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Case Report

Disseminated *penicillium marneffei* infection with fungemia and intestinal perforation in an HIV-negative patient in a non-endemic region

Hui He[1,3*], Yi Chen[1,2*], Ping Pan[1,2], Songzhe He[4], Wenjuan Tong[2], Shenghai Wu[2], Daojun Yu[1,2]

[1]The First Affiliated Hospital of Hangzhou, Zhejiang Chinese Medical University, Hangzhou, China; [2]Department of Clinical Laboratory, Hangzhou First People's Hospital, Hangzhou, China; [3]Department of Pathology, Zhoushan Hospital, Zhoushan, Zhejiang Province, China; [4]Department of Clinical Laboratory, Affiliated Hospital of Guilin Medical University, Guilin, China. [*]Equal contributors.

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Abstract: *Penicillium marneffei* is a rare invasive fungal infection that primarily occurs in the southeastern and eastern regions of Asia in HIV-positive individuals. Herein we present a rare case of disseminated *P. marneffei* infection with fungemia and intestinal perforation in an HIV-negative patient in China. Extensive diagnostic evaluations did not reveal the cause of the disease; we use the automated microbiology system, matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), polymerase chain reaction (PCR), and the sequencing analysis to detect and identify the pathogen in positive blood culture of patient. The pathogen was initially misdiagnosed as *Stephanoascus ciferrii* by automated microbiology system. However the sequencing analysis revealed the pathogen to be *P. marneffei*. The patient was treated with amphotericin B, and a significant improvement in his condition was achieved. However, after a month, the patient suffered from an intestinal perforation, which may have been caused by *P. marneffei*. Although *P. marneffei* has been found in select regions, and appears mostly in patients with a weak immune system, particularly HIV-positive individuals. It is important to note that in addition to patients who are HIV-positive, anyone with a weak immune system can be infected with *P. marneffei*. DNA sequencing is generally considered to be the gold standard for the identification of microorganisms.

Keywords: Invasive fungal infection, *penicillium marneffei*, HIV-negative patient, sequencing analysis, an intestinal perforation

Introduction

Penicillium marneffei (PM) is an opportunistic pathogen that can cause an invasive fungal infection [1]. PM is the only known thermal dimorphic fungus in the *Penicillium* species. Some reports in the literature have suggested that this species should be transferred to the genus *Talaromyces* along with other *Penicillium* species belonging to the subgenus *Biverticillium* [2]. Healthy individuals may be infected with PM; however, it primarily infects immunocompromised hosts such as patients with AIDS. PM is primarily found in the southeastern and eastern regions of Asia and in China [3]. This paper is the first report of a patient infected with PM who is HIV-negative in Zhejiang.

Disseminated PM infections, which remain poorly understood among most doctors, have complex and varied manifestations. Additionally, there are no diagnostic characteristics found upon chest imaging, which easily leads to misdiagnoses. The patient in this case was diagnosed with an early onset fungal infection. The result of his blood culture suggested a *Stephanoascus ciferrii* infection, which was identified by the Automated Microbiology System at the previous hospital he had visited. We extracted and amplified the fungal DNA and eventually found that it was PM, based on DNA sequencing. The patient had not only the standard symptoms of PM infection but also the complication of an intestinal perforation.

The clinical manifestations, treatment and outcome of the patient with PM and the morphological and molecular sequencing features of PM are presented and characterized in this report.

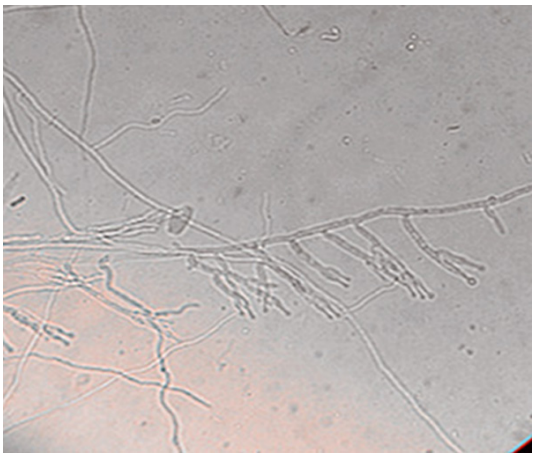

Figure 1. The three-day old colonies grew at 37°C (×400 magnification). The fungal hyphae were multi-branched in the shape of a broom.

