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Molecular identification of *Cryptococcus gattii* from cerebrospinal fluid using single-cell sequencing: A case study

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SUMMARY

A 31-year-old man presented with cryptococcal meningitis (CM) without typical clinical characteristics, but with abnormal walking, difficult leg lifting and frequent falling. He was admitted to Peking Union Medical College Hospital. After multiple tests failed to identify the pathogen, single-cell sequencing (scS) was used to test the cerebrospinal fluid (CSF). Comparing the sequence obtained from single-cell sequencing with the reference database, it was found that the infection was caused by *Cryptococcus gattii sensu stricto* (AFLP4/VGI genotype). *Cryptococcus* is difficult to cultivate from complex body fluids. The etiological agent of this patient was identified and the patient was treated. This is the first case in which scS was used to detect and identify fungal pathogen after conventional testing failed to identify the cause of the disease. This report demonstrates that the scS approach can be used to generate fungal genome sequences directly from the CSF of a CM patient. The scS technology could become a powerful tool to precise detect microscopically visible but uncultured pathogens in clinical samples.
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Introduction

Prompt recognition of the etiological agent of a central nervous system (CNS) infection is crucial for targeted treatment and patient survival.[1,2] A considerable proportion of the etiologic agents of CNS-related mycoses remains unidentified.[3,4] Cryptococcal meningitis (CM) is a life-threatening fungal infection associated with the human central nervous system, which is mainly caused by yeasts of the basidiomycetous genus *Cryptococcus*, particularly species that belong to the *Cryptococcus neoformans* and *Cryptococcus gattii* complexes.[5] The taxonomy and nomenclature of *C. neoformans* and *C. gattii* species complexes have undergone several changes and remain a subject of controversy.[6]

Compared to the high incidence rate of CM caused by *C. neoformans sensu stricto,* the incidence of the *C. gattii* complex is significantly less at a global level, but can be found more frequently in specific geographic or climatic zones.[7,8] The five genotype groups within *C. gattii sensu lato* identified based on their amplified fragment length polymorphism (AFLP) banding patterns were proposed as five separate species, including *C. gattii* (genotype AFLP4/VGI), *C. bacillisporus* (genotype AFLP5/VGIII), *C. deuterogattii* (genotype AFLP6/VGII), *C. tetragattii* (genotype AFLP7/VGIV), and *C. decagattii* (genotype AFLP10/VGIV).[9,10] Isolates of *C. gattii s.s.* and *C. deuterogattii* are the most frequently encountered globally,

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whereas *C. bacillisporus* and *C. decagattii* are mostly reported from the American continents, and *C. tetragattii* and VGV isolates of *C. gattii* seem to be restricted to southern Africa and India, respectively.[7,8,11]

Clinical symptoms and radiological signs of CM are notoriously non-specific, variable, and often absent.[12] Laboratory assays, such as Indian Ink staining and cryptococcal antigen (Cr-Ag) detection fulfill an important role for the diagnosis of CM in clinics worldwide.[13] However, these conventional diagnostic assays are generally used to target the cryptococcal capsule. This can be problematic if CM is caused by capsule-deficient *Cryptococcus* isolates.[13–15] Furthermore, the overwhelming majority of conventional assays on CM without pure cultures cannot distinguish members of the *C. neoformans*/*C. gattii* species complexes. As a result, proper treatments against *C. gattii sensu lato* may not be achieved.[8,16]

Routine molecular methods, such as multiple PCR-based assays, have not shown advantages when compared to Cr-Ag detection to diagnose CM[17]. Recently, next-generation sequencing (NGS) of CSF has been shown some potential advantages over traditional methods to identify culture-negative organisms among patients with CNS infections.[3,18] However, the effectiveness of NGS for identification of fungal pathogens can be challenging. Because fungi have hard cell walls different from other pathogens, making it difficult to extract their DNA from small quantities of complex clinical specimens. This is especially the case when there are questions related to whether the identified microbe represents a true pathogen or a contaminant. In addition, NGS data require appropriately trained personnel to interpret the results.[18]

