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Case Report

Identification of the first case of *Penicillium marneffei* infection in Shaanxi province by nanopore sequencing

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Abstract

Introduction: *Penicillium marneffei* is one of the most common opportunistic infectious fungi associated with AIDS (Acquired Immunodeficiency Syndrome). It is prevalent in Southeast Asia and southern China (such as Guangdong), but rare in inland provinces of China. *Penicillium marneffei* infections are often misdiagnosed.

Case Report: Here, we report a patient with *Penicillium marneffei* infection from Shaanxi Province, China, who was previously repeatedly diagnosed as Kala-azar in two hospitals. The patient received medical treatment due to fever and multiple papules, worked as a tour guide, and had travel history in Southeast Asian countries. We performed nanopore genome sequencing on blood samples of the patient and obtained 84,000 reads in 3.5 hours. The average length of the sequences was 7088.7 bases and the longest sequence was 87471 bases. Through comparison with the pathogenic bacteria database, 13 homologous *Penicillium marneffei* sequences were identified. Furthermore, by using specific quantitative real time-polymerase chain reaction of *Penicillium marneffei*, fungal ITS (Internal Transcribed Spacer) sequencing, and fungal biphasic culture, we further confirmed the pathogen as *Penicillium marneffei*. Meanwhile, the patient was confirmed to be HIV (Human Immunodeficiency Virus) positive. Thus, the patient was diagnosed with AIDS combined with *Penicillium marneffei* infection, which, to the best of our knowledge, is the first report of *Penicillium marneffei* infection in Shaanxi Province, China.

Conclusions: Metagenomic analysis based on nanopore sequencing provides an important reference for the diagnosis of *Penicillium marneffei* infection in this case.

Key words: Nanopore, metagenomics, *Penicillium marneffei*, ITS.

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Introduction

Penicillium marneffei (PM) is the only pathogenic biphasic fungus among more than 300 species of *Penicillium* [1]. Its main natural host is bamboo rat. It is generally believed that humans can be infected through contact with aerosols. PM is one of the common opportunistic infectious fungi of AIDS (Acquired Immunodeficiency Syndrome). It can cause Penicilliosis Marneffei (PSM), which is common in immunodeficiency patients. PSM is prevalent in Southeast Asia, and Guangdong, Guangxi, Hong Kong, and Taiwan in China [2]. In recent years, due to the increased global AIDS epidemic and the more frequent exchanges (such as tourism and business), the cases of AIDS combined with PSM have also been reported in inland provinces of China [3].

Information about the pathogen can be obtained through metagenome analysis of high-throughput sequencing, bioinformatics analysis and alignment with the pathogen database [4]. Nanopore sequencing is a third-generation sequencing technology developed in recent years. The MinION nanopore sequencer (Oxford Nanopore, London, UK) is compact and portable, with the advantages of rapid database construction, ultra-long read length, real-time data acquisition, and real-time analysis. It has unique advantages for the rapid identification of pathogens of infectious diseases [5]. Here, we report an AIDS patient with PSM. Nanopore sequencing, PCR, and biphasic culture were used for PSM diagnosis. To the best of our knowledge, this is the first case reported in Shaanxi Province, China.

Case report

Ethical approval and informed consent

The study was approved by the Ethics Review Board of Shaanxi Provincial Center for Disease Control

and Prevention, China. Written informed consent was obtained from the patient.

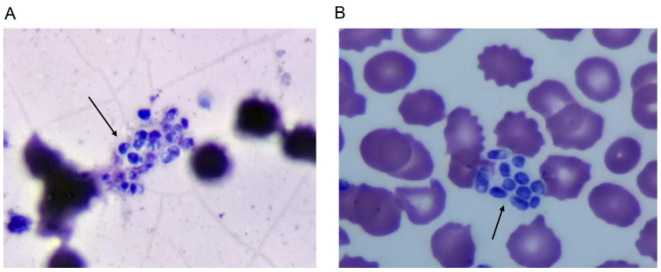
Clinical information

The patient was a 33-year-old male from Hanzhong City, Shaanxi Province, China. He worked as a tour guide, had travel history in Southeast Asian countries, and had unsafe homosexual and heterosexual behavior. In early November 2019, the patient had fatigue, night sweat, cough, bloating, diarrhea and other discomforts with unknown cause. He received oral Chinese medical treatment at a local clinic for 10 days. However, the treatment was not effective.

