

Heteroresistance of *Cryptococcus gattii* to Fluconazole[▽]

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We analyzed 71 clinical and environmental *Cryptococcus gattii* strains that had been isolated before or after the advent of azole antifungals to determine their level of heteroresistance to fluconazole (LHF). All strains of *C. gattii* manifested heteroresistance, with LHF's that ranged between 4 $\mu\text{g/ml}$ and 32 $\mu\text{g/ml}$. A considerably higher proportion of the *C. gattii* strains (86%) than *Cryptococcus neoformans* strains (46%) exhibited LHF's that were $\geq 16 \mu\text{g/ml}$. No significant correlation was observed between the molecular type or serotypes of strains and their respective LHF. The strains which expressed a higher LHF were also more resistant to xenobiotics than the strains with a low LHF, and the level of resistance to xenobiotics was significantly higher than that reported for *C. neoformans*. The heteroresistant subpopulation, whose level of drug resistance had been raised in a stepwise manner to 64 $\mu\text{g/ml}$, reverted to the original LHF upon daily transfers in drug-free medium. Importantly, the strains with high LHF's were significantly more virulent than those with low LHF's. Since all the clinical isolates that had not been exposed to azole drugs as well as the environmental strains manifested heteroresistance to fluconazole, heteroresistance of *C. gattii* to azoles is an intrinsic mechanism as in *C. neoformans* and is associated with the strain's virulence.

Cryptococcosis is caused by two species, *Cryptococcus neoformans* and *Cryptococcus gattii* (15); fluconazole (FLC), a triazole, is widely used for the treatment of cryptococcosis regardless of the causative species (29). Fluconazole-resistant strains of *C. neoformans* have been increasingly reported from cases of therapy failure in AIDS patients undergoing long-term maintenance therapy (1–3, 27, 36). In 1999, we reported heteroresistance to fluconazole, an adaptive mode of drug resistance, in strains of *C. neoformans* recovered from two patients. One patient had AIDS and suffered from a series of recurrent cryptococcosis episodes during azole maintenance therapy, while the other was a non-AIDS patient who had never been treated with azole drugs (26). Our recent expanded characterization of heteroresistance using over 100 strains of *C. neoformans* that had been isolated at least a decade prior to the advent of azole antifungals revealed that *C. neoformans* is intrinsically heteroresistant to fluconazole. The innate level of heteroresistance to fluconazole (LHF) ranged from 4 $\mu\text{g/ml}$ to 64 $\mu\text{g/ml}$, and 50% of serotype A strains and 21% of serotype D strains manifested high LHF's ($\geq 16 \mu\text{g/ml}$). Importantly, the LHF was observed to correlate with both the virulence of *C. neoformans* strains in mice and resistance to xenobiotics unrelated to fluconazole (31).

Strains of *C. gattii* infect immunocompetent patients more often than *C. neoformans* strains (25), and animal studies have revealed that some strains of *C. gattii* are more virulent than strain H99, a well-characterized highly virulent *C. neoformans* strain of serotype A (10). Although the clinical manifestations of the disease caused by *C. neoformans* and *C. gattii* are not

significantly different, the two species can readily be differentiated by their biochemical, genetic, ecological, and epidemiological characteristics (14). In light of the fact that strains of *C. gattii* infect immunocompetent individuals more often than *C. neoformans* strains and the two species are biologically and genetically distinguishable, we investigated the LHF's of *C. gattii* strains to compare them with those of the *C. neoformans* strains.

In this study, we have analyzed the LHF's of 71 clinical and environmental strains of *C. gattii*, which include serotypes B and C and are comprised of four major molecular types, VGII, VGIII, VGI, and VGIV. The strains were collected from different geographical areas, and all serotype B and the majority of serotype C clinical strains had been isolated at least 10 years prior to the availability of triazole antifungals for therapy of mycosis. As with *C. neoformans*, all strains of *C. gattii* manifested heteroresistance regardless of their chronology or source of isolation. Interestingly, a considerably higher percentage of the *C. gattii* strains (86%) expressed LHF's of $\geq 16 \mu\text{g/ml}$ than that for *C. neoformans* (46%) (27). The strains with high LHF's were more virulent in mice and more resistant to xenobiotics that are unrelated to fluconazole, as reported for *C. neoformans*. However, the degree of resistance to xenobiotics in the *C. gattii* strains with high LHF's was significantly higher than that for the strains of *C. neoformans* with high LHF's.

MATERIALS AND METHODS

***Cryptococcus gattii* strains.** A total of 71 clinical and environmental *C. gattii* strains of serotypes B and C that had been previously isolated from different geographic areas were included in this study (Table 1). All strains were purified by isolating a single colony, and their identity as *C. gattii* was confirmed according to routine diagnostic tests that included serotyping (Iatron Crypto serotyping kit), melanin formation, urease activity, growth at 37°C, and growth on L-canavanine–glycine–bromothymol blue medium (14). A set of standard strains for the molecular typing of *C. gattii* species were used as a reference: WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 161 (serotype B, VGIII), and WM

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TABLE 1. Clinical and environmental *Cryptococcus gattii* strains of serotypes B and C used in this study

