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Original article

Predominance of *Cryptococcus neoformans* var. *grubii* multilocus sequence type 5 and emergence of isolates with non-wild-type minimum inhibitory concentrations to fluconazole: a multi-centre study in China

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ABSTRACT

There are few data on the molecular epidemiology of cryptococcosis in China. Here we investigated the species distribution, molecular types and antifungal susceptibilities of 312 *Cryptococcus neoformans* species complex isolates from ten hospitals over 5 years. Isolates were identified by internal transcribed spacer (ITS) sequencing and by two matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems. Multilocus sequence typing (MLST) was used to verify species/variety and to designate molecular types. Susceptibility to six antifungal drugs was determined by the Sensititre YeastOne™ method. *Cryptococcus neoformans* was the predominant species (305/312 isolates (97.8%), all were ITS type 1, serotype A), of which 89.2% (272/305) were *C. neoformans* var. *grubii* MLST sequence type (ST) 5 and 6.2% (19/305) were ST31. Other *C. neoformans* var. *grubii* STs were rare but included six novel STs. Only two strains were *C. neoformans* var. *neoformans* (both serotype AD). *Cryptococcus gattii* was uncommon ($n = 7$, four ITS types) and comprised five MLST STs including one novel ST. For *C. neoformans* var. *grubii*, the proportion of isolates with non-wild-type MICs to fluconazole significantly rose in the fourth study year (from 0% (0/56 isolates) in the first year to 23.9% (17/71) in the fourth year), including five isolates with fluconazole MICs of ≥32 mg/L. The study has provided useful data on the species epidemiology and their genetic diversity and antifungal susceptibility. The proportional increase in isolates with non-wild-type MICs to fluconazole is noted. **X. Fan, CMI 2016;22:887.e1–887.e9**

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Introduction

The genus *Cryptococcus* comprises over 70 species and is responsible for life-threatening infections, particularly meningoencephalitis, in both immunocompromised and immunocompetent patients [1]. The *Cryptococcus neoformans* species complex, *C. neoformans* (including its varieties, *C.* var. *neoformans* and *C. neoformans* var. *grubii*) and *Cryptococcus gattii*, account for most cases of infections [2–4]. Other species such as *Cryptococcus laurentii* are rare [5,6].

Effective management of cryptococcal infections relies on appropriate antifungal therapy. The Infectious Diseases Society of America recommends amphotericin B and 5-flucytosine as the preferred agents for the initial or induction therapy, whereas the azoles (especially fluconazole) are generally used in the consolidation and maintenance phases of therapy or as primary prophylaxis [7]. However, in resource limited settings, azoles are often used as initial therapy [8]. Notably, antifungal susceptibility, particularly to fluconazole, has been noted to vary not only according to species but also with molecular type (genotype) and geographic region [3,9,10]. Therefore, knowledge of local epidemiology patterns of disease, including the molecular type and antifungal susceptibilities of the causative *Cryptococcus* species is essential to guide clinical management as well as population genetic studies [2,11].

Delineation of molecular types of *C. neoformans* and *C. gattii* may be performed by a number of techniques including sequencing of the rDNA internal transcribed spacer (ITS), PCR-fingerprinting, amplified fragment length polymorphism, restriction fragment length polymorphism and multilocus sequence typing (MLST) [2,4,12]. Of these, MLST lends itself as a highly discriminatory tool that allows objective comparison of results between centres. One such standardized MLST scheme is recommended by the International Society of Human and Animal Mycoses [12] as the preferred method for cryptococcal strain typing and there is consensus to use the nomenclature VNI to VNIV and VGI to VGIV for assigning genotypes of *C. neoformans* and *C. gattii*, respectively [12].

In Asia, *C. neoformans* genotype VNI (*C. neoformans* var. *grubii*, serotype A, ITS genotype ITS1) is reported to be the commonest genotype (81.0%) followed by *C. gattii* genotype VGI (serotype B/C, ITS genotypes ITS3/ITS7) (13.2%), with other genotypes being rare [2]. However, there is a higher prevalence of *C. gattii* VGI in India (29.3%) [2]. Little is known about the molecular epidemiology of cryptococcosis in China where previous studies were performed decades ago, or were restricted to a single/small number of institutions [13–16]. Although the first multicentre survey of invasive yeast infections in China (China Hospital Invasive Fungal Surveillance Net (CHIF-NET)) provided some epidemiological data for cryptococcosis, the molecular epidemiological aspects were not detailed [17,18]. Further, the programme determined drug susceptibility only to fluconazole and voriconazole. In the present study, we provide a contemporary snap shot of the species distribution, and investigate the genetic diversity and *in vitro* antifungal susceptibility of *C. neoformans* species complex isolates causing cryptococcosis from ten hospitals in China during a 5-year period.

Material and methods

Ethics statement

The study was approved by the Human Research Ethics Committee of Peking Union Medical College Hospital (S-263). Written informed consents were obtained from all patients, which included permission to study patient isolates for scientific research.