He was then admitted to the Hanzhong City Central Hospital in Shaanxi Province on November 19th. The routine blood tests indicated that the numbers of white blood cells, red blood cells and platelets were reduced. Bone marrow aspiration results suggested that granular hyperplasia was reduced, and suspicious inclusion bodies of Leishman-Donovan body were visible. Thus, the diagnosis of "Kala-azar" was suggested, and the patient was recommended to visit a better hospital.

He visited the Air Force Military Medical University on November 22nd. The routine blood test showed white blood cell count of 0.95×10^9/L, hemoglobin of 62 g/L, and platelet count of 55×10^9/L. On November 25th, the patient developed cough, white sticky sputum, and wheezing after physical activity, but no fever. On November 28th, another bone marrow aspiration was done and showed inclusion bodies of Leishman-Donovan body, and the diagnosis of "Kala-azar" was still considered. On December 2nd, the patient developed fever, with a temperature of 38.8 °C, accompanied by chills and diarrhea (about 2-3 times a day), watery stools, conscious cough, and gradually worsening asthma symptoms. On December 4th, the patient was tested for Leishmania antibody at Wangfeng Health Center in Hancheng City, and the result was negative, while the HIV (Human Immunodeficiency Virus) antibody and syphilis antibody screening results were positive. The patient was admitted to Xi'an Infectious Hospital on December 5th. Physical examination results were: temperature of 38.5 °C, pulse of 130 times/min, respiration of 22 times/min, and blood pressure of 100/60 mm Hg. HIV confirmation tests were positive. The routine blood test showed that lymphocyte count was 578/μL, CD4+ T lymphocyte count was 12/μL, CD8+ T lymphocyte count was 324/μL, and CD4+/CD8+ ratio was 0.04. The anticoagulant blood samples and bone marrow samples were further tested in our laboratory.

Figure 1. Wright's staining of blood and bone marrow samples.



Wright's staining was performed on blood and bone marrow samples. Results were observed under a microscope and yeast-like cells were observed (1000x). A: Blood smear. B: Bone marrow smear. Arrows indicate dark blue oval yeast-like cells.

Laboratory test

Microscopic examination: The blood and bone marrow samples were observed under the oil microscope after Wright staining. The results showed that there were dark blue oval yeast-like cells with a septum in the middle and dense stained large nuclei (Figure 1).

Nanopore sequencing, metagenome analysis and quantitative real-time PCR (qPCR) verification

The DNA extracted from blood samples was sequenced on MinION nanopore sequencer (Oxford Nanopore, London, UK). The actual sequencing time was 3.5 hours. A total of 84,000 reads were obtained. The highest quality score was 18.3, with an average of 12.4. The average length of all reads was 7088.7 bases, the longest was 87471 bases, the shortest was 562 bases, and the total data volume was 495 Mb. The sequencing data were uploaded to EPI2Me and analyzed with WIMP (What's In My Pot?) workflow [6]. In the generated data, 63617 sequences were human genome data and 13 were data of *Talaromyces*

Table 1. Sequence information of *Penicillium marneffei* aligned in Nanopore sequencing data.

Sequence	Length (bp)	Chromosome location	Chromosome length (bp)	Start	End
Read1	5339	CP015869.1	4250610	2258650	2263911
Read2	5476	CP015871.1	3290340	2044001	2049462
Read3	1773	CP015871.1	3290340	1677623	1679366
Read4	3584	CP015871.1	3290340	3045388	3048954
Read5	6089	CP015872.1	3256112	896675	902845
Read6	632	CP015873.1	3239194	2921924	2922485

marneffei. The most complete genome of PM (GCA_003971505.1_ASM397150v1_genomic) was downloaded from NCBI database (https://www.ncbi.nlm.nih.gov) [7]. PM has a total of 8 chromosomes. In the sequencing data of this patient, 6 high-quality sequences were aligned to those of PM, in which the longest reads were 6089 base and the shortest were 632 bases. The 6 sequences did not overlap each other, and 3 of them were located on the same chromosome CP015871.1 (Table 1 and Figure 2). The sequencing result was further validated by qPCR [8] and fungal Internal Transcribed Spacer (ITS) sequencing [9], as previously described.

