BRAZ J INFECT DIS. 2012;**16(2)**:175-179

The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Brief Communication

Antifungal susceptibilities of *Cryptococcus* species complex isolates from AIDS and non-AIDS patients in Southeast China

Meng Li[a,¥], *Yong Liao*[b,¥], *Min Chen*[a], *Weihua Pan*[a*], *Lixing Weng*[c]

[a]*Shanghai Key Laboratory of Molecular Mycology & PLA Key Laboratory of Fungal Diseases, Chang Zheng Hospital, Second Military Medical University, Shanghai, China*
[b]*Department of Dermatology, General Hospital of Beijing Military Command, Beijing, China*
[c]*School of Geography and Biological Information, Nanjing University of Posts and Telecommunications, Nanjing, Jiangsu, China*

ARTICLE INFO

Article history:
Received 19 October 2011
Accepted 11 December 2011

Keywords:
Cryptococcus neoformans
Cryptococcus gattii
Antifungal agents

A B S T R A C T

Cryptococcus spp. are common causes of mycoses in immunocompromised patients. To determine the drug susceptibilities of clinical *Cryptococcus* spp. isolates, the characteristics of 61 clinical *Cryptococcus* spp. complex isolates and their antifungal susceptibilities were investigated, including 52 *C. neoformans* and 9 *C. gattii* isolates collected at Shanghai between 1993 and 2009. Antifungal susceptibility of clinical isolates to amphotericin B, fluconazole, itraconazole, and flucytosine were determined by the microdilution method M27-A2 and the ATB FUNGUS 3 kit. The 90% minimum inhibitory concentration (MIC90) and susceptibility ranges were as follows: 1 (0.0625-1) µg/mL for amphotericin B, 4 (0.125-16) µg/mL for fluconazole, 0.25 (0.0313-4) µg/mL for itraconazole, and 4 (0.125-8) µg/mL for flucytosine. Fluconazole, itraconazole, and flucytosine have excellent *in vitro* activity against all tested clinical *Cryptococcus* spp., and we also found a high rate of tolerance to amphotericin B (MICs ranging from 0.55-1 µg/mL). Furthermore, *C. neoformans* isolates from acquired immune deficiency syndrome (AIDS) patients were less susceptible to fluconazole and flucytosine than those from non-AIDS patients. These data suggest that use of amphotericin B may lead to tolerance or resistance of the pathogen over time. There were also no significant associations between species, genotypes, and *in vitro* susceptibilities of these clinical isolates.

Infections by opportunistic pathogenic fungi, particularly *Candida* spp., *Cryptococcus* spp., and *Aspergillus fumigatus*, have become a serious medical problem in immunocompromised patients, who are highly susceptible to such infections. The *Cryptococcus* species complex consists of fatal fungal pathogens, which remain the most important cause of cryptococcal meningitis worldwide, in spite of the introduction of highly active antiretroviral treatment (HAART) to acquired immunodeficiency syndrome (AIDS) patients, in 1996.[1]

Cryptococcus neoformans and *C. gattii* are recognized within the *Cryptococcus* spp. complex, and they are closely related to basidiomycetous yeasts.[2] *C. neoformans* contains *C. neoformans*

**Corresponding author at:* Shanghai Key Laboratory of Molecular Mycology & PLA Key Laboratory of Fungal Diseases, Chang Zheng Hospital, Second Military Medical University, Shanghai 200003, China
E-mail address: panweuhua@medmail.com.cn (Weihua Pan)
¥ Both authors contributed equally to this work.

var. *neoformans* (serotype D), the hybrid isolates (serotype AD), and C. *neoformans* var. *grubii* (serotype A), which most commonly may cause meningoencephalitis, predominantly in immunocompromised hosts. *C. gattii* is divided into serotypes B and C, which are probable causes of cryptococcosis in immunocompetent hosts. Recently, PCR fingerprint patterns based on M13 or (GACA)4 primers have been used as the major genotyping technique in the ongoing global molecular epidemiologic survey of the *Cryptococcus* spp. complex, dividing over 600 clinical and environmental isolates into eight major molecular types: VNI (var. *grubii*, serotype A), VNII (var. *grubii,* serotype A), VNIII (serotype AD), VNIV (var. *neoformans*, serotype D), VGI, VGII, VGIII, and VGIV (*C. gattii*, serotypes B and C).[3]

