Mycopathologia (2021) 186:199–211
https://doi.org/10.1007/s11046-020-00500-6

ORIGINAL ARTICLE

Multilocus Sequence Typing of Clinical Isolates of *Cryptococcus* from India

Immaculata Xess · Mragnayani Pandey · Yubhisha Dabas · Reshu Agarwal · Shukla Das · Padma M. V. Srivastava · Rajeev Thakur · Shyama Sharma · Prashant Mani · Ashutosh Biswas · Dipankar Bhowmik · Bimal K. Das · Rakesh Singh · Preetilata Panda · Gagandeep Singh · A. C. Phukan · Margaret Yhome · Chithra Valsan · Anupma Jyoti Kindo

Received: 29 July 2020 / Accepted: 18 October 2020 / Published online: 20 January 2021

Abstract Cryptococcosis is a life-threatening infection caused by *Cryptococcus neoformans* and *C. gattii* species complex. In the present study, to understand the molecular epidemiology of 208 clinical isolates of *Cryptococcus* from different parts of India, multilocus sequence typing (MLST) using ISHAM MLST consensus scheme for *C. neoformans/C. gattii* species complex was used. MLST analysis yielded a total of 10 Sequence Types (STs)—7 STs for *C. neoformans* and 3 for *C. gattii* species complex. The majority of isolates identified as *C. neoformans* belonged to molecular type VNI with predominant STs 31 and 93. Only 3 isolates of *C. gattii* species complex were obtained, belonging to ST58 and ST215 of VGI and ST69 of VGIV. Phylogenetic analysis revealed less diversity among the clinical Indian isolates compared to the global MLST database. No association between prevalent STs and HIV status, geographical origin or minimum inhibitory concentration (MIC) could be established.

Keywords *Cryptococcus* · *Multilocus* sequence typing (MLST) · Antifungal susceptibility · India

Handling Editor: J. P. Xu.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11046-020-00500-6) contains supplementary material, which is available to authorized users.

I. Xess (✉) · M. Pandey · Y. Dabas · R. Agarwal · P. Mani · B. K. Das · G. Singh
Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi, Delhi 110029, India
e-mail: immaxess@gmail.com

S. Das · S. Sharma
Department of Microbiology, University College of Medical Sciences (UCMS) & Guru TegBahadur (GTB) Hospital, New Delhi, Delhi, India

P. M. V. Srivastava
Department of Neurology, All India Institute of Medical Sciences (AIIMS), New Delhi, Delhi, India

R. Thakur
Department of Microbiology, Institute of Human Behavior& Allied Sciences (IHBAS), Delhi, India

A. Biswas
Department of Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi, Delhi, India

D. Bhowmik
Department of Nephrology, All India Institute of Medical Sciences (AIIMS), New Delhi, Delhi, India

R. Singh
Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India

Introduction

Cryptococcosis, a life-threatening fungal infection, is mainly caused by *Cryptococcus neoformans s*and *C. gattii* species complexes affecting both immunocompromised and immunocompetent hosts. The mode of infection is through inhalation of basidiospores from environment. Clinical manifestations include pulmonary infection, chronic meningitis or dissemination depending upon the host immune status. Both species comprise genetically diverse subgroups that differ in ecology and epidemiology [1, 2]. *C. neoformans* includes 2 varieties with 3 serotypes: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), and a hybrid (serotype AD) and *C. gattii* species complex including serotypes B and C [3]. Recently, seven species have been recognized in *C. neoformans/C. gattii* species complex viz. *C. neoformans, C. deneoformans, C. gattii, C. bacillisporus, C. deuterogattii, C. tetragattii, C. decagattii* [4, 5]. With worldwide distribution, *C. neoformans* var. *grubii* (serotype A) is responsible for approximately 95% of cryptococcal infections and 98% of infections among HIV infected populations [6]. Until recently, *C. gattii* species complex (serotypes B and C) were considered restricted to tropical and subtropical regions, with Eucalyptus trees as an ecological niche. However, the environmental shifting of this species had been highlighted by a recent outbreak in the Pacific Northwest of North America [7, 8]. Additionally, it was also thought to have a predilection for infecting apparent immunocompetent hosts which had been challenged by African studies where most of the patients infected were immunosuppressed individuals [9]. Diagnosis of cryptococcosis is primarily based on direct demonstration of the capsule in India ink mount, isolation on culture and detection of capsular antigen by latex agglutination test (LAT) or lateral flow assay (LFA) in cerebrospinal fluid (CSF) and serum.

Several molecular typing methods like M13-PCR fingerprinting, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), multilocus microsatellite typing (MLMT) and multilocus sequence typing (MLST) have been used to study the molecular epidemiology of both *C. neoformans s*and *C. gattii* species complex [10]. Based on M13-PCR fingerprinting, these two species complexes are further divided into eight major molecular types: VNI and VNII (serotype A; *C. neoformans var. grubii)*, VNIII (hybrid serotype AD), VNIV (serotype D; *C. neoformans var. neoformans*), VGI, VGII, VGIII VGIV and VGV (serotypes B and C; *C. gattii* species complex) [11–16]. VNI and VGI are the most prevalent genotype for *C. neoformans* var. *grubii* and *C. gattii* species complex, respectively [17–19], and VNB, a genotype in *C. neoformans* var. *grubii* , was discovered in Botswana [10]

According to 2018 World Health Organization (WHO) guidelines, for treating cryptococcal meningitis among people living with HIV, the preferred regime for induction therapy is a combination of amphotericin B deoxycholate with flucytosine followed by fluconazole and for the consolidated/maintenance phase, fluconazole is recommended [20]. Despite the introduction of HAART (Highly Active Anti Retroviral Therapy), cryptococcosis remains one of the common opportunistic infections among people living with HIV [21–24]. The previous (2002–2007) data published from our institute showed the cryptococcal distribution of 60% in HIV positive and 40% in HIV negative individuals [25]. Extensive studies on environmental isolates had been conducted in India [26–32]. However, data on epidemiological typing of clinical isolates from India is still limited. Therefore, this study was planned to provide more insight into the molecular epidemiology of clinically isolated

P. Panda
Department of Microbiology, Maharaja Krishna Chandra Gajapati (MKCG) Medical College & Hospital, Brahmapur, Odisha, India

A. C. Phukan
Department of Microbiology, North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS), Shillong, Meghalaya, India

M. Yhome
Department of Microbiology, Naga Hospital Authority Kohima (NHAK), Kohima, Nagaland, India

C. Valsan
Department of Microbiology, Jubilee Mission Medical College and Research Institute, Thrissur, Kerala, India

A. J. Kindo
Department of Microbiology, Sri Ramachandra Medical College and Research Institute (SRMC & RI), Chennai, Tamil Nadu, India

Cryptococcus and their susceptibility profiles in the post-HAART era.

