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In Vitro Antifungal Susceptibilities and Amplified Fragment Length Polymorphism Genotyping of a Worldwide Collection of 350 Clinical, Veterinary, and Environmental *Cryptococcus gattii* Isolates▽

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The *in vitro* susceptibilities of a worldwide collection of 350 *Cryptococcus gattii* isolates to seven antifungal drugs, including the new triazole isavuconazole, were tested. With amplified fragment length polymorphism (AFLP) fingerprinting, human, veterinary, and environmental *C. gattii* isolates were subdivided into seven AFLP genotypes, including the interspecies hybrids AFLP8 and AFLP9. The majority of clinical isolates ($n = 215$) comprised genotypes AFLP4 ($n = 76$) and AFLP6 ($n = 103$). The clinical AFLP6 isolates had significantly higher geometric mean MICs for flucytosine and fluconazole than the clinical AFLP4 isolates. Of the seven antifungal compounds examined in this study, isavuconazole had the lowest MIC_{90} (0.125 μg/ml) for all *C. gattii* isolates, followed by a 1 $\log_2$ dilution step increase (MIC_{90}, 0.25 μg/ml) for itraconazole, voriconazole, and posaconazole. Amphotericin B had an acceptable MIC_{90} of 0.5 μg/ml, but fluconazole and flucytosine had relatively high MIC_{90}s of 8 μg/ml.

The basidiomycetous yeast *Cryptococcus gattii* is responsible for life-threatening invasive disease in apparently healthy humans and animals (7, 19). A typical *C. gattii* infection is acquired through the respiratory tract, from which it can further disseminate to the central nervous system, resulting in fatal meningitis (7, 19, 32). Cryptococcosis caused by the primary pathogenic yeast *C. gattii* was, until a decade ago, a rarely encountered infection outside tropical and subtropical regions (17, 26, 27). However, this changed due to an unprecedented outbreak that emerged in the temperate climate of Vancouver Island (British Columbia, Canada) that subsequently expanded farther into the Pacific Northwest (1, 8, 10, 16). Its sibling species, *Cryptococcus neoformans*, differs ecologically and epidemiologically from *C. gattii* since it occurs on a global scale and is linked with disease occurring in immunocompromised individuals, such as HIV-positive patients and transplant patients who receive immune-suppressive medicines (7, 10, 18, 19, 31).

Cryptococcus gattii can be discerned from *C. neoformans* using a wide range of microbiological and molecular techniques (7, 20). A convenient method is the use of canavanine-glycine-bromothymol blue (CGB) medium, which allows *C. gattii* but not *C. neoformans* to grow and which changes the pH indicator in the medium from green-yellowish to blue (18). With the increasing use of molecular techniques, such as PCR fingerprinting, restriction fragment length polymorphism (RFLP) analysis of the *PLB1* and *URA5* loci, and amplified fragment length polymorphism (AFLP) fingerprint analysis, as well as several multilocus sequence typing (MLST) approaches, it became clear that *C. gattii* could be divided into five distinct genotypes, named AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV, and AFLP10 (the last one of which is a recently observed novel genotype) (2, 6, 7, 13, 16, 20, 21, 23). Until recently, a serotype agglutination assay was widely used to distinguish *C. neoformans* (serotypes A and D) from *C. gattii* (serotypes B and C) (7, 27). In general, serotype B strains are found in each of the five *C. gattii* AFLP genotypes, but it seems that *C. gattii* serotype C strains are restricted to genotypes AFLP5/VGIII and AFLP7/VGIV (2, 6, 16, 21, 27).

In addition, it was found that *C. gattii* and *C. neoformans* can form interspecies hybrids, named genotype AFLP8 (*C. neoformans* var. *neoformans* AFLP2/VNIII serotype D × *C. gattii* AFLP4/VGI serotype B) and AFLP9 (*C. neoformans* var. *grubii* AFLP1/VNI serotype A × *C. gattii* AFLP4/VGI serotype B). These interspecies hybrids have, until now, been isolated only from clinical samples, and they might have a higher virulence potential than regular *C. gattii* or *C. neoformans* isolates (4, 5; F. Hagen, K. Tintelnot, and T. Boekhout, unpublished data).

Treatment of cryptococcosis depends on, besides the immune status of the patient, the severity and localization of the infection (11). Severe cases of cryptococcosis in immunocompetent and -compromised patients are treated according to the guidelines of the Infectious Diseases Society of America, according to which treatment consists of an induction therapy for

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TABLE 1. Distribution of *Cryptococcus gattii* strains on the basis of AFLP genotype profiles, geographical origin, and clinical, veterinary, or environmental source

