to microbiological clearance (17, 37). Conversely, our ECVs could aid in the separation of WT strains from those with reduced azole susceptibility or non-WT isolates. However, more information is needed regarding azole resistance in *C. neoformans* and *C. gattii*, especially for posaconazole and voriconazole, as well as the relationship between non-WT strains and resistance mechanisms.

In conclusion, the ECVs of fluconazole (8 to 32 μg/ml), itraconazole (0.25 to 1 μg/ml), posaconazole (0.25 to 0.5 μg/ml), and voriconazole (0.12 to 0.25 μg/ml) proposed in this study for the *C. neoformans-C. gattii* species complex are species- and molecular type-specific, but these differences were mostly within one dilu-

tion. Further investigation should determine the relationship between molecular mechanisms of triazole 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 expand the information provided in the present study. In the absence of CBPs, these ECVs may be clinically useful in detecting non-WT isolates that have reduced susceptibility to itraconazole, posaconazole, and voriconazole or that harbor fluconazole resistance mechanisms. ECVs should be included in the revised version of the CLSI M27-A3 document.

ACKNOWLEDGMENTS

We acknowledge all members of the Pacific Northwest *Cryptococcus gattii* Public Health Working Group and the members of the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) for their contribution, i.e., submission of isolates. We also acknowledge the efforts of the clinicians and laboratory technicians who provided isolates for this study. We thank all technical personnel in the participating laboratories for generating the data used in this study. We also thank Naureen Iqbal and Carol Bolden at the CDC and María de los Angeles Martínez at Escuela Nacional de Ciencias Biológicas, IPN, México, for their technical assistance.

The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC).