Cryptococcosis is mainly found in AIDS patients worldwide, but in China it occurs most commonly in non-AIDS patients, and the proportion of non-AIDS patient cases were reportedly between 80.5-91.5%.[4] The declining incidence of cryptococcosis in developed countries can be attributed to effective antiretroviral therapy.[5] However, the rate of infection is still increasing in developing countries, especially in China, which is mainly caused by a growing immunocompromised population resulting from immunosuppressive therapies and AIDS.

Several classes of antifungal drugs effectively treat cryptococcal infections, but the pathogen can develop resistance to these agents. In developed countries, many studies on the *in vitro* antifungal susceptibility of clinical strains of *C. neoformans* and *C. gattii* have been performed.[6] Clinical isolates of *Cryptococcus* spp. were shown to remain highly susceptible (99%) to amphotericin B at a minimum inhibitory concentration (MIC) of ≤ 1 µg/mL, susceptible to flucytosine at a MIC of ≤ 4 µg/mL, susceptible to fluconazole at a MIC of ≤ 8 µg/mL. Despite the apparent importance of drug resistance of clinical pathogens, its surveillance in developing countries is still poor or ignored in comparison with developed countries. In China, there have been few studies on the drug susceptibility of *C. neoformans*. Clinical isolates of *C. neoformans* from Taiwan were serotyped and their *in vitro* susceptibility to amphotericin B, fluconazole, and voriconazole were analysed.[7] In 2004, Zhu et al. tested the 50% minimum inhibitory concentration (MIC50) of 81 *C. neoformans* isolates from mainland China: 4 (2-128) µg/mL for fluconazole and 0.03 (0.002-0.13) µg/mL for flucytosine, and estimated the fungicidal effects between different drug combinations.[8]

There have been some reports of resistant *C. neoformans* isolates that are not susceptible to amphotericin B, fluconazole, flucytosine, or itraconazole during treatment. The emergence of resistance to these antimycotic drugs suggests the need for vigilance and large-scale surveillance of the *in vitro* chemosensitivity of clinical strains. Therefore, it is important to obtain susceptibility data of various clinical isolates at different times. The current study aimed to evaluate the *in vitro* susceptibility of clinical isolates of *C. neoformans* and *C. gattii* from mainland Chinese against four commonly used antifungal drugs. We also sought to determine if there was any correlation between origin, genotypes, and *in vitro* susceptibility in *Cryptococcus* spp. complex isolates.

A total of 61 clinical *Cryptococcus* species complex isolates were collected mainly from the southeast regions of mainland China, comprising Shanghai (n = 20), Guangdong (n = 12), Jiangsu (n = 9), Zhejiang (n = 5), Henan (n = 5), Anhui (n = 2), Jiangxi (n = 2), Fujian (n = 2), Sichuan (n = 2), Beijing (n = 1), Heilongjiang (n = 1), and these samples were recovered from the cerebrospinal fluid (n = 57), sputum (n = 2), feces (n = 1) and skin ulcer (n = 1). These clinical isolates were collected from patients with either cryptococcal meningitis or cryptococcal infection (one isolate per patient). All patients were admitted to our hospital between 1993 and 2009. Initial isolates were obtained at diagnosis. The majority was isolated from the cerebrospinal fluid. All isolates were identified by standard methods, including caffeic acid agar, positive urease test, or the API-20C AUX system (bioMérieux – France), and were maintained in frozen stock vials at –70°C. Each isolate was recovered at least twice from the frozen stock vials onto Sabouraud glucose agar (SDA) to ensure purity and viability, and a single colony was selected for analysis. The molecular type of the isolates was identified. The clinical *Cryptococcus* spp. were evaluated based on molecular characterization of genotype. The proportion of each genotype using the PCR fingerprint method was compared with previous Chinese report. Among the strains isolated, 52 strains of *C. neoformans* were assigned to VNI-III (45 of VNI, five of VNII and two of VNIII) and nine of *C. gattii* to VGI.