Strain	Date of isolation	VG type	Geographic area	Origin	Reference or source	LHF ($\mu\text{g/ml}$)
Serotype B, clinical ($n = 25$)						
NIH76	1965	III	USA	Patient	This study	32
NIH179	<1970 ^a	III	USA	Patient	This study	16
NIH184	1965	III	USA	Patient	This study	16
NIH189	1970	III	USA	Patient	This study	16
NIH190	1970	III	USA	Patient	This study	16
NIH198	1970	III	USA	Patient	This study	16
NIH200	1970	II	USA	Patient	This study	4
NIH254	1970	I	USA	Patient	This study	32
NIH281	1970	II	USA	Patient	This study	4
NIH286	1968	I	USA	Patient	This study	32
NIH367	1970	II	USA	Patient	This study	16
NIH408	1970	I	USA	Patient	This study	16
NIH409	1970	II	USA	Patient	This study	32
NIH412	1970	II	USA	Patient	This study	4
NIH435	1971	I	USA	Patient	This study	16
NIH444	1972	II	USA	Patient	14a	32
NIH487	1970	I	USA	Patient	This study	32
NIH535	1972	IV	USA	Patient	This study	4
NIH653	<1975	I	USA	Patient	This study	32
NIH744	1975	III	USA	Patient	This study	32
NIH754	1976	II	USA	Patient	This study	4
NIH767	1975	II	USA	Patient	This study	32
NIH771	1976	III	USA	Patient	This study	32
B3939	<1970	I	Africa	Patient	15	16
NIH1123	<1970	I	USA	Patient	This study	32
Serotype B, environmental ($n = 25$)						
RB-1	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-3	1999–2002	II	Vancouver, Canada	Douglas fir	13	8
RB-4	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-5	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-9	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-11	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-13	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-14	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-15	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-17	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-18	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-22	1999–2002	II	Vancouver, Canada	Alder	13	16
RB-26	1999–2002	II	Vancouver, Canada	Alder	13	32
RB-33	1999–2002	II	Vancouver, Canada	Douglas Fir	13	32
RB-34	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-35	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RB-8	1999–2002	II	Vancouver, Canada	Douglas fir	13	32
RAM-002		II	Australia		13	32
RAM-005		II	Australia		13	32
RAM-15		II	Australia		W. Meyer	32
VPB571-058		II	Australia		W. Meyer	32
B4506	1989	III	Borosa Valley, Australia	Eucalyptus	17	8
B5763		I	India	Eucalyptus	5	8
B5765		I	India	Eucalyptus	5	16
B5788		I	India	Eucalyptus	5	8
Serotype C, clinical ($n = 17$)						
NIH113	<1966	I	USA	Patient	This study	32
NIH139	<1966	I	USA	Patient	This study	32
NIH178	<1966	I	USA	Patient	This study	32
NIH187	<1966	I	USA	Patient	This study	32
NIH191	<1970	I	USA	Patient	This study	32
NIH257	<1970	III	USA	Patient	This study	4
NIH298	<1970	III	USA	Patient	This study	32
NIH312	<1970	III	USA	Patient	This study	32
NIH401	<1970	III	USA	Patient	This study	32
NIH403	<1970	I	USA	Patient	This study	16
NIH642	1973	III	USA	Patient	This study	32

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TABLE 1—Continued

Strain	Date of isolation	VG type	Geographic area	Origin	Reference or source	LHF ($\mu\text{g/ml}$)
NIH18	1971	I	USA	Patient	This study	32
NIH34	1962	I	USA	Patient	This study	16
NIH917	1977	I	USA	Patient	This study	32
H0058-1-78	1989	III	Arauca, Colombia	Patient	E. Castaneda	32
H0058-1-2023	2003	III	N. Santander, Colombia	Patient	E. Castaneda	32
H0058-1-2086	2004	III	Caldas, Colombia	Patient	E. Castaneda	32
Serotype C, environmental ($n = 4$)						
H0058-1-818	1998	III	Cacuta, Colombia	Almond	E. Castaneda	32
H0058-1-1686	2003	IV	El Colegio, Colombia	Almond	E. Castaneda	32
H0058-1-1875	2003	II	Bogota, Colombia	Eucalyptus	E. Castaneda	32
H0058-1-1941	2003	II	Santa Martha, Colombia	Almond	E. Castaneda	32

^a <, strain was isolated before the year indicated.

779 (serotype C, VGIV) (24). The isolates were stored in glycerol (25%) at -80°C until use and were maintained on YPD (2% glucose, 1% yeast extract, 2% peptone) agar at 25°C during this study.

Genotyping of strains. Yeast cultures were grown overnight in YPD broth at 30°C , and the genomic DNA was extracted as previously described (35). The molecular genotype of the strains was determined by PCR analysis of the microsatellite DNA fingerprint patterns generated by the M13 primer and the restriction fragment length polymorphisms (RFLPs) of the *URA5* gene sequence. PCR fingerprints using the microsatellite specific primer M13 were generated as described previously (24). Amplification products of the reaction were analyzed by electrophoresis on 1.4% agarose gels stained with ethidium bromide (10-mg/ml stock) and visualized under UV light. Based on the major bands in the patterns of the *C. gattii* standard reference strains, the strains were assigned to molecular types VGI to VGIV. To confirm the molecular type determined by M13 fingerprint patterns, the *URA5* gene sequence was amplified by PCR and digested with enzymes HhaI and Sau96I prior to analysis of RFLPs (6, 24).