AFLP genotype profile	No. (%) of strains[a]											
	Geographical origin							Source of isolation				Total
	Africa	Asia	Australia	Europe	North America	South America	Unknown	Clinical	Veterinary	Environmental	Unknown	
AFLP4	16 (11.7)	14 (10.2)	8 (5.8)	74 (54.0)	9 (6.6)	15 (10.9)	1 (0.7)	76 (55.5)	29 (21.2)	30 (21.9)	2 (1.5)	137 (39.1)
AFLP5			1 (4.5)	2 (9.1)	10 (45.5)	7 (31.8)	2 (9.1)	15 (68.2)		5 (22.7)	2 (9.1)	22 (6.3)
AFLP6	3 (1.8)	6 (3.6)	11 (6.5)	19 (11.2)	75 (44.4)	55 (32.5)		103 (60.9)	27 (16.0)	38 (22.5)	1 (0.6)	169 (48.3)
AFLP6A		4 (5.5)*	1 (1.4)*	5 (6.8)*	56 (76.7)*	7 (9.6)*		36 (49.3)*	18 (24.7)*	18 (24.7)*	1 (1.4)*	73 (43.2)*
AFLP6B	3 (3.4)*	2 (2.3)*	10 (11.4)*	14 (15.9)*	11 (12.5)*	48 (54.5)*		61 (69.3)*	7 (8.0)*	20 (22.7)*		88 (52.1)*
AFLP6C					8 (100)*			6 (75.0)*	2 (25.0)*			8 (4.7)*
AFLP7	7 (63.6)	2 (18.2)		2 (18.2)				10 (90.9)	1 (9.1)			11 (3.1)
AFLP8	2 (33.3)			2 (33.3)		2 (33.3)		6 (100)				6 (1.7)
AFLP9				2 (66.7)	1 (33.3)			3 (100)				3 (0.9)
AFLP10						2 (100)		2 (100)				2 (0.6)
Total	28 (8.0)	22 (6.3)	20 (5.7)	101 (28.9)	95 (27.1)	81 (23.1)	3 (0.9)	215 (61.4)	73 (20.9)	57 (16.3)	5 (1.4)	350 (100)

[a] An asterisk indicates strains that belong to one of the subgenotypes within AFLP6, namely, AFLP6A, AFLP6B, or AFLP6C; these strains are the cumulative number of strains in the main genotype AFLP6 group.

2 weeks with a combination of amphotericin B and flucytosine, followed by a 10-week consolidation therapy with fluconazole (11, 24).

Cryptococcus neoformans has been extensively studied for its *in vitro* susceptibility to a wide variety of antifungal compounds, including the new triazoles posaconazole, voriconazole, ravuconazole, and isavuconazole (12, 14, 28, 29, 33). Despite the ongoing *C. gattii* outbreak, only a few studies using relatively small sets of *C. gattii* isolates have been performed to investigate their *in vitro* susceptibilities to amphotericin B, flucytosine, fluconazole, and the new triazole antifungals (12, 15, 28–30). A few studies divided the *C. gattii* isolates into groups according to their serotype or genotype (15, 29).

Therefore, we studied the *in vitro* susceptibilities of each of the *C. gattii* genotypes from a large worldwide collection, subdivided by AFLP genotyping, to amphotericin B, flucytosine, fluconazole, itraconazole, voriconazole, posaconazole, and the new experimental broad-spectrum antifungal triazole isavuconazole.

MATERIALS AND METHODS

Strains and media. A worldwide collection of 350 *C. gattii* strains, represented by 215 clinical isolates (61.4%), 57 veterinary isolates (16.3%), 73 environmental isolates (20.9%), and 5 isolates from an unknown source (1.4%), were included in this study. Strains were obtained from the public culture collections of the CBS-KNAW Fungal Biodiversity Centre (Utrecht, Netherlands), BCCM-IHEM Scientific Institute of Public Health (Brussels, Belgium), as well as the working group collections of the authors. According to their geographical origins, the *C. gattii* strains were grouped as being of African (n = 28; 8.0%), Asian (n = 22; 6.3%), Australian (n = 20; 5.7%), European (n = 101; 28.9%), North American (n = 95; 27.1%), South American (n = 81; 23.1%), and unknown (n = 3; 0.9%) origin. The group of North American strains contained a set of 75 (24.1%) *C. gattii* strains related to the ongoing Vancouver Island outbreak. The distributions of the *C. gattii* strains, according to their geographical origins and sources of isolation, are listed in Table 1. The strains were checked for purity and were cultivated on malt extract agar medium (MEA; Oxoid, Basingstoke, United Kingdom). After inoculation, the cultures were incubated for 2 days at 30°C. A working collection was made by growing the *C. gattii* strains on MEA slants for 2 days at 30°C, after which the strains were stored at 4°C.

Genomic DNA extraction. An extraction procedure previously described by Bolano et al. (3) was further optimized to obtain high-quality genomic DNA. *C. gattii* strains were subcultured for 2 days at 30°C on yeast extract (1%)–peptone (1%)–D-glucose (2%) agar supplemented with 0.5 M NaCl to prevent the formation of a polysaccharide capsule. Approximately 150 μl of *C. gattii* cells was incubated for 3 h in 1.6 ml urea solution (10.7 M) at room temperature. After centrifugation at 4,000 × *g* for 2 min, the pellet was resuspended in 750 μl of lysis buffer (10% [wt/vol] sodium dodecyl sulfate, 10% [wt/vol] sodium Sarkosyl in TE [Tris-EDTA] buffer, pH 8.0) and 750 μl phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0). The cells were disrupted by bead beating at full speed (TissueLyser; Qiagen, Venlo, Netherlands) for 5 min. After centrifugation at 17,000 × *g* for 15 min at 4°C, approximately 750 μl supernatant was added to an equal volume of ice-cold 96.2% ethanol and 100 μl ice-cold 3.0 M sodium acetate. Precipitation of genomic DNA was accelerated by incubating the solution at −20°C for 1 h. Genomic DNA was precipitated by centrifugation at 17,000 × *g* for 15 min at 4°C, the pellet obtained was dissolved in 100 μl Tris-EDTA buffer (pH 8.0), and 1 μl RNase (Invitrogen, Breda, Netherlands) was added, followed by an incubation step at 37°C to degrade any remaining RNA. The quality and quantity of genomic DNA were measured using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), and final working solutions, each with a concentration of 100 ng/μl, were prepared.