Materials and Methods

Clinical Isolates

A prospective study for 3 years (2012–2015) was conducted in which 86 *Cryptococcus* isolates from 77 patients were obtained. Prospectively, patients were enrolled from the following hospitals**:** All India Institute of Medical Sciences (AIIMS), New Delhi (n = 35), Guru Teg Bahadur Hospital (GTB), New Delhi, (n = 20), Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry (n = 11), Institute of Human Behaviour & Allied Sciences (IHBAS), New Delhi,(n = 8), Maharaja Krishna Chandra Gajapati Medical College & Hospital (MKCG), Brahmapur, Odisha (n = 5), North Eastern Indira Gandhi Regional Institute of Health and Medical Sciences (NEIGRIHMS), Shillong, Meghalaya (n = 4), Sri Ramachandra Medical College and Research Institute (SRMC & RI), Chennai, Tamil Nadu (n = 1), Naga Hospital Authority Kohima (N-HAK), Kohima, Nagaland (n = 1), Jubilee Mission Medical College Hospital and Research Institute (JMMCRI), Thrissur, Kerala (n = 1). Also, to observe any differences in the sequence types and susceptibility patterns previously collected 122 *Cryptococcus* isolates from 86 patients (AIIMS:99 isolates from 63 patients and IHBAS: 23 isolates from 23 patients) during 2005–2011 were also included. Therefore, 208 isolates so obtained from 163 patients were included in this study. *Cryptococcus* was mainly isolated from CSF (196/208, 94.23%). The remaining 12 isolates were from scalp abscess (3/208, 1.44%), sputum (3/208, 1.44%) and urine (3/208, 1.44%), whereas 2 were from blood and one from bronchoalveolar lavage (BAL) (S1 Table). This study was approved by the institute's ethics committee i.e. All India Institute of Medical Sciences, New Delhi, India (Ref no. IEC/NP-364/2012 and RP-15/2012). The study was conducted using clinical samples obtained from patients for routine microbiological investigations. We were not directly involved with patient enrollment. The treating physician took verbal consent at the time of patient admission to the hospital.

Reference Strains

A set of standard laboratory reference strains provided by Dr. Wieland Meyer, The West mead Institute for Medical Research, Australia representing each of the 8 major molecular types were used for molecular typing: WM148 (VNI), WM626 (VNII), WM628 (VNIII), WM 629 (VNIV), WM179 (VGI), WM178 (VGII), WM175 (VGIII) and WM779 (VGIV).

Phenotypic Identification

Following growth on brain heart infusion agar, phenotypic identification of the isolates was made by standard mycological methods including urea hydrolyzing test, sensitivity to actidione, phenol oxidase production on birdseed agar [33–35]. Canavanine–Glycine–Bromothymol blue (CGB) agar was used to distinguish *C. gattii/neoformans* species complexes [36]

Molecular Characterization

DNA Extraction

DNA was isolated using the protocol as described earlier with slight modifications [22]. The cells were treated in pH 5.0 lysing buffer (1.0 M sorbitol–0.1 M sodium citrate) containing 10 mg/ml lysing enzyme (from *Trichoderma harzianum*; Sigma Aldrich, St. Louis, MO) for 3 h at 30 °C to generate protoplasts. After that, genomic DNA was extracted using the Qiagen DNeasy Mini Kit (Qiagen, Valencia, CA, USA), as per the manufacturer's instructions.

MLST Analysis

Six genetic loci (*CAP59, GPD1, LAC1, PLB1, SOD1, and URA5*) and non-coding IGS1 region were undertaken for MLST analysis as per ISHAM consensus. PCR in 50 µL reaction volume was performed for each of the seven MLST loci using the primers described earlier with slight modifications in protocols [10, 14, 37–39]. The PCR products' sequencing was outsourced to XCelris (XCelris Lab. Ltd., Ahmedabad, India). Sequences were edited using DNA BASER (v 4.36.0) and then aligned in MEGA 6.0 (www.megasoftware.net). The allele profile for each isolate was generated by assigning allele type (AT) to

each of the seven loci. All sequence types (ST) were then described according to the ISHAM MLST scheme for *C. neoformans/C. gattii species* complex (https://mlst.mycologylab.org/.) Data from https://mlst.mycologylab.org were used for the global comparison.

Phylogenetic Relationship

Phylogenetic analysis was performed using the maximum likelihood method with 1000 bootstrap replicates implemented in MEGA v6.00. To represent comparison between original sources and allelic profiles of *C. neoformans* isolates, a minimum spanning tree was generated by Phyloviz v1.0 using goeBURST algorithm (https://goeburst.phyloviz.net/). For comparison with global isolates, data of commonly reported *C. neoformans* STs from multiple countries were retrieved from the ISHAM-MLST database (https://mlst.mycologylab.org). A total of 113 different STs (85 VNI, 17 VNB, 10 VNII, and 1 VNIV) were included for the same.

Nucleotide Diversity

For *C. neoformans*, DNA polymorphisms including haplotype and nucleotide diversity, number of polymorphic and mutation sites were calculated using DnaSP v5.10 (https:// www.ub.edu/dnasp/).

Antifungal Susceptibility Testing

Antifungal susceptibility testing was performed using the broth microdilution assay, according to Clinical Laboratory Standards Institute (CLSI) approved standard M27-A3 guidelines suggested for yeasts [40]. Quality control isolates (*Candida parapsilosis* ATCC 22,019 and *Pichia kudriavzevii* ATCC 6258) were included. The antifungal drugs tested were: amphotericin B (AMB), flucytosine (5FC), fluconazole (FLU) and voriconazole (VOR) (Sigma Chemical Corporation, St. Louis, MO).

Statistical Analysis

Fisher's exact test was used for statistical analysis. A p-value of ≤ 0.05 was considered statistically significant.