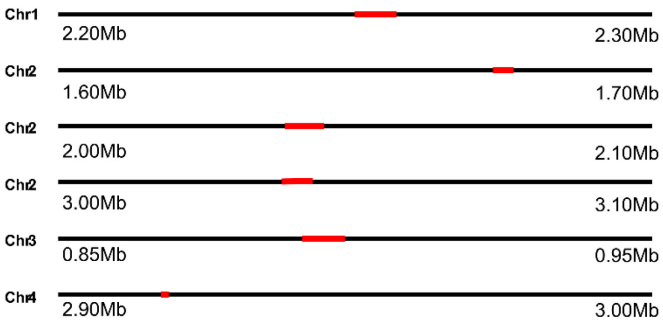
Pathogen separation and cultivation

The patient's anticoagulant blood sample (200 μL) was inoculated into Sabouraud Glucose Medium and incubated at 25 °C and 37 °C, respectively. After 2 days, the mold-like colonies were observed on the plate cultured at 25 °C. However, there were cream-colored, brick-red colonies on the plates incubated at 37 °C. After 7 days of culture, the colonies gradually increased. At 25 °C, the colonies became yellow, white, and green mold-like colonies, and red pigment that was produced and penetrated into the medium (Figure 3A). However, at 37 °C, yeast-like colonies were observed (Figure 3B). Ten colonies of different color and morphology were selected and subjected to qPCR amplification and sequencing of ITS sequences, which further identified these colonies as PM.

Diagnosis, interventions and outcomes

Therefore, based on the epidemiological history, clinical symptoms and signs, as well as laboratory results, the patient was diagnosed with AIDS and PSM.

Figure 2. The chromosome alignment of 6 sequences of Penicillium marneffei obtained in this study.



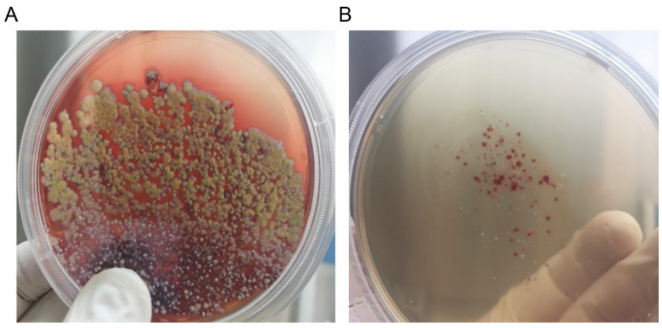
There were 8 chromosomes in Penicillium marneffei. Among the 13 sequences of Penicillium marneffei obtained in this study, the high-quality sequences on the alignment covered 6 chromosomes, and the sequence positions were not overlapped.

For treatment, the patient was given supportive therapy, immunomodulatory therapy, anti-infective therapy, and anti-fungal therapy. On January 19th, the patient's body temperature and the results of various tests returned to normal, and the patient was discharged clinically. The patient was instructed to continue oral HIV/AIDS treatment after discharge. After follow-up for half a year, the patient is in good health. The patient is in constant follow-up.

Discussion

Some fungi that cause deep infections (such as PM and *Histoplasma capsulatum*) are very similar in morphology to *Leishmania*, which causes Kala-azar. They can infect mononuclear phagocytes and parasitize macrophages. Moreover, their pathogenic mechanism and clinical symptoms are also similar, and they can cause fever, hepatosplenomegaly, and three-lineage cell reduction. Therefore, such infectious diseases are often misdiagnosed. Previously, we treated a case of suspected Kala-azar in 2018, who was finally diagnosed with *Aureobasidium pullulans* infection. Here, in this study, the patient had undergone bone marrow examinations in two hospitals, and the results showed suspected Leishman-Donovan bodies. The diagnosis of Kala-azar was suggested. However, the patient's *Leishmania* antibody was negative. Thus, Kala-azar was excluded. Through microscopic examination, we found that the structure of the corpuscle in the blood was different from that of the Leishman-Donovan body, but presented yeast-like structure. The pathogen was confirmed as PM by Nanopore sequencing, metagenome analysis, PCR verification and fungal

Figure 3. Fungal biphasic culture results of the patient's blood sample.



