Application of laser capture microdissection and PCR sequencing in the diagnosis of *Coccidioides* spp. infection: A case report and literature review in China

Xinyu Yang†, Yinggai Song†, TianYu Liang, Qiqi Wang, Ruoyu Li and Wei Liu

Department of Dermatology and Venereology, National Clinical Research Center for Skin and Immune Diseases, Research Center for Medical Mycology, Beijing Key Laboratory of Molecular Diagnosis on Dermatoses, Peking University First Hospital, Beijing, People’s Republic of China

**ABSTRACT**

*Coccidioidomycosis* is endemic to California, Arizona, and Mexico. In recent years, the reported cases of *coccidioidomycosis* have increased in nonendemic regions. Here, we reported a case of imported pulmonary *coccidioidomycosis* in a Chinese patient. A 63-year-old man presented with dry cough and fatigue for 6 months, and a computed tomography scan revealed a solitary nodule in the right lower lung and small nodules in both lungs. The diagnosis of *coccidioidomycosis* was initially confirmed by histopathologic examination. The pathogen *Coccidioides* spp. was identified by laser capture microdissection (LCM) combined with subsequent molecular techniques based on the positive histopathologic features. Additionally, we reviewed 47 reported cases of *coccidioidomycosis* in China. The number of reported cases is increasing, and the incidence of disseminated infection has exhibited a trend of shifting towards healthy young adults in China. Since clinical presentations and imaging findings lack specificity, a majority of domestic cases of *coccidioidomycosis* were initially misdiagnosed as tumours or tuberculosis. Moreover, the diagnosis of endemic mycoses may be challenging because of their rarity and the limited availability of diagnostic tests. The diagnosis was mainly confirmed by histopathological examination. The species involved were identified based on positive cultures in only 4 cases. To our knowledge, this is the first study to use LCM and molecular techniques to identify *Coccidioides* spp. in the histopathologically positive but uncultivable specimen. Comparing with previous reported studies, LCM combined with nucleic acid amplification techniques improve the ability of species identification for the timely diagnosis of *coccidioidomycosis*.

**ARTICLE HISTORY**

Received 21 September 2020; Revised 19 January 2021; Accepted 9 February 2021

**KEYWORDS**

*Coccidioidomycosis; Coccidioides* spp; laser capture microdissection; molecular diagnosis

**CONTACT**

Wei Liu, liuwei@bjmu.edu.cn, Department of Dermatology and Venereology, National Clinical Research Center for Skin and Immune Diseases, Research Center for Medical Mycology, Beijing Key Laboratory of Molecular Diagnosis on Dermatoses, Peking University First Hospital, Beijing, People’s Republic of China

†These authors contributed equally.

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group, on behalf of Shanghai Shangyixun Cultural Communication Co., Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

*Coccidioidomycosis* is a fungal disease caused by *Coccidioides immitis* and *Coccidioides posadasii*. *Coccidioidomycosis* is endemic to the Americas, including South America. Most cases are reported in California and Arizona, but it is found in many arid regions throughout North and South America where it is underreported [1]. With the continuous development of international communication and tourism, reported imported cases have been increasing in nonendemic areas [2]. *Coccidioidomycosis* can clinically manifest as an asymptomatic infection, primary pulmonary infection or even a disseminated infection [1]. Since the disease is rare and its clinical signs are nonspecific, it is easily misdiagnosed as a tumour or other disease in nonendemic areas.

The diagnosis of *coccidioidomycosis* is mainly based on exposure history, clinical manifestations and laboratory tests [3]. *Coccidioidomycosis* should be considered in the differential diagnosis of patients who have respiratory, skin, neurological and other symptoms and have recently travelled to endemic areas. Laboratory tests for the disease include histopathological examination, the isolation and culture of pathogens, serological examination, and the use of molecular methods for species identification [1,3]. Fungal culture and histopathology analyses are the gold standards for the diagnosis of coccidioidomycosis. The histopathology of *coccidioidomycosis* is characterized by spherules 20–200 μm in diameter containing endospores observed via microscopy [3]. Periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS) staining is very sensitive for detecting a small number of spherules. Fungal culture usually requires 3 weeks and should be carried out in a biosafety level 3 laboratory (BSL-3), which may lead to
delayed diagnosis and potential laboratory transmission [3]. Additionally, in nonendemic areas, serologic testing is not routinely available [3].