Results

Demographic Data and Molecular Types

Among 163 patients enrolled in the study, 126 were male and 37 were female. The HIV status was positive in 67 (67/163, 41.1%), negative in 90 (90/163, 55.21%) and status was unknown in 6 cases (6/163, 3.68%). Of the total of 208 isolates, 205 isolates were identified as *C. neoformans* belonging to VNI except one which belonged to VNII. Among the 3 *C. gattii* species complex, 2 were of molecular type VGI and one VGIV.

MLST Determination

A total of 10 sequence types (STs) were revealed following analysis of 208 *Cryptococcus* isolates by MLST. The aligned sequences were 4003 base pairs with 366 polymorphic sites. The 39 allele types (ATs) were obtained from all seven loci, one of which was novel to the Indian population (novel AT 5 of LAC1 gene belonging to ST 194). MLST analysis divided *C. neoformans* into seven STs: ST31 (n = 159), ST93 (n = 27), ST77 (n = 9), ST3 (n = 7), ST174 (n = 1) and ST194 (n = 1) of VNI and ST40 (n = 1) of VNII. In addition, three STs of *C. gattii* species complex from non-HIV population were identified; ST58 (n = 1) and ST215 (n = 1) of VGI and ST69 (n = 1) of VGIV (Fig. 1). Irrespective of the geographical distribution and HIV status, the most common ST type for *C. neoformans* was 31 (Table 1).

Phylogenetic Analysis of *C. neoformans*

Phylogenetic analysis of the global dataset using maximum likelihood method revealed 2 different clusters within the C. *neoformans* VNI population. VNB and VNII isolates clustered separately in different groups, VNIV was used as an out-group. Cluster I of the VNI population includes 23 different STs with the majority of European isolates. Cluster II is composed of 62 different STs with Asian isolates constituting the majority. Though isolates from different continents were dispersed in both the clusters, our isolates from the present study along with other Asian isolates fall under cluster II only (Fig. 2).

To understand the pattern of evolutionary descent among clusters of related genotypes, goeBURST

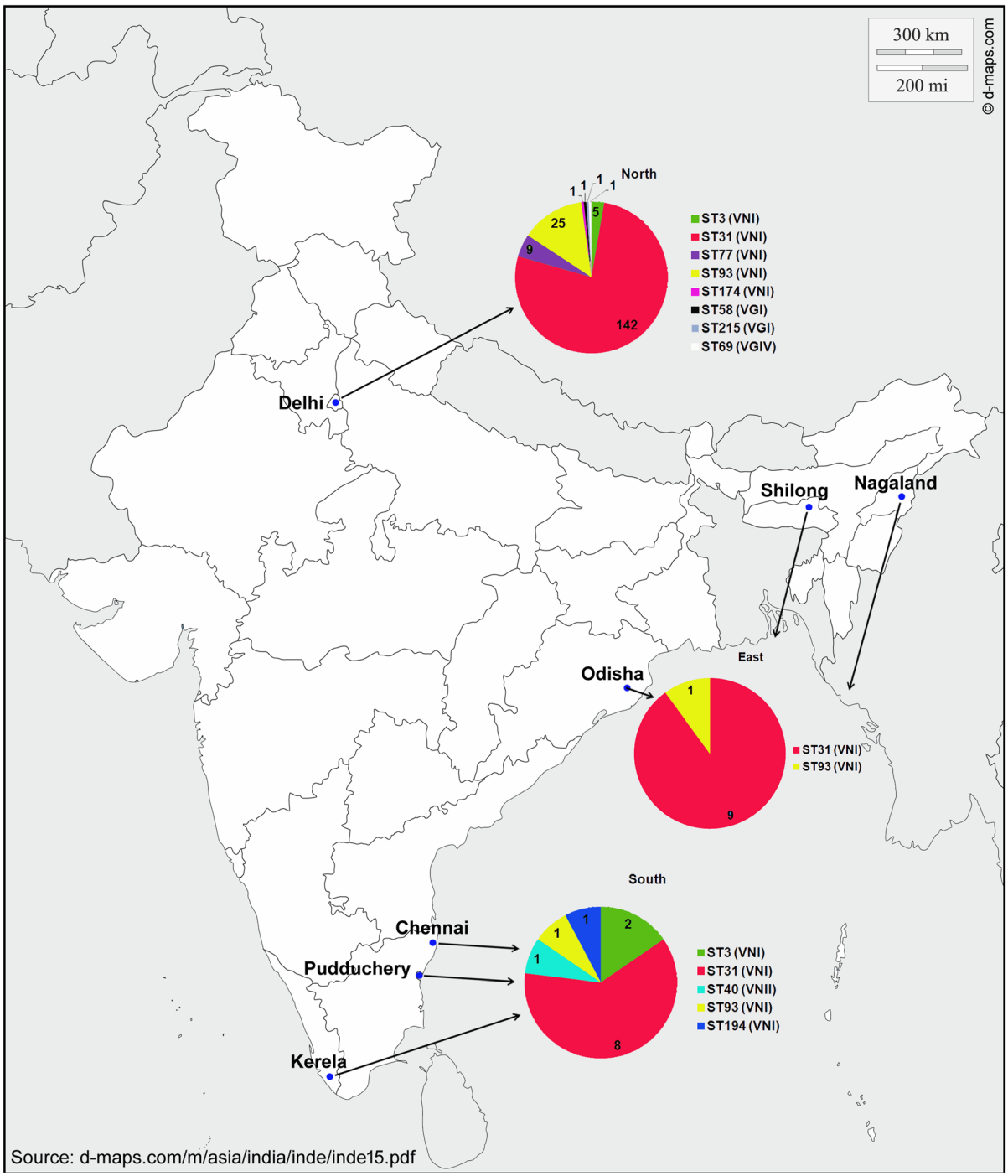


Fig. 1 Distribution of *Cryptococcus* sequence types (ST) in different parts of India. India map had been adapted from https://d-maps.com/m/asia/india/inde/inde15.pdf

analysis was performed. The goeBurst analysis differentiated our isolates into one main cluster and three singlet on STs VizST93, ST194 and ST40 (VNII) with ST174 as the founder one (Fig. 3a).