REFERENCES

1. **Aller AI, et al.** 2000. Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. Antimicrob. Agents Chemother. **44**: 1544–1548.
2. **Berg J, Clancy CJ, Nguyen MH.** 1998. The hidden danger of fluconazole primary prophylaxis for patients with AIDS. Clin. Infect. Dis. **26**:186–187.
3. **Bicanic T, Harrison A, Niepieklo A, Dyakopu N, Meintjes G.** 2006. Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of fluconazole resistance and immune reconstitution. Clin. Infect. Dis. **43**:1069–1073.
4. **Bovers M, Hagen F, Boekhout T.** 2008. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species-complex. Rev. Iberoam. Micol. **25**: S4–S12.
5. **Brandt ME, et al.** 2001. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. Antimicrob. Agents Chemother. **45**:3065–3069.
6. **Byrnes EJ, et al.** 2010. Emergence and pathogenicity of highly virulent *Cryptococcus gattii* genotypes in the northwest United States. PLoS Pathog. **6**:e1000850. doi:10.1371/journal.ppat.1000850.
7. **Byrnes EJ, et al.** 2011. A diverse population of *Cryptococcus gattii* molecular type VGIII in Southern Californian HIV/AIDS patients. PLoS Pathog. **7**:e1002205. doi:10.1371/journal.ppat.1002205.
8. **Castañón-Olivares LR, et al.** 2009. Genotyping of Mexican *Cryptococcus neoformans* and *C. gattii* isolates by PCR-fingerprinting. Med. Mycol. **47**: 713–721.
9. **Chandenier JK, et al.** 2004. In vitro activity of amphotericin B, fluconazole and voriconazole against 162 *Cryptococcus neoformans* isolates from Africa and Cambodia. Eur. J. Clin. Microbiol. Infect. Dis. **23**:505–508.
10. **Chong HS, Dagg R, Malik R, Chen S, Carter D.** 2010. In vitro susceptibility of the yeast pathogen *Cryptococcus* and other azoles varies with molecular genotype. J. Clin. Microbiol. **48**:4115–4120.
11. **Chowdhary A, et al.** 2011. In vitro antifungal susceptibilities and genotypes of 308 clinical and environmental isolates of *Cryptococcus neoformans* var. grubii and *C. gattii* serotype B from North West India. J. Med. Microbiol. **60**:961–967.
12. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts, third edition. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
13. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts, third informational supplement. CLSI document M27-S3. Clinical and Laboratory Standards Institute, Wayne, PA.
14. **Dalhoff A, Ambrose PG, Mouton JW.** 2009. A long journey from minimum inhibitory concentration testing to clinically predictive breakpoints: deterministic and probabilistic approaches in deriving breakpoints. Infection **37**:296–305.
15. **Dannaoui E, et al.** 2006. Results obtained with various antifungal susceptibility testing methods do not predict early outcome in patients with cryptococcosis. Antimicrob. Agents Chemother. **50**:2264–2470.
16. **DeBedout C, et al.** 1999. In vitro antifungal susceptibility of clinical isolates of *Cryptococcus neoformans* var. *neoformans* and *C. neoformans* var. *gattii.* Rev. Iberoam. Micol. **16**:36–39.
17. **Desnos-Ollivier M, et al.** 2010. Mixed infections and in vivo evolution in the human fungal pathogen *Cryptococcus neoformans*. mBio **1**:e00091–10. doi:10.1128/mBio.00091–10.
18. **Espinel-Ingroff A.** 2008. Mechanisms of resistance to antifungal agents: yeasts and filamentous fungi. Rev. Iberoam. Micol. **25**:101–106.
19. **Espinel-Ingroff A, et al.** 2010. Wild-Type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38–A2). J. Clin. Microbiol. **48**: 3251–3257.
20. **Espinel-Ingroff A, et al.** 2011. Wild-Type MIC distributions and epidemiological cutoff values for caspofungin and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrob. Agents Chemother. **55**:2855–2859.
21. **Espinel-Ingroff A, et al.** 2011. Wild-Type MIC distributions and epidemiological cutoff values for amphotericin B and *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). Antimicrob. Agents Chemother. **55**:5150–5154.
22. **Espinel-Ingroff A, et al.** 2012. *Cryptococcus neoformans-Cryptococcus gattii* complex: An international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for amphotericin B and flucytosine. Antimicrob. Agents Chemother. **56**:3107–3113.
23. **Gomez-Lopez A, et al.** 2008. In vitro susceptibility of *Cryptococcus gattii* clinical isolates. Clin. Microbiol. Infect. **14**:727–730.
24. **Govender NP, Patel J, van Wyk M, Chiller TM, Lockhart SR.** 2011. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates obtained through population-based surveillance in South Africa in 2002–2003 and 2007–2008. Antimicrob. Agents Chemother. **55**:2606–2611.
25. **Hagen F, et al.** 2010. In vitro antifungal susceptibilities and amplified fragment length polymorphism genotyping of a worldwide collection of 350 clinical, veterinary, and environmental *Cryptococcus gattii* isolates. Antimicrob. Agents Chemother. **54**:5139–5149.
26. **Hagen F, et al.** 2012. Extensive genetic diversity within the Dutch clinical *Cryptococcus neoformans* population. J. Clin. Microbiol. **50**:1918–1926.
27. **Harris JR, et al.** 2011. *Cryptococcus gattii* in the United States: clinical aspects of infection with an emerging pathogen. Clin. Infect. Dis. **53**: 1188–1195.
28. **Illnait-Zaragozi MT, et al.** 2008. In vitro activity of the new azole isavuconazole (BAL4815) compared with six other antifungal agents against 162 *Cryptococcus neoformans* isolates from Cuba. Antimicrob. Agents Chemother. **52**:1580–1582.
29. **Joseph-Horne T, Halloman DW, Loeffin R, Kelly SL.** 1995. Cross-resistance to polyene and azole drugs in *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **39**:1526–1529.
30. **Kahlmeter G, et al.** 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J. Antimicrob. Chemother. **52**:143–148.
31. **Kidd SE, et al.** 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. U. S. A. **101**:1758–1763.
32. **Lamb DC, Corran A, Baldwin BC, Kwon-Chung J, Kelly SL.** 1995. Resistant P45051 AI activity in azole antifungal tolerant *Cryptococcus neoformans* from AIDS patients. FEBS Lett. **368**:326–330.
33. **Lockhart SR, et al.** 2012. Epidemiological cutoff values for triazole drugs in *Cryptococcus gattii*: correlation of molecular type and *in vitro* susceptibility. Diagn. Microbiol. Infect. Dis. **73**:144–148.
34. **Menichetti F, et al.** 1996. High-dose fluconazole therapy for cryptococcal meningitis in patients with AIDS. Clin. Infect. Dis. **22**:838–840.
35. **Ostrosky-Zeichner L, Marr KA, Rex JH, Cohen SH.** 2003. Amphotericin B: time for a new "gold standard." Clin. Infect. Dis. **37**:415–425.
36. **Pan W, et al.** 2012. Resistance of Asian *Cryptococcus neoformans* serotype