Case report

A 21-year-old Chinese man with a 1-month history of a cough and expectoration, and 1-week history of an intermittent fever, was referred to our hospital for further evaluation on April 21, 2014. When his symptoms appeared, the patient began to cough and expectorate white phlegm with no obvious cause of a specific disease. At one-week prior to admission, he began to experience an intermittent fever without night sweats and had a maximum body temperature of 39.3°C. At the previous hospital examination, his sputum sample was detected repeatedly for acid-fast bacilli; however, the results were negative. His lung CT scan showed signs of inflammation in association with pleural effusions, which could have been caused by tuberculosis. The abdominal CT scan showed that his spleen was enlarged with multiple lesions, which may have been caused by the infection or cholecystitis. The fiberoptic bronchoscope examination showed a white necrotic material widely distributed in the right main bronchus, right-middle bronchus, and right upper lobe and did not find acid-fast bacilli via a brush biopsy. DNA load of tuberculosis bacilli in the bronchoalveolar lavage fluid sample of the patient were less than 10^2 copies/ml, and the test of his bronchoalveolar lavage fluid bacterial culture and T-SPOT was negative. The blood culture revealed that the patient was infected with *S. ciferrii*, which was identified by the Automated Microbiology System. Based on these medical examinations, the patient was treated with fluconazole and teicoplanin. However, the patient's condition did not improve with this treatment as he still had a fever that reached 39°C; thus, he was referred to our hospital for further treatment.

The patient was born and raised in Shaoxin, Zhejiang Province, and he reported never living in or visiting other locations. He was diagnosed with mycotic stomatitis, baldness, and leukonychia in his childhood. No other family members or close friends of the patient were diagnosed with similar diseases. During the physical examination, the patient appeared thin and chronically ill. There were no bleeding spots or rashes under his skin. The initial blood analysis revealed the following: hemoglobin, 95 g/L; white cell count, 3.3×10^9/liter (neutrophils, 3.1; lymphocytes, 0.2); and platelets, 73×10^9/liter. The biochemical test like alanine transaminase (ALT) and aspartate transaminase (AST) were high. The test for the presence of HIV antibodies was negative. The patient's blood and sputum cultures were positive and the fungus was isolated. The Automated Microbiology System (bioMérieux, Marcy L'Etoile, France) identified the isolate as S. ciferrii, as did the previous hospital. Its biological encode was 67577737737377771, and the credibility was 86%. We used MALDI-TOF (Bruker Daltonics, Germany) to identify the fungus, but we were not successful.

Subsequently, the patient was then given antifungal therapy consisting of a compound injection of fluconazole (200 mg once daily) and diflucan (400 mg once daily) combined with piperacillin-tazobactam (4.5 g every 8 hours) to treat the bacterial infection. Fourteen days after admission to our hospital, several sporadic blisters were raised on his back, which gradually spread to both his upper limbs and chest. The fluid inside the blisters was inoculated on a Sabouraud's Medium and blood plate, and no fungal or bacterial growth was found. The dermatologist theorized that it may have been chickenpox, and the patient was treated with famciclovir tablets (300 mg every 12 hours). The patient's condition did not improve. We believe that the Automated Microbiology System may have given us the wrong result, as the morphological characteristics of this fungal culture are not like those of *S. ciferrii,* which has a colony that is round, orderliness, velvet, moist and creamy [4].