The *in vitro* activities of amphotericin B, itraconazole, fluconazole, and flucytosine were tested using the microdilution method M27-A2 (CLSI 2002).[9] Standard antifungal powders of all tested drugs were obtained from Sigma (St. Louis, USA). Fluconazole and flucytosine were dissolved in sterile water; amphotericin B and itraconazole in dimethyl sulphoxide (DMSO); and before use, they were further diluted in RPMI 1640 medium (Sigma – St. Louis, USA) and buffered to a pH of 7.0 with morpholinepropanesulphonic acid (MOPS). The final concentrations of the different antifungal agents were 0.0313-16 µg/mL for amphotericin B and itraconazole, and 0.125-64 µg/mL for fluconazole and flucytosine. Suspensions of yeast from 72-h cultures were prepared in sterile saline (0.85%) adjusted using a spectrophotometer reading at a 530 nm wavelength to a cell density of approximately 1-5 x 10^6 cfu/mL. This suspension was diluted at 1:50 followed by a 1:20 dilution in RPMI 1640 to obtain a final concentration of 1-5 x 10^3 cfu/mL. To perform *in vitro* susceptibility assays, 96-well plates were covered with 100 µL of different concentrations of the antifungal agents and added to 100 µL of the yeast suspension. The plates were incubated at 35°C for 72 h and the MIC values were determined. The end-point for amphotericin B MICs was defined as the 100% inhibition point compared to a growth control. End-points for azoles and 5-fluorocytosine (5FC) MICs were defined as a prominent reduction of growth (≥ 50%) compared to a drug-free control well. *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control in each assay to check the accuracy of drug dilutions and validity of the results.

The susceptibilities of all isolates were again determined by the ATB FUNGUS 3 kit (bioMérieux – France). Suspensions of yeast from 72-h cultures were prepared in sterile saline (0.85%) adjusted with a turbidity equivalent to 2 McFarland standard units, which is equivalent to an approximate cell density of 1-5 x 10^6 cfu/mL. To perform the assay, 20 µL of the cell suspension was transferred into an ampule of ATB F2 medium and 135 µL ATB F2 medium was dispensed into the ATB FUNGUS 3 strips which consist of 16 pairs of cupules, of which 15 pairs contained five antifungal agents at several concentrations, and a positive growth control was included that was free of any agent. The five agents investigated were flucytosine, amphotericin B, fluconazole, itraconazole, and voriconazole. The concentrations of these agents were 4 and 16 µg/mL for flucytosine, 0.5-16 µg/mL for amphotericin B, 1-128 µg/mL for fluconazole and 0.125-4 µg/mL for itraconazole, and 0.06-8 µg/mL for voriconazole. These strips were incubated at 35°C for 72 h and the MICs read visually, according to the manufacturer's instructions.

Microdilution testing of four antifungal agents was performed using microdilution method M27-A2. The results obtained from the ATB FUNGUS 3 kit were similar to those obtained by the microdilution method. Table 1 shows MIC, MIC_{50}, and MIC_{90} ranges of the four antifungals tested against 61 *Cryptococcus* species complex isolates. Most *Cryptococcus* spp. showed uniform patterns of susceptibility to the four tested agents. When all strains were taken into consideration, they were susceptible to fluconazole, itraconazole, and flucytosine. The individual MIC ranges and MIC90 were 0.0313-4 µg/mL and 0.25 µg/ml for itraconazole, 0.125-16 µg/mL and 4 µg/mL for fluconazole, 0.125-8 µg/mL and 4 µg/mL for flucytosine.

The difference in MIC_{90} for fluconazole between AIDS and non-AIDS patients had been previously reported, but there were no data about the effect of flucytosine. When isolates were analyzed according to the origin of the patients (Table 1), 45 isolates from HIV-negative patients showed a lower geometric mean for fluconazole (1.1589/3.1228; $p = 0.001$) and flucytosine (1.4038/2.9720; $p < 0.001$), compared with the 16 isolates from AIDS patients. Remarkably, there were no significant differences in susceptibility between the species for four agents (data not shown).