Isolates

Cryptococcus isolates were collected consecutively from unique patients (one strain per patient) from the CHIF-NET study, a laboratory-based, national multicentre surveillance programme during a 5-year period from August 2009 to July 2014 [17]. If patients had two isolates of the same organism cultured during the surveillance, only the first isolate cultured was studied. A total of 312 isolates were collected from patients in the ten study hospitals (Fig. 1), and no *Cryptococcus* isolates were excluded from the study because of patient decline for participation. Isolates were initially identified at each study centre by routine mycological methods (Vitek 2 YST or API20C AUX; both bioMérieux, Marcy l'Etoile, France) and then forwarded to a central reference laboratory (Department of Clinical Laboratory, Peking Union Medical College Hospital) for species identification, molecular typing and antifungal susceptibility testing. Species identification was performed by sequencing of the ITS region and by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) [17,19]. The species identification obtained at the reference laboratory was taken as the definitive identification.

Identification of Cryptococcus species and variety

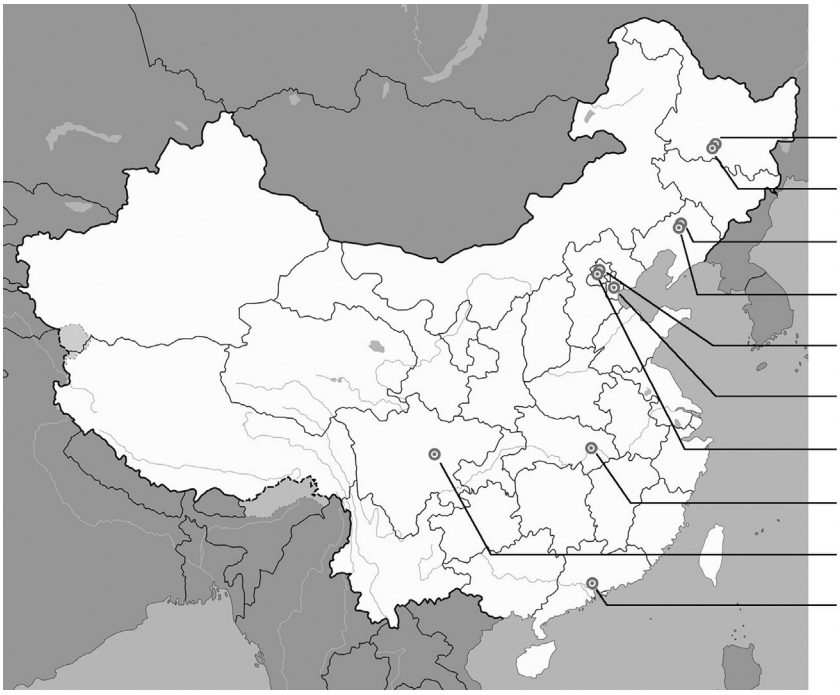
DNA extraction and amplification of the ITS region was performed as previously described using the primer pair ITS1 and ITS4 [17]. The PCR products were sequenced in both directions using the DNA analyser ABI 3730XL system (Applied Biosystems, Foster City, CA).

The obtained ITS sequences of *Cryptococcus* isolates were compared against those contained in the Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre database by using BioloMICSNet software (http://www.cbs.knaw.nl/collections/BioloMICSSequences.aspx). Further, ITS types for all isolates were assigned as previously described [4].

MLST, serotype and mating type analysis

MLST analysis was performed to delineate the subtype or genotype of the isolates. Briefly, seven housekeeping gene loci (*CAP59*, *GPD1*, *IGS1*, *LAC1*, *PLB1*, *SOD1* and *URA5*), were studied according to the protocol of Meyer *et al.* [12]. The PCR products were sequenced in both directions using the DNA analyser ABI 3730XL system (Applied Biosystems). Nucleotide sequences were analysed manually to ensure high-quality sequences, then queried against the online MLST database (http://mlst.mycologylab.org) to assign alleles for each locus. The sequence type (ST) was then defined according to isolates' allelic profiles. Molecular types (i.e. VNI to VNIV for *C. neoformans* and VGI to VGIV for *C. gattii*) were assigned according to isolates' STs and were queried against the online MLST database (http://mlst.mycologylab.org). Phylogenetic analysis depicting the genetic relationships between isolates based on MLST loci alleles were carried out with the categorical analysis method, and minimum spanning tree analysis based on strains' ST profiles were performed using BioNumerics software (version 7.5, Applied Maths, Kortrijk, Belgium). Novel allele types in each novel ST were confirmed twice by sequencing in both directions and have been deposited in the MLST database.

Serotyping of the isolates was performed as described previously using serotype A (JOHE2596/JOHE3241) and serotype D (JOHE2596/JOHE3240) specific primer sets [20], and mating type determined as described by Li *et al.* [21].



Hospital	No. of *Cryptococcus neoformans* var. *grubii* isolates (% of total)				
	Total	ST5	ST31	Other STs	Fluconazole non-WT
H1	17	14 (82.4)	0	3 (17.6)	4 (23.5)
H4	8	5 (62.5)	1 (12.5)	2 (25.0)	2 (25.0)
LR	1	1 (100)	0	0	0
Z1	11	9 (81.8)	2 (18.2)	0	0
PU	47	45 (95.7)	2 (4.3)	0	4 (8.5)
TZ	31	31 (100)	0	0	0
BD	12	12 (100.0)	0	0	0
TJ	35	31 (88.6)	3 (8.6)	1 (2.9)	1 (2.9)
HX	133	119 (89.5)	10 (7.5)	4 (3.0)	10 (7.5)
GZ	8	5 (62.5)	1 (12.5)	2 (25.0)	2 (25.0)
Total	303	272 (89.8)	19 (6.3)	12 (4.0)	23 (7.6)

