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Genotypic diversity and antifungal susceptibility of *Cryptococcus neoformans* species complex from China, including the diploid VNIII isolates from HIV-infected patients in Chongqing region

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Abstract

Although previous studies on the genotypic diversity and antifungal susceptibility of the *Cryptococcus neoformans* species complex (CNSC) isolates from China revealed ST5 genotype isolates being dominant, the information of the CNSC isolates from Chinese HIV-infected patients is limited. In this study, 171 CNSC isolates from HIV-infected patients in the Chongqing region of Southwest China were genotyped using the International Society for Human and Animal Mycology-multilocus sequence typing (ISHAM-MLST) consensus scheme, and their antifungal drug susceptibilities were determined following CLSI M27-A3 guidelines. Among 171 isolates, six sequence types (STs) were identified, including the dominant ST5 isolates, the newly reported ST15, and four diploid VNIII isolates (ST632/ST636). Moreover, a total of 1019 CNSC isolates with STs and HIV-status information were collected and analyzed from mainland China in the present study. A minimum spanning analysis grouped these 1019 isolates into three main subgroups, which were dominated by the ST5 clonal complex (CC5), followed by the ST31 clonal complex (CC31) and ST93 clonal complex (CC93). The trend of resistance or decreasing susceptibility of clinical CNSC isolates to azole agents within HIV-infected patients from the Chongqing region is increasing, especially resistance to fluconazole (FLC).

Lay Abstract

In this paper, novel ST15 and 4 diploid VNIII isolates (ST632/ST636) were found in 171 CNSC isolates in southwest China, including evidence for resistance to fluconazole. Moreover, we clustered the 1019 clinical CNSC isolates reported so far from Mainland China into three major subgroups.

Introduction

The *Cryptococcus neoformans* species complex (CNSC) is the most common etiologic agent of cryptococcosis, which is listed in the first place in the critical group of WHO fungal priority pathogens list[1]. Over the past 30 years, rapid developments in molecular marker technologies such as multilocus sequence typing (MLST) and whole genome sequence type (WGST) have improved the understanding of cryptococcal epidemiology and genetic population diversity[2]. Currently, two separate species have been recognized in CNSC, including *C. neoformans* (serotype A; genotype AFLP1/VNI, AFLP1A/VNB/VNII and AFLP1B/VNII), *C. deneoformans* (serotype D; genotype AFLP2/VNIV), and the hybrid isolates (serotype AD; genotype AFLP3/VNIII)[3,4]. Currently, over 660 sequence types (STs) have been identified in the VNI lineage using the consensus multilocus sequence typing (MLST) scheme of International Society for Human and Animal Mycology (the ISHAM-MLST scheme) (https://mlst.mycologylab.org/page/CN%20Search). These STs in VNI were further divided into three distinct sub-lineages (VNIa, VNIb, and VNIc) based on phylogenomic analysis[5]. In addition, CNSC has two opposite mating types, *MAT*α and *MAT*a, with *MAT*α being the prevalent type and more virulent in animal models[6,7].

During decades of profound global environmental change and expanding immunocompromised populations, human pathogenic fungi, including CNSC, are evolving resistance to current antifungals[8,9]. For example, *C. neoformans* can acquire cross-resistance

to amphotericin B (AMB) and flucytosine (5-FC) by adaptation to fluconazole (FLC) through genomic aneuploidy[8]. Therapeutic failure of cryptococcosis is increasing during the consolidation and maintenance phases because of the emergence of FLC-resistant CNSC isolates[10,11].

Although CNSC tends to infect immunocompromised hosts[1], the majority of cryptococcosis cases from immunocompetent people have been reported in China[12,13]. However, the high prevalence of cryptococcosis cases from immunocompetent people in China was likely due to biased reporting. Specifically, HIV-infected patients are generally transferred to be managed in specialized infectious disease hospitals under China's policies and regulations, but so far, relatively few studies on cryptococcosis have been reported from this kind of infectious disease hospitals in China[14]. As a result, the genetic characteristics of CNSC isolates from China's HIV-infected population is still limited[14].