Mating typing, serotyping, and genotyping. The mating types of all isolates were determined by partial amplification of the *STE12* locus and either the *MAT***a** or the *MAT*α allele using two different primer sets, as described by Bovers et al. (5). Briefly, 1 μl genomic DNA (100 ng/μl) was added to 24 μl PCR mixture consisting of 17.8 μl double-distilled H_2O, 0.75 μl $MgCl_2$ (50 mM; Bioline, London, United Kingdom), 2.5 μl 10× PCR buffer (Bioline), 1.9 μl deoxynucleoside triphosphates (1 mM; Bioline), 0.1 μl *Taq* DNA polymerase (5 U/μl; Bioline), and 0.5 μl of the forward and reverse primers (Biolegio, Nijmegen, Netherlands) for either the *STE12*α allele (forward primer STE12α-F809 [5′-TTGA CCTTTTRTTCCGCAATG-3′] and reverse primer STE12α-R1607 [5′-TTTCT TCTCCCCTGTTTATAGGC-3′]) or the *STE12***a** allele (forward primer STE12**a**-F537 [5′-GTTCTTTGGAATGGCTTATTTCATAT-3′] and reverse primer STE12**a**-R1299 [5′-GMCTTGCGTGGATCATATCTA-3′]). PCRs were carried out with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min and then 72°C for 5 min. The reaction mixture was finally cooled to 21°C. As a control, *C. gattii* mating type α strains CBS6956 and WM276 (CBS10510) and mating-type **a** strains CBS1930 and E566 (CBS11233) were included.

A Cryptocheck agglutination test kit (Iatron Laboratories, Tokyo, Japan) was used to determine the serotypes of those *C. gattii* isolates with contradictory serotype data.

The genotype of each *C. gattii* isolate was determined using AFLP fingerprint analysis, as described previously by Boekhout et al. (2). *C. gattii* isolates WM178 (CBS10082; AFLP6/VGII), A1M-R265 (CBS10514; AFLP6A/VGIIa), A1M-R272 (CBS10865; AFLP6B/VGIIb), A6M-R38 (CBS11545; AFLP6C/VGIIc), WM161 (CBS10081; AFLP5/VGIII), WM179 (CBS10078; AFLP4/VGI), WM779 (CBS10101; AFLP7/VGIV), CBS10488 (AFLP8), CBS10496 (AFLP9), and IHEM14941S (CBS11687; AFLP10) were used as controls to assign the AFLP genotypes to all *C. gattii* isolates (2, 8, 13, 20, 21).

In vitro susceptibility testing. The MICs of amphotericin B (Bristol Myers Squibb, Woerden, Netherlands), flucytosine (Valeant Pharmaceuticals, Zoetermeer, Netherlands), fluconazole and voriconazole (Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (Janssen Cilag, Tilburg, Netherlands), posaconazole (Schering-Plough Corp., Kenilworth, NJ), and isavuconazole (Basilea Pharmaceutica, Basel, Switzerland) were determined using the broth microdilution method, in accordance with the guidelines in CLSI document M27-A3 (9). Drug-free and sterile controls were included. *Paecilomyces variotii* (ATCC 22319) and *Candida krusei* (ATCC 6258, CBS573) were used for quality control. The microtiter plates were incubated for 72 h at 35°C under aerobic conditions.

Data analysis. Values for the MIC_{50} and MIC_{90} were obtained by ordering the MIC data for each antifungal in ascending arrays and selecting the median and 90th quantile, respectively, of the MIC distribution. Geometric mean MICs were computed using Microsoft Office Excel 2007 software, for which purpose values of $<x$ were set equal to $0.5x$ and values more than $>y$ were set equal to $2y$ (x and y are the lowest and highest MICs, respectively, in the range). A two-tailed Mann-Whitney-Wilcoxon test was applied to compare the MIC values between different *C. gattii* genotypes; and a multivariate analysis of variance (MANOVA) test was applied to compare geographical origins, AFLP genotype profiles, and MIC values (StatistiXL software, version 1.8, StatistiXL, Nedland, WA, Australia). A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Mating typing, serotyping, and genotyping. On the basis of the AFLP genotypes, the 350 *C. gattii* isolates were divided into five haploid AFLP genotypes ($n = 341$; 97.4%) and two hybrid AFLP genotypes ($n = 9$; 2.6%), namely, AFLP4 ($n = 137$; 39.1%), AFLP5 ($n = 22$; 6.3%), AFLP6 ($n = 169$; 48.3%), AFLP7 ($n = 11$; 3.1%), AFLP8 ($n = 6$; 1.7%), AFLP9 ($n = 3$; 0.9%), and AFLP10 ($n = 2$; 0.6%). The majority of the 215 clinical *C. gattii* isolates belonged to either genotype AFLP4 ($n = 76$; 35.4%) or genotype AFLP6 ($n = 103$; 47.9%). Strains that clustered within genotype AFLP6 could be further divided into the three subgenotypes AFLP6A ($n = 73$; 43.2%), AFLP6B ($n = 88$; 52.1%), and AFLP6C ($n = 8$; 4.7%) (Table 1).

The serotypes of the genotype AFLP7 strains were investigated in more detail, since several of these isolates were, according to the literature, known to be serotypes B and C (6, 21, 23). However, we determined that all *C. gattii* AFLP7 isolates were serotype C. The two genotype AFLP10 strains were determined to be serotype B. Within the other AFLP genotypes, no contradictions from the literature were found, and serotyping was omitted for these groups.

The mating types for all *C. gattii* isolates were determined by amplification of either the *STE12***a** or the *STE12*α allele. This revealed that within the group of 341 haploid *C. gattii* strains (genotypes AFLP4 to AFLP7 and AFLP10), the mating type α strains ($n = 296$; 86.8%) were dominant over the mating type **a** strains ($n = 45$; 13.2%).