Although the majority of human fungal pathogens are culturable, clinical specimens often contain visible spores or hyphae, but may fail to yield viable cultures in many clinical cases.[19] Thus, molecular identification of visible spores or hyphae from clinical specimens, such as CSF, can provide critical information for a reliable diagnosis of CNS-related mycoses. Notably, single-cell sequencing (scS) recently has been shown to be a powerful approach for exploring biological systems with unprecedented resolution. For example, the scS technology has been successfully used to do preimplantation genetic diagnosis and analysis of circulating tumor cells.[20] Moreover, scS technology was used to analyze the convalescent patients' B cells and identify potent neutralizing antibodies against SARS-CoV-2 during the COVID-19 pandemic in 2020.[21] This is helpful for prescribing specific targeted therapy on diseases. Although scS has demonstrated a broad potential, it has seldomly applied to detect fungal pathogens. As mentioned above, due to the hard fungal cell wall, it is typically more difficult to extracted DNA from a small number of pathogen cells than the mammalian cells associated with clinical specimens. Here, we firstly used scS and laser dissection technology to directly identify *C. gattii* from CSF from a CM patient with atypical clinical characteristics.

Methods and materials

Case presentation

An otherwise healthy 31-year-old man with more than 1-month history of intermittent fever (37.5 °C - 38.5 °C), slight headaches, muscle weakness of both legs, and new onset seizures, was admitted to Peking Union Medical College Hospital in China on November 13, 2014. He was a telecommunications engineer with no previous medical history, including no history of recurrent infections, and no travel history outside of mainland China. Clinical manifestations of the patient did not improve after taking neurotrophic therapy with vitamin B1 (10 mg/d per os), vitamin B6 (10 mg/d per os) and citicoline (0.5 g/d intravenous drip) for approximately one month prior to his admission to the hospital. His neck was supple with no sign of meningeal irritation. He did not have any skin lesion or rashes and was able to open his eyes to painful stimuli. His pupils were symmetrical and equally reactive with intact oculocephalic and corneal reflexes. No facial asymmetry or grimace was noted. Both HIV and tuberculosis tests were negative, and the chest radiograph did not show evidence of any abnormalities.

Upon admission, MRI scan of the head indicated the presence of multiple abnormal lesions in the medulla oblongata and the brain. Because of intermittent fever episodes and headache for approximately 40 days, lumbar punctures were performed on separate occasions starting November 14, 2014. After arriving at the hospital, CSF samples were obtained and intracranial pressure was monitored. CSF analysis revealed a glucose concentration of 2.0–2.3 mmol/L, protein concentrations ranging from 0.59–0.61 g/L and white blood cell counts ranging from 57 to 70×10^6/L. The intracranial pressure ranged from 155 to 255 mmH_2O. No fungal pathogens grew on malt extract agar (MEA; Oxoid, U.K.), Czapekdox agar (CDA; Oxoid, U.K.) and potato dextrose agar (PDA; Oxoid, U.K.). The titers of Cr-Ag by the IMMY LFA (ImmunoMycologics, Inc, Norman, OK, U.S.A) were 1/1 positive in the first CSF specimen that was collected from the first lumbar puncture, but the remaining CSF samples were all negative. We also observed the possible presence of *Cryptococcus* cells in the CSF following India ink staining of the first lumbar puncture sample (November 14, 2014). However, these cells appeared to be capsule-deficient *Cryptococcus* cells observed by microdissection system at 40x magnification. At this time, we tried to identify the suspected pathogen directly using PCR amplification and sequencing of the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster based on the total DNA extracted from CSF sample. Although we obtained the PCR amplification product, the generated sequence result was not readable, due to the presence of multiple peaks likely caused by the mixed DNA sample.

Due to worsening clinical manifestations under antiviral and neurotrophic therapy, the patient was subjected to combination antifungal therapy [amphotericin B (AMB), 1–10 mg/day; flucytosine (5-FC), 6.0 g/day] for approximately five weeks beginning on Nov 14, 2014. During this period, the clinical symptoms (including fever and headaches) improved significantly. Since Jan 21, 2015, the patient refused to receive further antifungal therapy, because he was afraid of side effects related to AMB. The patient was discharged from hospital and was treated with fluconazole (FLU, 400 mg/day).