If clinical specimens cannot be cultured, the application of molecular techniques to detect *Coccidioides* spp. directly from formalin-fixed, paraffin-embedded (FFPE) tissue is useful for the molecular diagnosis of coccidioidomycosis [4]. For example, a specific target region of the internal transcribed spacer (ITS) or the proline-rich antigen (PRA) coding region of the fungal genome may be amplified by PCR for species identification. Furthermore, Real-time PCR is a rapid and sensitive method for analyzing single-nucleotide polymorphisms (SNPs) from polymorphic gene fragments to identify possible species [5].

In recent years, laser capture microdissection (LCM)-based analytic techniques have been applied for the microbiological diagnosis of infectious diseases [6, 7]. LCM is a novel and powerful technique for selectively harvesting cells of interest directly from histological sections under direct microscopy [8]. It is widely used for genomic analysis, gene expression profiling and the evaluation of host-microorganism interactions [9].

In this study, we reported a case of imported coccidioidomycosis confirmed by histopathology. To further determine the pathogen, suspected pathogenic fungi from FFPE lung tissue sections subjected to PAS staining were collected by LCM. Genomic DNA was extracted, and downstream PCR and Sanger sequencing were performed to identify the pathogen. Since coccidioidomycosis is rare in China, we summarized all reported 47 cases so as to better understand the etiology and clinical features of this disease.

**Case**

A 63-year-old man presented to our hospital with a 6-month history of dry cough and fatigue. Six months earlier, the patient had presented with dry cough and fatigue without an obvious cause. He did not exhibit fever, headache, or night sweats. A chest computed tomography (CT) scan at a local hospital revealed a 1.4 × 1.3 × 1.1 cm solitary solid nodule in the right lower lobe. The patient was suspected of having bacterial infection and was treated with intravenous cephalosporin for 10 days but showed no improvement. He was previously healthy except for a 20-year history of asthma. One year prior to his appearance at the hospital, he had worked as a miner for 6 months in Mexico, and eventually disappeared during treatment. The patient is still undergoing follow-up.

**Materials and methods**

**Laser capture microdissection of FFPE tissue sections**

To further identify the pathogen at the species level, FFPE tissue was cut into 6 μm-thick sections, which were then affixed to microscope slides, precoated with polyethylene naphthalate, for PAS staining. Laser microdissection and laser pressure catapulting were performed to capture PAS-positive fungal components such as hyphae and spores in the tissue sections under light microscopy using a Leica LMD6000 system (Leica, Wetzlar, Germany) following the manufacturer’s instructions. The universal fungal ITS primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTATTGATATGC-3’) were used as outer primers for the first round of PCR [10]. PCR was carried out in a 25 μl volume containing 20 ng of genomic DNA, each of the primers at 0.08 μM, and 12.5 μl of 2× Taq PCR MasterMix (Tiangen Bio-tech Ltd, Beijing, China). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. Then, the PCR products were used as templates for
nested PCR with the Coccidioides-specific primers ITSC1 (5’-CATCATAGCAAAAAATCAAAC-3’) and ITSC2 (5’-AGGCCCGTCCACACAAG-3’) [11]. The cycling conditions were as follows: initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 7 min. A. fumigatus genomic DNA and sterile water were used as templates to validate the specificity of the nested PCR assay.

**Cloning and sequencing of amplified products**

The PCR products from each round of PCR were ligated with T-Vector pMD19 (Takara, Otsu, Japan) using T4 DNA ligase (Takara, Otsu, Japan). The ligated products were then transformed into Escherichia coli DH5α competent cells (Yeasen, Shanghai, China). The transformed cells were plated on LB agar plates containing 100 μg/ml ampicillin and incubated at 37°C for 16–18 h. Single colonies were then randomly picked. Sixty positive clones from the first-round PCR products and 12 positive clones from the second-round PCR products were screened by colony PCR. Then, the 72 colonies were individually inoculated into LB liquid broth with 100 μg/ml ampicillin and grown at 37°C with shaking at 220 rpm for 12 h. The plasmids were subsequently extracted using the TIANprep Mini Plasmid Kit (Tiangen Biotech Ltd, Beijing, China) and sent to the BGI Company (Beijing, China) for sequencing using the M13F (5’-CAGGAAACAGCTATGAC-3’) and M13R (5’-GTTTTCCCAGTCACGAA-3’) primers [12]. The sequences of the inserted fragment were aligned against the CBS database (https://wi.knaw.nl/page/Pairwise_alignment) and GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) for species identification.

**Real-time PCR assay using Coccidioides-specific TaqMan probes**

Real-time PCR is a highly sensitive and specific method for quickly detecting Coccidioides directly from clinical specimens [13]. Castano-Olivares et al. [14] described a method in which a probe labelled with the VIC™ fluorophore (5’-VIC-CCCAATGTGATCCCA-3’) was hybridized to nucleotides 404–467 of the

---

**Figure 1.** Chest computed tomography (CT) scans. CT images of the lung window (A) and soft tissue window (B) revealed a 1.4 × 1.3 × 1.1 cm solitary solid nodule in the left lower lobe (arrowhead), mild ground-glass opacity and multiple small nodules in both lungs.