Analysis of the level of heteroresistance to fluconazole (LHF). FLC powder was provided by Pfizer Global Research & Development (Groton, CT). Stock solutions were prepared in the solvent dimethyl sulfoxide (Sigma) at a concentration of 50 mg/ml. Etests (AB BIODISK, Solna, Sweden) were performed to determine the level of fluconazole susceptibility using YPD agar (31). The LHF was determined for all strains by spot testing on YPD agar supplemented with different concentrations of FLC (4 to 128 $\mu\text{g/ml}$). The lowest concentration of the drug at which minor resistant subpopulations emerged was identified as each strain's LHF. To determine the proportion of each strain's population that could tolerate different concentrations of FLC, representative strains from each LHF category were tested (Table 2). Briefly, cell suspensions (1×10^3 to 4×10^3 CFU/ml) of each selected strain were suspended in sterile saline and plated on either plain YPD agar or YPD agar supplemented with various concentrations of FLC. The growth patterns were recorded after incubation at 30°C for 72 h. The percentages of the resistant subpopulations that emerged at the lowest concentration of FLC were recorded (31).

Stability of FLC resistance *in vitro*. Subclones from the representative strains within each category of LHF that had adapted to tolerate 64 $\mu\text{g/ml}$ FLC were grown and suspended in YPD broth. Fifty microliters of the suspension was then repeatedly transferred into 5 ml of fresh YPD broth daily and incubated at 30°C for 24 h. The proportions of the subpopulations expressing resistance to FLC (16 to 64 $\mu\text{g/ml}$) were determined by periodically plating a 50 μl suspension of each subculture on either YPD agar alone or YPD agar plus FLC and incubated at 30°C for 72 h. The number of colonies resistant at each level of FLC concentration was recorded.

Resistance to other xenobiotics. Four to five strains from different LHF categories were randomly chosen for analysis of their resistance to xenobiotics unrelated to azole drugs (shown in bold in Table 2). Each strain was first inoculated in YPD broth and incubated overnight at 30°C , washed, serially diluted in sterile saline, and spotted (3 μl) onto YPD agar alone and YPD agar containing different xenobiotics. The compounds tested were trichostatin A, rhizoxin, and gliotoxin at various concentrations. All compounds were obtained from Sigma (Sigma-Aldrich, St. Louis, MO), unless specified.

Virulence in mice. Virulence among strains with different LHFs was compared using a murine model of pulmonary cryptococcosis. Cultures of the strains to be tested were grown overnight in standard broth medium (YPD) at 30°C . The

cultures were then diluted to the desired concentration in a 0.9% NaCl solution. Six-week-old anesthetized female BALB/c mice (weight, 20 g) were infected by intranasal inoculation of a 20- μl droplet containing 5×10^7 yeast cells. Representative strains from Canada, Australia, and India that had exhibited different LHFs were used for comparisons of virulence. The strains used had been determined to have comparable levels of production of capsule, melanin, urease, and growth at 37°C . Ten animals were used for each strain. The survival of mice was recorded daily for 35 days, and the survival data were statistically analyzed using the log rank method.

RESULTS AND DISCUSSION

Relationship between serotype, molecular type, and LHF of *C. gattii* strains. It is well known that *C. neoformans* (serotypes A, D, and AD) and *C. gattii* (serotypes B and C) strains differ from each other in several characteristics that include epidemiology, ecology, biochemistry, and genetics. Differences in the two species are also recognized by the risk factors of the host. While HIV infection is the major risk factor for cryptococcosis due to *C. neoformans* strains, this is not the case for infection by strains of *C. gattii* (16, 28). In the present study, we report differences between the two species in the LHFs. A total of 71 clinical and environmental *C. gattii* isolates of serotypes B and C from different geographic sources were used in this study (Table 1). As was the case with *C. neoformans*, all 71 strains of *C. gattii* exhibited innate levels of heteroresistance to fluconazole regardless of serotype, source, or time of isolation relative to the advent of azoles for the treatment of fungal diseases. All the clinical strains of serotype B (25 strains) and C (17 strains), excluding the three serotype C strains obtained from Colombia, had been isolated in or before 1977, at least a decade prior to the availability of triazole drugs. These strains had been maintained in our laboratory as lyophilized stocks. Of the 25 environmental isolates of serotype B, 17 were from Canada (13), 5 from Australia (17), and 3 from India (5). All 4 environmental strains of serotype C were isolated in Colombia.

Heteroresistance to fluconazole is the phenomenon described for *C. neoformans* as the emergence of a minor subpopulation of resistant cells within a single colony of the susceptible strain that can tolerate FLC concentrations higher than the strain's MIC levels (31). The levels of resistance to fluconazole were determined by Etest analysis (Fig. 1) and spot test analysis of serial dilutions (Fig. 2) for each strain on growth media supplemented with different concentrations of

TABLE 2. Percent heteroresistant subpopulation of clinical and environmental *C. gattii* strains at different fluconazole concentrations^a