To prevent the patient from deteriorating and to get better treatment as soon as possible, it was urgent to identify the mysterious pathogen. After consultation with several experts, the scS (single-cell sequencing) method was chosen and the remaining CSF sample from the first lumbar puncture (remaining no more than 1 mL) was immediately processed for scS identification in the Beijing Key Laboratory of New Molecular Diagnosis Technologies for Infectious Disease on Jan 21, 2015. After a successful single-cell DNA amplification, library preparation, and sequencing, we performed end trimming, data filtering, sequence assembly, and bioinformatics analysis of the sequencing readouts. Moreover, the patient felt headaches on Jan 23, 2015, and the second MRI scan of the head performed suggested brain anomalies. Culture and the Cr-Ag lateral flow immunoassays (LFA) were both negative based on a follow-up lumbar puncture examination. Fortunately, on Jan 25, 2015, the scS results showed that the pathogen belonged to *C. gattii sensu stricto*. Because the patient could not tolerate side effects of AMB, the patient was treated with voriconazole (VOR, 400 mg/day) and 5-FC (6.0 g/day) for nearly 7 months when both CSF pleocytosis and head MRI examinations showed that the patient was recovered. The details of this case history are summarized in Fig. 1.

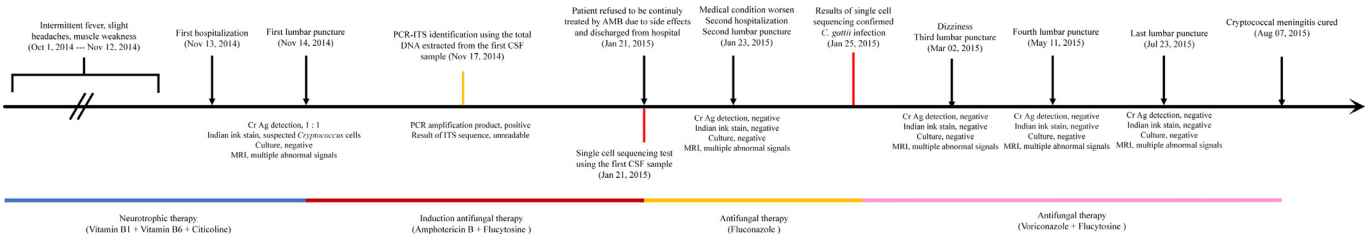

Fig 1. A timeline of clinical course of the patient with CM caused by *C. gattii sensu stricto* in October 2014 and ending after his recovery in August 2015.

Sample preparations

For scS, we first isolated the yeast cells from the CSF using laser microdissection. Specifically, the CSF was mounted onto a laser microdeposition-coated slide (Leica Membrane Slides PEN 2.0 µM). The yeast-like cells were isolated using a laser microdissection system (Leica LMD7000) at 63 × magnification at a laser intensity of 40 M (laser power: 40 ± 3). The dissected cells were placed into a sterile micro-centrifuge tube, and a total of 10 yeast-like cells were collected. The isolated cells were suspended in 200 µL of 0.8 M D-sorbitol solution and incubated at 4 °C for 2 h.

DNA extraction and library preparation

An equal volume (200 µL) of pretreatment buffer consisting of 50 mM Tris, 5 mM EDTA, and 5% β-mercaptoethanol was added to the suspended cells and incubated at 35 °C for 1 h. After incubation, the tubes were centrifuged at 5000 rpm for 5 min to collect the yeast cells, and the supernatant was discarded. The cells were then washed with 200 μL ddH_2O, centrifuged at 5000 rpm for 5 min, and the supernatant discarded. Approximately 200 μL of a mixed enzyme solution containing snail enzyme (6 mg/mL) and lysozyme (4 mg/mL) was added, and the cells were incubated at 37 °C for 6 h. The cells were again centrifuged at 5000 rpm for 5 min to collect the cells, and the supernatant was discarded. The precipitated cells were washed with 50 μL of PBS, centrifuged at 5000 rpm for 5 min, and the supernatant was discarded; approximately 4 μL of the residual liquid was left at the bottom of the tube. Finally, 3 μL of Buffer D2 (Qiagen, Duesseldorf, Germany) from the REPLI-g Single Cell kit (Qiagen, Duesseldorf, Germany) was added to the cell suspension. The contents of the tube were subsequently mixed and incubated at 65 °C for 10 min.Finally, 3 μL of the stop solution from the reagent kit was added, mixed and kept on ice.

