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ORIGINAL RESEARCH

# Molecular Epidemiology and Antifungal Susceptibility of *Cryptococcus neoformans*: A First Report of Clinical Isolates from Shanxi Province, China

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**Purpose:** *Cryptococcus neoformans* is a critical fungal pathogen, and continuous surveillance of its molecular epidemiology and antifungal susceptibility is essential for clinical management. However, relevant data from many regions of China (including Shanxi Province) remain limited. This study aimed to provide the first molecular epidemiological description and antifungal susceptibility profile of *C. neoformans* clinical isolates from Shanxi Province, China, and to establish crucial baseline data for this region.

**Patients and Methods:** A retrospective analysis was conducted on all clinical isolates of *C. neoformans* (n=8) collected between 2014 and 2024 from a major regional hospital in Shanxi Province, China. All the isolates were subjected to molecular typing (serotype, mating type, genotype, and sequence type) and antifungal susceptibility testing.

**Results:** All 8 clinical isolates were identified as *C. neoformans*, with 100% consistency in key genetic features: serotype A, MATα mating type, VNI genotype, and ST5 sequence type. Antifungal susceptibility testing revealed low minimum inhibitory concentrations (MICs) for five common antifungals (fluconazole, amphotericin B, 5-fluorocytosine, itraconazole, voriconazole), with all 8 isolates (100%) falling within the wild-type epidemiological cut-off values (ECVs), notably, four of the eight strains (50%) showed higher MIC values (2 μg/mL) for fluconazole compared with the remaining isolates.

**Conclusion:** This study provides the first baseline data on the molecular epidemiology and antifungal susceptibility of *C. neoformans* in Shanxi Province. The exclusive presence of the susceptible ST5 lineage is consistent with the predominant pattern reported in China. These findings underscore the importance of establishing region-specific surveillance systems to monitor potential future shifts in the molecular epidemiology or antifungal resistance of *C. neoformans*, thereby supporting effective clinical management of related infections.

**Keywords:** *Cryptococcus neoformans*, molecular epidemiology, antifungal susceptibility, baseline data, Shanxi Province, China

## Introduction

Cryptococcosis is a life-threatening fungal infection caused by *Cryptococcus neoformans* and *Cryptococcus gattii* species[1] that mainly affect the central nervous system and lungs.[2] Human infections typically occur through inhalation of airborne fungal spores originating from environmental reservoirs, such as pigeon droppings and eucalyptus trees.[3,4] Cryptococcal meningitis is the most common form of the disease and disproportionately affects individuals with HIV infection. It is estimated that 152,000 cases of cryptococcal meningitis linked to HIV occur each year worldwide, with nearly 112,000 deaths resulting therefrom, constituting 19% of total AIDS-related mortalities globally.[5] Owing to its substantial disease burden and high mortality, *C. neoformans* was designated by the World Health Organization (WHO)



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in 2022 as a pathogen of critical priority in the WHO fungal priority pathogens list,[5,6] highlighting its significance to global public health.

The taxonomy of these pathogens has been substantially refined. The *C. neoformans* species complex encompasses two major haploid species: *C. neoformans* (serotype A, molecular type VNI/VNII), formerly known as *C. neoformans var. grubii*, which is the primary cause of cryptococcosis worldwide and predominantly affects immunocompromised populations, and *C. deneoformans* (serotype D, molecular type VNIV), synonymous with *C. neoformans var. neoformans*, which are widely distributed but most prevalent in Europe, can infect both immunocompromised and immunocompetent hosts.[7] Conversely, the *Cryptococcus gattii* species complex (sensu lato) comprises five primary species: *C. gattii* (VGI), *C. deuterogattii* (VGII), *C. bacillisporus* (VGIII), *C. tetragattii* (VGIV), and other unnamed species (VGV),[8] which are more frequently encountered in tropical and subtropical regions and are notable for causing severe diseases in immunocompetent hosts. In addition to haploid reproduction, *Cryptococcus* species can undergo hybridization, further contributing to genetic diversity. These taxonomic refinements provide an essential framework for interpreting the molecular epidemiological data from different geographic regions.

To date, several molecular methods have been applied for the detection of specific genetic sequences of members of the *C. neoformans/ C. gattii* species complex. This has enabled precise species identification and has revealed differences in geographic distribution, epidemiology, and antifungal susceptibility among species. In 2009, the International Society for Human and Animal Mycology (ISHAM) recommended multilocus sequence typing (MLST) as the preferred method to characterizing *Cryptococcus* strains.[9] In China, the predominant *Cryptococcus* group is *C. neoformans*, genotype VNI, serotype A and ST5, which primarily infect individuals without no underlying disease.[10] Early differential diagnosis and timely therapeutic intervention are pivotal for improving patient outcome. According to the Invasive Fungal Surveillance Net (CHIF-NET), approximately 10% of the strains analyzed in China exhibit reduced susceptibility or non-wild-type phenotypes to fluconazole (FCZ).[11] Although most strains remained susceptible, this trend highlights the need for continued antifungal surveillance.[12]

Taiyuan, the capital of Shanxi Province in northern China, has a temperate continental monsoon climate that influences the regional fungal distribution and infection patterns. However, epidemiological data on *Cryptococcus* infections in this region are scarce. This knowledge gap creates critical clinical and public health challenges: local clinicians lack region-specific baseline data to guide empirical antifungal therapy and targeted surveillance, and the absence of Shanxi data limits a comprehensive understanding of national *Cryptococcus* epidemiological trends and geographic variations in drug susceptibility. In this study, we retrospectively analyzed clinical *Cryptococcus* isolates from a tertiary hospital in Shanxi Province between 2014 and 2024, characterizing their molecular features and antifungal susceptibility profiles. By contextualizing these regional findings with national data, we aimed to establish foundational baseline information to support evidence-based clinical management and targeted epidemiological surveillance of cryptococcosis in northern China.