Strain	Serotype	Source	Fluconazole concn ($\mu\text{g/ml}$)					VG type
			2	4	8	16	32	
NIH200	B	Clinical	100	0.1				II
NIH754	B	Clinical	100	0.04				II
NIH412	B	Clinical	100	0.07				II
NIH535	B	Clinical	100	0.1				III
NIH198	B	Clinical	100	100	100	1.9		I
B3939	B	Clinical	100	100	100	0.36		III
NIH184	B	Clinical	100	100	100	35		II
NIH444	B	Clinical	100	100	100	100	0.5	III
NIH189	B	Clinical	100	100	100	0.7		III
NIH190	B	Clinical	100	100	100	0.8		III
NIH367	B	Clinical	100	100	100	9		III
NIH409	B	Clinical	100	100	100	100	0.4	III
NIH286	B	Clinical	100	100	100	100	0.06	I
NIH771	B	Clinical	100	100	100	100	9	III
B4506	B	Env	100	100	12			III
B5788	B	Env	100	100	5.7			I
B5763	B	Env	100	100	0.03			I
B5765	B	Env	100	100	100	0.05		I
RB3	B	Env	100	100	55			II
RB14	B	Env	100	100	100	100	0.8	II
RB22	B	Env	100	100	100	0.15		II
RAM15	B	Env	100	100	100	100	10	II
RAM002	B	Env	100	100	100	100	0.53	II
VPB572-058	B	Env	100	100	100	100	1.6	II
NIH18	C	Clinical	100	100	100	100	0.44	I
NIH34	C	Clinical	100	100	100	0.22		I
NIH257	C	Clinical	100	2				III
NIH113	C	Clinical	100	100	100	100	0.99	I
H0058-1-78	C	Clinical	100	100	100	100	2.3	III
H0058-1-2023	C	Clinical	100	100	100	100	7.3	III
NIH191	C	Clinical	100	100	100	100	20	I
NIH298	C	Clinical	100	100	100	100	9	III
H0058-1-1941	C	Env	100	100	100	100	1.4	II
H0058-1-818	C	Env	100	100	100	100	16	III
H0058-1-1686	C	Env	100	100	100	100	0.75	IV

^a Strains in bold indicate those selected for resistance assays to different xenobiotics as shown in Fig. 5.

fluconazole to determine the LHF. Emergence of resistant subpopulations within the patch was indicative of heteroresistance at that concentration of fluconazole (Fig. 2, lanes 1A to C). In *C. neoformans*, 79% of the serotype D strains (22 of 28) and 50% of the serotype A strains (51 of 102) which had previously not been exposed to azole drugs showed LHF of $\leq 8 \mu\text{g/ml}$ fluconazole (31). As shown in Table 1, 86% of the *C. gattii* strains (61 of 71) which had not been exposed to azole drugs had LHF of $\geq 16 \mu\text{g/ml}$. All clinical strains isolated before 1990 were considered to be fluconazole-naïve strains.

Of the serotype B clinical strains isolated prior to the advent of triazole antifungals, 80% (20 of 25) exhibited LHF of $\geq 16 \mu\text{g/ml}$, while 92.9% of the serotype C clinical strains isolated during the same era (16 of 17) exhibited similar LHF (Tables 3 and 4). The LHF of a majority of the serotype C strains (18 of 21), regardless of their origin, was $32 \mu\text{g/ml}$ (Table 4). These results indicated that overall, more strains of *C. gattii* had high LHF than did strains of *C. neoformans* (31).

In *C. neoformans*, strains of serotype A tend to have a higher LHF than that of the strains of serotype D (31). In *C. gattii*,

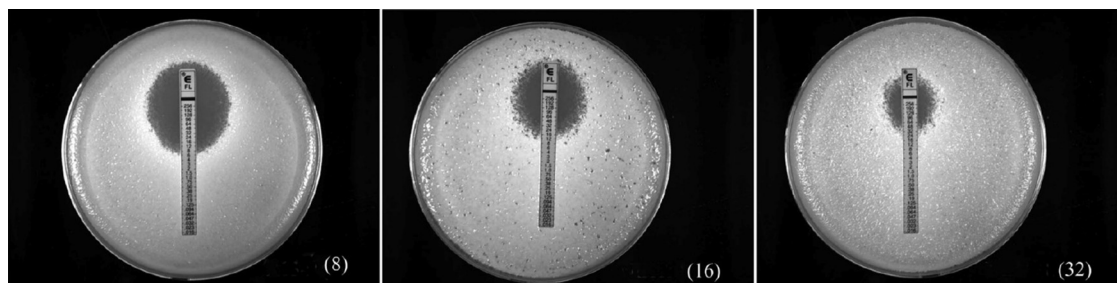


FIG. 1. Determination of fluconazole resistance by Etest analysis. Three representative cultures, exhibiting resistance to 8, 16, and $32 \mu\text{g/ml}$ fluconazole, are shown.

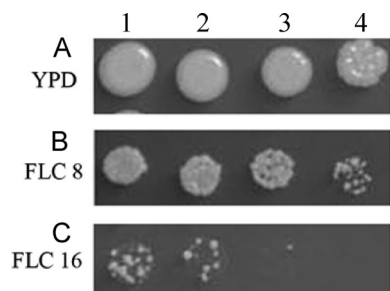


FIG. 2. Determination of LHF by spot test analysis. Spot test analysis representing growth of *C. gattii* strains on YPD agar medium alone (A) and YPD agar medium supplemented with 8 µg/ml (B) or 16 µg/ml (C) fluconazole. Lanes 1 to 4 represent 10-fold serial dilutions of culture. This example shows the spot test of a strain determined to have the LHF of 16 µg/ml FLC.

