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CASE REPORT

A Rare Case of Keratitis Caused by *Graphium basitruncatum* in China

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Abstract: *Graphium basitruncatum (G. basitruncatum)* has been rarely reported in human infections. In this study, a rare fungal keratitis case caused by *G. basitruncatum* in China was reported. Its microbiological and molecular characteristics were described. The strain was isolated from corneal of a 35-year-old male farm worker. Routine biochemical and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) methods failed to identify the pathogenic strain. Internal transcribed space (ITS) second-generation sequencing was performed. ITS sequence of the isolate showed 99.77% similarity with *Graphium basitruncatum JCM 9300*,which identified the strain as *G. basitruncatum* . The strain had physiological characteristics including strong adaptability (able to grow on various cultured medias) and thermal tolerance (able to grow at 42°C), which may prompt its environmental adaptability and potential for opportunistic human infection. The patient had a favorable outcome after receiving treatment. In conclusion, this case indicates that *G. basitruncatum* should be recognized as an emerging opportunistic pathogen that can cause human infections.

Keywords: keratitis, *Graphium basitruncatum*, antifungal susceptibility test

Introduction

The fungal kingdom is highly diverse with 2.2–3.8 million species and is widely distributed in the environment.[1] Although the vast majority of fungi do not exhibit pathogenic traits, some species cause infections in humans, ranging from superficial to life threatening.[2,3] Fungal keratitis is a severe corneal infection that often results in blindness and eye loss and affects over a million people annually.[4] With advances in molecular techniques, the epidemiology of fungal infections in the eye is changing. Many fungi species have not previously been described as pathogens, but now contribute to the disease burden.

G. basitruncatum was first identified from forest soil in Solomon Is in 1971.[5] It is a species of ascomycete fungi in the order *Microascales*, family *Graphiaceae*, genus *Graphium*. It is phylogenetically distinct from other 19 species in *Graphium genus*.[6] Many species included in the genus *Graphium* are known as plant pathogens. However, the biology and distribution of *G. basitruncatum* are poorly known.[7] A few human cases of infection (in a heart transplant patient, a man with acute leukemia and an immunosuppressed child post stem-cell transplantation, respectively) with *G. basitruncatum* have been reported previously.[7–9]

In this study, a rare case of keratitis caused by *G. basitruncatum* in a male farm worker in Southwestern China was reported. The accurate identification of this rare species using routine laboratory assays remains challenging. We performed experiments to investigate the physical and chemical factors that affect the growth of this organism.

Case Description

A 35-year-old male farm worker, whose left eye had been scratched by tree leaves 30 days prior, complained of a foreign body sensation, lacrimation, photophobia, redness, pain, and no other obvious discomfort.

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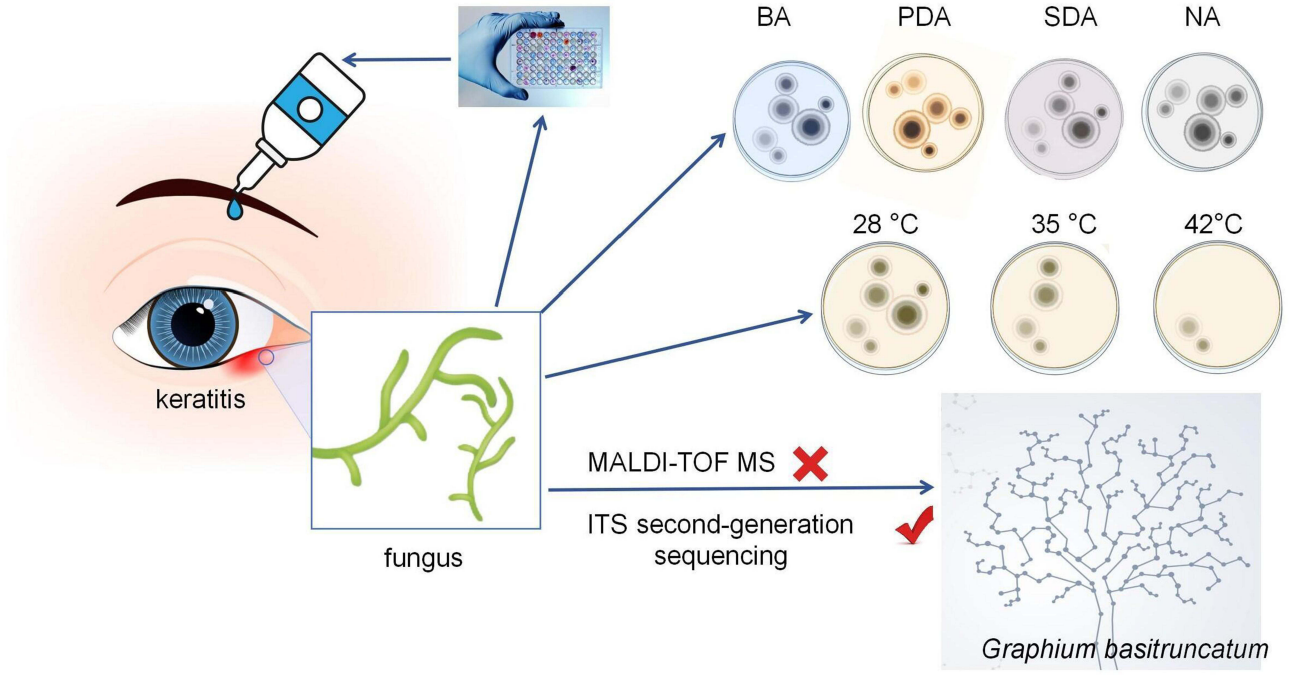
Graphical Abstract