The characteristics of genotypic diversity and antifungal susceptibility of CNSC have been noted to vary between geographic regions[15,17]. Chongqing region is located in Southwest China, with a high degree of Chinese HIV-infected incidence rate and a population of 32,124,000 people[16]. To the best of our knowledge, no study have been conducted on the CNSC isolates from HIV-infected patients from the specialized hospitals in China[14,17]. The present study aimed to estimate genotypic diversity and antifungal susceptibility of clinical CNSC isolates in China, particularly for the isolates from HIV-infected patients the Chongqing region. This study could lay a foundation for understanding fine-scale spatial and temporal genetic structures of Chinese CNSC isolates from HIV-infected patients from the perspective of population genetics, particularly for the ST5 isolates.

Materials and methods

Isolates and patients

Fifty-eight HIV-infected patients confirmed by positive culture for clinical specimens were included between 2017 and 2018. All these patients signed informed consent following the ethics statement "2018-010-01-KY", which was approved by the ethics committee Chongqing public health medical center (CPHMC) according to the criterion of IRB (Figure S1). The document of informed consent for the patients was also included. One clone from each cryptococcal culture was collected for each patient by molecular sequencing of the ribosomal RNA intergenic spacer 1 (IGS1) of the rDNA region[18]. Then, the cryptococcal isolates were collected in chronological order and stored in tubes containing 2 mL of sterile distilled water at − 70 °C. The cryptococcal strains H99 (Serotype A; *MAT*α), KN99a (Serotype A; *MAT*a), JEC21 (Serotype D; *MAT*α), and JEC20 (Serotype D; *MAT*a) were included as reference strains for identifying the molecular type and mating type. In addition, we conducted a comprehensive review for studies published in English using the PubMed. For CNSC, we used medical subject headings (MeSH) and/or keyword terms in the title/abstract for each pathogen and criterion. The final search used ["China" & "Cryptococc*" & ("multi locus sequence typing" OR "MLST" OR "sequence typing"); time: 2008-2022].

Serotype, mating type, and ploidy

The genomic DNA of cryptococcal isolates was extracted as previously described[19]. To determine the serotype and mating type of CNSC isolates, PCR amplification using specific primer sets (*STE20Aα*, *STE20Aα*, *STE20Da*, and *STE20Dα*) was performed following protocols described previously[20]. Cell flow cytometry was performed using BD FACSAriaIII to test ploidy of the isolates following protocols described previously[21], with haploid strain H99 and hybrid strain PH140 as reference isolates.

Genotype

Each locus (*CAP59*, *GPD1*, *LAC1*, IGS1, *PLB1*, *SOD1* and *URA5*) for the isolates was amplified in a 25 μL PCR volume according to the ISHAM – MLST scheme. New specific primers to amplify the two alleles of every seven MLST loci were used to genotype AD hybrids within CNSC[22]. The PCR sequence reads were manually edited using SeqMan v8.0.2 (DNASTAR, Madison, WI, USA). The MLST sequences were aligned individually using the MAFFTv.7.0 (www.ebi.ac.uk/Tools/msa/mafft/) and queried against the online MLST database (http://mlst.mycologylab.org) to assign a seven-digit ST for each isolate.

The minimum spanning analysis

A total of 1019 clinical CNSC isolates were collected and selected from the present study and previous studies [11,20,23-28], which included all 46 reported STs in China, to establish a minimum spanning tree for the clinical CNSC isolates in China. The goeBURST analysis was conducted by PHYLOVIZ 2.0 (http://goeburst.phyloviz.net) software and a minimum spanning tree was generated based on goeBURST Full MST function in the software, to confirm the haplotypes obtained via phylogenetic analysis and to compare their origins and their allelic profiles[29]. A clonal complex (CC) was defined as a group of STs that differ from each other by a single or double locus variants.