A is confined to few microsatellite genotypes. PLoS One **7**:e32868. doi: 10.1371/journal.pone.0032868.
37. **Perfect JR, Cox JM.** 1999. Drug resistance in *Cryptococcus neoformans*. Drug Resist. Updat. **2**:259–269.
38. **Perfect JR, et al.** 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. Clin. Infect. Dis. **50**:291–322.
39. **Perkins A, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2005. Rates of antifungal resistance among Spanish clinical isolates of *Cryptococcus neoformans* var. *neoformans.* J. Antimicrob. Chemother. **56**:1144–1147.
40. **Pfaller MA, et al.** 2009. Wild type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute broth microdilution methods. J. Clin. Microbiol. **47**:3142–3146.
41. **Pfaller MA, et al.** 2009. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: 10.5-year analysis of susceptibilities of noncandidal yeast species to fluconazole and voriconazole determined by the CLSI standardized disk diffusion testing. J. Clin. Microbiol. **47**:117–123.
42. **Pfaller MA, Andes D, Diekema DJ, Espinel-Ingroff A, Sheehan D.** 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: time to harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist. Updat. **14**:180–195.
43. **Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Jones RN.** 2011. Wild-type MIC distributions and epidemiological cutoff values for fluconazole, posaconazole and voriconazole when testing *Cryptococcus neoformans* by CLSI broth microdilution methods. Diagn. Microbiol. Infect. Dis. **71**:252–259.
44. **Pfaller MA, et al.** 2012. Wild-type MIC distributions and epidemiological cutoff values for amphotericin B, flucytosine, and itraconazole and *Candida* spp. as determined by CLSI broth microdilution. J. Clin. Microbiol. **50**:2040–2046.
45. **Posteraro B, et al.** 2003. Identification and characterization of a *Cryptococcus neoformans* ATP binding cassette (ABC) transporter-encoding gene, Cn*AFR1*, involved in the resistance to fluconazole. Mol. Microbiol. **47**:357–371.
46. **Revankar SG, et al.** 2004. Cloning and characterization of the lanosterol 14-α-demethylase (*ERG11*) gene in *Cryptococcus neoformans*. Biochem. Biophys. Res. Commun. **324**:719–728.
47. **Rodriguez-Tudela JL, et al.** 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **52**:2468–2472.
48. **Sheng C, et al.** 2009. Three-dimensional model of lanosterol-α-demethylase from *Cryptococcus neoformans:* active-site characterization and insights into azole binding. Antimicrob. Agents Chemother. **53**:3487–3495.
49. **Sionov E, et al.** 2012. Identification of a *Cryptococcus neoformans* cytochrome P450 lanosterol 14-α-demethylase (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole. Antimicrob. Agents Chemother. **56**:1162–1169.
50. **Thompson GR, et al.** 2009. Antifungal susceptibilities among different serotypes of *Cryptococcus gattii* and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **53**:309–311.
51. **Trilles L, Meyer W, Wanke B, Guarro J, Lazera M.** 2012. Correlation of antifungal susceptibility and molecular type within *Cryptococcus neoformans/C. gattii* species complex. Med. Mycol. **50**:328–332.
52. **Turnidge J, Kahmeter G, Kronvall G.** 2006. Statistical characterization of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin. Microbiol. Infect. **12**:418–425.
53. **Varma A, Kwon-Chung KJ.** 2010. Heteroresistance of *Cryptococcus gattii* to fluconazole. Antimicrob. Agents Chemother. **54**:2303–2311.
54. **Venkateswarlu K, Taylor M, Manning NJ, Rinaldi MG, Kelly SL.** 1997. Fluconazole tolerance in clinical isolates of *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **41**:748–751.
55. **Witt MD, et al.** 1996. Identification of patients with acute AIDS-associated cryptococcal meningitis who can be effectively treated with fluconazole: the role of antifungal susceptibility testing. Clin. Infect. Dis. **22**:322–328.