On the first examination, the patient's vital signs were normal, however his visual acuity was 0.4 in the left eye with diffuse conjunctival hyperemia. There was an 8 mm gray-white infiltration with irregular borders, edema in the central cornea, and invasion of the shallow and middle stroma (Figure 1). No abnormalities were observed in the right eye.

On imaging logical examinations, anterior segment coherence optical tomography (AS-OCT) showed that the central cornea was infiltered. Corneal scraping was performed at the bottom and edge of the corneal ulcer and dripped with 10% potassium hydroxide. Broad hyaline fungal aseptate was observed by fluorescent staining of corneal scrapings (Figure 2A). Corneal scraping revealed sparse septate hyphae on Gram staining (Figure 2B). The remaining scrapings were directly inoculated into Potato Dextrose Agar (PDA, Dijing Biotech, Guangzhou, China) and incubated at 28°C. After 3 days, small white colonies, apparently of a single fungal species, appeared on the culture media (Figure 2C). A follow-up molecular identification confirmed that the strain was *G. basitruncatum*.

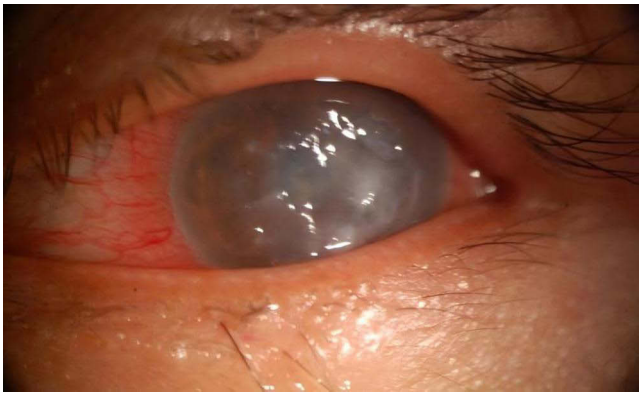


Figure 1 Slit-lamp examination of the left eye showing a 8 mm gray–white lesion infiltrate at the center cornea, invasion of shallow and middle stroma.