Antifungal susceptibility

To assess the *in vitro* antifungal susceptibility, five antifungal drugs, namely, amphotericin B (AMB, Sigma-Aldrich, Basingstoke, UK), 5-flucytosine (5-FC, Sigma-Aldrich, Basingstoke, UK), fluconazole (FLC, Sigma-Aldrich, Basingstoke, UK), itraconazole (ITZ, Sigma-Aldrich, Basingstoke, UK) and voriconazole (VOR, Sigma-Aldrich, Basingstoke, UK) were used for the isolates according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 method[30]. The minimum inhibitory concentration (MIC) was determined after 72 h of incubation at 35 °C. *Pichia kudriavzevii* (formerly *Candida*

krusei) strain ATCC6258 and *Candida parapsilosis* strain ATCC22019 were used as quality controls. According to recommendations from previous studies[31,32], the epidemiological cut-off values (ECVs) of resistance in CNSC were set at 8 μg/mL for 5-FC and FLC; 0.25 μg/mL for ITR and VOR; and 1 μg/mL for AMB.

Statistical analysis

This study evaluated the differences among groups using the Mann – Whitney U test or t-test for continuous variables (expressed as the median [IQR]) or mean value ± standard deviation (Std.) and χ^2 tests for categorical variables, as appropriate. Statistical analyses were conducted using GraphPad Prism version 8.0.1. A *P* value of less than 0.05 was considered statistically significant.

Results

Serotype, genotype, ploidy and mating type of the CNSC isolates from Chongqing

The 171 CNSC isolates were collected in chronological order from 58 HIV-infected patients hospitalized at Chongqing Public Health Medical Center, Southwest China between 2017 to 2018. The majority of the isolates were recovered from cerebrospinal fluid (CSF) (83.6%, 143/171), followed by blood (15.8%, 27/171) and skin (0.6%, 1/171). The mean age of these patients was 47.3 ± 14.2 years (range: 11–75 years), with the majority within the age range of 41-50 years (37.9%, 22/58). The male/female gender ratio was 3.8 (46/12). Notably, analysis of the isolates collected in chronological order from each patient, as well as isolates from different sources, revealed mixed infections with multiple STs presented in approximately 17.2% (10/58) of the patients in this study.

Approximately 97.7% (167/171) of the isolates were identified as *C. neoformans* (Serotype A/VNI; *MAT*α), followed by four diploid isolates (2.3%; 4/171; Serotype AD/VNIII; *MAT* aA/Dα) generated in crosses between Serotype A/*MAT*a and Serotype D/*MAT*α isolates. Among the VNI isolates, five STs were identified, with the predominant

isolate being ST5 (91.6%; 153/167), followed by ST31 (6.6%; 11/167), ST15 (0.6 %; 1/167), ST79 (0.6 %; 1/167), and ST191 (0.6 %; 1/167). All four VNIII isolates had the same genotype ST632/ST636. The MLST sequences of all our 171 CNSC isolates were deposited in the Genbank under the accession numbers: OQ127704 – OQ127870 and OQ128345 – OQ129370. Details are shown in Table S1.

Genotypic analyses of the clinical CNSC isolates from China

A total of 1019 clinical CNSC isolates were collected in mainland China, including 488 isolates (482 VNI isolates, one VNII isolate, four VNIII isolates, and one VNIV isolate; 18 STs) from HIV-infected patients (Table S2) and 531 isolates (530 VNI isolates and one VNIV isolate; 28 STs) from HIV-uninfected patients, respectively. The minimum spanning tree (Figure 1) including 1019 isolates with all 46 reported STs from China (Figure 2) revealed that the ST5 clonal complex (CC5) clustered with its descendants (ST359 and ST298) and largely dominant in CNSC isolates from both HIV-infected and HIV-uninfected populations in China. The second-ranked ST31 clonal complex (CC31) also clustered with its descendants (ST77 and ST32). The third-ranked ST clonal complex of clinical CNSC isolates from China is ST93. Details are shown in Figure S2.