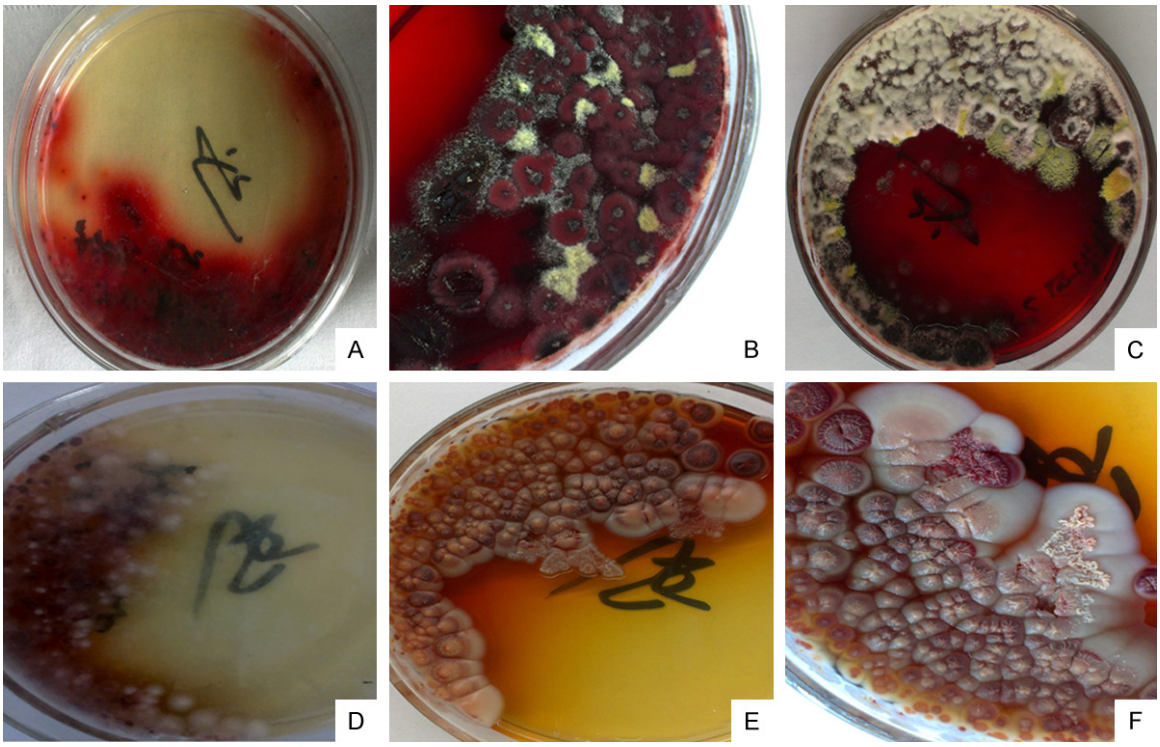

Figure 2. Colony morphology on Sabouraud's Medium at 25°C and 37°C. A: Two-day-old colonies grown on Sabourand's Medium at 25°C; B: Three-day-old colonies grown on Sabourand's Medium at 25°C; C: Seven-day-old colonies grown on Sabourand's Medium at 25°C. D: Three-day-old colonies grown on Sabourand's Medium at 37°C. E: Five-day-old colonies grown on Sabourand's Medium at 37°C. F: Seven-day-old colonies grown on Sabourand's Medium at 37°C.

In this case, isolate was grown on Sabouraud's Medium at 25°C and 37°C for one week. We examined the fungus that grew at 37°C under the microscope and observed that the fungal hyphae were multibranched in the shape of a broom (**Figure 1**). When the culture grew at 25°C, a grey villous fungal colony in mold form appeared, with a diameter of 2-4 mm, and small burgundy pigments percolated through the culture medium after 48 h (**Figure 2A**). Subsequent to another 72 h, the fungal colony increased in size with additional burgundy pigments percolating through the culture medium (**Figure 2B**). Midway through the growth period (4-7 days), the fungal colony appeared firm and became isabelline with a black microvillus (0.5-1.5 cm) in the middle, which could not be easily picked. Subsequent to another 7 days, the fungal colony exhibited restricted growth with an isabelline villus, and the entire culture medium became burgundy (**Figure 2C**). The fungus that incubated at 37°C grew slower than that incubated at 25°C; after 3 days, the fungus began to develop a white yeast-like colony (1-4 mm) with hazel pigments surrounding it (**Figure 2D**). After 5 days, the middle of the colony raised and formed surrounding wrinkles similar to gyrus (**Figure 2E**). After 7 days, it appeared to have restricted growth (**Figure 2F**).