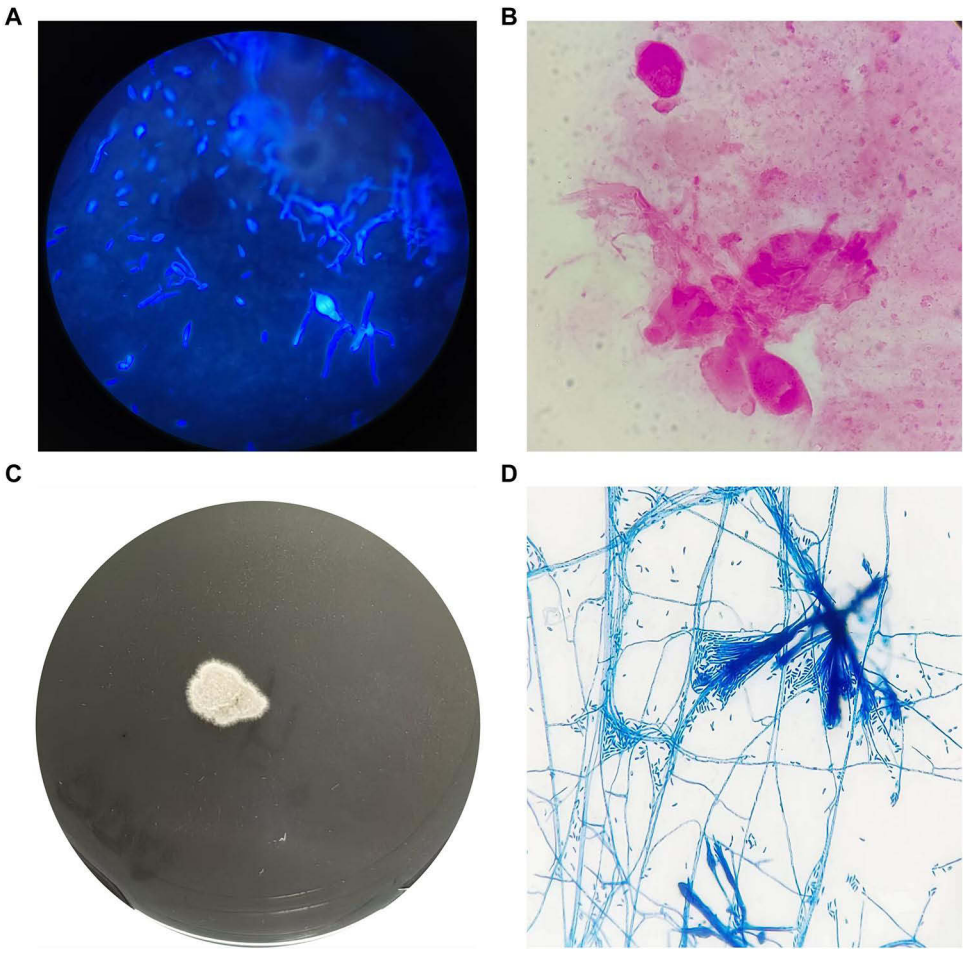


Figure 2 (**A**) The picture shows the fungal hyphae, fluorescence staining, ×400; (**B**)The picture shows sparsely septate hyphae, gram staining, ×1000; (**C**)The picture shows *G. basitruncatum* on potato dextrose agar (PDA) medium plates after 3 days of incubation at 28°C; (**D**) Microscopic examination of *G. basitruncatum* fungi. Lactophenol cotton- blue staining, ×1000.

After hyphae were detected, the patient was administered voriconazole 1% eye drops 1 times per hour, natamycin 0.25% eye drops once per hour, levofloxacin 0.5% eye drops four times a day, atropine sulfate 0.1% eye drops three times a day, and gatifloxacin 0.3% eye ointment once a night. Itraconazole 0.3% eye ointment once a night. Fifteen days later, the corneal ulcer was controlled, and the patient was discharged from the hospital. Topical antifungals were tapered and discontinued, and outpatient follow-up was conducted. Three months later, the corneal ulcer healed and no medication was required. However, the patient's visual acuity was not restored.

Methods

Morphology and Physiological Studies

The clinical strain *G. basitruncatum 631* isolated from the patient was used for follow-up investigations. The strain was subcultured on blood agar (BA, Dijing Biotech, Guangzhou, China), sabouraud glucose agar (SDA, Dijing Biotech, Guangzhou, China), potato dextrose agar (PDA, Dijing Biotech, Guangzhou, China), and nutrient agar (NA, Dijing Biotech, Guangzhou, China) and incubated at 37°C to observe growth on different media. Growth rates at different temperatures (28°C, 37°C, and 42°C) were determined for the strain studied on BA and PDA.

Molecular Identification and ITS Second-generation Sequencing

The cultured isolate was analyzed using a matrix-assisted laser desorption/ionization-time of flight mass spectrometry system (MBT HT Filamentous Fungi IVD Module, Bruker, Germany) following the manufacturer's instructions. For

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each run, *Escherichia coli* strain ATCC 25922 and *Candida albicans Berkhout* ATCC90028 were used as calibration controls. Genomic DNA was extracted using a Rapid Fungal Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The internal transcribed spacer regions (ITS) region of the rRNA was amplified with the primer pair ITS1 (5′- TCCGT AGG TGA ACC TGCGG- 3′) and ITS4 (5′- TCCTC CGC TTA TTG ATATGC- 3′). The sequences obtained were analyzed in the Mycobank (https://www.mycobank.org/Pairwise_alignment).