# Materials and Methods

## Clinical Isolates Information

The clinical isolates investigated in this study were obtained from cerebrospinal fluid (CSF) or blood cultures of patients diagnosed with *Cryptococcus* infections at Shanxi Bethune Hospital between 2014 and 2024. The Shanxi Bethune Hospital, established in 2011, is a provincial tertiary teaching hospital that currently functions as a major regional referral center in northern China.

Documented clinical information included age, sex, symptomatology, diagnosis, medical history (encompassing underlying disorders and exposure to poultry), prognosis, timing of specimen collection, and outcomes of routine CSF and biochemical examinations. Our inclusion criteria were as follows: (1) clinical cases with specimens from sterile anatomical sites (eg, cerebrospinal fluid and blood) and no concurrent polymicrobial infection at the same site; (2) positive results of *Cryptococcus* culture; (3) inclusion of only one index isolate per patient (duplicate isolates were excluded), with all strains viably preserved and successfully recovered; and (4) availability of complete clinical and microbiological data (eg, comorbidities, demographics, and clinical outcomes).

## Activation and Identification

Clinical specimens (CSF or blood) were initially processed using a BacT/ALERT 3D automated microbial detection system. For primary fungal isolation, the resulting isolates were inoculated on plates containing Sabouraud Dextrose Agar (SDA; 1% peptone, 4% dextrose, and 1.5% agar) (Autobio Diagnostics, Zhengzhou, Henan, China) and incubated at 35°C for 24–72 h.[13] The isolates were identified as *C. neoformans* using MALDI-TOF mass spectrometry.[14] They were preserved in cryovials filled with Brain Heart Infusion (BHI) broth containing 20% glycerol, which served as a cryoprotective agent. To obtain axenic cultures for downstream analyses, the resuscitated colonies were aseptically transferred to SDA plates, purified using the streak plate method, and subsequently incubated at 35°C for 48 h.

## DNA Extraction

Fungal DNA was extracted using the QIAamp Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions, with minor modifications.[15] Specifically, a fungal colony was collected from the edge of a demarcated plate using an inoculation loop, and then transferred to a sterile microcentrifuge tube. The colony was resuspended in 180 µL Buffer ATL supplemented with 10 µL lysozyme and 50 mg glass beads, vortexed for 30 min, and incubated at 37°C for 30 min to facilitate cell wall disruption. Proteinase K and lysis buffer were added in accordance with the manufacturer's standard protocol, followed by incubation at 56°C for 30 min, and heat inactivation at 95°C for 15 min. After supplementation with additional lysis buffer, mechanical bead beating, ultrasonication, and treatment with pre-chilled (4°C) ethanol,[16] the resulting mixture was loaded onto a QIAamp Mini spin column. The column was washed twice as per the manufacturer's instructions, and genomic DNA was eluted with 200 µL of elution buffer and stored at −20°C until further use.

## *Cryptococcus* Variant Identification

To confirm the identity of the isolated fungal strains, the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene was amplified using the universal primers ITS1 and ITS4 (Table 1). The resulting amplicons were bidirectionally sequenced on a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the obtained sequences were aligned and compared against reference sequences of *Cryptococcus* strains retrieved from the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast). This comparative sequence analysis enabled accurate determination of fungal variant types.[17] The methodology is detailed in the Supplementary Methods 1.

## *Cryptococcus* Genotype Identification

To clarify the genotypic characteristics of the isolates, we amplified the superoxide dismutase 1 (SOD1) gene, which serves as a core molecular marker for cryptococcal genotyping.[18–20] The sequences obtained were aligned with reference sequences from the following representative *Cryptococcus* strains: WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype D, VNIII), WM629 (serotype AD, VNIV), WM179 (serotype B, VGI), WM178 (serotype B, VGII), WM175 (serotype B, VGIII), and WM779 (serotype C, VGIV),[21] which are internationally recognized reference strains widely used in cryptococcosis research. The detailed methodology is provided in the Supplementary Methods 2.

## *Cryptococcus* Mating Type Identification

The mating type was determined using a multiplex PCR assay with primers (Table 1) targeting the pheromone-encoding sequences within the MAT loci of *Cryptococcus*, where the MATα-specific target was the α-pheromone-encoding sequence in the MATα locus, and the MATa-specific target was the a-pheromone-encoding sequence in the MATa locus. These genes were specific to α and a mating types, respectively, as previously described.[20,22,23] The amplified MAT locus-specific PCR products were subjected to electrophoresis on 1% agarose gels for the initial validation. Specifically, the well-characterized *Cryptococcus neoformans* reference strain H99 (MATα) was used as a positive control to confirm PCR specificity, whereas a no-template control (NTC) was included to rule out potential cross-contamination or reagent contamination. To further substantiate the mating-type assignments, the PCR amplicons were subjected to Sanger sequencing and the resulting sequences were aligned and compared against the H99 reference allele