Finally, we exacted the genomic DNA from the 4-day-old culture incubated at 37°C using the microwave method according to a protocol described previously [5], and PCR amplification was performed with universal fungal primers (ITS3 GCATCGATGAAGAACGCAGC, ITS4 TCCTCCGCTTATTGATATGC; LR12R GAACGCCTCTAAGTCAGAATCCG, InvSR1R ACTGGCAGAATCAACCAGGTA). The PCR conditions were 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and finally 72°C for 7 min. The amplified products, obtained with the above primer pairs, were sequenced with an ABI Prism automated DNA sequencer. These sequences were used to identify the fungus and were aligned with the help of the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The aligned sequences (18S rRNA gene) were used

Figure 3. Fast minimum evolution analysis of nucleic acid sequences of 18S rRNA. Gene from this isolate revealed it was *Penicillium marneffei*. The phylogenetic tree was calculated for each locus and sequenced using BLAST pairwise alignments.

for a phylogenetic analysis (phylogenetic tree), which was produced using the BLAST pairwise alignments. The sequence analysis determined that the best matches were with PM (**Figure 3**). And antifungal susceptibility testing was performed to determine the activity of fluconazole (FLC), voriconazole (VRC), itraconazole (ITC), ketoconazole (KEC), amphotericin B (AMB), caspofungin (CAS), micafungin (MICA), and flucytosine (5-FC) against this isolate using the broth microdilution assay, as previously described [6, 7] according to an adaptation of the methodology recommended for *Candida* spp. and *Cryptococcus neoformans* and following the CLSI approved standard M38-A2. The results showed that the isolate was sensitive to amphotericin B; however, it resisted other antifungal regimens such as benzimidazoles (voriconazole and ketoconazole), triazoles (fluconazole and itraconazole) and flucytosine.

Subsequently, the patient was treated with amphotericin B at an initial dose of 0.3 mg/kg daily. This dose was then increased gradually to 0.6 mg/kg daily, which has been demonstrated to successfully treat PM according to both the literature and antifungal susceptibility testing. The patient's condition appeared to improve as he had a milder cough and the fever and expectoration disappeared. However, a month later, the patient presented with a fever complicated with hypogastralgia. The imageological examination found the patient was suffering from a ruptured hollow viscus with an alimentary tract hemorrhage and suppurative peritonitis. The patient was immediately given a partial small bowel resection and a small intestine transverse anastomosis via laparoscopy and laparotomy. The postoperative pathological diagnosis was a small bowel perforated complicated with focal gangrenous inflammation, suppurative inflammation, and abscess formation around the small bowel. We speculated that the bowel perforation was caused by thrombosis in the small vessels, which arose from the disseminated PM infection. The patient continued to be treated with amphotericin B and was discharged when all his vital signs were stable. During 6-month follow-up, no similar symptoms were observed and PM was no longer found in his blood and sputum.

Discussion

PM appears mostly in patients with a weak immune system, particularly HIV-positive individuals, and is the third most common infection in the HIV-positive population after extra-pulmonary tuberculosis and cryptococcal meningitis [8]. Although the patient in this report was not infected with HIV, several tests such cellular, humoral immunological functions and so on revealed that his immune system is not normal. Penicilliosis marneffei (PSM) includes the topical and disseminated types. Most patients who are infected with PM present with the disseminated type, which affects multiple organs including the lungs, skin, liver and spleen, and the involvement of bone and joint complicated with osteolysis may occasionally follow [9]. The clinical manifestations of the disseminated PM include fever, mild anemia, splenomegaly, pneumonia and skin damage including molluscum contagiosum and multiple subcutaneous nodules, and so on, which are lack of characteristic presentations [10]. If the first symptoms of patients who are infected with PM are fever and cough, the imageological diagnosis often reveals that the lung damage is similar to that caused by tuberculosis, and patients are usually misdiagnosed with tuberculosis. The patient in this report was also initially misdiagnosed with tuberculosis as the main clinical manifestations were fever and cough. In addition to these manifestations, the patient also had anemia, hepatosplenomegaly and polyserositis, which conform to the manifestations of PM infection. The patient also reported pain in his knees, which may be another symptom of PM infection. But he had not received an imaging examination of his knees, and the knee pain cannot be confirmed to be caused by PSM. The patient was treated with a combination therapy of amphotericin and voriconazole immediately upon the diagnosis of PSM and his condition appeared to improve. However, a month later, the patient suddenly had an intestinal perforation. In addition, the D-dimer of the patient was 5930.0 μg/L in the hospital examination. Detection of the plasma level of the D-dimer has great clinical significance in a variety of thrombotic disease diagnoses [11], treatments and prognoses. A high level of D-dimer always suggests that there are abnormal changes of coagulation