Fig. 1. Geographic distribution of 10 hospitals participated in the present study, percentage for *Cryptococcus neoformans* var. *grubii* isolates of different sequence types (STs), and for fluconazole non-wild-type (non-WT, MIC >8 mg/L) *Cryptococcus neoformans* var. *grubii* isolates in each hospital. Hospital names and abbreviations: PU, Peking Union Medical College Hospital; TJ, Tongji Hospital; GZ, The First Affiliated Hospital of Sun Yat-Sen University; H1, The First Affiliated Hospital of Harbin Medical University; BD, Peking University First Hospital; HX, West China Hospital; TZ, Tianjin Medical University General Hospital; Z1, The First Hospital of China Medical University; LR, The People's Hospital of Liaoning Province; H4, The Fourth Affiliated Hospital of Harbin Medical University.

Antifungal drug susceptibility testing

Susceptibility to six antifungal drugs (fluconazole, voriconazole, itraconazole, posaconazole, amphotericin B and 5-flucytosine) was investigated using the Sensititre YeastOne™ YO10 method (Thermo Scientific, Cleveland, OH, USA). Briefly, isolates were sub-cultured onto Sabouraud dextrose agar and incubated at 35°C for 48 h. After this, 20 μL of 0.5 McFarland yeast suspension was transferred into 11 mL of inoculum broth and then 100 μL of the inoculated broth was transferred to each well of the manufacturer's plate. Plates were incubated at 35°C and the MIC endpoints were read at 72 h. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC22019 were used as quality control organisms for each run.

As there are currently no standard clinical breakpoints for *Cryptococcus* spp., we used the epidemiological cut-off values as recommended by previous studies: fluconazole, 8 mg/L; voriconazole, 0.12 mg/L; itraconazole and posaconazole, 0.25 mg/L; amphotericin B, 1 mg/L; and 5-flucytosine, 8 mg/L [9–11].

Statistical analysis

All statistical analyses were performed using IBM SPSS software (version 22.0; IBM SPSS Inc., New York, NY, USA). Categorical variables were compared using the χ^2 or Fisher's exact test, and continuous variables by the Mann–Whitney *U* test. A p value of 0.05 was considered significant.

Results

Cryptococcus isolates

A total of 312 *C. neoformans* species complex among 4191 (7.4%) yeast isolates (including *Candida*, *Cryptococcus* and other yeasts) were collected over the 5-year period. The proportion of *Cryptococcus* among all causative yeast species varied between hospitals ranging from 0.5% (1/189 in hospital LR) to 19.4% (136/700 in hospital HX); its prevalence over the 5 years was stable ranging from 6.0% (61/1022 isolates in the fifth year) to 8.5% (73/857 isolates in the third year) (p 0.183, not significant). Isolates from cerebrospinal fluid made up 67.6% (211/312) of isolates followed by blood (74/312, 23.7%). Isolates cultured from tissue biopsy specimens ($n = 13$), abscess ($n = 7$), ascitic fluid ($n = 4$), bile ($n = 1$), bronchoalveolar lavage fluid ($n = 1$) and bone marrow ($n = 1$) were rare.

Distribution of Cryptococcus species by ITS sequencing

The ITS sequencing assigned 312 *C. neoformans* species complex isolates into six ITS types, including two ITS types from 305 *C. neoformans* isolates and four from seven *C. gattii* isolates (Table 1). Within *C. neoformans*, 99.3% (303/305) isolates were *C. neoformans* var. *grubii*, and only two strains were *C. neoformans* var. *neoformans*. Molecular serotyping found all *C. neoformans* var. *grubii* to be serotype A, and the two *C. neoformans* var. *neoformans* isolates were serotype AD (Table 1). In addition, 99.3% (303/305) *C. neoformans* isolates were mating type α, and 0.7% (2/305) isolates were mating type a (Table 1). All *C. gattii* isolates were mating type α (Table 1).

Among seven *C. gattii* isolates, six had ITS types delineated previously, i.e. ITS types 3, 4 and 7 (Table 1) [4]. The remaining isolate, strain ID no. 12TJ267, exhibited a previously undocumented ITS type and was called as 'ITS type SH' in the present study (Table 1). Its ITS sequence was 100% identical to a previously reported *C. gattii* isolate (strain ID no. S8012) [22].

MLST analysis

In general, the results of MLST analysis supported the species identification and variety of *Cryptococcus* assigned by ITS

Table 1
Species distribution and molecular epidemiology of 312 *Cryptococcus neoformans* species complex isolates collected in the present study