Table 1 Sequence types (STs) according to HIV status

HIV status	Sequence types										Total
	C. *neoformans*							C. *gattii* species complex			
	ST3	ST31	ST77	ST93	ST40	ST174	ST194	ST69	ST215	ST58	
HIV positive	3	74	2	7	1	0	0	0	0	0	87
HIV negative	4	78	7	20	0	1	1	1	1	1	114
Unknown HIV status	0	7	0	0	0	0	0	0	0	0	7
Total	7	159	9	27	1	1	1	1	1	1	208

Eight groups were formed when our isolates were compared with the global *C. neoformans* MLST dataset using goeBURST analysis. Groups were defined according to a single locus variant (SLV), and all the STs present in one group should be SLV for any one of the ST in that group. The founder ST of each group has been shown in figure as black circle. Most of the Asian isolates were found in group 1 and group 4. Isolates in the present study were grouped with other Asian isolates in group 1. Group 2 consists of the majority of STs originated from Europe. All the VNII isolates formed a separate group (Group 3). Group 5 contained four STs of VNI from all the continents with the majority from Europe. Most of the African VNI sequence types were clustered together in groups 6 and 8. Group 7 was formed by 2 VNII isolates from Europe. Most of the VNB isolates and few VNI, VNII and VNIV from different parts of the world were scattered as singletons (STs without group) (Fig. 3b).

Phylogenetic Analysis of *C. gattii* Species Complex

Phylogenetic analysis using Maximum-likelihood among the global *C. gattii* species complex revealed ST215 and ST58 from this study along with isolates from China clustering together in VGI. VGIV included ST69 from this study and ST243 previously reported from India (Fig. 4).

Association Between Sequence Types, HIV Status and Geographical Origin

No significant correlation between STs and HIV status (p value = 0.249), STs and geographical origin (p value = 0.087) could be established.

Nucleotide Diversity for *C. neoformans*

Among seven loci analyzed (*CAP59, GPD1, IGS1, LAC1, SOD1, PLB1 and URA5*), the highest nucleotide diversity (p) of 0.26685 was observed in *IGS1*, followed by *GPD1* (p = 0.00123) and *LAC1* (p = 0.00071). The haplotypes number (alleles) varies for different loci: 1 for *CAP59, PLB1, SOD1* and *URA1*, 2 for *LAC1* and 3 for *GPD1* and *IGS1*. The haplotypic diversity ranged from 0.33 for *LAC1* to 0.733 for *IGS1* (Table 2).

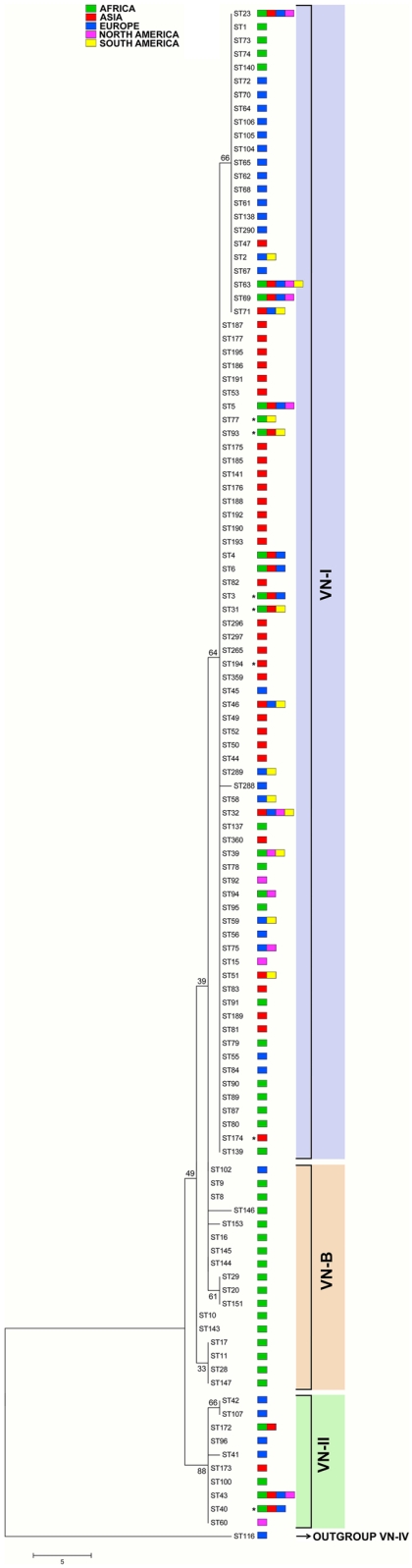
Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing of 208 isolates was performed for 4 drugs, namely AMB,

5FC, FLU and VOR. All of the isolates, irrespective of the species, showed low MICs for all the antifungals tested (Table 3).

Discussion

In the present study, 98.5% of the isolates were *C. neoformans* with molecular types VNI, VNII and 1.4% were *C. gattii* species complex with molecular types VGI, VGIV. With worldwide distribution, VNI had been reported as the most common molecular type reported for *C. neoformans* [18]. Molecular type VNII had been reported from varied continents including North America, South America, Europe, Africa and Asian countries like Thailand, India and Japan [10, 18, 19, 41–43]. It also accounted for 1%, 4.5% and 13.7% of the isolates from Taiwan, Malaysia and China, respectively [44–46]. Another frequent molecular type VNB, mainly considered endemic in



◀**Fig. 2** Phylogenetic analysis of *C. neoformans* using Maximum likelihood method. Each colored rectangle represents geographical origin of isolates. * indicates STs found in the present study. The numbers at each branch depict bootstrap values 50%, based on 1,000 replicates

Botswana, had now been reported from Rwanda, RD Congo, South Africa, Italy, Portugal, Brazil, Columbia but never from Asian countries [10, 13, 47]. The molecular epidemiology of 205 isolates in the present study showed the same paradigm with 204 isolates (99.5%) belonging to molecular type VNI and one isolate (0.5%) to VNII [18].

MLST of *C. neoformans* revealed a total of seven STs, of which one ST was not previously reported from our country, India. In the present study the predominant STs identified were ST31 followed by ST93, accounting for 77.5% and 13.1%, respectively. These 2 STs have previously been reported from India, as well as from other Asian countries like China, Japan, Korea, Qatar, Kuwait, Thailand and Indonesia, and also from Africa [18, 19, 47–52]. ST93 was reported as the most common ST type accounting for 63.6% among 143 clinical and environmental Brazilian isolates [53]. ST93 has recently been documented as a predominant ST (76.7%) in clinical and environmental samples from India [54]. ST77 has mostly been reported from clinical samples in India, along with few cases from Thailand, Uganda, Brazil and France [18, 19, 53, 55]. The remaining few genotypes in our study ST174 have also been reported previously from India and Kuwait, and ST3 from Thailand isolated from clinical isolates [18, 19]. ST194 has not been reported previously from our country India but from another Asian country China [18]. The predominant STs reported from Asian countries like China, Hong Kong, Japan and Thailand include ST4, ST5 and ST6; however, none of them were identified in our study [18, 48, 51]

In the present study, the highest nucleotide diversity was demonstrated at *IGS1* (0.26685) followed by *GPD1* (0.00123) and *LAC1* (0.00071) locus *for C. neoformans*. Similarly the highest diversity at *IGS1* locus (0.0045) followed by *LAC1* (0.0018) and *GPD1* (0.0014) was reported by Khayhan K et al. on Asian isolates [18].