***In vitro* susceptibility testing.** MIC ranges, MIC_{50}s, geometric mean MICs, and MIC_{90}s related to AFLP genotype are presented in Table 2. For each antifungal for each AFLP genotype examined, the MIC_{50} and geometric mean MIC values differed by <1 $\log_2$ dilution step, indicating that in all cases the MIC_{50}s obtained by inspection reasonably reflect the central tendency of antifungal susceptibility of the population.

Isavuconazole had the lowest MIC_{90}s for all *C. gattii* isolates surveyed by at least 1 $\log_2$ dilution step. The overall ranges of MICs for each of the seven drugs were <0.016 to 1 μg/ml for amphotericin B, 0.125 to >64 μg/ml for flucytosine, 0.25 to >64 μg/ml for fluconazole, <0.016 to 1 μg/ml for itraconazole and voriconazole, and < 0.016 to 0.5 μg/ml for posaconazole and isavuconazole. The geometric mean MIC values varied widely when they were calculated for all 350 *C. gattii* strains (Table 2). The highest geometric mean MIC was 3.573 μg/ml for fluconazole, followed by geometric mean MICs of 2.896 μg/ml for flucytosine and 0.272 μg/ml for amphotericin B and much lower geometric mean MICs for itraconazole and the new triazoles voriconazole, posaconazole, and isavuconazole (0.124, 0.108, 0.113, and 0.051 μg/ml, respectively).

Comparison of geometric mean MIC values of the *C. gattii* strains obtained from clinical, environmental, and veterinary sources revealed that the last category of strains had decreased susceptibility to all seven antifungal drugs investigated except flucytosine in comparison to the susceptibilities of the clinical and environmental strains. When the susceptibilities of the clinical versus veterinary *C. gattii* strains and the environmental versus veterinary *C. gattii* strains were compared, these differences were significant for itraconazole ($P = 0.026$ and $P = 0.02$, respectively) and the novel triazoles posaconazole ($P = 0.008$ and $P = 0.02$, respectively), voriconazole ($P = 0.013$ and $P = 0.008$, respectively), and isavuconazole ($P = 0.004$ and $P = 0.002$, respectively).

A MANOVA showed that there was a significant difference ($P < 0.001$) between the susceptibility values obtained for the seven antifungal compounds and the geographical origins of the *C. gattii* strains (Table 3). It was observed that the MIC values of amphotericin B and flucytosine contributed to this significant difference ($P = 0.003$ and $P = 0.02$, respectively). This difference was caused by *C. gattii* strains from Europe, which differed significantly in their susceptibility to amphotericin B compared to the susceptibilities of strains from Africa ($P = 0.002$), Asia ($P = 0.014$), Australia ($P = 0.041$), North America ($P < 0.001$), and South America ($P = 0.019$), which were found to be less susceptible. For flucytosine, North American *C. gattii* strains had a significantly higher geometric mean MIC than African ($P < 0.001$), Asian, ($P < 0.001$), European ($P < 0.001$), and South American ($P = 0.001$) strains (Table 3). When all *C. gattii* genotype AFLP4 and AFLP6 strains (Table 2) were analyzed by MANOVA, it was observed that it was not the geographical origin but the genotype that contributed to the significant differences in antifungal susceptibility patterns ($P < 0.001$). The antifungal drugs amphotericin B ($P < 0.001$), fluconazole ($P < 0.001$), itraconazole ($P = 0.038$), voriconazole ($P = 0.001$), and isavuconazole ($P = 0.001$) were found to be highly significant contributors to this phenotypic difference between AFLP4 and AFLP6 *C. gattii* strains.

Clinical strains that belonged to the genotype AFLP6 cluster had a significantly ($P < 0.05$) higher geometric mean MIC than strains of genotype AFLP4 for six of the seven antifungal compounds tested. However, both genotypes had almost identical geometric mean MICs for posaconazole (0.112 and 0.124 μg/ml, respectively; $P = 0.391$). Strikingly, clinical AFLP6 isolates had high geometric mean MICs of 4.961 and 5.638 μg/ml for flucytosine and fluconazole, respectively, compared to those for clinical isolates within genotype AFLP4 (1.401 and 2.467 μg/ml, respectively).

C. gattii strains within the Vancouver outbreak-related genotype, genotype AFLP6, were divided into the three defined subgenotypes AFLP6A ($n = 73$; 43.2%), AFLP6B ($n = 88$;

TABLE 2. MIC ranges, MIC_{50}s, MIC_{90}s, and geometric mean MICs for all 350 *C. gattii* strains and AFLP genotypes