Single-cell sequencing

Approximately 40 μL of the reaction solution was added to the amplification reaction mixture (prepared according to the instruction as specified in the REPLI-g Single Cell kit), which consisted of the following: 9 μL of ddH_2O, 29 μL of the REPLI-gsc Reaction Buffer REPLI-g, and 2 μL of the REPLI-g scDNA polymerase. Following incubation for 8 h at 30 °C, REPLI-g DNA polymerase was inactivated by incubating at 65 °C for 3 min. The liquid in the tube containing MDA-amplified DNA was generated using a NEBNext® UltraDNA Library Prep Kit for Illumina® (NEB, San Francisco, California, U.S.A.). Finally, the genome of the collected yeast-like cells was sequenced based on the gDNA library. Sequences were obtained by generating 300 bp Paired End reads using the Illumina MiSeq platform (Illumina, Salem, Massachusetts, U.S.A).

Sequence assembly and identification

The trimming of reads and the removal of Illumina adapters were performed by Trimmomatic 0.36. The leading and trailing bases with a quality value below 5 were removed and a sliding window trimming was performed with a window size of 15 bp. An average quality value threshold of 4 and reads with length above 60 bp were kept for further analyses. After trimming and filtering, a total of 6925,939 read pairs (94.95%, 6925,939/7294,295) fulfilled our criteria. The filtered reads were then assembled *de novo* using SPAdes 3.10.1 with metagenomic assembly mode and Kmers of 21, 33, and 55 were tested to achieve the best assembly effect.[20] The assembled contigs were then identified by Kraken[22] using a combined genome database of Archaea, Bacteria, Fungi, *Homo sapiens* (GRCh38.p13), Protozoa and Viruses. The assembly sequence has been submitted to the NCBI SAR database, with the accession number of SRX6952669.

Phylogenetic analysis

We extracted the internal transcribed spacer (ITS) sequences from the obtained sequence and compared the ITS sequences with those present in GenBank. Our comparison showed that the yeast-like cells belonged to the human pathogenic *Cryptococcus* complex. To further identify its phylogenetic placement, nine *C. gattii* reference genomes and the *C. neoformans* JEC21 genome were downloaded from NCBI: WM276 (ASM18594v1), EJB2 (ATAL01), Ru294 (ASCO01), E566 (ASCT01), NT-10 (AZGX01), R265 (AAFP02), Ram5 (ASCM01), CA1873 (ASCQ01), IND107 (ATAM01) and JEC21 (ASM9104v1). Blastn of blast+ 2.2.30 was used to align the 4575 contigs classified by Kraken2 as *C. gattii sensu stricto* to each of the 10 reference genomes. Blastn was run with the best hit score edge of 0.05 and the best hit overhang of 0.25, and only the best hits were kept. A total of 2963 contigs had consistent matches in all 10 reference genomes. The sequences of these 2963 contigs and sequences of their corresponding regions of the 10 reference genomes were extracted by Bedtools 2.25.[23] The extracted sequences were concatenated by FASconCAT-G v1.0[24] and further aligned by Mafft 7.271.[25] A phylogenetic tree subsequently was constructed based on the alignment of the DNA obtained in this study. The 10 reference strains were analyzed using MEGA 7.0.21 with the neighbor joining (NJ) method with 1000 bootstraps.[26]

Results

Assembly and identification of the scS sequence

A total of 12,384 contigs with a contig N50 of 1151 bp were obtained by SPAdes using Kmer of 55. This has a better assembly effect than when using the Kmers of 21 and 33. Among the 12,384 contigs, 5644 contigs (64.35%, 5135,098 bp/7979,241 bp) were identified by Kraken2 with a combined genome database of sequences from *Archaea, Bacteria, Fungi, Homo sapiens, Protozoa* and *Viruses*. The distributions of the 5644 identified contigs were as follows: fungi (81.06%, 4575/5644), viruses (11.09%, 626/5644), bacteria (4.82%, 272/5644), homo sapiens (1.51%, 85/5644), protozoa (1.43%, 81/5644) and archaea (0.09%, 5/5644). Among these 5644 classified contigs, 4575 contigs accounting for 4274,048 bp were identified by Kraken2 as *C. gatti sensu stricto*. These 4575 contigs