***In vitro* antifungal drug susceptibility**

The MIC values of the isolates in the present study were determined for five antifungal compounds, namely AMB, 5-FC, FLC, ITR and VOR. For the 171 isolates, the *in vitro* antifungal MIC_{90} and susceptibility ranges were 1.0 μg/mL (0.0625-1.0 μg/mL) for AMB, 8 μg/mL (0.125-16 μg/mL) for FLC, 8 μg/mL (0.25-32 μg/mL) for 5-FC, 0.06 μg/mL (0.015-0.5 μg/mL) for VOR, and 0.5 μg/mL (0.0313-2.0 μg/mL) for ITR. The highest percentage of isolates showed resistance was to ITR (approximately 18.7%; 32/171), followed by resistance to 5-FC (approximately 4.7%; 8/171), resistance to FLC (approximately 2.3%; 4/171), and resistance to VOR (approximately 1.8%; 3/171). No isolate showed resistance to AMB, but a

considerable proportion of the isolates showed decreased susceptibility (approximately 11.1%; MIC_{90} = 1 μg/mL). This phenomenon was also frequent in the isolates to FLC (approximately 9.4%; MIC_{90} = 8 μg/mL) and to 5-FC (approximately 6.4%; MIC_{90} = 8 μg/mL). One ST5 isolate (CQ57) exhibited cross-resistance to both FLC and 5-FC. Moreover, a considerable proportion of serial isolates from one HIV-infected patient in the study exhibited hetero-resistance (i.e., isolates CQ52, CQ53, CQ54, CQ55, CQ57, CQ58, CQ59, CQ61, CQ62 and CQ65 from Patient 18). Details are shown in Tables 1 and S3.

Discussion

Previous genetic analyses revealed statistically significant differentiations among continental and national populations of CNSC[17]. A comprehensive analysis of the genetic diversity of clinical CNSC isolates from different regions of China is still lacking, despite the rising number of CNSC STs that have been reported during the last decade[16,33]. In the present study, the majority of the patients from the Chongqing region were males aged 41−50 years, which was similar to previous studies conducted in China[34]. Sex hormones have been implicated in sex biases to cryptococcal infections[35]. Most of the isolates in the present study were recovered from CSF (83.6%), consistent with reports from other geographic regions showing the central nervous system (CNS) as the organ most frequently affected by cryptococcosis worldwide, including in China[36]. Approximately 17.2% of the patients in this study were co-infected by serial *C. neoformans* isolates with different STs. To our knowledge, this is the first report revealing the characteristics of cryptococcosis in China, which were reported once each from Cameroon[37] and Peru[38].

At the level of molecular type, the dominance of haploid VNI (*MAT*α) in this study (97.7%) was consistent with previous findings that VNI isolates have caused 95% of global cryptococcosis among HIV-infected populations annually[39], including China[25,36]. All the VNI isolates from Chongqing belonged to VNIa[5], largely due to the dominance of ST5 in

clinical CNSC from China and the close relationships between other STs and ST5[33]. Notably, four VNIII isolates (Serotype AD) were found in Chongqing. These isolates have mainly been reported from Europe, are diploid or aneuploid, and contain two sets of chromosomes and two mating type alleles, *MAT*α and *MAT*a[22,26,27]. All the VNIII isolates obtained in this study were diploid (*MAT* aA/Dα) by cell flow cytometry, our MLST analysis also revealed that they were identical to ST632/ST636, which belongs to a major VNIII genotype likely originated in sub-Saharan Africa[41]. VNIV isolates most often cause skin lesions[42] and occur mainly in Europe[40]; these isolates are rarely reported in China. While only two VNIV isolates (ST260/*MAT*α and ST535/*MAT*α) have been reported so far from Mainland China (the Hunan[24] and Sichuan[25] provinces), VNIV may be under-sampled but could be important in China, not only because they can cause infections by themselves but also they act as parental strains and hybridize with VNI strains to generate VNIII hybrids. In addition, one haploid VNII isolate (*MAT*α; ST43) was reported recently in an HIV-infected patient from eastern China according to the analysis, which is a rare genotype in clinical CNSC worldwide[19,26], consistent with potentially additional novel genetic diversity of CNSC in China.