The patient's blood samples were inoculated on Sabouraud Glucose Medium and cultured at 25 °C and 37 °C for 7 days, respectively. A: After 7 days incubation at 25 °C, there were mold-like colonies, which produced red pigments. B: After 7 days incubation at 37 °C, yeast-like colonies were observed.

biphasic culture. In addition, the patient had an epidemiological history of unsafe homosexual and heterosexual behavior, and travel to Southeast Asia. The patient's clinical symptoms and signs were consistent with those of AIDS, and laboratory tests for HIV antibodies were positive. Eventually, the patient was diagnosed with AIDS and PSM.

Invasive fungal disease is an important manifestation of HIV infection at the AIDS stage [10]. In China, the first PM infected patient was identified in Guangxi in 1982 [11], and PSM is generally prevalent in Guangdong, Guangxi, Yunnan, Hong Kong, and Taiwan. However, recent PSM cases have also been reported in inland provinces of China [12]. In Shaanxi Province, the number of HIV-positive gay men has increased from 51 cases in 2008 to 1066 cases in 2017 [13]. In areas with high HIV infection rates, PM infections are also high [2]. However, PSM cases have not been reported in Shaanxi Province previously. Bamboo rats are natural hosts of PM, and they are mostly distributed in Southeast Asia and southern China [2]. In this study, the patient had HIV infection and had a history of travel to Southeast Asia. However, the source of infection still needs to be determined.

Metagenomic analysis and high-throughput sequencing can help to identify potential pathogens [4]. In this study, we used Nanopore MinION sequencer for high-throughput sequencing. The sequencing library was constructed in 2 hours, and the sequencing was completed in 3.5 hours. In the end, we got 84,000 reads in total, and the longest read length reached 87471 bases. EPI2Me is a cloud-based online analysis system built by Nanopore Oxford. The WIMP WorkFlow includes a RefSeq bacterial, fungal, and viral database [6], which can be used to align sequencing data with the RefSeq database for classification and identification [14]. We uploaded the metagenomic sequence to EPI2Me for online analysis and obtained the alignment results in 10 minutes. The results showed that in addition to the human genome, 13 sequences of PM were identified. Compared with the whole genome of PM, 6 high-quality sequences of PM were identified, which did not overlap each other. Therefore, at the genomic level, we confirmed that there was PM in the patient's blood specimens. Thus, metagenomic sequencing in non-endemic areas has important guiding significance for the identification of unknown pathogens.

The fungal ITS has a wide range of homology and can be used for fungal identification and phylogenetic analysis. Using fungal ITS universal primers to amplify and sequence sterile body fluids, fungal infections can be quickly identified [8]. In this study, the metagenomic sequencing results were further confirmed by qPCR and PCR amplification and sequencing of ITS sequences.

The gold standard for the diagnosis of fungal infections is pathogen isolation and cultivation. Pathogenic fungi often have the characteristics of biphasic culture [1], which grow hyphae at room temperature (25-28 °C) and can produce specific pigments, while exhibiting yeast-like morphology at 37 °C. It is generally believed that biphasic pigment-producing fungi have certain pathogenicity and toxicity. PM is the only biphasic growth of all 300 species of *Penicillium*, and can produce a large amount of red pigment [15]. In this study, the cultured colonies had characteristics of biphasic culture. Ten colonies of different size and morphology were selected and identified by ITS sequencing, which further confirmed that the cultured colony was PM. However, the isolation and cultivation of fungi takes a long time. Thus, it can be used for final confirmation after the rapid molecular detection methods (such as metagenomic sequencing).

Conclusions

In conclusion, we reported the first case of AIDS combined with PM in Shaanxi Province, China, with metagenomic analysis based on nanopore sequencing, qPCR, fungal ITS sequencing, and fungal biphasic culture. These results indicate that metagenomic analysis based on nanopore sequencing is of great significance for rapid identification of unknown pathogens.

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