Antifungal Susceptibility Testing

Antifungal susceptibility testing was performed according to CLSI 38 methods.[10] The concentration of suspension was 0.5 Maxwell turbidity. The incubator temperature was 28°C and reading results after 48 h. Nine antifungal agents were tested, including amphotericin B, flucytosine, micafungin, caspofungin, fluconazole, isavuconazole, voriconazole, posaconazole, itraconazole (Dier Biotech, Zhuhai, China). According to the current CLSI document, susceptibility breakpoints for *G. basitruncatum* have not been established.

Results

The clinical strain *Graphium 631* was isolated from a 35-year-old male patient with keratitis. The colonies were initially fluffy, flat, and white, and became gray-brown in color (Figure 2C). Distinctive microscopic features included hyphae in prominent fascicles. Synnemata are darkly pigmented and erect, and occur solitarily or in clusters. Conidia were hyaline, one-celled, smooth, subglobose to ovoid, and usually aggregated in slimy heads at the apex of the synnemata (Figure 2D). White cotton-like filamentous colonies were able to grow on all culture media (Figure 3). The strain grew well at temperatures ranging from 28°C to 42°C, with the fastest growth rate obtained at 28°C (Figure 4).

MALDI-TOF MS analysis failed to identify strain 631. To accurately identify the fungus, ITS rDNA was sequenced and the amplified DNA fragments were 523 bp. A search in the Mycobank revealed that the isolate showed 99.77% similarity, and 100% query cover with *Graphium basitruncatum JCM 9300* (accession number AB038427.1). The isolates are considered for species because of ITS ≥ 99.6%.[11]