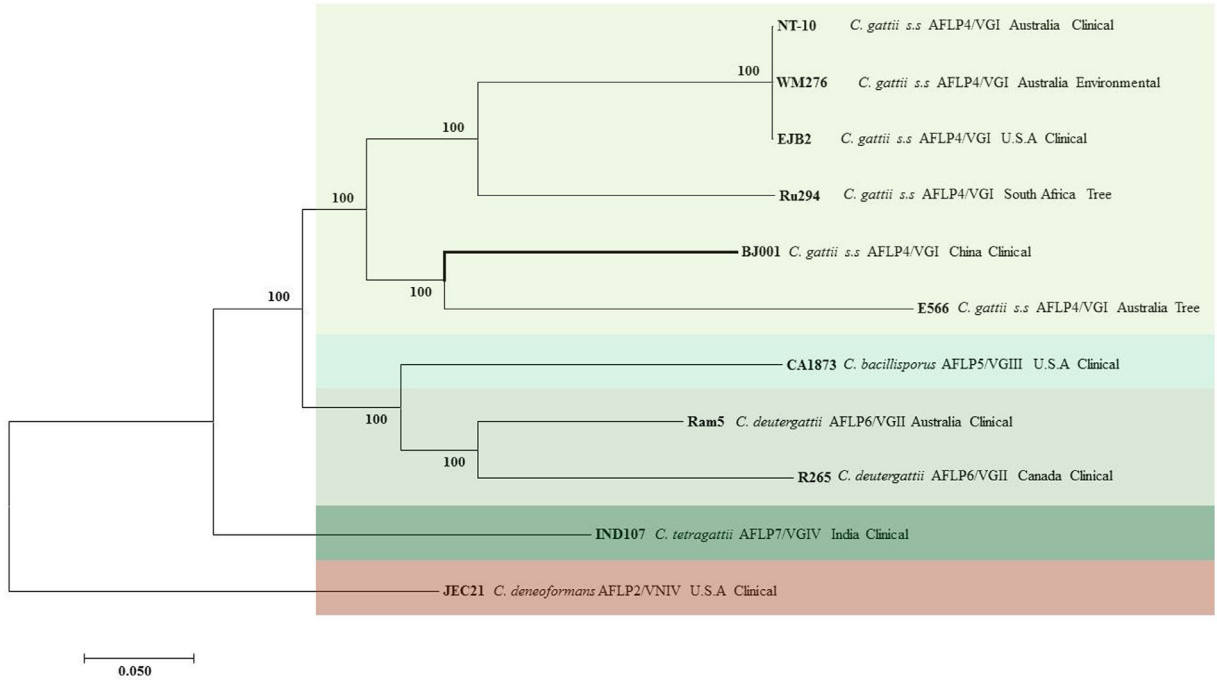

Fig 2. Phylogenetic tree showing the relationships between strain BJ001 and 9 reference *C. gattii* strains based on 2963 contigs using Neighbor-Joining (NJ) method with 1000 bootstraps test. One reference *C. deneoformans* strain JEC21 was used as outgroup.

accounted for almost the total length of the 5644 classified contigs (83.23%, 4274,048 bp /5135,098 bp). The detailed assembly information is shown in Table S1. The complete Kraken identification results are shown in Table S2.

Phylogenetic analysis

A total of 2963 contigs in our assembly were found to have consistent matches with the 10 reference strain genomes. These contigs were further used to construct the phylogenetic tree. The 2963 contigs accounted for 70.3% (3004,566 bp / 4274,048 bp) of the 4575 contigs identified by Kraken2 as *C. gatti sensu stricto*. The phylogenetic analysis revealed that the yeast-like cells in this patient belonged to the genotype AFLP4/VGI of the *C. gattii* species complex, closely related to *C. gattii sensu stricto*. strain E566 (Fig. 2). Following this deduction, we could identify the suspected pathogenic isolate as *C. gattii sensu stricto* with a high level of confidence. As mentioned above, the routine clinical diagnostic tests of our CSF samples obtained initially from this patient failed to identify the etiological agent responsible for his condition. In contrast, scS in combination with a bioinformatics pipeline allowed us to confirm that the infection was caused by *C. gattii sensu stricto* which equals the AFLP4/VGI genotype.