and the fibrinolysis system in patients. It was thought that thrombosis occurred in the small vessels of the intestine, which was caused by disseminated PM. Spontaneous intestinal perforation is seldom seen in clinical practice. It is normally caused by typhoid intestinal perforation, enterophthisis, and segmental enteritis, among other factors. To the best of our knowledge, there has been no case of disseminated PM infection with intestinal perforation so far. As this is the first case, it leads us to recommend that when disseminated PM infection is diagnosed, the level of the patient's D-dimer should be monitored and that keen vigilance regarding intestinal perforation should be maintained.

The early identification and treatment of PSM is important because it is a life-threatening invasive fungal infection. However, the main clinical manifestations lack specificity. Its definitive diagnosis depends on a fungal culture from the blood, bone marrow, purulent discharge and so on. The fungus had been found in this patient's blood multiple times, and unfortunately it was misdiagnosed two times as S. ciferrii by the Automated Microbiology System in our hospital. In the published literature in China and abroad, S. ciferrii is rarely described [4].

The Automated Microbiology System differentiates bacteria based on biochemical characteristics for identification purposes, and thus its results are not sufficiently reliable when the automated microbiology system only has a few samples of a rare species. The reliability in this case was only 86%, and the patient did not respond well to diflucan and teicoplanin, which are the first choice for treating S. ciferrii infection. Because of this situation, the results of fungal identification were questioned. We picked a signal fungal colony and cultured the fungi at temperatures of 25°C and 37°C. Surprisingly, the surface morphology appeared quite different in the two environments, which suggested it was a thermal dimorphic fungus. Whereas S. ciferrii is yeast belonging to the Candida genus, which produces a colony that is round, orderliness, velvet, moist and creamy, the appearance of this fungus was quite different. This evidence indicated that this fungus was not S. ciferrii.

We also used MALDI-TOF MS to identify the fungus; however, it failed to identify the fungus. The reason why this method failed to identify PM may be that its database did not include the specific standardized characteristics of PM.

The gold standard for the identification of microorganisms remains DNA sequencing. We confirmed that the fungus was PM based on its DNA sequence. Many studies have demonstrated that PCR technology has a strong specificity and high sensitivity and that it can identify PM even though its morphology has changed. Sequence analysis represents a new method for the identification of PM that can be used in addition to phenotypic observation, histological examination, and immunological techniques, thus offering promising prospects in clinical microorganism identification. As PSM has raised concerns, molecular biology-based analytical techniques to identify PM have advanced at a rapid speed because they have the advantages of high specificity and sensitivity.

The case described here suggested that although PM has been found in select regions, predominantly southern Asia and southern China, medical staff in other areas should be vigilant with respect to its incidence. It is important to note that in addition to patients who are HIV-positive, anyone with a weak immune system can be infected with PM. When a patient presents with a fever of unknown origin, pulmonary infection, and multiple organ damage, these symptoms should raise the suspicion of an opportunistic fungal infection. When a fungal infection is suspected, the fungus should be cultured in 25°C and 37°C environments. A result indicating that the pathogen is a thermally regulated dimorphic mold may suggest a PM diagnosis; furthermore, the observation of a brush structure during microscopic examination combined with the results of other comparative fungal examinations, especially molecular biological analyses, can be used to make an early diagnosis of PSM. If PSM can be diagnosed at its early stage, the patient can receive the appropriate treatment in a timely manner, thus reducing the rate of mortality significantly.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Daojun Yu, Department of Clinical Laboratory, Hangzhou First People's Hospital, 261 Huansha Road, Hangzhou 310006, China. Tel: +86-571-56007155; Fax: +86-571-56007155; E-mail: yudaojun98@163.com

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