Species	Serotype	Mating type	ITS type	Multilocus sequence typing								Molecular type	No. of isolates
				ST	*CAP59*	*GPD1*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*URA5*		
C. neoformans													305
var. *grubii*	A	α	ITS type 1	5	1	3	1	3	2	1	1	VNI	272
	A	α	ITS type 1	31	1	1	10	3	2	1	1	VNI	19
	A	α	ITS type 1	63	7	1	1	18	1	1	1	VNI	2
	A	α	ITS type 1	69	7	5	1	3	3	1	1	VNI	2
	A	α	ITS type 1	6	1	1	1	3	2	1	5	VNI	1
	A	α	ITS type 1	359	1	25	1	5	2	1	1	VNI	1
	A	a	ITS type 1	534[a]	53	11	84	42	10	61	43	VNI	1
	A	α	ITS type 1	535[a]	52	3	1	5	2	1	1	VNI	1
	A	α	ITS type 1	536[a]	1	41	1	5	2	1	1	VNI	1
	A	α	ITS type 1	537[a]	1	3	1	5	2	1	54	VNI	1
	A	α	ITS type 1	539[a]	55	3	1	5	2	1	1	VNI	1
	A	a	ITS type 1	538[a]	54	42	1	5	38	60	55	VNIV	1
var. *neoformans*	AD	α/α	ITS type 2	N/A[b]	Het[b]	1	60	23	Het[b]	1	Het[b]	VNIII	2
C. gattii													**7**
	ND	α	ITS type 3	57	16	5	3	5	5	65	12	VGI	3
	ND	α	ITS type 3	51	16	5	3	5	5	32	12	VGI	1
	ND	α	ITS type 4	2[a]	14	6	3	21	2	58	2	VGII	1
	ND	α	ITS type 7	332	16	51	70	13	13	34	24	VGI	1
	ND	α	ITS type SH[c]	159	16	14	3	5	5	45	12	VGI	1

ITS, rDNA internal transcribed spacer region; ND, not done; N/A, not applicable; ST, sequence type; Het, heterozygote.
[a] Novel STs identified in the present study.
[b] As the two *C. neoformans* var. *neoformans* isolates identified in the present study were serotype AD isolates and were heterozygote at gene loci *CAP59*, *PLB1* and *URA5*, thus the isolates' ST could not be assigned.
[c] The ITS type of strain 12TJ267 collected in the present study was not involved in ITS genotype system developed by Katsu *et al.* [4] But its ITS sequence was 100% identical to a previously reported *C. gattii* isolate (strain ID no. S8012) that described by Chen *et al.* [22] The *IGS1*, *LAC1* gene loci sequences were also 100% identical between strain 12TJ267 and S8012.

sequencing. Overall, *C. neoformans* presented a low degree of genetic diversity. Twelve STs were identified among 303 *C. neoformans* var. *grubii* isolates, including six that were novel (Table 2). VNI strains accounted for 302 of the 303 isolates (99.7%), only one strain of the 303 (0.3%) was VNIV. In addition, 89.8% of isolates (272/303) were of *C. neoformans* ST5, and this ST was the predominant ST in all ten hospitals (Fig. 1). The majority of *C. neoformans* var. *grubii* isolates from cerebrospinal fluid, blood, and all from other specimen types were ST5 (Table 2). ST31 was the next most common ST, identified in six of ten hospitals (Fig. 1). None of the other STs comprised more than three isolates, and their geographic distribution was scattered (Table 1, Fig. 1). Further combined phylogenetic analysis for 423 *C. neoformans* var. *grubii* isolates from this study (303 isolates) and two previous studies by Wu *et al.* (41 isolates) [23] and Dou *et al.* (79 isolates) [13] in China based on isolates' MLST locus alleles also illustrated that isolates belonging to ST5 and its closely related STs of clonal complex (CC) 5 were predominant in China (390/423 isolates, 92.2%), followed by CC31 isolates (24/423 isolates, 5.7%) (Fig. 2). We could not assign an ST to the two *C. neoformans* var. *neoformans* (molecular type VNIII) isolates because of heterozygosity at the *CAP59*, *PLB1* and *URA5* loci, but analysis of the other gene loci employed supported its 'variety' level (Table 1).

In comparison, *C. gattii* isolates were more genetically diverse, with five STs identified among seven isolates. Three of seven (42.9%) isolates were *C. gattii* ST57, and one isolate each belonged to the remaining four MLST STs including a novel ST (Table 1). In addition, for the ITS type SH, *C. gattii* isolates 12TJ267, its *IGS1*, *LAC1* gene loci sequences were also 100% identical to that of *C. gattii* strain S8012, which further supports its identification results by analysis of the ITS region (Table 1). Six of the seven (85.7%) *C. gattii* isolates were molecular type VGI, and one isolate (14.3%) was VGII (Table 1, Fig. 3); however, the latter was genetically divergent from two well-known VGII clones (represented by *C. gattii* strain R265 of ST20 for the major clone and strain R272 of ST7 for the minor clone) responsible for the *C. gattii* outbreaks in Vancouver (Fig. 3).

Antifungal drug susceptibilities

The susceptibilities to antifungal drugs are summarized in Table 3. Among 303 *C. neoformans* var. *grubii* isolates, the wild-type (WT) phenotype was seen in 97.7%–100% of isolates for voriconazole, itraconazole, posaconazole, amphotericin B and 5-flucytosine, and there were no significant trends for MIC_{50}, MIC_{90} and geometric mean MIC over the 5 years (all the p values >0.5) (Fig. 4). However, 7.6% (23/303) of isolates were non-wild-type (non-WT, MIC >8 mg/L) to fluconazole; these isolates were from six of the ten participating hospitals (Table 1, Fig. 1). The majority of fluconazole non-WT isolates (21/23, 91.3%) belonged to ST5, and one isolate each (4.3%) was ST31 and ST69 (Table 2).