though, there were more serotype C than serotype B strains that had high LHF. However, the difference was not as great as that between serotype A and D strains of *C. neoformans* (31). The genetic diversity between strains of serotype A and serotype D is well documented, and these strains are clearly distinguishable by the commonly used molecular typing tools, such as M13-based PCR fingerprint patterns or multilocus sequence typing (4, 18, 22). The molecular types of 71 *C. gattii* strains were determined by their M13 PCR fingerprint patterns and their *URA5* RFLPs. The results showed that all four major molecular types of *C. gattii*, VGII, VGIII, VGI, and VGIV, were present in a decreasing order of frequency (Table 1). Figure 3 shows the different molecular types among the representative isolates from five different geographical areas as discerned from their M13 PCR fingerprint patterns (Fig. 3A) as well as their corresponding *URA5* RFLPs (Fig. 3B). The *C. gattii* isolates from Vancouver used in this study were all of the VGIIa molecular type. No apparent correlation was observed between molecular type and LHF, although the three environmental strains of the VGI molecular type isolated from India consistently showed lower LHF than those of the VGI strains from other geographical regions. A correlation between serotype and the LHF was not significant, although a slightly higher proportion of serotype C had ≥ 16 µg/ml LHF than did the strains of serotype B. Strains of serotype A are comprised of molecular types VNI and VNII, while strains of serotype D are exclusively of the VNIV molecular type (24). However, the molecular types of *C. gattii* strains, VGI to VGIV, are not serotype associated and can occur in strains of serotype B or C

TABLE 3. Population analysis profile of *C. gattii* serotypes B isolates screened on FLC-containing medium

Isolate source	Level of heteroresistance to fluconazole (µg/ml)	No. of isolates tested (%)
Clinical	4	5 (20)
	16	9 (36)
	32	11 (44)
Environmental	8	4 (16)
	16	2 (8)
	32	19 (76)

^a A total of 25 clinical isolates and 25 environmental isolates were tested.

TABLE 4. Population analysis profile of *C. gattii* serotype C isolates screened on FLC-containing medium

Isolate source	Level of heteroresistance to Fluconazole (µg/ml)	No. of isolates tested (%)
Clinical	4	1 (5.8)
	16	2 (11.8)
	32	14 (82.4)
Environmental	32	4 (100)

^a A total of 17 clinical isolates and 4 environmental isolates were tested.

(23). This may explain the correlation between the serotype and the LHF of *C. neoformans* strains and an absence of such a correlation between molecular type and either serotype or LHF in strains of *C. gattii*.

Heteroresistance by definition is the ability of a subpopulation to adapt to higher concentrations of the drug in a stepwise manner, resulting in homogeneous resistant populations. Furthermore, resistance acquired in such a manner is also lost in a stepwise manner upon repeated transfer into drug-free medium, resulting in reversion to the original LHF (31). To determine whether such elevations in drug resistance were transitional in nature, cultures of representative strains from Canada (RB-14, 32 µg/ml LHF), Australia (RAM-15, 32 µg/ml LHF), and India (B-5765, 8 µg/ml LHF) were grown at the fluconazole concentration of each one's LHF and then sequentially transferred to media with increasing concentrations of fluconazole. The subpopulations of Canadian and Australian isolates with an LHF of 32 µg/ml were able to adapt to levels of fluconazole as high as 256 µg/ml, while the Indian isolates with an LHF of 8 µg/ml could only achieve a maximum resistance level of 64 µg/ml (data not shown). The strains that had adapted to 64 µg/ml FLC were cultured in YPD medium supplemented with 64 µg/ml fluconazole and then transferred daily into drug-free YPD broth and incubated at 30°C. Aliquots of these cultures were removed daily and plated on YPD media with different concentrations of the drug to monitor the loss of resistance. All the strains lost the elevated level of resistance to fluconazole and returned to their original, innate levels of resistance. The numbers of transfers in drug-free medium that were required for the loss of resistance correlated with the original LHF: strains with LHF of 32 µg/ml that had adapted to 64 µg/ml FLC required 42 transfers to return to the original level of resistance, while only 28 transfers were required for the strain from India with 8 µg/ml LHF (Fig. 4). As represented in Table 2, all strains exhibited heteroresistance to fluconazole but at various concentrations, 4 µg to 32 µg, indicating that *C. gattii* strains are innately heteroresistant to fluconazole. While for *C. neoformans*, only 50% and 21% of serotype A and serotype D strains, respectively, exhibited LHF of ≥ 16 µg/ml, for *C. gattii*, 82% and 95% of serotype B and serotype C strains, respectively, exhibited LHF of ≥ 16 µg/ml. The overall LHF of *C. gattii* strains, therefore, were significantly higher than those observed for strains of *C. neoformans*.

Relationship between LHF and resistance to toxins and antibiotics produced by environmental microorganisms. In *C. neoformans*, a correlation between the LHF of a strain and its resistance to several xenobiotics has been reported (31). We