Indian *C. neoformans* along with other Asian isolates were found to be less diverse when compared

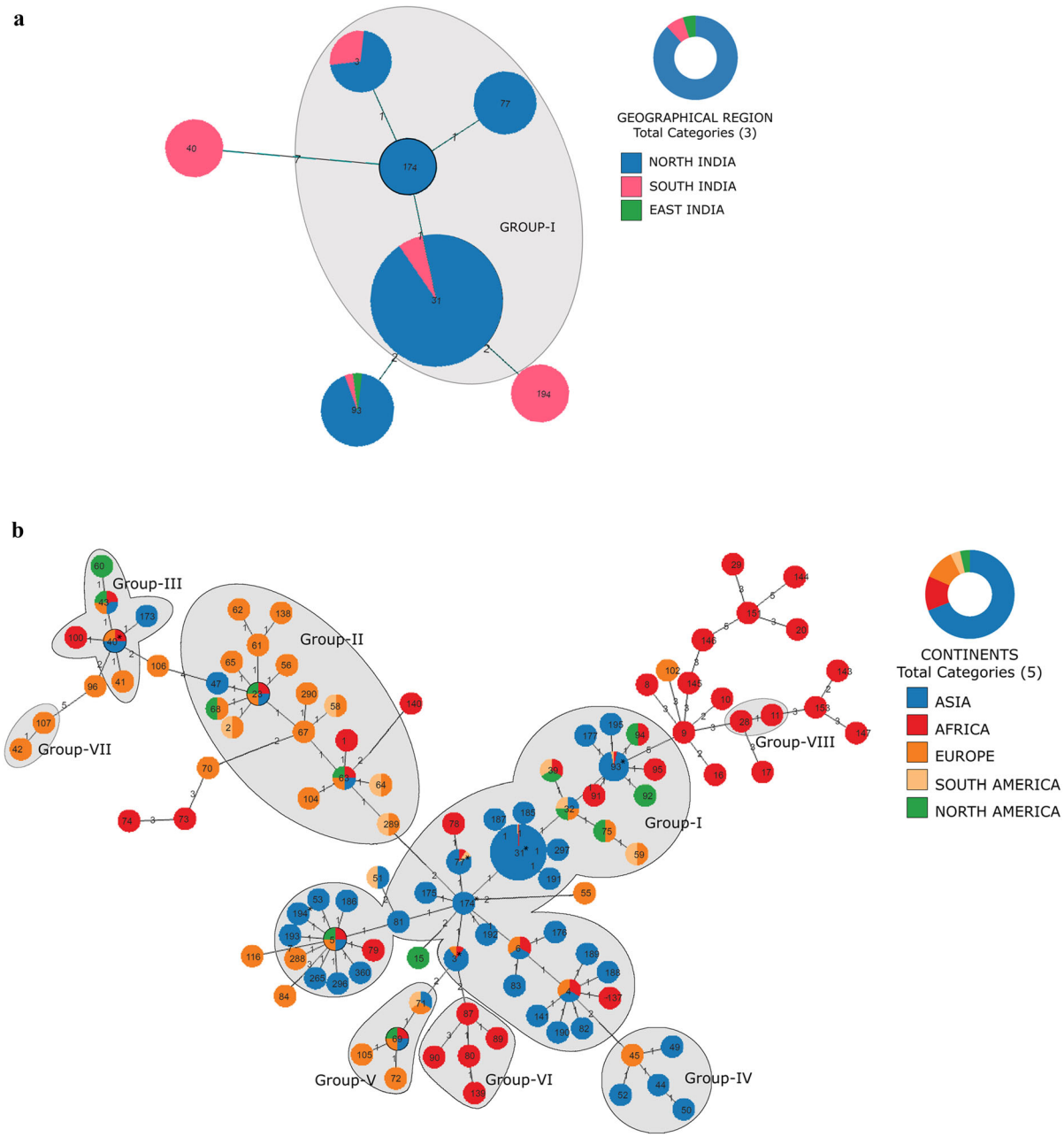


Fig. 3 **a** Minimum spanning tree showing C. *neoformans* isolates from different parts of India using goeBURST algorithm. Each colour of the circle represents a different part of India with sequence type. **b** Minimum spanning tree showing C. *neoformans* isolates from different continents using goeBURST algorithm. Each colour of the circle represents different continents with sequence type

with African, North and South American, and European population. Some isolates from Africa, North and South America and Europe shared the same Asian haplotypes. In contrast, the allele of very few Asian isolates displayed haplotypes similar to those seen in isolates of other continents [18].

A significant correlation between STs and HIV negative had been observed in various studies for *C. neoformans* [17, 18, 43, 56]. However, in contrast to

the above statement, no such correlation between ST and HIV status could be established in the present study (p value > 0.5). Similar to the previous report, in the present study, no change in STs distribution over the years was observed [51].

Five molecular types were recognized within *C. gattii* species complex: VGI, VGII, VGIII, VGIV and VGV with VGI as the predominant one. VGII is mostly associated with outbreaks and has been reported from British Columbia, Western Australia, Brazil, Venezuela, Pacific Northwest of the USA and Asia [14, 22, 57–59]. VGIII has been reported in Mexico, Colombia and the USA [8, 60–62]. VGIV has been reported mainly from India, Africa and South America [14, 47, 63]. In our study, two *C. gattii* species complex isolates belong to VGI with ST58 and ST215 and one to VGIV with ST69. ST58 had previously been reported from Columbia [62]. However, to the best of our knowledge ST215 (VGI) and ST69 (VGIV) have never been reported from India till date.