In addition, the proportion of isolates that demonstrated non-WT MICs to fluconazole was significantly higher in the fourth study year (17/71, 23.9%, see Supplementary material, Table S1) compared with the first 3 years (0%–2.1%, p <0.001) (Fig. 4). These isolates were cultured from patients in different hospitals and medical services and were not clustered together. They included 1.7% (5/303) isolates that were *C. neoformans* ST5 with fluconazole MICs of ≥32 mg/L (Table 2) from three hospitals and which were identified in the fourth year. In comparison, the two *C. neoformans* var. *neoformans* isolates and seven *C. gattii* isolates had WT MICs to all drugs tested except for one *C. gattii* isolate with a fluconazole MIC of 16 mg/L (Table 1).

To explore any clinical variables that may have been associated with the proportional increase in isolates with non-WT MICs to fluconazole in the fourth year of the study, we reviewed the medical records and drug charts of 11/17 (64.7%) patients infected by these isolates. For seven patients (7/11, 63.6%), there was no history of fluconazole receipt within the preceding 30 days or prior to this

Table 2
Distribution of *Cryptococcus neoformans* var. *grubii* isolates of different sequence types (STs) in different specimen types, and prevalence of fluconazole non-wild-type (non-WT, MIC >8 mg/L) and fluconazole highly non-susceptible (MIC ≥32 mg/L) isolates

Sequence type	No. of *C. neoformans* var. *grubii* isolates (% of total)				
	Specimen type			Fluconazole susceptibility	
	Cerebrospinal fluid	Blood	Other specimen types	non-WT	MIC ≥32 mg/L
ST5	181 (89.6)	64 (86.5)	27 (100)	21 (91.3)	5 (100)
ST31	15 (7.4)	4 (5.4)	0	1 (4.3)	0
ST63	1 (0.5)	1 (1.4)	0	0	0
ST69	0	2 (2.7)	0	1 (4.3)	0
ST6	0	1 (1.4)	0	0	0
ST359	0	1 (1.4)	0	0	0
ST534	0	1 (1.4)	0	0	0
ST535	1 (0.5)	0	0	0	0
ST536	1 (0.5)	0	0	0	0
ST537	1 (0.5)	0	0	0	0
ST538	1 (0.5)	0	0	0	0
ST539	1 (0.5)	0	0	0	0
Total	202 (100)	74 (100)	27 (100)	23 (100)	5 (100)

time. However, the remaining four patients (4/11, 36.4%) had received fluconazole within the preceding 30 days before diagnosis of cryptococcosis; the reasons for fluconazole prescription were not stated, but presumably for empiric therapy of a possible fungal infection. In comparison, none of the four patients who were infected by 5-flucytosine non-WT isolates identified in CHIF-NET13, had previously received 5-flucytosine treatment (see Supplementary material, Table S1). After the diagnosis of cryptococcosis was established, nine of 11 patients received targeted anti-cryptococcosis therapy with amphotericin B plus 5-flucytocine and/or fluconazole, and two patients received no antifungal therapy. Three of 11 (27.3%) patients responded favourably to antifungal therapy, three (27.3%) patients died, and five patients (45.4%) discharged themselves against medical advice and outcome data were not available (see Supplementary material, Table S1).

Discussion

Worldwide, *C. neoformans* and *C. gattii* are major species causing cryptococcosis, although the proportion of species- and variety-specific distribution differs between geographic regions. Our study, for the first time, provides a description and understanding of the species epidemiology of cryptococcosis from ten centres in China over half a decade, more specifically of the genetic diversity and antifungal susceptibility of a large number of *C. neoformans* species complex strains.

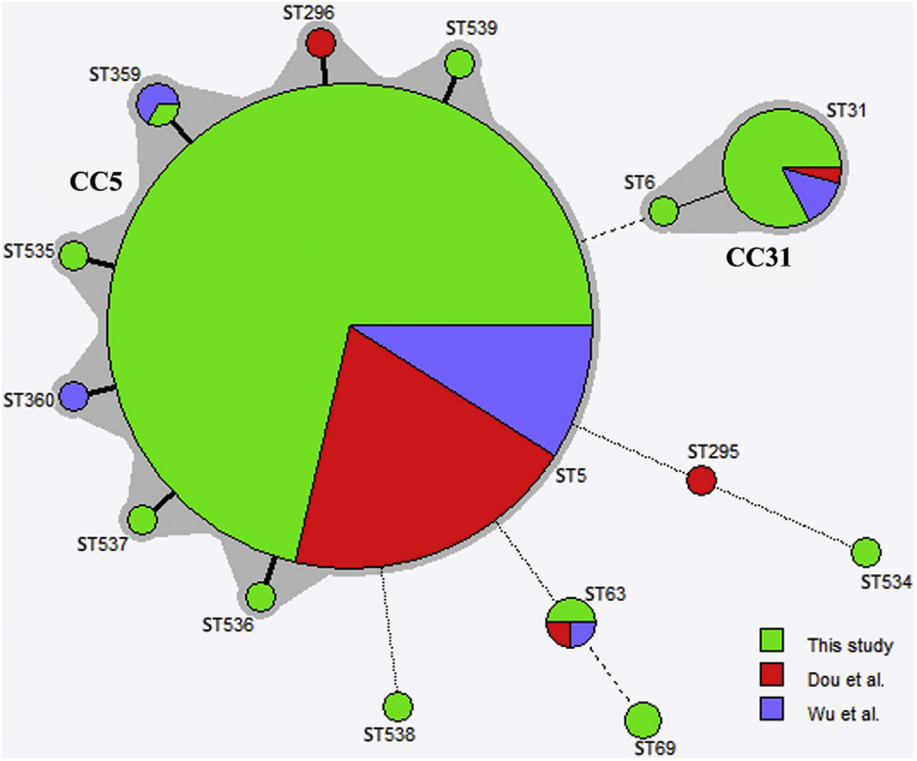
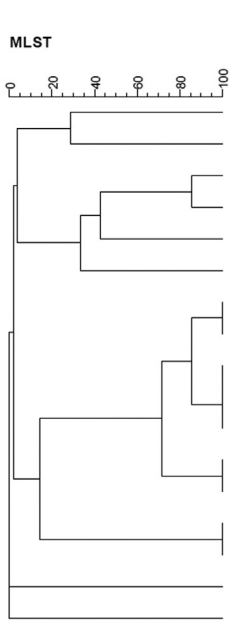