Strain	Drug	MIC (μg/ml)			
		Range	50%	Geometric mean	90%
All *C. gattii* strains (n = 350)	Amphotericin B	<0.016–1	0.25	0.272	0.5
	Flucytosine	0.125–>64	2	2.896	8
	Fluconazole	0.25–64	4	3.573	8
	Itraconazole	<0.016–1	0.125	0.124	0.25
	Voriconazole	<0.016–1	0.125	0.108	0.25
	Posaconazole	<0.016–0.5	0.125	0.113	0.25
	Isavuconazole	<0.016–0.5	0.063	0.051	0.125
C. gattii AFLP4 (n = 137)	Amphotericin B	<0.016–0.5	0.25	0.238	0.5
	Flucytosine	0.125–16	1	1.667	4
	Fluconazole	0.25–16	2	2.550	8
	Itraconazole	<0.016–0.5	0.125	0.119	0.25
	Voriconazole	<0.016–0.25	0.063	0.090	0.25
	Posaconazole	<0.016–0.5	0.125	0.123	0.25
	Isavuconazole	<0.016–0.5	0.031	0.044	0.125
C. gattii AFLP5 (n = 22)	Amphotericin B	0.125–0.5	0.25	0.214	0.5
	Flucytosine	0.5–4	2	1.938	4
	Fluconazole	0.25–8	2	2.064	4
	Itraconazole	<0.016–0.25	0.063	0.078	0.125
	Voriconazole	<0.016–0.125	0.063	0.059	0.125
	Posaconazole	0.031–0.25	0.063	0.076	0.125
	Isavuconazole	<0.016–0.063	0.031	0.023	0.063
C. gattii AFLP6 (n = 169)	Amphotericin B	0.125–1	0.25	0.312	0.5
	Flucytosine	0.5–>64	4	5.033	8
	Fluconazole	0.5–64	4	5.137	16
	Itraconazole	<0.016–1	0.125	0.148	0.5
	Voriconazole	<0.016–1	0.125	0.142	0.25
	Posaconazole	<0.016–0.5	0.125	0.119	0.25
	Isavuconazole	<0.016–0.5	0.063	0.067	0.25
C. gattii AFLP6A (n = 73)	Amphotericin B	0.125–0.5	0.25	0.299	0.5
	Flucytosine	1–16	4	5.268	8
	Fluconazole	0.5–32	4	4.235	8
	Itraconazole	<0.016–1	0.125	0.123	0.5
	Voriconazole	<0.016–1	0.125	0.117	0.25
	Posaconazole	<0.016–0.5	0.125	0.105	0.25
	Isavuconazole	<0.016–0.5	0.063	0.050	0.25
C. gattii AFLP6B (n = 88)	Amphotericin B	0.125–1	0.25	0.319	0.5
	Flucytosine	0.5–>64	4	4.609	8
	Fluconazole	1–16	8	5.438	16
	Itraconazole	<0.016–0.5	0.25	0.162	0.25
	Voriconazole	<0.016–0.5	0.125	0.152	0.25
	Posaconazole	<0.016–0.25	0.125	0.124	0.25
	Isavuconazole	<0.016–0.25	0.063	0.078	0.25
C. gattii AFLP6C (n = 8)	Amphotericin B	0.25–0.5	0.25	0.354	0.5
	Flucytosine	4–16	8	8.724	16
	Fluconazole	8–64	16	16.000	64
	Itraconazole	0.25–0.5	0.25	0.297	0.5
	Voriconazole	0.25–1	0.25	0.354	1
	Posaconazole	0.125–0.5	0.25	0.229	0.5
	Isavuconazole	0.063–0.5	0.25	0.193	0.5
C. gattii AFLP7 (n = 11)	Amphotericin B	0.125–0.5	0.5	0.343	0.5
	Flucytosine	1–8	2	2.269	8
	Fluconazole	0.5–32	4	4.537	16
	Itraconazole	<0.016–0.25	0.125	0.104	0.25
	Voriconazole	0.031–0.25	0.125	0.117	0.25
	Posaconazole	0.031–0.25	0.125	0.110	0.25
	Isavuconazole	<0.016–0.25	0.063	0.052	0.125
C. gattii AFLP8–10 (n = 11)	Amphotericin B	0.125–0.5	0.25	0.220	0.25
	Flucytosine	1–8	1	1.656	8
	Fluconazole	0.5–8	2	2.130	8
	Itraconazole	<0.016–0.125	0.063	0.043	0.125
	Voriconazole	<0.016–0.25	0.031	0.059	0.125
	Posaconazole	<0.016–0.125	0.063	0.043	0.125
	Isavuconazole	<0.016–0.063	0.016	0.020	0.063

TABLE 3. *In vitro* susceptibilities of the *C. gattii* strains according to geographical origin

C. gattii strain origin	Drug	MIC (μg/ml)			
		Range	50%	Geometric mean	90%
Africa (n = 28)	Amphotericin B	0.125–0.5	0.25	0.320	0.5
	Flucytosine	1–16	2	2.378	8
	Fluconazole	0.25–32	4	4.308	8
	Itraconazole	<0.016–0.25	0.125	0.111	0.25
	Voriconazole	<0.016–0.25	0.125	0.114	0.25
	Posaconazole	<0.016–0.25	0.125	0.122	0.25
	Isavuconazole	<0.016–0.5	0.063	0.058	0.125
Asia (n = 22)	Amphotericin B	0.125–0.5	0.25	0.312	0.5
	Flucytosine	0.5–16	2	2	8
	Fluconazole	0.5–16	2	2.741	8
	Itraconazole	0.031–0.5	0.125	0.1	0.250
	Voriconazole	0.031–0.25	0.063	0.08	0.250
	Posaconazole	0.063–0.5	0.125	0.107	0.250
	Isavuconazole	0.016–0.25	0.031	0.044	0.125
Australia (n = 20)	Amphotericin B	0.125–0.5	0.25	0.297	0.5
	Flucytosine	1–32	2	3.138	16
	Fluconazole	0.5–16	2	2.639	8
	Itraconazole	0.063–0.5	0.125	0.134	0.25
	Voriconazole	0.031–0.25	0.125	0.091	0.25
	Posaconazole	0.031–0.5	0.125	0.121	0.25
	Isavuconazole	0.016–0.125	0.063	0.049	0.125
Europe (n = 101)	Amphotericin B	<0.016–1	0.25	0.223	0.5
	Flucytosine	0.125–>64	2	2.263	8
	Fluconazole	0.5–32	4	3.301	8
	Itraconazole	<0.016–0.5	0.125	0.137	0.25
	Voriconazole	<0.016–1	0.125	0.112	0.25
	Posaconazole	<0.016–0.5	0.125	0.131	0.25
	Isavuconazole	<0.016–0.5	0.063	0.055	0.125
North America (n = 95)	Amphotericin B	0.125–0.5	0.25	0.300	0.5
	Flucytosine	1–16	4	4.334	8
	Fluconazole	0.5–64	4	4.240	8
	Itraconazole	<0.016–1	0.125	0.121	0.25
	Voriconazole	<0.016–1	0.125	0.114	0.25
	Posaconazole	<0.016–0.5	0.125	0.103	0.25
	Isavuconazole	<0.016–0.5	0.063	0.047	0.25
South America (n = 81)	Amphotericin B	0.125–1	0.25	0.277	0.5
	Flucytosine	0.5–16	4	2.865	8
	Fluconazole	0.25–16	4	3.548	16
	Itraconazole	<0.016–1	0.125	0.126	0.25
	Voriconazole	<0.016–0.5	0.125	0.111	0.25
	Posaconazole	<0.016–0.5	0.125	0.104	0.25
	Isavuconazole	<0.016–0.5	0.063	0.051	0.25