At the level of the ISHAM sequence type, five STs were identified among the VNI isolates (n = 167) from the Chongqing region, with the predominant isolate being ST5 (91.6%), followed by ST31 (6.6%) and three other STs (ST15, ST79, and ST191). This result is not surprising because the dominance of ST5 has been repeatedly reported in Chinese clinical CNSC isolates[33,43]. Contemporary factors such as wind, animal and human migrations and other anthropogenic activities could all have facilitated the dispersals of genotypes, causing wide distributions of certain genotypes[17]. Moreover, this study identified ST15 (isolate CQ166) in blood obtained from a 57-year-old male patient. To the best of our knowledge, this is the first report in China of a clinical CNSC isolate with ST15; this isolate has only been reported from environmental samples in Brazil and Colombia[44-45].

As of January 2022, there were 657 total STs deposited into the *Cryptococcus* MLST database for CNSC in the world[17]. As of June 2023, our analysis on 1019 clinical CNSC isolates revealed that 46 STs exist in China, with ST5 being the most common genotype. These results suggest a low genetic diversity of CNSC in Far East Asia, including China[17,46]. Among the 46 STs, 28 and 18 STs were respectively reported from HIV-infected (n = 488) and HIV-uninfected (n = 531) populations in China, which suggest the genetic diversity of Chinese CNSC isolates from the HIV-infected population might be higher than that of isolates from HIV-uninfected population. All of the CNSC isolates from HIV-uninfected patients in China belong to *MAT*α[33,43]. Thus, clinical CNSC isolates from China, particularly those from the HIV-uninfected population, might have been derived by clonal expansion or same-sex reproduction between *MAT*α isolates[47].

Our minimum spanning analysis grouped all clinical CNSC isolates from China into three subclades, namely, the predominant ST5 clonal complex (CC5), followed by the ST31 clonal complex (CC31) and ST93 clonal complex (CC93). CC5 and CC93 are dominant across the globe and identical to the VNIa-5 and VNIa-93 subclades, respectively[5,17]. Moreover, ST5 seems to have a correlation with the immunocompetent population in China, whereas ST31 seems to be dominant in environmental CNSC isolates[48,49]. The mitochondrial morphology changes could partially explain the stronger ability of ST5 to infect HIV-uninfected people[5]. Except for ST5 and ST31, other STs such as ST359 (n=16), ST53 (n=5) and ST186 (n=5) also account for a considerable proportion of clinical CNSC isolates in China, which all belonged to the VNIa-5 subclade[5,32], one of the most common VNIa subclades worldwide. ST4 should belong to the VNIa-4 subclade, which is predominant in Southeast Asian countries such as Thailand and Laos[33,46] although it has not been reported from clinical CNSC in China. Although the ST93 subclade is also primarily found in the world, particularly in Africa[5,34], two ST93 isolates have been reported in China[19]. This

finding is consistent with both long-distance gene flow and geographic differences in the distributions of STs among countries, regions or continents[19,45,46].

Globally, the rate of antifungal resistance in human pathogenic yeasts continues to rise [9,35,36]. According to the current clinical guidelines for cryptococcosis[55], treating cryptococcosis is challenging due to resistance to azole antifungals, particularly to FLC[15,31,32]. The ARTEMIS DISK Global Antifungal Surveillance Study reported that FLC resistance in *C. neoformans* has progressively increased from 7.3% to 11.7% between 1997 and 2007[56], especially in isolates obtained from cryptococcosis patients with relapses[57]. In China, increasing resistance or decreasing susceptibility to FLC in clinical and environmental CNSC isolates has also been reported within the last decade, particularly among the ST5 isolates[11,27,43,48]. In the present study, approximately 2.3% of the isolates showed FLC resistance (MIC > 8 μg/mL) and one ST5 isolate (CQ57) was cross-resistant to both FLC and 5-FC, a result similar to those reported from the United States in the 1990s[58,59]. Environmental fungicides, such as tebuconazole, can cause *in vitro* cross-resistance between the agrochemical and clinical azoles such as FLC and ITR, due to the increased expression of the *ERG11*, *AFR1*, and *MDR1* genes[9].Approximately 4.7% of our isolates from Chongqing region showed 5-FC resistance, which was higher than the value given in the previous report (approximately 2.0%) from the United States in the 1990s[59]. Moreover, acquired resistance to AMB was not observed in the present study, which is uncommon in CNSC currently. However, approximately 11.1% of the isolates in the present study showed decreasing susceptibility to AMB (MIC = 1 μg/mL), which was similar to previous results from China[23]. Using FLC for long-term maintenance or monotherapy often results in hetero-resistant subpopulations[61], which might be acquired through the acquisition of different mutations, including aneuploid chromosomes[8].