**Table 1** Primer Information for Gene Amplification of *Cryptococcus neoformans* Species Used in This Study

| Target Gene Region | Primer Sequence (5′–3′) | PCR Amplification Conditions: | Fragment Size(bp) | Reference Method (Supplementary) |
|---|---|---|---|---|
| ITS | ITS1: TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCTTATTGATATGC | 94°C 5min; 30 cycles: 94°C 60s, 50°C 60s, 72°C 60s; 72°C 10min | 550 - 600 | Method 1 |
| STE20Aα | F: CTTCACTGCCATCTTCACCA R: GACACAAAGGGTCATGCCA | 95°C 3min; 30 cycles: 94°C 60s, 57.5°C 60s, 72°C 60s; 72°C 10min | 101 | Method 3 |
| STE20Aa | F: CGCCTTCACTGCTACCTTCT R: AACGCAAGAGTAAGTCGGGC | 95°C 3min; 30 cycles: 94°C 60s, 57.5°C 60s, 72°C 60s; 72°C 10min | 117 | Method 3 |
| CAP59 | F: CTCTACGTCGAGCAAGTCAAG R: TCCGCTGCACAAGTGATACCC | 94°C 3min; 35 cycles: 94°C30s, 56°C 30s; 72°C 1min; 72°C 10min | 559 | Method 4 |
| GPD1 | F: CCACCGAACCCTTCTAGGATA R: CTTCTTGGCACCTCCCTTGAG | 94°Cmin; 35 cycles: 94°C 45s, 63°C 1min; 72°C 2min; 72°C 10min | 543 | Method 4 |
| IGS1 | F: ATCCTTTGCAGACGACTTGA R: GTGATCAGTGCATTGCATGA | 94°C 3min; 35 cycles: 94°C 30s, 60°C 30s; 72°C 1min; 72°C 10min | 723 | Method 4 |
| LAC1 | F: AACATGTTCCCTGGGCCTGTG R: ATGAGAATTGAATCGCCTTGT | 94°C 3min; 30 cycles: 94°C 30s, 58°C 30s; 72°C 1min; 72°C 10min | 469 | Method 4 |
| PLB1 | F: CTTCAGGCGGAGAGAGGTTT R: GATTTGGCGTTGGTTTCAGT | 94°C 3min;30 cycles: 94°C 45s, 61°C 45s; 72°C 1min; 72°C 10min | 532 | Method 4 |
| SOD1 | CNF: AAGCCTCCTCATCCATATTCTT CNR: TTCAACCACGAATATGTA CGF: GATCCTCACGCCATTACG CGR: GAATGATGCGCTTAGTTGGA | 94°C 3min; 35 cycles: 94°C 30s, 52°C 30s; 72°C 1.5min; 72°C 10min | 535 | Method 2 / Method 4 |
| URA5 | F: ATGTCCTCCCAAGCCCTCGAC R: TTAAGACCTCTGAACACCGTACTC | 94°C 3min; 35 cycles: 94°C 45s, 63°C 1min; 72°C 2min; 72°C 10min | 601 | Method 4 |

**Abbreviations**: F, forward; R, reverse; CN, *Cryptococcus neoformans*; PCR reaction conditions: Initial denaturation; Cycle number: Denaturation, Annealing, Extension; Final extension; s, seconds; min, minutes.

(GenBank accession number: AF542529.2) using the NCBI BLAST database, focusing on sequence identity and coverage to ensure reliable genotype assignment. The detailed methodology is provided in the supplementary Methods 3.

## *Cryptococcus* MLST Identification

Seven housekeeping genes (CAP59, GDP1, LAC1, PLB1, URA5, IGS1, and SOD1) of *Cryptococcus* were amplified according to the ISHAM consensus MLST protocol for *Cryptococcus* species,[21] consistent with previous studies on Chinese clinical isolates.[20,24] The procedures are described in detail in Supplementary Methods 4. The sequences were submitted to the MLST online database (http://mlst.mycologylab.com) for analysis.

## Phylogenetic Analysis

The sequences of seven housekeeping genes from clinical isolates and reference strains (one strain chosen per distinct sequence type and genotype archived in the MLST database) were concatenated using the BioEdit software to generate integrated sequence datasets for subsequent analyses. ClustalW was used for sequence alignment. Phylogenetic trees were constructed using MEGA 11.0, based on the neighbor-joining method with 1000 bootstrap replications.

## National Comparison of *Cryptococcus*

A comprehensive literature review of global peer-reviewed studies published in the last 25 years was conducted, focusing on reports that detailed the number of *C. neoformans* isolates and confirmed their typing by MLST. The gathered data were used to generate provincial maps, illustrate strain distribution, and refine the evolutionary tree of the system.

## Antifungal Susceptibility Testing

Before testing, the isolates were sub-cultured on SDA at 35 °C for 48 h to guarantee optimal growth conditions. Subsequently, the turbidity of the fungal suspensions was measured using a McClure turbidimeter to standardize the inoculum concentration in the susceptibility assay. Minimum inhibitory concentrations (MICs) of amphotericin B (AMB), 5-fluorocytosine (5FC), fluconazole (FCZ), itraconazole (ITR), and voriconazole (VCZ) were determined using the BioMérieux ATB FUNGUS 3 system. The experimental procedures, including sample preparation, reagent mixing, and signal measurement, were performed in strict accordance with the manufacturers' protocols. After 48 h of incubation at 35°C, the strips were visually examined. *Candida Krusei* (ATCC6258) and *Candida parapsilosis* (ATCC22019) served as quality control specimens. Owing to the absence of well-established breakpoints specifically for *Cryptococcus* within the CLSI framework, epidemiological cutoff values (ECVs) were used to categorize the isolates as either wild-type or non-wild-type based on a comparison with the MICs. ECVs were established in CLSI document M59.[25] Specifically, for *C. neoformans* (VNI) species, the ECVs were 0.5 µg/mL for AMB, 8 µg/mL for 5FC and FCZ, and 0.25 µg/mL for ITR and VCZ.[26] Strains with MICs surpassing these thresholds were classified as non-wild type, indicating potential antifungal resistance.[27]