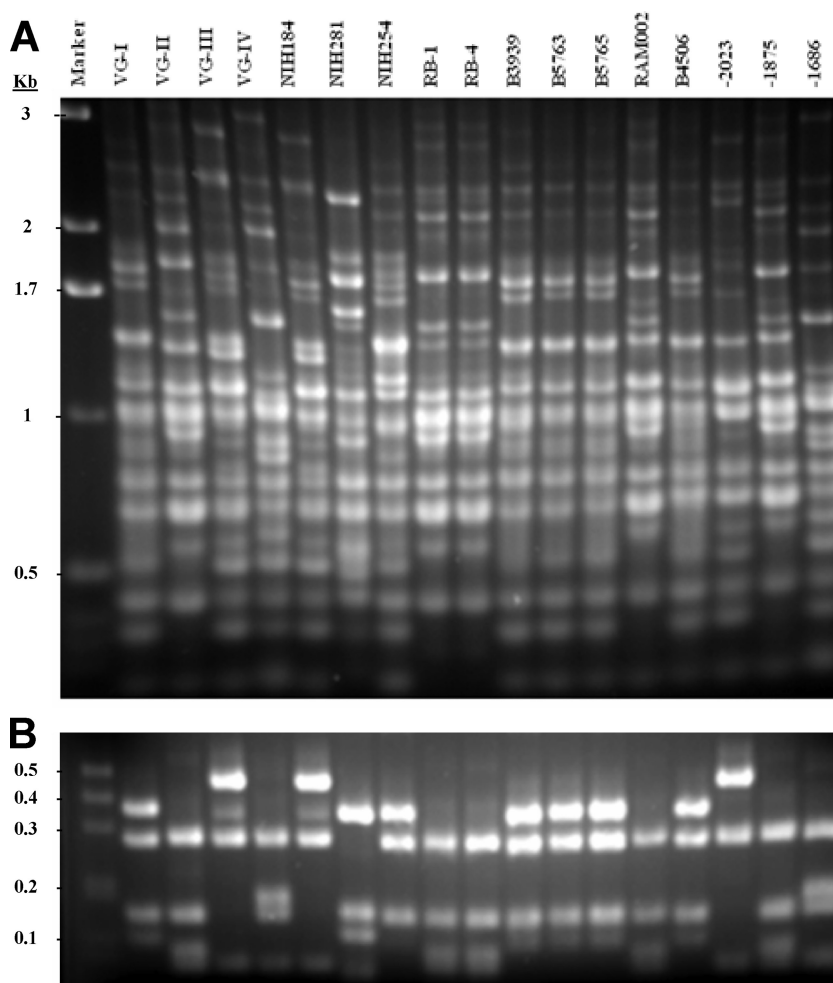


FIG. 3. (A) Examples of PCR fingerprint patterns obtained by using the M13 primer for clinical and environmental strains of *C. gattii* that represent the four different molecular types. Lane 1, size marker; lanes 2 to 5, reference strains representing the four molecular types VG-I to VG-IV. (B) Examples of *URA5* gene restriction fragment length polymorphism profiles from clinical and environmental isolates of *C. gattii* representing the four different molecular types. Lanes are as in Fig. 1A.

tested several of the xenobiotics produced by microorganisms that *C. gattii* strains are likely to confront in the environment. Strains of *C. gattii* most commonly inhabit tree hollows (8) where numerous other microorganisms may cohabitate. The trees with which *C. gattii* strains are found to be associated include alder, cedar, Douglas fir, almond, and particularly, the hollows of the Eucalyptus species (2, 9, 10, 15). Since all environmental isolates were recovered from trees such as alder and Douglas fir, prior exposure to agricultural fungicides, such as clotrimazole and ketoconazole, in areas of their recovery is not likely.

The tree hollows may harbor various ubiquitous saprophytic microorganisms, such as *Penicillium*, *Aspergillus*, and common soil bacteria. We tested trichostatin A, a histone deacetylase inhibitor produced by *Streptomyces platensis*, rhizoxin, a microtubule inhibitor produced by *Burkholderia* species, and gliotoxin, an antibiotic commonly produced by *Aspergillus* and *Penicillium* species (31). Figure 5 shows the susceptibilities of *C. gattii* strains with various levels of heteroresistance against these three xenobiotics. All the strains of *C. gattii* were ob-

served to be highly resistant to these xenobiotics at the minimum concentration which completely inhibited the growth of *C. neoformans* strains (31). We raised the concentration of gliotoxin (3.2 $\mu\text{g/ml}$) and trichostatin A (64 $\mu\text{g/ml}$) 2-fold each and the concentration of rhizoxin (0.5 $\mu\text{g/ml}$) 5-fold in comparison to the minimum concentration that had been reported to inhibit growth of *C. neoformans* strains in order to achieve the minimum concentration at which *C. gattii* strains are inhibited. As was the case with strains of *C. neoformans*, the *C. gattii* strains with higher LHF tended to be more resistant to the xenobiotics tested than the strains with low LHF. *C. gattii* strains with a LHF of 4 $\mu\text{g/ml}$ were generally more sensitive to all three compounds than strains with LHF of 8 and 16 $\mu\text{g/ml}$ FLC. The strains with a 32- $\mu\text{g/ml}$ LHF, however, were not necessarily more resistant than the strains with a 16- $\mu\text{g/ml}$ LHF and showed considerable variations in the degree of resistance (Fig. 5). These results show that the overall resistance of *C. gattii* to these xenobiotics is at least two times higher than that of the strains of *C. neoformans*. Although both species of *Cryptococcus* are recovered from soil samples and may share

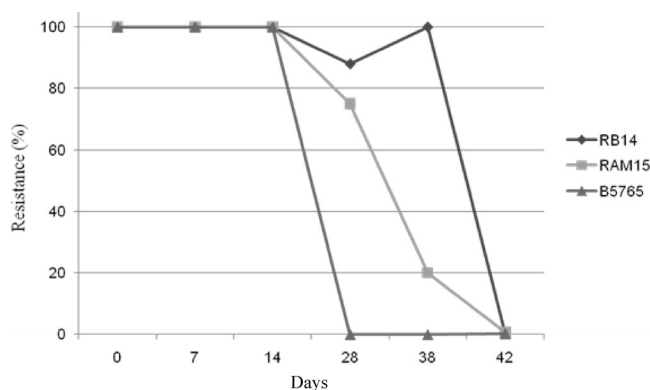


FIG. 4. Loss of the acquired tolerance to fluconazole by heteroresistant subpopulations of *C. gattii* strains upon daily transfer of the cultures grown at 30°C in YPD medium without fluconazole.

some common environmental niches, *C. gattii* strains are not known to be associated with pigeon droppings, the major environmental source of *C. neoformans*. With such ecological differences, *C. gattii* may have been predisposed to develop the ability to tolerate higher levels of certain xenobiotics than can *C. neoformans* strains.