Fig. 2. Minimum spanning tree analysis for 423 *Cryptococcus neoformans* var. *grubii* isolates from this study (303 isolates) and two previous studies by Wu *et al.* (41 isolates) [23] and Dou *et al.* (79 isolates) [13] in China based on isolates' multilocus sequence typing loci alleles. Each circle corresponds to a sequence type (ST), and size of circle represents number of isolates for each ST. Different colour in circle represents different studies, and grey halo surrounding the circles denote STs belong to the same clonal complex. The lines between circles indicate the similarity between profiles (bold line, six of seven loci alleles in common; dashed line, four alleles; dotted line, fewer than three alleles).



MLST										
CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Strain No.	ST	Genotype	Reference
1	17	16	16	14	19	7	WM 178	21	VGII standard	Meyer et al. 2009
1	1	4	4	1	14	7	R265	20	VGII	Fraser et al. 2005
14	6	3	21	2	58	2	12HX537	2	VGII	This study
14	6	27	21	2	58	2	C73	44	VGII	Wu et al. 2015
2	6	10	4	2	15	2	R272	7	VGII	Fraser et al. 2005
8	6	22	4	2	87	3	PU8	182	VGII	Dou et al. 2015
16	5	3	5	5	32	12	14H1388	51	VGI	This study
16	5	3	5	5	32	12	WM 179	51	VGI standard	Meyer et al. 2009
16	5	3	5	5	65	12	13GZ378	57	VGI	This study
16	5	3	5	5	65	12	14HX699	57	VGI	This study
16	5	3	5	5	65	12	14HX706	57	VGI	This study
16	14	3	5	5	45	12	12TJ267	159	VGI	This study
16	14	3	5	5	45	12	HB3	159	VGI	Wu et al. 2015
16	51	70	13	13	34	24	11GZ174	332	VGI	This study
16	51	70	13	13	34	24	PU99	332	VGI	Dou et al. 2015
18	18	14	3	6	28	19	WM 175	60	VGIII standard	Meyer et al. 2009
17	10	8	18	3	37	11	WM 779	70	VGIV standard	Meyer et al. 2009

Fig. 3. Phylogram indicating the genetic relationships between the *Cryptococcus gattii* isolates from the present study, two previous studies by Wu *et al.* [23] and Dou *et al.* [13], reference strains of *C. gattii* genotypes I–IV proposed by Meyer *et al.* [12], and two VGII clone isolates reported in the Vancouver outbreak (strain R265 of ST20 represented the major clone and strain R272 of ST7 represented the minor clone) [43].

Table 3
Antifungal susceptibilities for *Cryptococcus* species collected in this study

Cryptococcus species	Fluconazole	Voriconazole	Itraconazole	Posaconazole	Amphotericin B	5-Flucytosine
C. neoformans var. *grubii* ($n = 303$)						
range	0.5–64	0.008–0.5	0.015–0.5	0.008–0.5	0.25–1	0.06–16
GM	4.28	0.034	0.057	0.084	0.60	3.42
MIC_{50}	4	0.03	0.06	0.06	0.5	4
MIC_{90}	8	0.12	0.12	0.25	1	8
WT (%)	92.4	98.3	99.0	97.7	100	98.7
Non-WT (%)	7.6	1.7	1.0	2.3	0	1.3
C. neoformans var. *neoformans* ($n = 2$)						
range	2	0.015	0.03–0.06	0.03–0.06	0.5–1	0.5–4
WT (%)	100	100	100	100	100	100
Non-WT (%)	0	0	0	0	0	0
C. gattii ($n = 7$)						
range	1–16	0.008–0.12	0.015–0.25	0.03–0.25	0.5	0.5–1
GM	4	0.033	0.055	0.09	0.5	0.61
WT (%)	85.7	100	100	100	100	100
Non-WT (%)	14.3	0	0	0	0	0

GM, geometric mean; MIC, minimum inhibitory concentration; WT, wild-type; non-WT, non-wild-type.

To date, ITS sequencing remains the most often used reference standard method for identification of most fungi species [17]. Within the *C. neoformans* species complex, ITS sequencing is also sufficiently discriminatory to not only distinguish between *C. neoformans* and *C. gattii*, but between the two varieties of *C. neoformans* [4]. Moreover, ITS sequencing can also subtype the *C. neoformans* species complex, particularly *C. gattii*, and ITS types correlate with other genotyping methods, although with lower discriminatory power [4,12].