52.1%), and AFLP6C (n = 8; 4.7%). Significant differences in the geometric mean MICs between *C. gattii* AFLP6A and AFLP6B strains were found for all antifungal drugs except amphotericin B (P = 0.398) and flucytosine (P = 0.091); however, the geometric mean MICs were within 1 $\log_2$ dilution step. There was also no significant difference in amphotericin B MICs when AFLP6A and AFLP6C strains (P = 0.353) and AFLP6B and AFLP6C strains (P = 0.607) were compared, while all other antifungal compounds tested showed significant differences between these subgenotypes (Table 2). However, the number of strains within subgenotype AFLP6C is too small to compare its antifungal susceptibility characteristics with those of the larger group of strains within genotypes AFLP6A and AFLP6B.

DISCUSSION

Despite the fact that *C. gattii* has become an important primary pathogen due to the expanding outbreak in British Columbia (Canada) and the Pacific Northwest (United States), there is a lack of in-depth studies on the susceptibilities of large sets of *C. gattii* strains to conventional and new antifungal compounds (10). In particular, the relation between susceptibility profiles and the different genotypes within the *C. gattii* complex has rarely been studied and has been studied with only low numbers of isolates (15). In this study, we have compared the susceptibilities of 350 *C. gattii* isolates collected from different geographical regions and sources (e.g., clinical, veterinary, and environmental) to four clinically used antifungal

compounds, i.e., amphotericin B, flucytosine, fluconazole, and itraconazole, and the three novel triazoles voriconazole, posaconazole, and isavuconazole. The older antifungal drugs, amphotericin B, flucytosine, and fluconazole, had lower levels of activity against all *C. gattii* isolates than itraconazole and the novel triazoles, as visualized by their geometric mean MIC values (Table 2). The observation that flucytosine and fluconazole had poorer antifungal activity against *C. gattii*, as well as against its sibling, *C. neoformans*, was a consistent finding in previous studies (12, 14, 15, 25, 29, 30). For testing of *C. neoformans* susceptibility to amphotericin B, flucytosine, and fluconazole, so-called surrogate breakpoints were introduced by Pfaller et al. (25). *C. neoformans* was regarded to be highly susceptible to amphotericin B when the MIC *in vitro* was ≤1 μg/ml. In this study, none of the 350 *C. gattii* strains showed higher *in vitro* MICs, and thus, all isolates remained highly susceptible. Similar findings were reported in three other studies, which tested lower numbers of isolates (15, 29, 30). On the contrary, decreased *in vitro* susceptibility (≥16 μg/ml) to fluconazole was observed for 26 strains (7.4%), and only one clinical isolate within the AFLP6C cluster appeared to have high-level resistance (MICs, ≥64 μg/ml) to fluconazole *in vitro*. With respect to flucytosine, the number of strains within the group of isolates with decreased susceptibility (MICs, ≥8 μg/ml) was 84 (24.3%), of which 9 (2.57%) belong to the category of high-level-resistant isolates (MICs, ≥32 μg/ml).

The majority of *C. gattii* isolates in the present study belong to either genotype AFLP4 (n = 137; 39.1%) or AFLP6 (n = 169; 48.3%), while the remaining 44 (12.6%) *C. gattii* isolates belong to one of the minor genotypes (AFLP5 or AFLP7 to AFLP10) that are more restricted to certain geographical regions and which less frequently cause infections (7, 27). The preponderance toward *C. gattii* strains with an AFLP4 or AFLP6 genotype can be interpreted as being the result of the analysis of a nonrepresentative selection of strains. This apparent overrepresentation might be due to the inclusion of preserved culture collection strains from well-studied geographical localities, such as Australia and the *C. gattii* outbreak region in North America (27). These localities were repeatedly found to harbor more genotype AFLP4 or AFLP6 strains rather than strains of genotype AFLP5 or AFLP7 (15, 16, 27). The South American continent has been shown to be a melting pot of known haploid AFLP genotypes compared to the diversity of genotypes in other localities (21, 23, 27). However, preserved *C. gattii* strains from the less well studied regions, Africa, Asia, and Europe, were found to follow the trend that strains with an AFLP4 genotype profile were more frequently isolated than others (13, 27). Hence, it appears that *C. gattii* AFLP4 and AFLP6 strains are collected and preserved more frequently and that the availability and exchangeability of strains can influence studies toward a specific genotype (13, 27). It might influence the outcome of susceptibility values when only the geographical origin is taken into consideration, while a combined analysis of the geographical origin with a genotyping method (e.g., RFLP, AFLP, or MLST) will reveal more subtle differences, as has been shown in the current study.

