Clinical Microbiology and Infection 22 (2016) 887.e1–887.e9

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

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

X. Fan [1, 14], M. Xiao [1, 14], S. Chen [2, 14], F. Kong [2], H.-T. Dou [1], H. Wang [1], Y.-L. Xiao [3], M. Kang [3], Z.-Y. Sun [4], Z.-D. Hu [5], Z. Wan [6], S.-L. Chen [7], K. Liao [8], Y.-Z. Chu [9], T.-S. Hu [10], G.-L. Zou [11], X. Hou [1], L. Zhang [1], Y.-P. Zhao [12], Y.-C. Xu [1, *], Z.-Y. Liu [13, *]

[1] *Department of Clinical Laboratory, Peking Union Medical College Hospital, and Graduate School, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China*
[2] *Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, Westmead Hospital, University of Sydney, Westmead, New South Wales, Australia*
[3] *Department of Laboratory Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan, China*
[4] *Department of Clinical Laboratory, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China*
[5] *Department of Clinical Laboratory, Tianjin Medical University General Hospital, Tianjin, China*
[6] *Department of Clinical Laboratory, Peking University First Hospital, Beijing, China*
[7] *Department of Clinical Laboratory, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*
[8] *Department of Clinical Laboratory, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong, China*
[9] *Department of Clinical Laboratory, The First Hospital of China Medical University, Shenyang, Liaoning, China*
[10] *Department of Clinical Laboratory, The People's Hospital of Liaoning Province, Shenyang, Liaoning, China*
[11] *Department of Clinical Laboratory, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*
[12] *Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing, China*
[13] *Department of Infectious Disease, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing, China*

ARTICLE INFO

*Article history:*
Received 4 February 2016
Received in revised form
12 June 2016
Accepted 5 July 2016
Available online 16 July 2016

Editor: E. Roilides

*Keywords:*
Antifungal susceptibility
China
*Cryptococcus*
Invasive fungal infections
Molecular epidemiology
Species distribution

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**


* Corresponding authors. Y.-C. Xu, Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing 100730, China and Z.-Y. Liu, Department of Infectious Disease, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing 100730, China.
*E-mail addresses:* xycpumch@139.com (Y.-C. Xu), zhengyinl@hotmail.com (Z.-Y. Liu).
[14] Authors Xin Fan, Meng Xiao and Sharon Chen contributed equally to this work.

http://dx.doi.org/10.1016/j.cmi.2016.07.008
1198-743X/

## 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].