**Figure 2.** Histopathological examination of biopsy tissue. PAS staining (A) and GMS staining (B) showing multiple thick-walled spherules and endospores (arrowhead) in the nodules.
*C. immitis* PRA gene and a probe labelled with the FAM<sup>TM</sup> fluorophore (5'-6FAM-CCCAATTGA-CATCCCA-3') was hybridized to nucleotides 415–478 of the *C. posadasii* PRA gene. Genomic DNA extracted from LCM-isolated tissue was used as the template for Real-time PCR with the primers PRAF1 (5'-GTCGTTGACCAGTGCTCCAA-3') and PRAR1 (5'-CGGCGGTGGTGTCAACT-3') and the above-mentioned specific probes. An Applied Biosystems ViiA7 Real-time PCR system was used. Each reaction mixture volume (20 μl) contained 1× ABI TaqMan PCR Master Mix (Applied Biosystems, Rochester, NY), each primer at 900 μM, 2 μl of template, and each fluorescein-labelled probe at 125 μM. The cycling conditions were as follows: an initial denaturation step at 94°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 s and annealing/elongation at 60°C for 60 s. All samples were run in triplicate.

**Literature review of coccidioidomycosis in China**

We searched the literature on coccidioidomycosis in Chinese patients from PubMed (http://www.ncbi.nlm.nih.gov), the China Knowledge Resource Integrated (CNKI) database (http://www.cnki.net), and the Wanfang database (http://www.wanfangdata.com.cn/index.html) published between 1958 and 2020. The search terms used were “coccidioidomycosis” and “China”. The epidemiological features, underlying diseases, clinical manifestations, diagnostic methods, treatment and prognosis of all patients were summarized and analyzed.

**Results**

**Fungi were isolated by LCM from FFPE tissue sections**

Round purple-red fungal spores scattered within alveoli were visible in the PAS-stained FFPE tissue sections under the inverted microscope of an LCM instrument (Figure 3A). Fungal spores were further microdissected under UV light and collected in a sterile environment (Figure 3B).

**Coccidioides and other filamentous fungi were detected by PCR in LCM-isolated FFPE tissue**

Using the universal fungal primers ITS1 and ITS4, the first-round PCR yielded two bands between approximately 500 and 750 bp (Figure 4A). The DNA in the two bands was purified and cloned. Sixty positive clones were selected for plasmid DNA extraction and sequencing. A variety of fungal species were identified, including Alternaria spp., Aspergillus spp., Cladosporium spp., and Fusarium spp.

At the same time, the products from the first-round PCR were used as the templates and the ITSC1 and ITSC2 were used as *Coccidioides*-specific primers for nested PCR amplification, yielding two bands of approximately 250 bp (Figure 4B). Each of the PCR products was purified and cloned. Twelve positive colonies were sequenced. A 237-bp sequence of the inserted fragment obtained from positive colonies was deposited in the NCBI database under GenBank accession number MT899215, which showed 97.92% similarity with *C. immitis* sequences in both CBS and GenBank databases.

**Coccidioides spp. was detected by Real-time PCR in LCM-isolated FFPE tissue**

The Real-time PCR results showed the two amplification curves presented in Figure 5. The cycle threshold (Ct) value of the first amplification curve labelled with the VIC fluorophore was 6, possibly corresponding to *C. immitis*. The Ct value of the other amplification curve labelled with the FAM fluorophore was 30, possibly corresponding to *C. posadasii*. And the amount of *C. immitis* may be greater than that of *C. posadasii* since the Ct value of *C. immitis* was significantly
lower than that of *C. posadasii* [15]. These results suggest that both *C. immitis* and *C. posadasii* may present in LCM-isolated FFPE tissue.

**Literature review of coccidioidomycosis in China**

Following literature review, 38 articles reporting 47 coccidioidomycosis patients were included in this study (Table 1). The new patient reported herein was also included, leading to a total of 48 patients [16–52]. Twenty patients (42.6%) had a definitive history of travel or residence in endemic areas, 22 patients (46.8%) reported no exposure history, and 5 patients had unknown exposure history.