Several other studies have also reported differences between the two species with regard to their susceptibilities to amphotericin B and various other azoles (7, 12, 25, 33, 34). Khan et al. (12) reported on antifungal susceptibilities based on the largest number of environmental isolates from both species and showed that *C. gattii* strains had significantly higher MICs for both amphotericin B and azoles. The contradictory reports

regarding the *in vitro* susceptibility of *C. neoformans* and *C. gattii* strains to some antifungals may be due to the methods used by different laboratories. Although the Etest is recommended as a good alternative to the microbroth and broth macrodilution method of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) for antifungal susceptibilities of yeast, the results have sometimes been contradictory and do not always corroborate the MIC values (12). Larger inoculums with agitation have been proposed as an alternative method for MIC determinations (11). In *Candida* species, the standardized CLSI methodology has proven to be reliable, but not for *Cryptococcus*. It is noteworthy that drugs which show differences in resistance between the two species are those that inhibit or bind ergosterol in the cell membrane. Treatment with these drugs of cryptococcosis due to *C. gattii* has been more difficult than of disease caused by *C. neoformans* (25, 33). At present, the mechanism for the susceptibility difference between the two species against these antimycotics is unknown. In our experiments, a higher percentage of *C. gattii* isolates exhibited high heteroresistance levels to fluconazole than *C. neoformans* strains. The *C. gattii* strains with high LHF were also found to express higher resistance to xenobiotics such as rhizoxin. This inherently higher LHF of *C. gattii* strains likely influences the MICs of the strains, resulting in the variability in resistance to different compounds between different studies. So, the LHF of the strains should be taken into account when investigating resistance of the strains for different compounds.

Virulence and LHF. A total of six strains, including two strains each from Canada and Australia with an LHF of 32 µg/ml and two strains from India with an LHF of 8 µg/ml, were

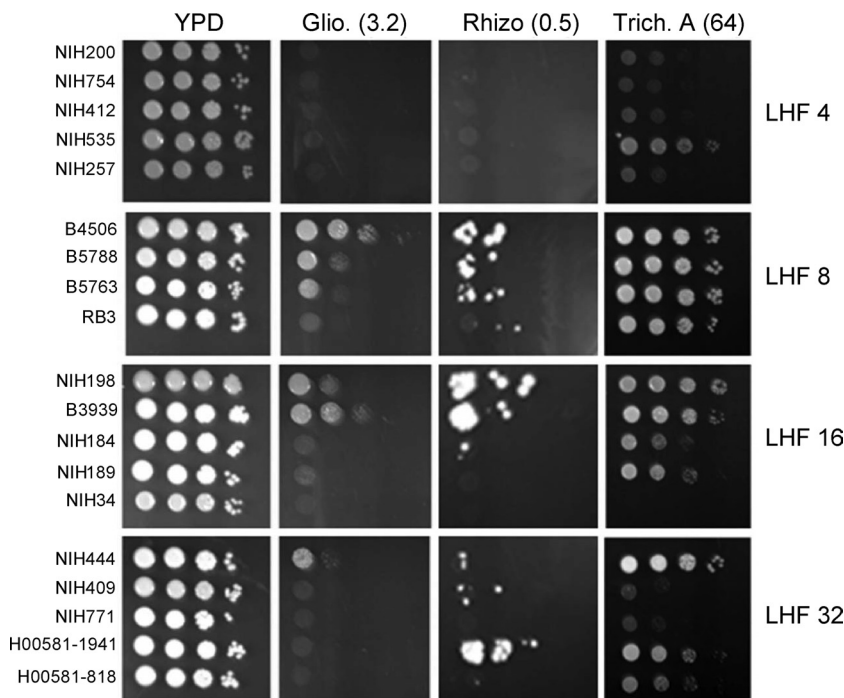


FIG. 5. Spot test analysis to determine resistance of the *C. gattii* strains to different xenobiotics: gliotoxin (Glio., 3.2 µg/ml), rhizoxin (Rhizo., 0.5 µg/ml), and trichostatin A (Trich. A, 64 µg/ml). The strains selected represent the four groups of strains that exhibited heteroresistance at 4, 8, 16, and 32 µg/ml fluconazole (boldface in Table 2).