Our study has several limitations. First, owing to the limited and sparsely distributed number of reported environmental CNSC isolates from China, we did not include the data in the present study for comparison. Further work should be conducted on environmental CNSC isolates from China, which can help identify potential differences between clinical and environmental populations of CNSC in Eastern Asia, including China[33,44]. Second, the mechanisms for the observed MIC changes, including serial isolates from the same patients, were not explored.

In summary, although the genotypic diversity of clinical CNSC isolates in China is extremely low, novel ST genotypes continue to emerge over the last decade, particularly the diploid VNIII (ST632/ST636) isolates from HIV-infected patients in the Chongqing region. Alarmingly, the MIC values of clinical CNSC isolates to antifungal drugs, particularly to FLC, has been increasing in China in recent decades. Our study calls for greater surveillance on cryptococcosis and increasing efforts to combat the spread of drug-resistant CNSC in China.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LYZ, SSW and NH participated in laboratory test, statistical analysis and drafted the manuscript. MYL, YTL, TZ, YP, CHH, XL, ZZ, MH and MZG participated in laboratory test and statistical analysis on this study. JPX and MC directed the study and revised the manuscript. GJL and MC designed the study and drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

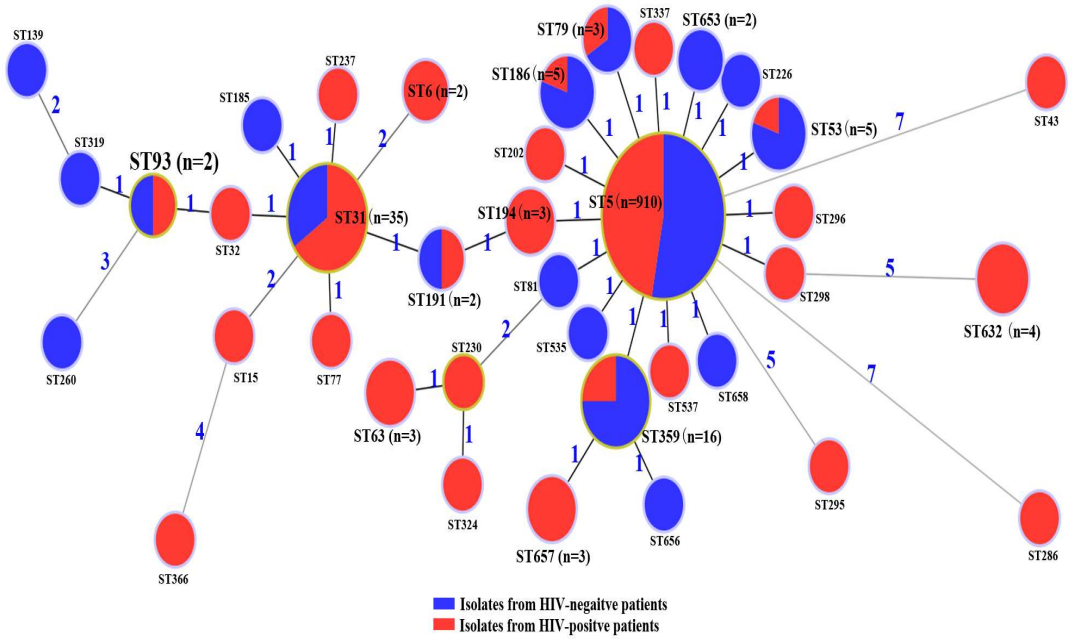

Figure 1. Minimum-spanning tree based on goeBURST analysis of the 46 detected STs and their relative distribution between HIV-infected and HIV-uninfected patients of 1019 Chinese clinical CNSC isolates. STs with sample size greater than one are all indicated in the figure. STs with dark green annulus: sub-group founder identified by goeBURST; STs with blue annulus: common nodes identified by goeBURST