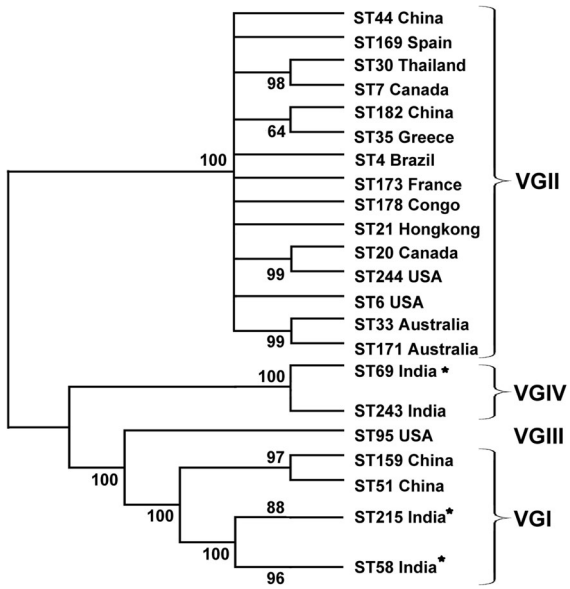


Fig. 4 Phylogenetic analysis of C. *gattii* from India and different countries of world using Maximum likelihood method. * Indicates *C. gattii* isolates found in the present study

In the present study, antifungal susceptibility testing was done on all isolates. The MIC_{50} and MIC_{90} values for all the antifungals tested are in accordance with previously published data [64, 65]. In the current study, the MIC_{50}, 2 μg/ml and MIC_{90}, 4 μg/ml for fluconazole were found similar to the published surveys from the USA and South Africa [64, 65]. These lower MICs for fluconazole [22, 66–68] and 5-flucytosine [29] observed are incongruent to the previous data from our country India. Higher MIC value than the epidemiological cut-offs reported earlier was found for one isolate to voriconazole (MIC 0.25 μg/ml) and for 2 isolates to amphotericin B (MIC 1 μg/ml) [69, 70].

In our study, no significant differences in susceptibility to the tested antifungal drugs were observed among isolates with different STs. This might be due to the high degree of clonality observed in this region. The limitations of the study included the lack of clinical isolates from the western part of the country and lack of environmental isolates, and due to the financial constraints we could not include AFLP in our methodology.

Table 2 Genetic variability of *C. neoformans* in the present study

Locus	Length (bp)	Number of polymorphic sites	Number of haplotypes	Haplotype diversity	Number of mutations	Nucleotide diversity
CAP59	560	0	1	0.000	0	0.00000
GPD1	544	2	3	0.600	2	0.00123
IGS1	722	362	3	0.733	362	0.26685
LAC1	471	1	2	0.333	1	0.00071
PLB1	533	1	1	0.533	1	0.00100
SOD1	536	0	1	0.000	0	0.00000
URA5	637	0	1	0.000	0	0.00000
Total	4003	366	12	2.199	366	0.26979

Table 3 *In vitro* antifungal susceptibility results according to the sequence types

		Sequence types									
		ST3	ST31	ST40	ST58	ST69	ST77	ST93	ST174	ST194	ST215
No. of isolates	208	7	159	1	1	1	9	27	1	1	1
Fluconazole				1	1	2			2	2	2
$^{+}$GM	1.48	1.48	1.42				1.58	1.75			
MIC_{50}	2	1	2				2	2			
MIC_{90}	2	4	2				4	4			
Amphotericin B				0.03	0.06	0.03			0.03	0.03	0.03
$^{+}$GM	0.09	0.10	0.09				0.08	0.13			
MIC_{50}	0.125	0.125	0.125				0.06	0.125			
MIC_{90}	0.25	0.5	0.25				0.125	0.5			
5-Flucytosine				0.5	0.5	1			2	0.5	2
$^{+}$GM	1.40	0.90	1.46				0.57	1.85			
MIC_{50}	2	1	2				1	2			
MIC_{90}	4	2	4				4	4			
Voriconazole				0.06	0.03	0.125			0.125	0.125	0.06
$^{+}$GM	0.03	0.03	0.03				0.03	0.03			
MIC_{50}	0.03	0.03	0.03				0.03	0.03			
MIC_{90}	0.06	0.03	0.06				0.03	0.06			

$^{+}$geometric mean, *MIC* minimum inhibitory concentration

Conclusion

The present study for the first time from India provides detailed information on molecular epidemiology and antifungal susceptibility pattern of 208 clinical *Cryptococcus* strains isolated from different regions of the country. The majority of isolates identified were *C. neoformans* belonging to molecular type VNI with predominant STs 31 and 93. The phylogenetic analysis revealed our isolates were clustered along with other Asian isolates in a group. Worldwide, the highest diversity was seen in African isolates [18]. Only 3 isolates of *C. gattii* species complex were obtained, belonging to ST58 and ST215 of VGI and ST69 of VGIV. Low MIC to different antifungals tested was observed for all the included isolates. Hence, fluconazole still can be given both in prophylaxis and maintenance therapy in the Indian scenario. Interestingly, no association between prevalent STs and HIV status, geographical origin or MIC value could be established. Additionally, we noticed an increase in the isolation of *Cryptococcus* species from HIV negatives. The present study database including both molecular epidemiology and antifungal susceptibility testing can be further utilized in a better understanding of the pathogenesis and treatment modalities in the future.

Acknowledgements The authors are grateful to laboratory technologist Mr. Bhaskar Rana for maintaining the cultures

Funding This work was supported by Department of Biotechnology, India (BT/PR5193/MED/29/463/2012), and All India Institute of Medical Sciences, New Delhi, India (A-08).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Heitman J, et al. Cryptococcus: from human pathogen to model yeast. Washington, DC: ASM press; 2011.
2. Matsumoto MT, et al. Genotyping, serotyping and determination of mating-type of *Cryptococcus neoformans*