Indeed, in the present study, by ITS sequencing, the majority (97.1%) of cryptococcal isolates were *C. neoformans* var. *grubii* (ITS type 1), consistent with previous studies on the species distribution of *C. neoformans* species complex isolates in China (84%–99%) [13,15,16,23,24]. Similar species and variety distributions of clinical *Cryptococcus* isolates are also observed elsewhere in East Asia and Africa [2,25–27]. It has been reported that cryptococcosis is more severe in patients infected with *C. neoformans* serotype A (ITS type 1) compared with those infected with *C. neoformans* serotype D (ITS type 2) or AD hybrid [28,29]; only two isolates (0.6% of 312 studied isolates) were ITS type 2, highlighting the importance of genotyping at the laboratory level. Infections caused by *C. neoformans* var. *neoformans* and the serotype AD hybrid were also rare in previous Chinese studies [2,13] in contrast with studies from Europe (>25% overall) [20,30]. In one French study, host factors including corticosteroid receipt may contribute to the apparent higher risk of being infected by *C. neoformans* serotype D (*C. neoformans* var. *neoformans*) [31]; however, the present study was not designed to capture these variables.

In our study, the predominance of a single ITS type of *C. neoformans* and the relative low genetic diversity was further explored by MLST analysis. We identified 12 MLST STs among 303 *C. neoformans* var. *grubii* isolates, which is in general agreement with previous findings that the Asian *C. neoformans* var. *grubii* population has lower diversity than the African, American and

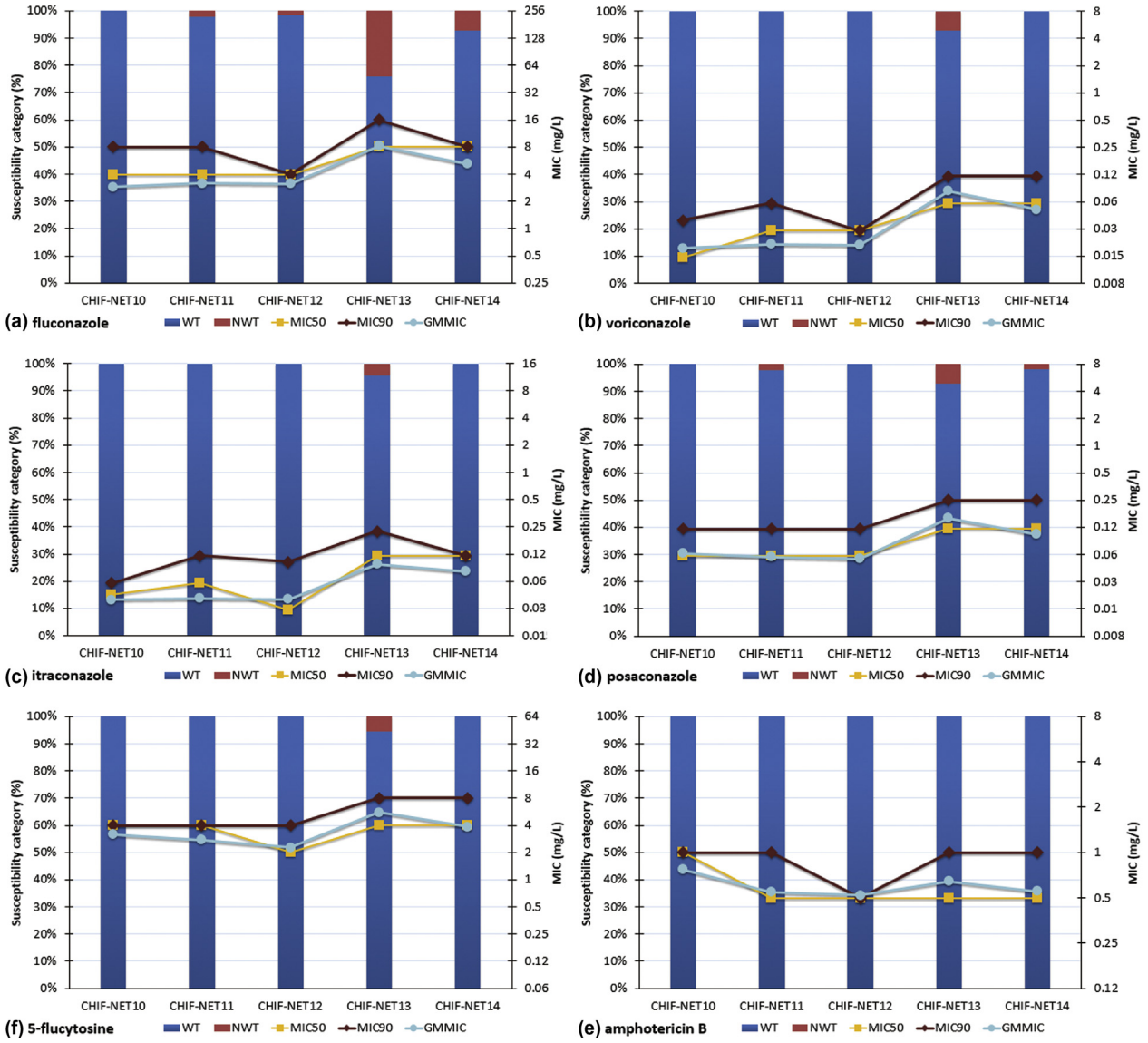


Fig. 4. Trends of susceptibility, including wild-type (WT) and non-wild-type (non-WT) rates, geometric mean MICs, MIC_{50} and MIC_{90} values of 303 *Cryptococcus neoformans* var. *grubii* isolates to six antifungal agents over 5 years (from August 2009 to July 2014).