Discussion

Compared to CM caused by *C. neoformans sensu stricto*, CM caused by *C. gattii sensu lato* is often associated with cerebral cryptococomma[27] and elevated CSF opening pressure.[28,29] However, our case did not exhibit these clinical characteristics or the typical positive Cr Ag test. Indeed, conventional assays failed to positively detect the causative agent. We only observed putative *Cryptococcus* cells in the first CSF sample using Indian ink staining and weak positive agglutination (1:1) resulted from the Cr-Ag interaction, neither criteria were specific enough for species identification or the separation of *C. neoformans* complex from *C. gattii* complex. Because a definite diagnosis of CM requires multiple lines of evidence, such as positive Indian ink staining, a viable culture, and/or a positive latex agglutination test (titers $>$ 1:50), the results from our routine lab tests did not result in a definitive diagnosis of the infection as CM. The patient's condition gradually worsened after one month of receiving antiviral and neurotrophic therapy in our hospital. Therefore, we tentatively started induction antifungal therapy. Subsequently, the reduction in symptoms after antifungal treatments suggested that a fungal infection was likely the cause. The likelihood of CM was confirmed after a scS analysis.

A prompt and reliable identification of the etiologic agents of CNS-related mycoses is critical for the diagnosis, treatment and prognosis of the infection.[19] However, routine molecular techniques, such as various PCR-based methods, have not shown added-values for the diagnosis of CM when compared to conventional assays[17]. Our result of direct sequencing of the clinical CSF sample targeting the internal transcribed spacer of the nuclear ribosomal rRNA gene also failed to identify the etiological agent of the infection in this study.

Notably, the scS technology in our study effectively and accurately generated enough sequence reads to facilitate direct detection and phylogenetic analysis of the cells of *C. gattii sensu stricto* in clinical CSF samples. The majority (approximately 82%) of the classified contigs ($n = 5644$) in our study were identified as fungi (81.73%, 4438/5644). Among all the classified contigs, 4438 of them were identified as *C. gattii sensu stricto.* This accounted for approximately 83% of the total length of the classified contigs (83.23%, 4274,048 bp/5135,098 bp). In our opinion, the scS technology is more effective than routine metagenome sequencing for identifying unexpected pathogens in clinical specimens. This is particularly true for the biopsy containing microscopically visible cells that fail to grow viable colonies onto standard laboratory media. One key reason is that the laser microdissection and scS technologies allow the end users to isolate and analyze individual pathogen cells directly from the clinical samples. In contrast, metagenome sequencing only yields a small fraction of the obtained sequences from the putative pathogen, with the majority sequences coming from the host cells.

We successfully isolated ample target cells following Indian ink staining using laser capture microdissection (LCM).[30,31] This pro-

vided the basis for us to successfully extract cryptococcal genomic DNA from the CSF and generate a genomic DNA library using a combination of snail enzyme, lysozyme and the REPLI-g Single Cell kit. In our opinion, the present study clearly provides a suitable basis to make additional refinements for extraction of fungal DNA from other clinical sample types. This will allow molecular identification of the etiologic agents of CNS-related and other invasive mycoses. Efficient methods for the extraction of fungal DNA from clinical specimens are urgently required for pathogen detection using molecular assays.[32]

The generated sequence reads facilitated our phylogenetic analyses to identify the targeted yeast cells as a specific lineage of *C. gattii sensu lato*. These were derived from the genotype AFLP4/VGI representing *C. gattii sensu stricto*, which is the primary species of *C. gattii sensu lato* occurring in mainland China.[33,34] Furthermore, the DNA of *C. gattii sensu stricto* in our study showed a close phylogenetic relationship with that of strain E566 originating from Australia. This suggests that VGI isolates from China have a close genetic relationship with those from Australia.[33,34] The environmental source of them could be related to wood production and the introduction of foreign tree species, such as *Eucalyptus* species, into China from Australia.[35,36]

In summary, using a scS approach, we successfully identified a *C. gattii sensu stricto* strain that causes CNS infection. To our knowledge, this is the first time that this technology has been used to diagnose a case of CNS-related mycosis caused by pathogenic fungi that could not be cultured. After further optimization of clinical sample separation, the scS technology will have great potential to accurately identify the etiologic agents of CNS-related mycoses, as well as other disease-causing fungal pathogens.

Author contributions

Y.Y., Y.X, and W.L conceived and designed the study. M.C., N.H., S.H., A.A, L.G., T.B., and JP.X drafted the manuscript. Y.Y., M.C.,H.G and M.X. did molecular diagnostic test. N.H., X.Z. and M.C. did the analysis of genome sequence data. P.W., H.G and Z.Z. did clinical detection. All authors played a significant role in data collection and analysis. M.C., N.H. and S.H. contributed equally to this work and share the first co-authorship.

Declaration of Competing interest

The authors declared that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.06.040.

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