# Results

## Patient Characteristics

To date, fewer than 50 cases of cryptococcosis have been diagnosed at our hospital. Because patients with severe conditions were transferred to tertiary medical centers in Beijing or other provinces during the early stage of hospital establishment and because fungal cultures had already been completed for some patients before their admission to our hospital, with clinical isolates preserved at the referring institutions, a total of eight clinical isolates were retrieved from the Laboratory Department of Shanxi Bethune Hospital, Shanxi Province. Each isolate represented a unique patient, and no duplicate isolates were included. Fundamental clinical data were documented for all patients. All the isolates were identified as *C. neoformans* (formerly *C. neoformans var. grubii*). The patients were predominantly male (n=5, 62.5%) and all were middle-aged or older. Seven isolates were obtained from cerebrospinal fluid (CSF), and one isolate (specimen No. 6) was recovered from blood. The clinical diagnoses included cryptococcal meningitis simplex (CM) (n=5, 62.5%), cryptococcal pneumonia simplex (PC) (n=1, 12.5%), and multisite disseminated infection (n=2, 25%). None of the patients had a history of poultry exposure. Intracranial pressure data were not available for this cohort. Routine cerebrospinal fluid examination revealed elevated protein concentrations and decreased glucose levels in most cases, consistent with the typical laboratory findings of cryptococcal meningitis. The CSF chloride levels were generally within the normal range, which is in accordance with previous reports.[28] The reference values for CSF protein, glucose, and chloride in adults are 0.15–0.45 g/L, 2.5–4.4 mmol/L, and 120–130 mmol/L, respectively. A summary of the clinical manifestations of the patients is presented in Table 2.

## *Cryptococcus* Genotypes

Multilocus sequence analysis was performed on all eight clinical isolates to determine species identity, involving amplification of the ITS region of rRNA, mating-type markers, and housekeeping genes. All isolates were identified as *C. neoformans*, exhibiting α-mating type (confirmed by PCR, Figure S1), VNI molecular type, and ST5 sequence type. These findings indicated a significant level of genetic uniformity across the study area. Consistent with the PCR typing results, BLAST analysis of the mating-type sequences against the reference strain H99 in the NCBI database revealed a sequence identity ranging from 94.92% to 98.33%. This high level of sequence homology further validated the accuracy and robustness of the MATα typing results. Detailed genotype data are summarized in Table 3.

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**Table 2** Clinical Aspects of Eight Patients with Cryptococcosis

| ID | Gender | Age | Specimen | Underlying Condition | Diagnosis | chlorine | Protein | sugar | India ink | Crag | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | mmol/L | g/L | mmol/L | | | |
| 1061 | M | 46 | CSF | No | CM | 119.1 ↓ | 2.030↑ | 3.01 | + | + | good |
| 701 | F | 75 | CSF | SS, DM, Chronic steroid usage | PC, CM | 124.2 | 0.875↑ | 0.24 ↓ | + | NT | LAMA |
| 705 | M | 40 | CSF | HIV(+) | CM | 106.6 ↓ | 0.859↑ | 1.80 ↓ | + | NT | good |
| 850 | M | 49 | CSF | KT, Chronic Immunosuppressive Agents | PC, CM | 106.0 ↓ | 1.025↑ | 1.76 ↓ | + | + | blindness |
| 864 | F | 71 | CSF | DM, Cirrhosis, HBsAg (+), HEV-IgM(+) | CM | 110.6 ↓ | 2.936↑ | 0.55 ↓ | + | NT | dead |
| 908 | M | 55 | Blood | HIV(+), Syphilis | PC | Unknown | Unknown | Unknown | NT | NT | Transfer for treatment |
| 1070 | F | 69 | CSF | DM, Hypertension | CM | 116.6↓ | 2.472↑ | 1.29↓ | + | + | dead |
| 1076 | M | 69 | CSF | RAU, Hypertension | CM | 129.8 | 0.909↑ | 0.88↓ | + | + | good |

**Abbreviations**: M, male; F, female; CSF: Cerebrospinal Fluid, CM, cryptococcal meningitis; PC, cryptococcal pneumonia; DM, diabetes mellitus; SS, systematic sclerosis; RAU, recurrent aphthous ulcer; KT, Kidney transplant; CrAg, cryptococcal antigen test; NT, for not tested; +, positive result; CSF chlorine, 120–130 mmol/L; CSF protein, 0.15–0.45 g/L; CSF sugar, 2.5–4.4 mmol/L; ↑, above normal reference range; ↓, below normal reference range; LAMA, Left hospital against medical advice.

**Table 3** Allele Type Numbers for MLST Loci and MLST Sequence Type Numbers for 8 Isolates of *Cryptococcus neoformans*

| Strains | Allele type | | | | | | | ST | genotype | Mating type |
|---|---|---|---|---|---|---|---|---|---|---|
| | CAP59 | GPD1 | IGS1 | LAC1 | PLB1 | SOD1 | URA5 | | | |
| 1 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 2 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 3 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 4 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 5 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 6 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 7 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |
| 8 | 1 | 3 | 1 | 5 | 2 | 1 | 1 | 5 | VNI | α |

**Abbreviation**: ST, sequence type.

## Phylogenetic Tree Analysis

Phylogenetic analysis was performed using MLST data acquired in this study, along with data previously documented in China. Our findings revealed that the *C. neoformans* isolates from Shanxi Province belong exclusively to the ST5 lineage (Table 3). In contrast, previous studies have reported substantial genetic diversity of *C. neoformans* across China, with more than 40 sequence types identified to date, including ST2, ST4, ST5, ST6, ST31, ST32, ST38, ST43, ST53, ST57, ST63, ST69, ST77, ST79, ST81, ST 93, ST139, ST174, ST185, ST186, ST191, ST194, ST195, ST202, ST226, ST230, ST237, ST265, ST278, ST289, ST295, ST296, ST297, ST298, ST319, ST324, ST337, ST359, ST360, ST534, ST535, ST536, ST537, ST538, ST539, ST653, ST656, ST657, and ST658 (Figure 1).[9,14,18,20,26,28–33] Despite this diversity, ST5 remains the dominant clinical lineage in most regions of China (Table 4). This distribution is similar to that observed in Hong Kong, Korea, and Japan, and no significant differences were noted across different immunized populations.[34–36] The results of the phylogenetic tree analysis (Figure 1) demonstrated that the VNI genotype occupied the primary branches of the evolutionary tree, exhibiting rich diversity of sequence types. These strains formed tightly clustered groups within the tree, confirming that VNI was the predominant molecular type of *C. neoformans* in China. Among these, 8 strains of *C. neoformans* from Shanxi Province clustered with multiple ST types in China, and all (100%) clustered with the VNIc subcluster.