Very few studies have compared the susceptibilities of *C. neoformans* and *C. gattii* among specific genotypes. The MICs for all isolates of each genotype against four agents are shown in Table 2. Although the geometric mean MICs were different, there was no statistically significant difference ($p > 0.05$) observed between genotypes VNI and VGI. The other genotypes, VNII and VNIII, were not compared because the number of isolates was too small. We found that within the VNI genotype group there were some isolates

Table 1 - *In vitro* susceptibility of *Cryptococcus* spp. isolates to amphotericin B, itraconazole, fluconazole, and flucytosine according to origin

Isolates and antifungal agents	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)
Non-AIDtS 45 isolates				
Amphotericin B	0.0625~1	0.5	1	0.5463
Itraconazole	0.0313~0.5	0.125	0.25	0.1095
Fluconazole	0.125~4	1	4	1.1589
Flucytosine	0.125~4	2	4	1.4038
AIDS 16 isolates				
Amphotericin B	0.125~1	0.5	1	0.4310
Itraconazole	0.0313~4	0.125	0.5	0.1683
Fluconazole	0.5~16	4	8	3.1228
Flucytosine	0.25~8	4	4	2.9720
Total (non-AIDS+AIDS)				
Amphotericin B	0.0625~1	0.5	1	0.5173
Itraconazole	0.0313~4	0.125	0.25	0.1208
Fluconazole	0.125~16	2	4	1.4550
Flucytosine	0.125~8	2	4	1.6675

Significance was determined using the Student's t-test ($p < 0.05$).

Table 2 - *In vitro* susceptibility of *Cryptococcus* spp. isolates to amphotericin B, itraconazole, fluconazole, and flucytosine according to genotypes

Genotype	Amphotericin B				Itraconazole				Fluconazole				Flucytosine			
	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	GM (µg/mL)
VNI	0.0625~1	0.5	1	0.5156	0.0313~0.5	0.125	0.25	0.1122	0.125~16	2	4	1.3608	0.125~8	2	4	1.6371
VNII	0.25~1	0.5	1	0.4353	0.0625~4	0.25	4	0.2872	0.5~8	2	8	2.2974	0.25~2	2	2	1.1487
VNIII	0.125~1	0.5	1	0.3536	0.0625~0.25	0.0625	0.25	0.125	0.5~1	0.5	1	0.7071	4~4	4	4	4
VGI	0.125~1	1	1	0.6300	0.0313~0.5	0.125	0.5	0.1072	0.5~4	2	4	1.8517	0.5~4	2	4	1.8517

from HIV-positive patients, while there were none from the VGI genotype group. To minimize interfering factors, we compared the VNI and VGI groups again, after removing the strains isolated from HIV-positive patients in VNI group, and still there was no significant difference ($p > 0.05$).

The lowest MIC_{50} and MIC_{90} (0.125/0.25 µg/mL) values were found for itraconazole, which were similar to the results of published studies from Asia, such as 0.032/0.125 µg/mL from India,[10] 0.125/0.5 µg/mL from Malaysia,[11] except that there was one isolate with a MIC of 4 µg/mL for itraconazole.

The MIC values for fluconazole documented in our study were similar to those previously reported. One study from India showed that the MIC_{50} and MIC_{90} of fluconazole were 4/16 µg/mL,[10] which were also similar to another Malaysian study;[11] two studies from Taiwan have shown that the MIC_{90} of fluconazole were 2 and 8 µg/mL; while many other studies showed higher MIC values and increasing rates of resistance to fluconazole, such as one study from the mainland China with high MICs (2-128 µg/mL).[12]

The MICs for flucytosine in our study were consistent with that of previous reports, but lower than some other studies. A study from Taiwan found a high MIC_{90} and susceptibility ranges of 16 (0.125-16) µg/mL. Compared with these Asian reports, our results found a lower MIC_{90} and susceptibility ranges of 4 (0.125-8) µg/mL; however, there was one study from Malaysia that showed a much lower value of MIC_{50} and MIC_{90} (0.023/0.25 µg/mL), which was compatible to one study presenting median MIC and susceptibility ranges 0.06 (0.008-2) µg/mL from mainland China.