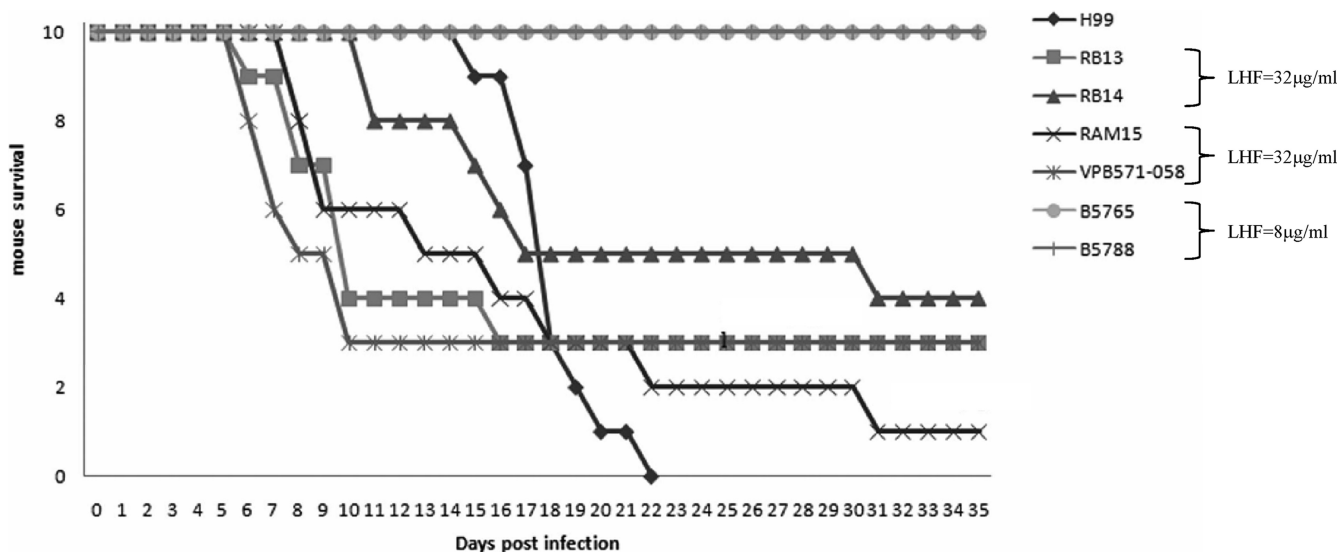


FIG. 6. Virulence in BALB/c mice following intranasal inoculation of 5×10^7 cells per mouse with the selected *C. gattii* strains from different geographic regions that exhibited different levels of heteroresistance to fluconazole.

used for the virulence study. The highly virulent *C. neoformans* serotype A strain H99 was used as a control. Mice infected with *C. gattii* strains exhibiting a LHF of 32 $\mu\text{g/ml}$ began to die significantly earlier than the mice infected with strains that had an LHF of 8 $\mu\text{g/ml}$ (Fig. 6). Although the mice infected by *C. gattii* strains with a 32- $\mu\text{g/ml}$ LHF started dying earlier than those infected by the H99 strain, their virulence was not found to be significantly different from that of H99, as determined by the log rank test ($P \geq 0.6$). These results demonstrate a correlation between LHF and virulence in *C. gattii* strains which is similar to what has been reported for strains of *C. neoformans* (27). In the previous work, mice were infected with *C. neoformans* strains with high and low LHFs and then either left untreated or treated with fluconazole (31). The CFU of strains recovered from the tissues of infected mice were propagated on media with and without different concentrations of fluconazole and showed that the original LHF for the strains was maintained. The mice infected with strains with high LHFs were found to die earlier regardless of treatment, while those mice infected with strains with lower LHFs survived much longer if treated with fluconazole. Although mice were not treated with fluconazole in the present study, it is predicted that more mice infected with *C. gattii* strains will be unresponsive to fluconazole treatment than those infected with *C. neoformans*. Heteroresistance, an adaptive mechanism employed by the organism to counteract the stress of increasing drug concentration in the environment, may enhance the organism's fitness to survive under other stresses as well. This overall fitness may be translated into higher virulence.

This study serves to highlight the stark differences between the two species with respect to the level of heteroresistance to fluconazole. It shows that a higher percentage of *C. gattii* strains manifest high LHFs than *C. neoformans* strains. Such an understanding would be beneficial for establishing therapeutic regimens and in predicting clinical outcomes with respect to developing resistance and relapses in cryptococcosis caused by *C. gattii*. Furthermore, strains of *C. gattii* were found to exhibit

markedly higher levels of resistance to different xenobiotics than *C. neoformans*, which also mirrored their LHFs. This resistant property of the *C. gattii* strains most likely reflects an acquisition from its ecological niche.

The mechanism of heteroresistance in *C. neoformans* is yet to be clearly established (31). It is very different from the heteroresistance reported for *Candida albicans*, which is also most often treated with triazoles. Unlike in *C. neoformans*, heteroresistance in *Candida albicans* is reported to develop following short exposures to FLC, both *in vitro* and *in vivo*, and has been associated with disseminated infection in patients that had received bone marrow transplants (19, 21). However, as in *C. albicans*, heteroresistance in isolates of *C. neoformans* and *C. gattii* that became resistant to azole drugs was transient, since the strains became susceptible after serial transfers in the absence of the drug (19, 20). Recent studies have revealed an association between alterations in ploidy and azole resistance in *C. albicans* (30) and *C. neoformans* (32). In resistant strains, the presence of an extra whole chromosome or an isochromosome was detected, and such chromosomal gain or loss was strongly associated with increased and decreased azole resistance (30, 32). In the *C. neoformans/C. gattii* species complex, heteroresistance to azoles is innate and the level of heteroresistance to fluconazole varies between strains. The present study reveals that a significantly higher number of *C. gattii* strains than *C. neoformans* strains manifest a ≥ 16 - $\mu\text{g/ml}$ LHF that is associated with higher virulence. It remains to be elucidated whether a mechanism similar to that described for *C. albicans* is responsible for heteroresistance in the *C. neoformans/C. gattii* species complex.

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