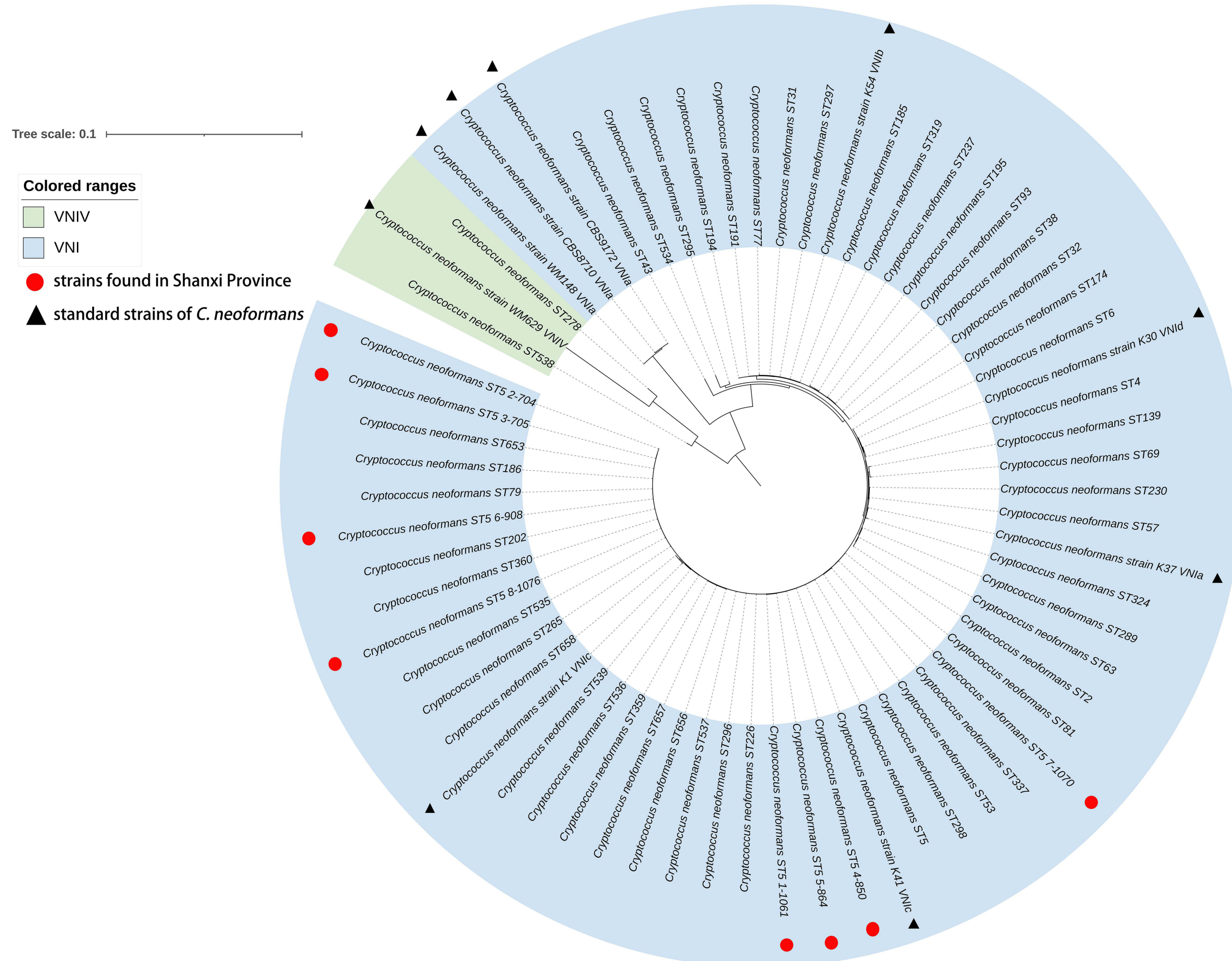


**Figure 1** Phylogenetic tree of ST types reported nationwide and strains found in this study. The black triangles represent standard strains of *C. neoformans*, red circles represent strains found in Shanxi Province, and the remainder represent strains already reported in China. Standard strains refer to well-characterized, internationally recognized reference strains with confirmed sequence types (STs), which were included as controls to validate the accuracy of the phylogenetic analysis.

## National Epidemiology of *C. neoformans* in China

An analysis of reported *C. neoformans* cases in China over the past 25 years has revealed significant geographical disparities. As shown in Figure 2, the incidence of infection was substantially higher in the southern provinces than in the northern regions and was similarly higher in the coastal areas than in the inland areas.

## Antifungal Susceptibility Testing

The in vitro antifungal susceptibility test implemented for the eight *Cryptococcus* strains revealed that the range of the MIC distribution was relatively narrow. This implied that the strains within this region were susceptible to the antifungal agents 5FC, AMB, FCZ, ITR, and VCZ. Representative raw images of microdilution plates used for antifungal susceptibility testing are shown in Figure S2. All strains were categorized as wild-type, in line with ECVs derived from prior investigations. Nevertheless, strains 2, 6, 7, and 8 demonstrated relatively higher MICs for FCZ than other strains. MIC data are presented in Table 5.