In previous evaluations of the effect of amphotericin B on *Cryptococcus* spp., most isolates appeared significantly susceptible to this agent.[13] A study from Malaysia showed low MIC_{50} and MIC_{90} (0.25/0.38 µg/mL). A similar result was reported from Taiwan, where the MIC_{90} and susceptibility ranges were 0.5 (0.125-1) µg/mL, in agreement with results obtained by Chandenier et al.,[14] who reported that both Asian and African isolates are susceptible to amphotericin B, whose MIC values did not exceed 0.125 µg/mL against the tested isolates. However, the results reported here show a slightly higher MIC than previous studies, with the MIC_{90} and susceptibility ranges of 1 (0.0625-1) µg/mL, respectively.

Our data are consistent with the results from the following report. A study from Brazil, using the time-kill method, showed that seven isolates (17.5% of all) were tolerant to amphotericin B (MICs ranged from 0.25-1 µg/mL) and correlated well with *in vitro* susceptibility and clinical response.[15]

Some studies have shown even higher MICs compared to our results, such as another study from Brazil displaying a high rate of resistance to amphotericin B (> 1 µg/mL), including 40% of *C. gattii* and 12% of *C. neoformans* isolates. Lozano-Chui et al.[16] defined three isolates from 12 clinical strains as resistant to amphotericin B, with MICs of 3.0-4.0 µg/mL that were subsequently found to be associated with therapeutic failure in the USA. Perkins et al.[17] reported 17 strains among Spanish clinical isolates (5.3% of all) that had MICs for amphotericin B of ≥ 2 µg/mL.

For these four agents, there were no significant differences in susceptibility between the species. This was consistent with the largest published series assessing species-specific MICs, which also showed no differences.[18] However, other studies have described species-specific differences in antifungal susceptibility that may lead to higher rate of complications, slower response, and longer duration of treatment for patients with *C. gattii* infection. There was only one previous study about the correlation of genotypes and antifungal susceptibilities of *C. gattii*, and it found significant differences in MICs between some subtypes.[19]

Amphotericin B deoxycholate has remained the mainstay of treatment for invasive fungal infections for many years[20] since it is active against a wide variety of fungi, including *C. neoformans*. Amphotericin B targets the ergosterol in the fungal plasma membrane to form a channel where the cell leaks potassium ions, resulting in a disruption of the proton gradient. In addition to this action, amphotericin B causes oxidative damage to the plasma membrane.

Although there are no breakpoints defined by the CLSI for amphotericin B and *C. neoformans*, it has been suggested an MIC value of 2 µg/mL is the resistance threshold for amphotericin B and the susceptibility pattern of *C. neoformans* strains is predictable, with MICs ranging from 0.12-0.5 µg/mL. Although our study did not identify any isolates with amphotericin B resistance, our data described elevated average MICs to amphotericin B, which was rarely observed in previous studies using Asian isolates. Unfortunately, very little was known about the clinical prognoses and outcomes of patients infected with these relatively high-MIC amphotericin B isolates. It was reported that treatment with amphotericin B may induce the development of clinical and *in vitro* amphotericin B resistance.[21] However, the reason for the low susceptibility to amphotericin B was not apparent in this study, and we suspect that the tolerant strains were isolated from patients that had likely been previously exposed to amphotericin B. These data suggest that the use of amphotericin B may lead to tolerance or resistance of the pathogen over time. We are currently gathering related clinical data to analyze the apparent cause of these tolerant strains and investigate the correlation between susceptibility results and clinical outcome.

Acknowledgements

We are thankful for the funds provided by the National Natural Science Foundation of China under Grants 81071335 and 90813010, and by the Shanghai Natural Science Foundation under Grant 08ZR1401400; Shanghai Science and Technology Commission Fund (No. 10dz2220100).

Conflict of interest

All authors declare to have no conflict of interest.

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