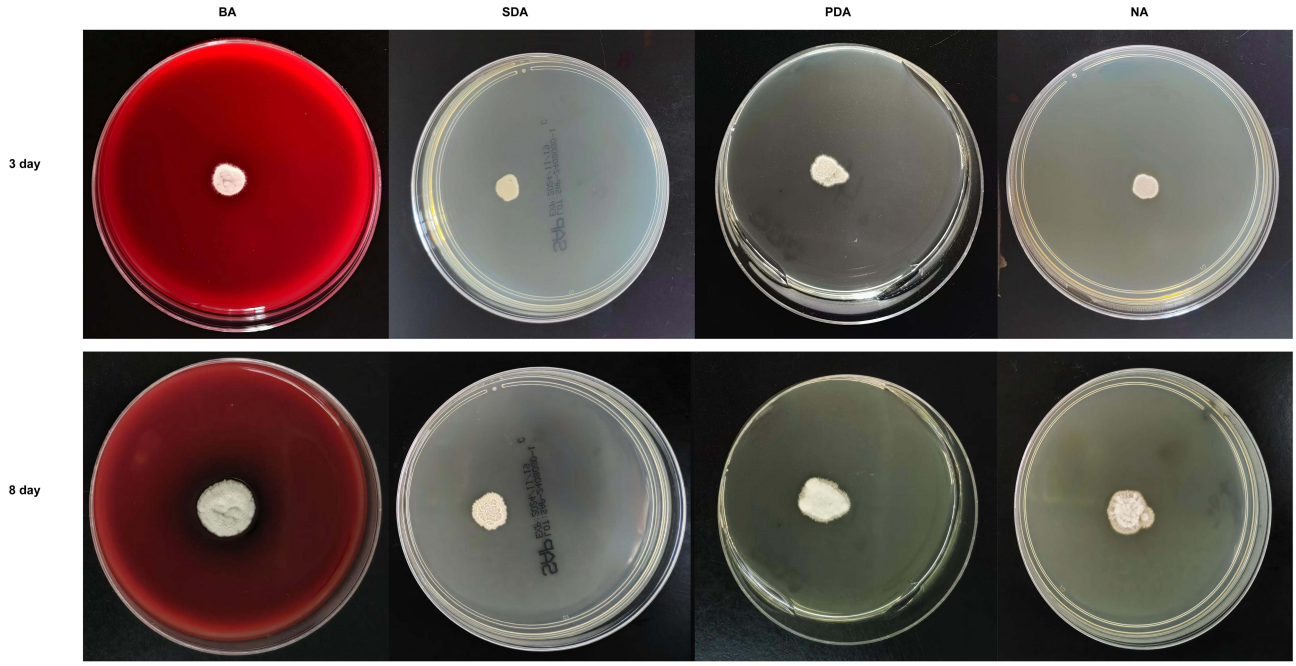

Figure 3 Blood agar (BA), potato dextrose agar (PDA), sabouraud glucose agar (SDA), and nutrient agar (NA) medium incubated at 35°C for 3 days and 8 days.

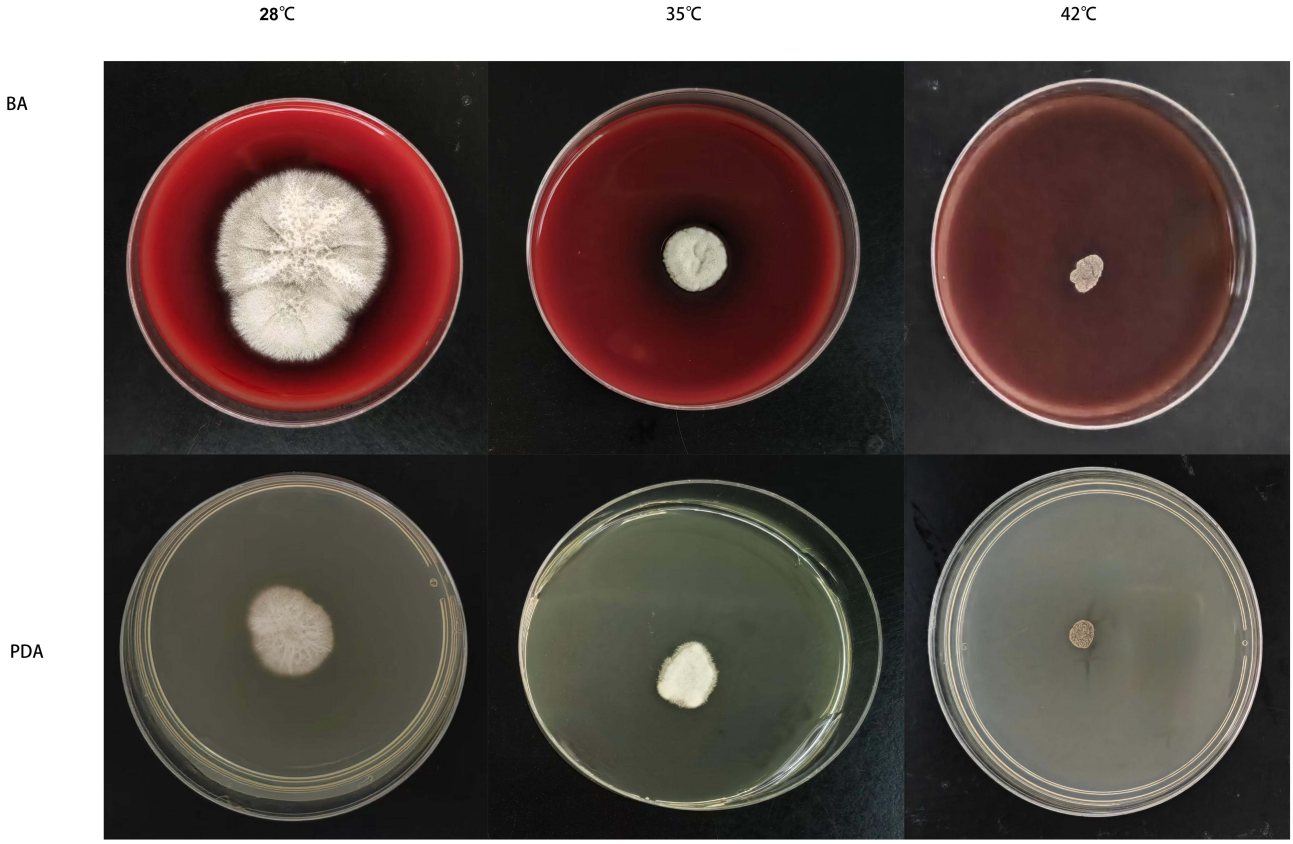

Figure 4 At 28°C,35°C, 42°C, blood agar (BA) and potato dextrose agar (PDA) growth of 8 days.