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**Table 4** Summary Distributions of Sequence Types Identified in Shanxi Province in Other Parts of China

| ST | Geographic location | | Percentage of the population |
|---|---|---|---|
| ST5 | china | Hong Kong | (85.7%)12/14[34] |
| | | Jiangxi | (89.5%)178/199[30] |
| | | Guangdong | (89.3%)75/84[14] |
| | | **Shanxi** | **(100%)8/8** |
| | | Beijing | (88.0%)22/25[29] |
| | | Hunan | (89.1%)98/110[13] |
| | | Sichuan | (89.6%)119/133[9] |
| | | Henan | (93.3%)14/15[29] |
| | | Heilongjiang | (76%)19/25[9] |
| | | Liaoning | (85.7%)10/12[9] |
| | | Zhejiang | (86.1%)155/180[31] |
| | | Tianjin | (100%)31/31[9] |
| | | Hubei | (88.6%)31/35[9] |
| | | Ningxia | (100%)1/1[20] |
| | | Gansu | (100%)1/1[20] |
| | | Shandong | (83.3%)5/6[29] |
| | | Hebei | (83.3%)10/12[29] |

**Note**: Bold values indicate the results obtained in the present study.

# Discussion

This study provides the first molecular epidemiological and antifungal susceptibility profile of *C. neoformans* in the Shanxi Province, northern China, thereby addressing an important regional knowledge gap. Several novel findings emerged from this investigation, offering new insights into both the local and national cryptococcal epidemiology.

## Predominance of the VNI/ST5 Lineage

Accurate typing of *Cryptococcus* is crucial to advance our understanding of this pathogen.[9] A striking feature of this study was that all eight Shanxi isolates shared the same molecular profile (VNI, serotype A, MATα, and ST5) (Table 3). Notably, phylogenetic analysis of our cohort confirmed that the VNI genotype (corresponding to *Cryptococcus neoformans* serotype A) dominates clinical *Cryptococcus* isolates in China. As illustrated in Figure 1, the vast majority of the Chinese clinical isolates clustered within the VNI clade, further validating its epidemiological predominance. Based on phylogenetic analysis, all VNI genotypes in this region (n=8, 100%) clustered with the VNIc subgroup, consistent with previous reports from China,[37] and no distinct internal sublineage differentiation has been identified.

Within the context of the VNI lineage, our study documented a 100% detection rate ST5 in a regional cohort. This proportion is comparable to or exceeds the ST5 rates reported in major northern Chinese medical centers. Specifically, a large multicenter study analyzing 305 clinical *C. neoformans* isolates via MLST reported an ST5 prevalence of 100% at the Peking University First Hospital, 95.7% at the Peking Union Medical College Hospital, and 100% at the Tianjin Medical University General Hospital. Notably, these ST5 prevalence rates in northern regions (including Shanxi) were significantly higher than those observed in southern Chinese regions included in the same multicenter