When only the clinical *C. gattii* strains from the two major genotypes, genotypes AFLP4 and AFLP6, were compared, it appeared that strains belonging to AFLP6 (n = 103) had significantly ($P < 0.05$) higher geometric mean MICs for six of the antifungal compounds tested than strains belonging to genotype AFLP4 (n = 76). However, both genotypes had almost identical geometric mean MICs for posaconazole (0.112 and 0.124 μg/ml, respectively). Strikingly, clinical AFLP6 isolates had higher geometric mean MICs of 4.961 and 5.638 μg/ml for flucytosine and fluconazole, respectively, than clinical isolates within genotype AFLP4 (1.401 and 2.467 μg/ml, respectively). Similar results were recently reported for a collection of 43 North American *C. gattii* isolates (15). In the current study, fluconazole and flucytosine had the lowest levels of activity against the *C. gattii* strains. Itraconazole, which has been used clinically to treat cryptococcosis, had a MIC_{90} of only 0.25 μg/ml, which is 1 or 2 $\log_2$ dilutions lower than the MIC_{90}s previously found by others (15, 30). Isavuconazole, posaconazole, and voriconazole demonstrated excellent potency against each isolate and all AFLP genotypes, including isolates with reduced fluconazole susceptibilities.

The results for voriconazole (MIC_{90}, 0.25 μg/ml) were comparable to those of other studies (15, 29, 30) and 1 $\log_2$ dilution higher than that found in one study (22). Posaconazole MICs differed by a maximum of 2 $\log_2$ dilution steps between this and previous studies. Isavuconazole had the lowest MIC_{90} (0.125 μg/ml) in this large study, and the MIC_{90} was within 1 $\log_2$ dilution of that recently reported (MIC_{90}, 0.063 μg/ml) by Thompson et al. (29). When isolates were tested by Etest, these authors also found a MIC_{90} of 0.125 μg/ml (28). Isavuconazole has even better *in vitro* activity against *C. neoformans*, a finding which also holds true for the other antifungal compounds (14).

All antifungal compounds tested were active against all *C. gattii* genotypes; however, some strains showed high MICs, as was the case for amphotericin B (up to 1 μg/ml), flucytosine (>64 μg/ml), fluconazole (64 μg/ml), and itraconazole and voriconazole (1 μg/ml). The two novel triazoles isavuconazole and posaconazole had relatively low MIC values compared to those of the other antifungal compounds tested. We conclude that amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole were the drugs that were the most active against *C. gattii* isolates *in vitro*. Isavuconazole, with its attractive pharmacological and toxicological properties and availability in oral and parenteral formulations, may be a welcome addition to the clinician's antifungal armamentarium to fight this pathogen.