In vitro antifungal test, the minimal inhibitory concentration of the isolate was following respectively: amphotericin B (=1 μg/mL), flucytosine (>64 μg/mL), micafungin (>8 μg/mL), caspofungin (>8 μg/mL), fluconazole (>128 μg/mL), isavuconazole (=2μg/mL), voriconazole (= 2μg/mL), posaconazole (= 0.5 μg/mL), itraconazole (>16 μg/mL). The results showed amphotericin B, isavuconazole, voriconazole and posaconazole were effective against *G. basitruncatum* in vitro experiment.

Discussion

G. basitruncatum has rarely been reported to be a human pathogen. To date, only three publications have described the isolation of *G. basitruncatum* in clinical settings. In the first case report from Canada, *G. basitruncatum* was isolated from an immunocompromised patient with acute leukemia. *G. basitruncatum* has been repeatedly isolated from a sterile source (blood) and has metastasized to the skin, resulting in necrotic fungal nodules. The patient showed clinical improvement with liposomal amphotericin B, although the improvement coincided with the recovery of neutrophils and was temporary. The patient died from a relapse of infection despite showing no evidence of leukemia recurrence.[8] The second report from America described *G. basitruncatum* fungemia in an immunosuppressed child after stem-cell transplantation. Although it is difficult to establish the relative importance of the different fungi cultured from this patient over the course of the disease process, the fact that *G. basitruncatum* was cultured from more than one body site and from samples collected on different days suggests that the patient was indeed infected with this organism.[9] In addition, a case of subcutaneous infection caused by this fungus in a heart transplant recipient was reported at the Hospital Universitario Fundación Favaloro, Argentina. Fungal infections are localized to the subcutaneous tissue. The patient did not adhere to treatment or medical control. Voriconazole was administered only once, and adequate serum levels were achieved (3885 ng/mL). During surgery, the lesion was completely excised, and no evidence of disease.[7] The findings from this study, together with previous reports, suggest that *G. basitruncatum* is capable of causing a broad range of

human infections, and the treatment for *G. basitruncatum* includes a combination of surgical debridement and topical and parenteral antifungal agents.

However, little is known regarding the basic characteristics of *G. basitruncatum*. Some experiments were performed to investigate the physical factors that affect the growth of this organism and found that *G. basitruncatum* survives in different media and at a wide range of temperatures (28°C–42°C). *G. basitruncatum* grows slowly, conventional morphological identification is time-consuming, and the results are insensitive. However, early diagnosis and treatment can preserve vision in fungal keratitis.[4] Microscopic examination of fluorescent staining of biopsy material is important for early diagnosis. The clinical *G. basitruncatum* strain isolated in this study was not identified by MALDI-TOF MS. As *G. basitruncatum* is rare and its spectral profile may not be included in the databases used in mainstream commercial products, such as Bruker Biotyper or Vitek MS.[12,13] This is likely the reason why the identification failed. However, in-house customization of the database can be performed to improve identification using MALDI TOF/MS.[14]

In summary, *G. basitruncatum* is an emergent fungus that is increasingly being recognized in human infections. Treatment includes a combination of surgical debridement, and topical and parenteral antifungal agents. The microscopic identification of species is difficult and requires DNA sequencing.

Abbreviations

Graphium basitruncatum: G. basitruncatum; AS-OCT, anterior segment coherence optical tomography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ITS, internal transcribed space; MIC, minimum inhibitory concentration; BA, subcultured on Blood agar; SDA, Sabouraud glucose agar; PDA, Potato dextrose agar; NA, Nutrient agar.

Data Sharing Statement

The original contributions of this study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics Approval

This research complies with the guidelines for human studies and is in accordance with the Declaration of Helsinki. The publication of this report was approved by the Medical Ethics Committee of the People's Hospital of Guangxi Zhuang Autonomous Region (Guangxi Academy of Medical Science). Written informed consent for the disclosure of his detailed information was obtained from the patient.

Consent for Publish

Written informed consent was obtained from the patient for the publication of this case report.

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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 authors declare that they have no conflicts of interest in this work.

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