European populations [27]. Further, we found that *C. neoformans* ST5 was the predominant ST (272/305, 89.2%) in *C. neoformans* isolates, followed by ST31 (19/305, 6.2%). Similar *C. neoformans* ST distributions were also observed by Dou *et al.* (ST5 accounted for 94.9% (75/79) isolates) [13] and Wu *et al.* (ST5 82.9% (34/41), ST31 7.3% (3/41)) [23] in previous studies in China. Moreover, in the previous study by Dou *et al.*, it was reported that among 22 HIV-positive and 43 HIV-negative *C. neoformans*-infected patients in China, 86.4% (19/22) and 97.7% (42/43) patients, respectively, were infected by *C. neoformans* ST5 [13]. Of note, *C. neoformans* ST5 has been the most common ST in all East Asian countries where epidemiology data were available, including China, Japan and South Korea [13,32–34]. However, in Thailand, ST4 and ST6 have been found to be the major MLST types, while ST93 is dominant in India and Indonesia [27,32].

In the present study, recovery of *C. gattii* remains uncommon (2.2% of isolates), contrasting with the epidemiology in regions endemic for *C. gattii* such as Australia and Papua New Guinea (64%–90%) [2] and, in the outbreak setting in North America [35,36]. In China, *C. gattii* cryptococcosis has been reported only relatively recently, mostly from patients living in subtropical and tropical regions [16,24], as were six of seven of our patients. That isolates from China may be distantly related from strains causing the outbreaks of infection [35,36] is supported by phylogenetic analysis of the VGII strain recovered herein. Xue *et al.* [16] stated that if more laboratories undertook MLST analysis, more cases of *C. gattii* would be diagnosed. However, of 312 infections in our study, MLST and ITS sequencing only identified seven cases, supporting the notion that *C. gattii* is uncommon in our region.

The epidemiology of *Cryptococcus* species is related to their ecology [24,37,38]. *Cryptococcus neoformans* has been isolated from soil, plant and animal samples, of which pigeon droppings have been found to be one of the more important reservoirs [38,39]. All ecological surveys in China, including those in avian habitats, have showed the predominance of *C. neoformans* var. *grubii* (serotype A) [24,38,39]. In comparison, an important ecological niche of *C. gattii* is eucalypt material. Eucalypt trees, including *Eucalyptus camaldulensis,* are also grown in large areas of China yet a previous survey

did not recover *C. gattii* from *E. camaldulensis* samples [38]; it is possible that both adaptation of the immediate eucalypt environment, and of the fungus to local climatic conditions may have affected likelihood of recovery of *C. gattii* [24].

During 5 years, there were no significant trends for changes in MIC_{50}, MIC_{90} and geometric mean of MIC values for *Cryptococcus* to any of six antifungal agents tested. However, in the fourth surveillance year, there was a significant increase in the proportion of fluconazole non-WT phenotype isolates in *C. neoformans* var. *grubii* (17/71 isolates, 23.9%) compared with the first 3 years (0%–2.1%, $p < 0.001$). Moreover, five isolates *C. neoformans* var. *grubii* isolates in the fourth year exhibited fluconazole MIC of ≥32 mg/L, all of which also had decreased susceptibly to voriconazole, itraconazole and posaconazole and belonged to the common clonal *C. neoformans* ST5. These five isolates were isolated from three different hospitals and were not clustered geographically. Before the present study, fluconazole MICs of ≥32 mg/L had not been reported in China, and their occurrence also appears to be rare worldwide [40–42]. However, as our review of the patients' medical records showed, only 36.4% patients infected by fluconazole non-WT *C. neoformans* isolates had previously received fluconazole treatment. Therefore, the proportional rise in strains with higher fluconazole MICs cannot be entirely attributed to the selective pressure during antifungal treatment. Future surveillance of both laboratory- and clinic-based data is warranted.

In conclusion, the present study provides useful data on the epidemiology, genetic diversity and antifungal susceptibility of *C. neoformans* species complex. *Cryptococcus neoformans* var. *grubii* was the predominant species, and ST5 is the commonest MLST type. Although overall antifungal susceptibility remained stable over the 5 years, increasing rates of fluconazole non-WT isolates were seen, especially among *C. neoformans* ST5 isolates.

Author contributions

XF, MX, HW, YPZ, YCX, ZYL conceived and designed the experiments. XF, MX, XH, LZ performed the experiments. XF, MX, SC, FK analysed the data. HW, YLX, MK, ZYS, ZDH, ZW, SLC, KL, YZC, TSH, GLZ contributed reagents/materials/analysis tools. XF, MX, SC, FK contributed to the writing of the manuscript.

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Appendix A. Supporting information

Additional Supporting Information may be found in the online version of this article at http://dx.doi.org/10.1016/j.cmi.2016.07.008.

Transparency declaration

The authors have declared that no competing interests exist.

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