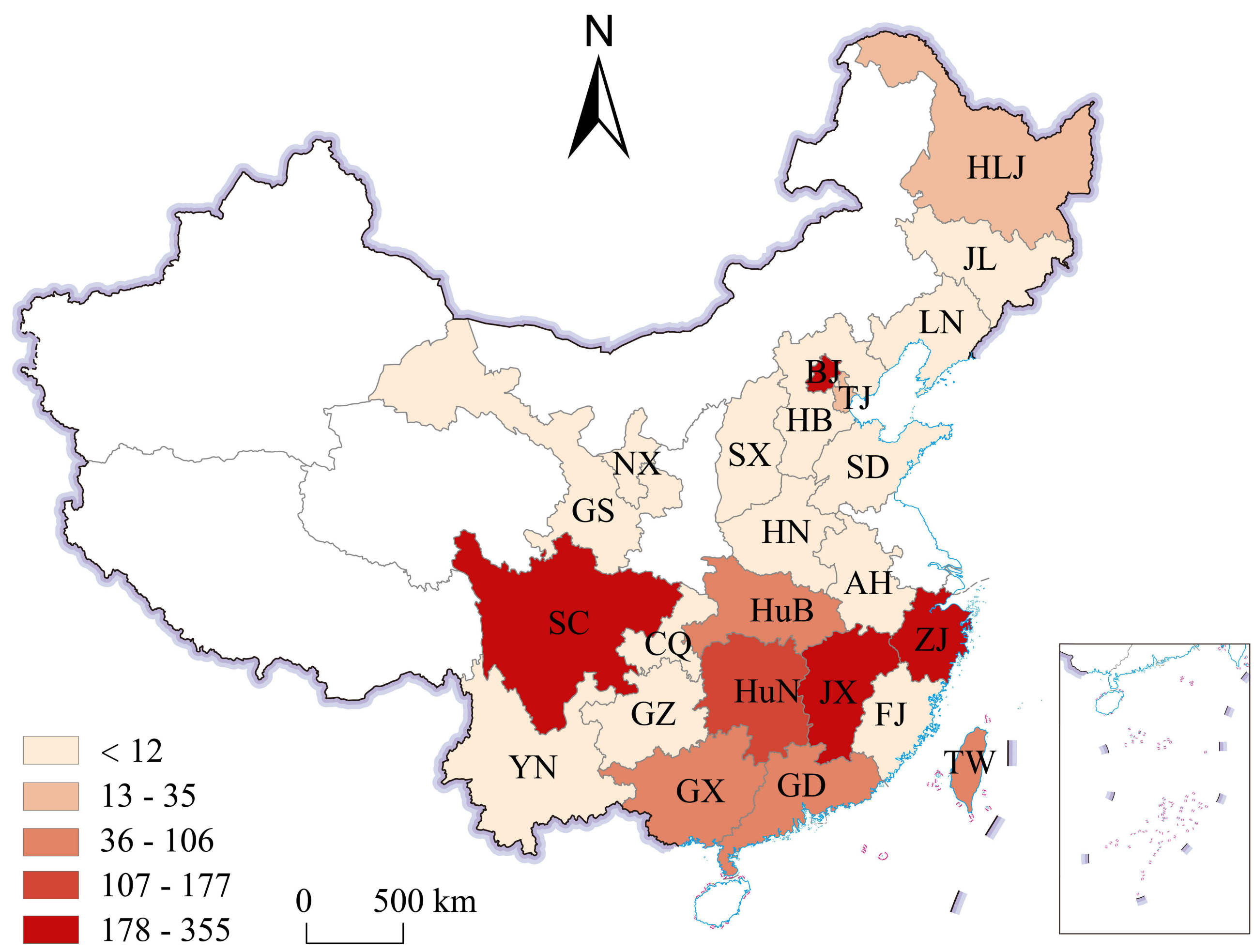


**Figure 2** Geographic distribution of reported *Cryptococcus neoformans* clinical isolates in China (2000–2024).Geographic distribution of documented *Cryptococcus neoformans* clinical isolates in China. Number and geographic distribution of the documented clinical isolates of *Cryptococcus neoformans* in China since 2000. Scale key: The colored gradient indicates the number of *Cryptococcus neoformans* clinical isolates per provincial administrative region, with numerical ranges corresponding to isolate counts as follows: <12 isolates, 13–35 isolates, 36–106 isolates, 107–177 isolates, 178–355 isolates. Abbreviations on the map correspond to the following provinces/regions (isolate count in parentheses): SX(Shanxi):8, GD(Guangdong):93, SC(Sichuan):355, HLJ(Heilongjiang):27, LN(Liaoning):19, TJ(Tianjin):34, BJ(Beijing):232, HuB(Hubei):41, TW(Taiwan):99, JX(Jiangxi):318, ZJ(Zhejiang):182, GX(Guangxi):92, HuN(Hunan):108, HN(Henan):12, HB(Hebei):12, FJ(Fujian):3, SD(Shandong):4, AH(Anhui):2, JL(Jilin):2, GZ(Guizhou):2, NX(Ningxia):1, GS(Gansu):1, YN(Yunnan):1, CQ(Chongqing):1. This map is based on the standard map with the map review number GS(2023)2767 from the Standard Map Service Website of the Ministry of Natural Resources, and the base map has not been modified.

investigation.[5,9,28,29] The highly concentrated genotype distribution observed in Shanxi may reflect a regional clonal structure that is potentially influenced by local ecological conditions or founder effects, although this interpretation remains speculative and is constrained by the limited sample size. Comparable trends have been observed in other East Asian countries, including South Korea and Japan, where ST5 prevalence reached 96% and 88.6%, respectively,[36,38] suggesting that the VNI/ST5 lineage may be well-adapted in East Asia.

Within the VNI/ST5 lineage, haploid MATα strains exhibited overwhelming predominance. In China, MATα strains of *C. neoformans* are predominant, and this mating type is closely associated with virulence. Several studies have demonstrated that these strains possess an enhanced ability to cross the blood-brain barrier and cause severe infections.[14,39] A multicenter study covering six major regions in China reported that MATα accounted for 89.6% of the isolates.[10] In the current study, all isolates were identified as MATα, which is consistent with the national data. However, given the limited number of isolates analyzed, the current data do not allow for meaningful assessment of regional differences in strain virulence, and further research is needed to evaluate the pathogenic potential of isolates from the Shanxi Province.[40]

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**Table 5** Antifungal Susceptibility Data of 8 Isolates Recovered in the Study

| ID | Strains | MIC (μg/mL) | | | | | R(%) |
|---|---|---|---|---|---|---|---|
| | | 5-FC | AMB | FCZ | ITR | VCZ | |
| 1061 | 1 | ≤4 | ≤0.5 | ≤1 | ≤0.125 | ≤0.06 | 0 |
| 701 | 2 | ≤4 | ≤0.5 | **2** | ≤0.125 | ≤0.06 | 0 |
| 705 | 3 | ≤4 | ≤0.5 | ≤1 | ≤0.125 | ≤0.06 | 0 |
| 850 | 4 | ≤4 | ≤0.5 | ≤1 | ≤0.125 | ≤0.06 | 0 |
| 864 | 5 | ≤4 | ≤0.5 | ≤1 | ≤0.125 | ≤0.06 | 0 |
| 908 | 6 | ≤4 | ≤0.5 | **2** | ≤0.125 | ≤0.06 | 0 |
| 1070 | 7 | ≤4 | ≤0.5 | **2** | ≤0.125 | ≤0.06 | 0 |
| 1076 | 8 | ≤4 | ≤0.5 | **2** | ≤0.125 | ≤0.06 | 0 |
| ECV (μg/mL) | | 8 | 0.5 | 8 | 0.25 | 0.25 | |

**Notes**: Boldface values denote elevated fluconazole MICs observed in this study. Representative raw images of the microdilution plates are shown in Figure S2.

**Abbreviations**: AMB, amphotericin B; 5-FC: 5-fluorocytosine; FCZ, fluconazole; ITR, itraconazole; VCZ, voriconazole; R, resistant; ECV, epidemiological cutoff value; MIC, minimum inhibitory concentration.

## Early Signals of Rising Fluconazole MICs

According to global guidelines for the diagnosis and Management of Cryptococcosis,[41] the primary antifungal agents used to treat cryptococcal infections in China include 5-FC, AMB, FCZ, ITR, and VCZ. Among these, the combination of AMB with FCZ and 5-FC is the most cost-effective, and FCZ is administered for a prolonged duration throughout the course of cryptococcal infection treatment. Antifungal susceptibility testing showed that all isolates were susceptible to first-line antifungal agents, with MIC values remaining below established ECVs. However, four isolates exhibited elevated MICs (2 μg/mL) for fluconazole, potentially reflecting an early shift toward reduced susceptibility. This pattern parallels the trends observed in southwestern China and Taiwan, where MICs for FCZ showed a gradual upward trajectory.[32,42] Similarly, national surveillance data indicate that resistance to FCZ is rising, increasing from 10.5% in 2010 to 34% in 2014.[11]

Although a one-dilution difference in fluconazole MICs was observed among isolates, all values remained within the wild-type range, and no statistically meaningful associations with clinical outcomes or temporal trends could be assessed owing to the limited sample size. Nevertheless, systematic surveillance is essential for detecting potential shifts in antifungal susceptibility patterns over time.