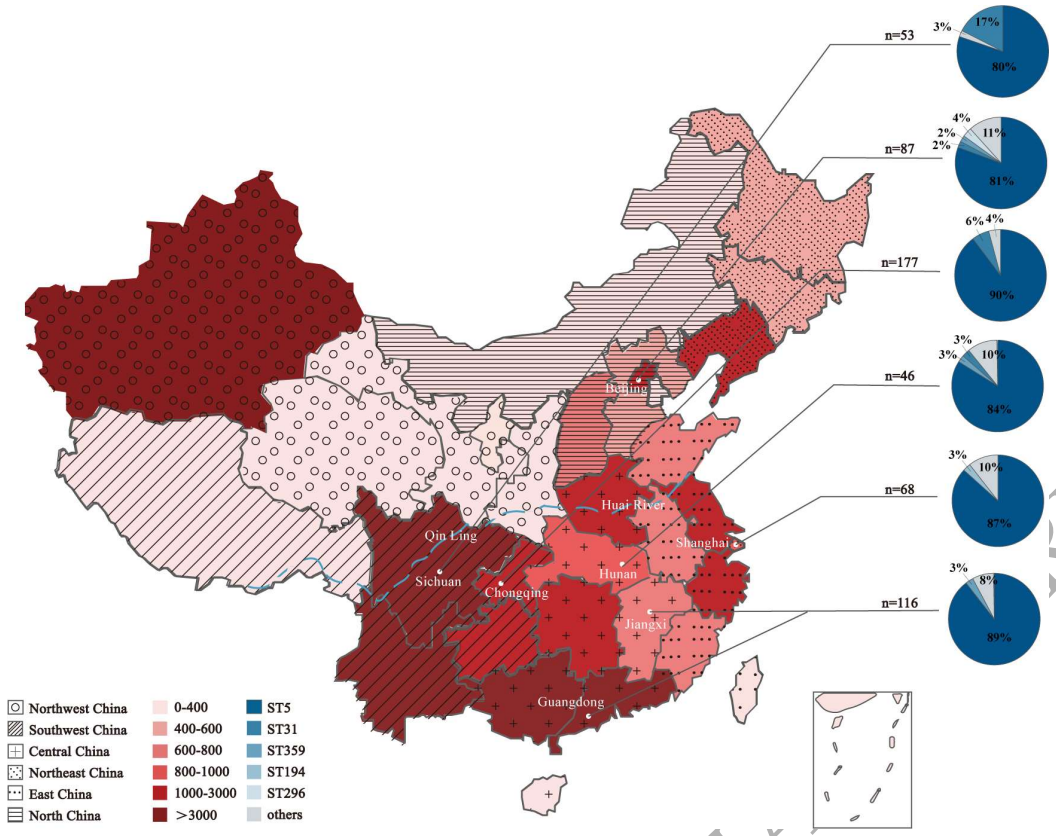

Figure 2. The distribution of *C. neoformans* STs from different geographical regions of China

Table legends

Table 1. MIC ranges, MIC_{50}, MIC_{90}, MIC distribution of antifungal drugs tested *in vitro* against 171 CNSC isolates from HIV-infected patients in Chongqing region, Southwest China

Antifungal agents	MIC ranges	Numbers of isolates with MIC (μg/mL) of											
		≤ 0.015	0.03	0.0625	0.125	0.25	0.5	1	2	4	8	16	32
Amphotericin B	0.0625-1	0	0	51	17	43	41	19	0	0	0	0	0
Fluconazole	0.125-16	0	0	0	8	18	14	34	48	28	17	4	0
Voriconazole	0.015-0.5	67	64	26	9	2	3	0	0	0	0	0	0
Itraconazole	0.0313-2	0	6	21	59	53	19	5	8	0	0	0	0
Flucytosine	0.25-32	0	0	0	0	19	21	30	53	29	11	0	0

MIC, minimal inhibitory concentration; MIC_{50}, MIC at which 50% of the isolates tested are inhibited; MIC_{90}, MIC at which 90% of the isolates tested are inhibited.