clinical isolates from Sao Paulo State, Brazil. Rev Inst Med Trop Sao Paulo. 2007;49(1):41–7.
3. Franzot SP, Salkin IF, Casadevall A. Cryptococcus neoformans var. grubii: separate varietal status for *Cryptococcus neoformans* serotype a isolates. J Clin Microbiol. 1999;37(3):838–40.
4. Hagen F, et al. Recognition of seven species in the *Cryptococcus gattii/Cryptococcus neoformans* species complex. Fungal Genet Biol. 2015;78:16–48.
5. Hagen F, Lumbsch HT, Arsenijevic VA, Badali H, Bertout S, Billmyre RB, Bragulat MR, Cabañes FJ, Carbia M, Chakrabarti A, Chaturvedi S. Importance of resolving fungal nomenclature: the case of multiple pathogenic species in the Cryptococcus genus. Msphere. 2017;2(4).
6. Casadevall A, Perfect JR. Cryptococcus neoformans. Washington, DC: ASM press; 1998.
7. Datta K, Bartlett KH, Marr KA. *Cryptococcus gattii*: emergence in western North America: exploitation of a novel ecological niche. Interdiscip Perspect Infect Dis. 2009;2009:176532.
8. Byrnes EJ 3rd, et al. A diverse population of *Cryptococcus gattii* molecular type VGIII in southern Californian HIV/AIDS patients. PLoS Pathog. 2011;7(9):e1002205.
9. Nyazika TK, et al. Cryptococcus tetragattii as a major cause of cryptococcal meningitis among HIV-infected individuals in Harare Zimbabwe. J Infect. 2016;72(6):745–52.
10. Litvintseva AP, et al. Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. grubii (serotype A), including a unique population in Botswana. Genetics. 2006;172(4):2223–38.
11. Bovers M, Hagen F, Boekhout T. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. Rev Iberoam Micol. 2008;25(1):S4-12.
12. Bovers M, et al. Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. Fungal Genet Biol. 2008;45(4):400–21.
13. Boekhout T, et al. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology. 2001;147(Pt 4):891–907.
14. Meyer W, et al. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9(2):189–95.
15. Sukroongreung S, et al. Serotypes of *Cryptococcus neoformans* isolated from patients prior to and during the AIDS era in Thailand. Mycopathologia. 1996;135(2):75–8.
16. Farrer RA, Chang M, Davis MJ, van Dorp L, Yang DH, Shea T, Sewell TR, Meyer W, Balloux F, Edwards HM, Chanda D. A new lineage of *Cryptococcus gattii* (VGV) discovered in the central Zambezian Miombo woodlands. Mbio. 2019;10(6).
17. Choi YH, et al. Prevalence of the VNIc genotype of *Cryptococcus neoformans* in non-HIV-associated cryptococcosis in the Republic of Korea. FEMS Yeast Res. 2010;10(6):769–78.
18. Khayhan K, et al. Geographically structured populations of *Cryptococcus neoformans* variety grubii in Asia correlate with HIV status and show a clonal population structure. PLoS ONE. 2013;8(9):e72222.
19. Kaocharoen S, et al. Molecular epidemiology reveals genetic diversity amongst isolates of the *Cryptococcus neoformans/C. gattii* species complex in Thailand. PLoS Negl Trop Dis. 2013;7(7):2297.
20. World Health Organization. Guidelines for the diagnosis, prevention, and management of cryptococcal disease in HIV-infected adults, adolescents and children, March 2018: supplement to the 2016 consolidated guidelines of the use of antiretroviral drugs for treating and preventing HIV infection. 2018; Available from: https://www.who.int/iris/handle/10665/260399.
21. Kumar S, et al. Cryptococcal meningitis in HIV infected: experience from a North Indian tertiary center. Neurol India. 2008;56(4):444–9.
22. Jain N, et al. Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. J Clin Microbiol. 2005;43(11):5733–42.
23. Baradkar V, et al. Prevalence and clinical presentation of cryptococcal meningitis among HIV seropositive patients. Indian J Sex Transm Dis. 2009;30(1):19–22.
24. Patel AK, et al. Management of cryptococcal meningitis in HIV-infected patients: experience from western India. Indian J Sex Transm Dis. 2010;31(1):22–6.
25. Saha DC, et al. Detection of cryptococcus by conventional, serological and molecular methods. J Med Microbiol. 2009;58(Pt 8):1098–105.
26. Prakash A, et al. Environmental distribution of Cryptococcus species and some other yeast-like fungi in India. Mycoses. 2017;61:305–13.
27. Chowdhary A, et al. First environmental isolation of *Cryptococcus gattii*, genotype AFLP5, from India and a global review. Mycoses. 2013;56(3):222–8.
28. Chowdhary A, et al. Environmental prevalence of *Cryptococcus neoformans* and *Cryptococcus gattii* in India: an update. Crit Rev Microbiol. 2012;38(1):1–16.
29. Chowdhary A, et al. In vitro antifungal susceptibility profiles and genotypes of 308 clinical and environmental isolates of *Cryptococcus neoformans* var. grubii and *Cryptococcus gattii* serotype B from north-western India. J Med Microbiol. 2011;60(7):961–7.
30. Chakrabarti A, et al. Isolation of *Cryptococcus neoformans* var. gattii from *Eucalyptus camaldulensis* in India. J Clin Microbiol. 1997;35(12):3340–2.
31. Randhawa HS, Kowshik T, Khan ZU. Decayed wood of *Syzygium cumini* and *Ficus religiosa* living trees in Delhi/New Delhi metropolitan area as natural habitat of *Cryptococcus neoformans*. Med Mycol. 2003;41(3):199–209.
32. Bedi NG, et al. Seasonal prevalence of *Cryptococcus neoformans* var. grubii and *Cryptococcus gattii* inhabiting *Eucalyptus terreticornis* and *Eucalyptus camaldulensis* trees in Jabalpur city of Madhya Pradesh, Central India. J Mycol Med. 2012;22(4):341–7.
33. Vidotto V, et al. A new caffeic acid minimal synthetic medium for the rapid identification of *Cryptococcus neoformans* isolates. Rev Iberoam Micol. 2004;21(2):87–9.
34. Zimmer B, Roberts GD. Rapid selective urease test for presumptive identification of *Cryptococcus neoformans*. J Clin Microbiol. 1979;10(3):380–1.
35. Randhawa H, et al. The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India. Sabouraudia. 2008;46(8):823–33.