## Clinical Characteristics and Potential Prognostic Disparities

Age and sex appeared to be associated with clinical outcomes in this small cohort. Consistent with national data, most patients were middle-aged or older males.[43] However, a notable trend emerged: both fatal cases occurred in older females, whereas all favorable outcomes were observed in male patients, including those aged <60 years. In addition, all three female patients presented with CM. These observations suggest that sex and age may influence clinical outcomes, which is consistent with recent reports of higher mortality among older female patients.[44] Consistently, elevated cerebrospinal fluid protein levels and reduced glucose concentrations were observed, whereas chloride levels generally remained within the normal range, which is consistent with previous reports.[28] The limited sample size (n = 8) restricts further analysis of genotype-phenotype correlations; nevertheless, the observed clinical pattern suggests that host factors warrant further investigation in northern China.

## Contrasting HIV Association and Host Risk Profiles

Although HIV infection remains the predominant risk factor for cryptococcosis globally, most cases in China occur among HIV-negative individuals. The proportion of HIV-positive cryptococcosis cases in China ranges from 12.9% to 24.7%,[45] significantly lower than that in Europe (80%), America (86%), and Africa (69%).[46–48] In this study, two of eight patients (25%) tested positive for HIV, and these two patients were diagnosed with CM and PC, respectively. The eight patients, seven of whom were diagnosed with CM, and the majority of these cases were identified in negative. Consistent with previous studies in China, an extremely high proportion (approximately 85%) of CM cases were identified in HIV-negative patients.[49] This situation contrasts with those observed in other countries. A plausible explanation for this discrepancy lies in the severe under-reporting of CM cases in China's HIV-infected population, as previously speculated.[49] Differences in reported HIV associations across regions may partially reflect variations in hospital-admission patterns and study settings. Most HIV-negative patients in this cohort had other recognized risk factors, including diabetes and renal transplantation. Only one HIV-negative patient had no identifiable immunosuppressive condition, which is a lower proportion than that reported in earlier Chinese studies.[10] These findings highlight the heterogeneity of the risk profiles among HIV-negative patients and the importance of broad clinical vigilance.

### Implications for Transmission

Our 25-year synthesis of national molecular epidemiological data confirmed that the incidence of *C. neoformans* is higher in southern China than in northern China, and in coastal regions than in inland regions (Figure 2). This distribution is likely driven by multiple factors. The warm, humid subtropical and tropical climates in these areas provide favorable conditions for environmental survival and propagation of *C. neoformans*. They remain widely distributed in soil, decayed wood, and pigeon excreta.[50] Although none of the patients reported bird exposure, the infection likely resulted from environmental inhalation of fungal spores.[51] Molecular epidemiological studies have demonstrated considerable genotypic diversity among environmental isolates, with numerous novel genotypes identified in pigeon droppings.[52] These genotypic disparities likely reflect the adaptive evolution of organisms across the ecological niches. Environmental studies in northern China have indicated that ST31 frequently dominates environmental niches, whereas ST5 predominates clinical isolates,[53] potentially reflecting host-selective adaptation. The intrinsic differences between ST5 and ST31, including variations in virulence and host susceptibility, may contribute to their distinct clinical and environmental distribution.

### Limitations

This study has several limitations. First, the absence of environmental sampling precluded the identification of the ecological factors underlying the observed ST5 homogeneity. Second, the small sample size limited genotype–phenotype analyses and prevented further investigation of ecological or epidemiological factors associated with the predominance of the ST5 lineage in this region. Third, incomplete intracranial pressure data and the lack of immunological markers, such as anti-GM-CSF antibody testing,[28,54,55] constrained the exploration of host-pathogen interactions.

### Future Directions and Conclusion

Given the predominance of ST5 in Shanxi and its potential implications for antifungal resistance, future studies should prioritize establishing a China-wide MLST network. This would enable the establishment of a correlation between genotype distribution (eg, ST5 prevalence in Shanxi vs. southern provinces) and antifungal resistance trends, ultimately supporting the development of region-specific strategies for cryptococcosis management.

## Conclusion

This study established the first baseline data on the molecular epidemiology and antifungal susceptibility of *C. neoformans* in Shanxi Province. The exclusive presence of the susceptible ST5 lineage is consistent with the predominant pattern reported in China. These findings carry several clinical implications. First, the occurrence of *C. neoformans* infection in immunocompetent individuals highlights that cryptococcosis should not be overlooked in patients without classical risk factors. Second, the predominance of ST5 and the generally low MIC values to antifungal agents support the continued use of standard antifungal therapy in our region. However, the observation of a trend toward higher fluconazole MIC values in some isolates underscores the importance of ongoing antifungal susceptibility monitoring, particularly in refractory infections. Third, our observations suggest that delayed diagnosis may contribute to poor clinical outcomes, emphasizing the importance of early diagnostic intervention. These findings underscore the importance of establishing region-specific surveillance systems to monitor potential future shifts in the molecular epidemiology or antifungal resistance of *C. neoformans*, thereby supporting effective clinical management of related infections.

## Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Shanxi Bethune Hospital (Approval Number: YXLL-2024-169). The patients' information was anonymized before the data analysis. The Ethics Committee waived the need for written informed consent from the participants and conducted the study in accordance with the Declaration of Helsinki.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The author(s) report no conflicts of interest in this work.

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