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REFERENCES

1. **Bartlett, K. H., S. E. Kidd, and J. W. Kronstad.** 2008. The emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest. Curr. Infect. Dis. Rep. **10:**58–65.
2. **Boekhout, T., B. Theelen, M. Diaz, J. W. Fell, W. C. Hop, E. C. Abeln, F. Dromer, and W. Meyer.** 2001. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology **147:**891–907.
3. **Bolano, A., S. Stinchi, R. Preziosi, F. Bistoni, M. Allegrucci, F. Baldelli, A. Martini, and G. Cardinali.** 2001. Rapid methods to extract DNA and RNA from *Cryptococcus neoformans*. FEMS Yeast Res. **1:**221–224.
4. **Bovers, M., F. Hagen, E. E. Kuramae, H. L. Hoogveld, F. Dromer, G. St.-Germain, and T. Boekhout.** 2008. AIDS patient death caused by novel *Cryptococcus neoformans* × *C. gattii* hybrid. Emerg. Infect. Dis. **14:**1105–1108.
5. **Bovers, M., F. Hagen, E. E. Kuramae, M. R. Diaz, L. Spanjaard, F. Dromer, H. L. Hoogveld, and T. Boekhout.** 2006. Unique hybrids between the fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii*. FEMS Yeast Res. **6:**599–607.
6. **Bovers, M., F. Hagen, E. E. Kuramae, and T. Boekhout.** 2008. Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. Fungal Genet. Biol. **45:**400–421.
7. **Bovers, M., F. Hagen, and T. Boekhout.** 2008. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. Rev. Iberoam. Micol. **25:**S4–S12.
8. **Byrnes, E. J., R. J. Bildfell, S. A. Frank, T. G. Mitchell, K. A. Marr, and J. Heitman.** 2009. Molecular evidence that the range of the Vancouver Island outbreak of *Cryptococcus gattii* infection has expanded into the Pacific Northwest in the United States. J. Infect. Dis. **199:**1081–1086.
9. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Datta, K., K. H. Bartlett, R. Baer, E. J. Byrnes, E. Galanis, J. Heitman, L. Hoang, M. J. Leslie, L. MacDougall, S. S. Magill, M. G. Morshed, K. A. Marr, and the *Cryptococcus gattii* Working Group of the Pacific Northwest.** 2009. Spread of *Cryptococcus gattii* into Pacific Northwest region of the United States. Emerg. Infect. Dis. **15:**1185–1191.
11. **Dromer, F., C. Bernede-Bauduin, D. Guillemot, O. Lortholary, and the French Cryptococcosis Study Group.** 2008. Major role for amphotericin B-flucytosine combination in severe cryptococcosis. PLoS One **3:**e2870.
12. **Gomez-Lopez, A., O. Zaragoza, M. Dos Anjos Martins, M. C. Melhem, J. L. Rodriguez-Tudela, and M. Cuenca-Estrella.** 2008. In vitro susceptibility of *Cryptococcus gattii* clinical isolates. Clin. Microbiol. Infect. **14:**727–730.
13. **Hagen, F., A. G. Burggraaf, A. Kamermans, J. Sweere, K. Tintelnot, D. Swinne, F. Dromer, R. Iatta, M. T. Montagna, J. M. Torres-Rodriguez, M. A. Viviani, A. Velegraki, M. F. Colom Valiente, M. Gari-Toussaint, and T. Boekhout.** 2009. The occurrence of the primary pathogenic yeast *Cryptococcus gattii* in Europe. Mycoses **52**(Suppl. 1)**:**57.
14. **Illnait-Zaragozi, M. T., G. F. Martínez, I. Curfs-Breuker, C. M. Fernández, T. Boekhout, and J. F. Meis.** 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.
15. **Iqbal, N., E. E. DeBess, R. Wohrle, B. Sun, R. J. Net, A. M. Ahlquist, T. Chiller, and S. R. Lockhart.** 2010. Correlation of genotype and in vitro susceptibilities of *Cryptococcus gattii* from the Pacific Northwest of the United States. J. Clin. Microbiol. **48:**539–544.
16. **Kidd, S. E., F. Hagen, R. L. Tscharke, M. Huynh, K. H. Bartlett, M. Fyfe, L. Macdougall, T. Boekhout, K. J. Kwon-Chung, and W. Meyer.** 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc. Natl. Acad. Sci. U. S. A. **101:**17258–17263.
17. **Kwon-Chung, K. J., and J. E. Bennett.** 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. Am. J. Epidemiol. **120:**123–130.
18. **Kwon-Chung, K. J., I. Polacheck, and J. E. 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.
19. **Ma, H., and R. C. May.** 2009. Virulence in *Cryptococcus* species. Adv. Appl. Microbiol. **67:**131–190.
20. **Meyer, W., D. M. Aanensen, T. Boekhout, M. Cogliati, M. R. Diaz, M. C. Esposto, M. Fisher, F. Gilgado, F. Hagen, S. Kaocharoen, A. P. Litvintseva, T. G. Mitchell, S. P. Simwami, L. Trilles, M. A. Viviani, and K. J. Kwon-Chung.** 2009. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med. Mycol. **47:**561–570.
21. **Meyer, W., A. Castañeda, S. Jackson, M. Huynh, E. Castañeda, and the IberoAmerican Cryptococcal Study Group.** 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg. Infect. Dis. **9:**189–195.
22. **Morera-López, Y., J. M. Torres-Rodríguez, T. Jiménez-Cabello, and T. Baró-Tomás.** 2005. *Cryptococcus gattii*: in vitro susceptibility to the new antifungal albaconazole versus fluconazole and voriconazole. Med. Mycol. **43:**505–510.
23. **Ngamskulrungroj, P., F. Gilgado, J. Faganello, A. P. Litvintseva, A. L. Leal, K. M. Tsui, T. G. Mitchell, M. H. Vainstein, and W. Meyer.** 2009. Genetic diversity of the *Cryptococcus* species complex suggests that *Cryptococcus gattii* deserves to have varieties. PLoS One **4:**e5862.
24. **Perfect, J. R., W. E. Dismukes, F. Dromer, D. L. Goldman, J. R. Graybill, R. J. Hamill, T. S. Harrison, R. A. Larsen, O. Lortholary, M. H. Nguyen, P. G. Pappas, W. G. Powderly, N. Singh, J. D. Sobel, and T. C. Sorrell.** 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.
25. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, G. V. Doern, and D. J. Diekema.** 2005. Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004). J. Clin. Microbiol. **43:**2163–2167.
26. **Sorrell, T. C.** 2001. *Cryptococcus neoformans* variety *gattii*. Med. Mycol. **39:**155–168.
27. **Springer, D. J., and V. Chaturvedi.** 2010. Projecting global occurrence of *Cryptococcus gattii*. Emerg. Infect. Dis. **16:**14–20.
28. **Thompson, G. R., III, A. W. Fothergill, N. P. Wiederhold, A. C. Vallor, B. L. Wickes, and T. F. Patterson.** 2008. Evaluation of Etest method for determining isavuconazole MICs against *Cryptococcus gattii* and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **52:**2959–2961.
29. **Thompson, G. R., III, N. P. Wiederhold, A. W. Fothergill, A. C. Vallor, B. L. Wickes, and T. F. Patterson.** 2009. Antifungal susceptibilities among different serotypes of *Cryptococcus gattii* and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **53:**309–311.
30. **Trilles, L., B. Fernandez-Torres, M. dos Santos Lazera, B. Wanke, and J. Guarro.** 2004. In vitro antifungal susceptibility of *Cryptococcus gattii*. J. Clin. Microbiol. **42:**4815–4817.
31. **Van Elden, L. J., A. M. Walenkamp, M. M. Lipovsky, P. Reiss, J. F. Meis, S. de Marie, J. Dankert, and A. I. Hoepelman.** 2000. Declining number of patients with cryptococcosis in the Netherlands in the era of highly active antiretroviral therapy. AIDS **14:**2787–2788.
32. **Velagapudi, R., Y. P. Hsueh, S. Geunes-Boyer, J. R. Wright, and J. Heitman.** 2009. Spores as infectious propagules of *Cryptococcus neoformans*. Infect. Immun. **77:**4345–4355.
33. **Yamazumi, T., M. A. Pfaller, S. A. Messer, A. Houston, R. J. Hollis, and R. N. Jones.** 2000. In vitro activities of ravuconazole (BMS-207147) against 541 clinical isolates of *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **44:**2883–2886.