36. Klein K, et al. Identification of *Cryptococcus gattii* by use of L-canavanine glycine bromothymol blue medium and DNA sequencing. J Clin Microbiol. 2009;47(11):3669–72.
37. Meyer W, et al. Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. Med Mycol. 2009;47(6):561–70.
38. Fraser JA, et al. Same-sex mating and the origin of the Vancouver island *Cryptococcus gattii* outbreak. Nature. 2005;437(7063):1360–4.
39. D'Souza CA, et al. Investigation of the basis of virulence in serotype a strains of *Cryptococcus neoformans* from apparently immunocompetent individuals. Curr Genet. 2004;46(2):92–102.
40. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition M27–A3. CLSI: W., PA, USA; 2008.
41. Simwami SP, et al. Low diversity *Cryptococcus neoformans* variety grubii multilocus sequence types from Thailand are consistent with an ancestral African origin. PLoS Pathog. 2011;7(4):e1001343.
42. Umeyama T, et al. Determination of epidemiology of clinically isolated *Cryptococcus neoformans* strains in Japan by multilocus sequence typing. Jpn J Infect Dis. 2013;66(1):51–5.
43. Mihara T, et al. Multilocus sequence typing of *Cryptococcus neoformans* in non-HIV associated cryptococcosis in Nagasaki Japan. Med Mycol. 2013;51(3):252–60.
44. Liaw SJ, Wu HC, Hsueh PR. Microbiological characteristics of clinical isolates of *Cryptococcus neoformans* in Taiwan: serotypes, mating types, molecular types, virulence factors, and antifungal susceptibility. Clin Microbiol Infect. 2009;16(6):696–703.
45. Tay ST, et al. Determination of molecular types and genetic heterogeneity of *Cryptococcus neoformans* and *C gattii* in Malaysia. Med Mycol. 2006;44(7):617–22.
46. Chen M, et al. Molecular epidemiology of *Cryptococcus neoformans* species complex isolates from HIV-positive and HIV-negative patients in southeast China. Front Med China. 2010;4(1):117–26.
47. Cogliati M. Global molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii*: an atlas of the molecular types. Scientifica (Cairo). 2013;2013:675213.
48. Dou HT, et al. Molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii* in China between 2007 and 2013 using multilocus sequence typing and the diversiLab system. Eur J Clin Microbiol Infect Dis. 2015;34(4):753–62.
49. Park SH, et al. Molecular epidemiology of clinical *Cryptococcus neoformans* isolates in Seoul Korea. Mycobiology. 2014;42(1):73–8.
50. Wu SY, et al. Molecular characterisation of clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from Sichuan province China. Mycoses. 2015;58(5):280–7.
51. Hatthakaroon C, et al. Molecular epidemiology of cryptococcal genotype VNIc/ST5 in siriraj hospital, Thailand. PLoS ONE. 2017;12(3):e0173744.
52. Smith KD, et al. Increased antifungal drug resistance in clinical isolates of *Cryptococcus neoformans* in Uganda. Antimicrob Agents Chemother. 2015;59(12):7197–204.
53. Ferreira-Paim K, et al. MLST-based population genetic analysis in a global context reveals clonality amongst *Cryptococcus neoformans* var. grubii VNI isolates from HIV patients in southeastern Brazil. PLoS Negl Trop Dis. 2017;11(1):e0005223.
54. Prakash A, et al. Genotypic diversity in clinical and environmental isolates of *Cryptococcus neoformans* from India using multilocus microsatellite and multilocus sequence typing. Mycoses. 2020;63(3):284–93.
55. Wiesner DL, Moskalenko O, Corcoran JM, McDonald T, Rolfes MA, Meya DB, Kajumbula H, Kambugu A, Bohjanen PR, Knight JF, Boulware DR. Cryptococcal genotype influences immunologic response and human clinical outcome after meningitis. MBio. 2012;3(5).
56. Chen J, et al. *Cryptococcus neoformans* strains and infection in apparently immunocompetent patients. China Emerg Infect Dis. 2008;14(5):755–62.
57. Trilles L, et al. Regional pattern of the molecular types of *Cryptococcus neoformans* and *Cryptococcus gattii* in Brazil. Mem Inst Oswaldo Cruz. 2008;103(5):455–62.
58. Lockhart SR, et al. *Cryptococcus gattii* in the United States: genotypic diversity of human and veterinary isolates. PLoS ONE. 2013;8(9):e74737.
59. Ngamskulrungroj P, et al. Genetic diversity of the cryptococcus species complex suggests that *Cryptococcus gattii* deserves to have varieties. PLoS ONE. 2009;4(6):e5862.
60. Castanon-Olivares LR, et al. *Crytococcus neoformans* var. gattii in an AIDS patient: first observation in Mexico. J Med Vet Mycol. 1997;35(1):57–9.
61. Escandon P, et al. Isolation of *Cryptococcus gattii* molecular type VGIII, from *Corymbia ficifolia* detritus in Colombia. Med Mycol. 2010;48(4):675–8.
62. Lizarazo J, et al. Retrospective study of the epidemiology and clinical manifestations of *Cryptococcus gattii* infections in Colombia from 1997–2011. PLoS Negl Trop Dis. 2014;8(11):e3272.
63. Litvintseva AP, et al. Prevalence of clinical isolates of *Cryptococcus gattii* serotype C among patients with AIDS in sub-Saharan Africa. J Infect Dis. 2005;192(5):888–92.
64. Brandt ME, et al. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. Antimicrob Agents Chemother. 2001;45(11):3065–9.
65. Govender NP, et al. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates obtained through population-based surveillance in South Africa in 2002–2003 and 2007–2008. Antimicrob Agents Chemother. 2011;55(6):2606–11.
66. Tewari A, et al. Comparative analysis of the Vitek 2 antifungal susceptibility system and E-test with the CLSI M27–A3 broth microdilution method for susceptibility testing of Indian clinical isolates of *Cryptococcus neoformans*. Mycopathologia. 2012;173(5–6):427–33.
67. Datta K, et al. Fluconazole and itraconazole susceptibility of clinical isolates of *Cryptococcus neoformans* at a tertiary care centre in India: a need for care. J Antimicrob Chemother. 2003;52(4):683–6.
68. Capoor MR, et al. Current scenario of cryptococcosis and antifungal susceptibility pattern in India: a cause for reappraisal. Mycoses. 2008;51(3):258–65.

69. Pfaller MA, et al. Wild-type MIC distributions and epidemiologic cutoff values for fluconazole, posaconazole, and voriconazole when testing *Cryptococcus neoformans* as determined by the CLSI broth microdilution method. Diagn Microbiol Infect Dis. 2011;71(3):252–9.
70. Espinel-Ingroff A, et al. *Cryptococcus neoformans-Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for amphotericin B and flucytosine. Antimicrob Agents Chemother. 2012;56(6):3107–13.