Among the 47 patients, the most common infection site was lung and 37 patients (78.73%) had respiratory symptoms such as dry cough or white, yellow, bloody

**Figure 4.** Electrophoresis of the nested-PCR amplification products in 2% agarose gels. (A) DNA products amplified in first-round PCR using the universal primers ITS1 and ITS4. Lane M, 2 kb DNA ladder. Lane 1, product amplified from *Aspergillus fumigatus* DNA as positive control. Lane 2, negative control, no amplification product by using DNA extracted from laser microdissected adjacent areas without fungal spores in the identical field of the same tissue section. Lane 3, products amplified from fungal DNA extracted from LCM-isolated FFPE tissue. Lane 4, no-template control. (B) Nested PCR products obtained by using the first-round PCR products as templates and ITSC1 and ITSC2 as the Coccidioides-specific primers. Lane M, 2 kb DNA ladder. Lane 1, nested PCR products obtained by using the first-round PCR products as templates. Lane 2, no product obtained by using first-round PCR products, amplified from *A. fumigatus* DNA, as templates.

**Figure 5.** Amplification curves of TaqMan-based Real-time PCR assay using Coccidioides-specific TaqMan probes and fungal DNA extracted from LCM-isolated FFPE tissue as template.
sputum. Thirty-one patients (66.0%) showed single system involvement, including 23 cases of lung infection, 4 cases of skin infection, and 1 case each of lymph node, cornea, stomach, and bone infection. In addition, 16 patients (34.0%) presented a disseminated infection involving the lungs, lymph nodes, central nervous system and other organs, 8 of which (50.0%) had been reported during the past 5 years (2016–2020). Disseminated infections were severe and mainly occurred in young individuals (87.5%). Additionally, 8 patients (17.0%), including 6 patients with single system involvement and 2 patients with disseminated infection, exhibited underlying diseases such as acquired immune deficiency syndrome (AIDS), drug abuse, chronic hepatitis and other diseases. However, 26 patients (55.3%) had no underlying diseases.

Chest imaging of pulmonary infection showed nodules, cavities, masses, hilar and peritracheal lymphadenopathy, diffuse ground-glass opacity and consolidation in the lung. X-ray or MRI visualization of bone infection mainly revealed severe osteolytic bone lesions and bone destruction. All of these radiologic findings of coccidioidomycosis were nonspecific.

The laboratory diagnoses of coccidioidomycosis cases in China were mainly based on histopathology, which was positive in 40 cases (85.1%) (Table 2). The pathogenic species were identified on the basis of positive culture in only 4 cases: two of the patients infected by C. posadasii were diagnosed by PCR or a combined matrix-assisted laser desorption/ionation time of flight mass spectrometry (MALDI-TOF MS) and next-generation sequencing (NGS), the third patient infected by C. immitis was identified by histopathology and NGS, and the last one infected by mixed infection of C. immitis and C. posadasii was identified using MALDI-TOF MS. Based on histopathologic features, Coccidioides spp. was identified by LCM combined with nested PCR and Real-time PCR in our patient.

Among the 47 patients, patients with single system involvement were usually treated with fluconazole alone and those with disseminated infections were usually treated with fluconazole in combination with amphotericin B. And the remission or cure rate was 94.5%. Six patients underwent surgery, 3 of whom were cured, while the results for other 3 were not reported. Another three patients with disseminated infections did not receive antifungal treatment because of not considering the diagnosis of coccidioidomycosis, and died.

Discussion

In this study, the patient developed nonspecific manifestations such as dry cough, fatigue, and pulmonary nodules, which were in consistent with domestic cases of pulmonary coccidioidomycosis. The diagnosis of coccidioidomycosis was confirmed by the presence of spherules containing endospores using histopathologic examination, and Coccidioides spp. was identified in limited FFPE tissue using a combination of LCM, nested PCR and Real-time PCR. Luna-isaac et al. [53] studied the relationship between Coccidioides spp. and their clinical manifestations as well as geographical distribution in Mexico, and he reported that C. immitis and C. posadasii were mainly distributed in northeastern and northwestern Mexico, respectively [53]. These two organisms were genetically distinct but unable to be distinguished on the basis of morphological and clinical features, immune response, or epidemic regions [1, 53]. Therefore, we speculated that this patient reported herein may inhale two species of Coccidioides spores during long-term contact with soil.

Additionally, round PAS-positive fungal microconidia, which may be species of Coccidioides spp., Aspergillus spp., Penicillium spp., or other fungi in the stained tissue, were shown in Figure 3. Hence, these microconidia cannot be identified as Aspergillus spp. alone, since it is difficult to identify species of microconidia based on the round PAS positive appearance observed on the tissue section. The histopathological features of Coccidioides in the biopsy specimens are spherules filled with 2- to 4-μm-diameter endospores. The mycelial form is very uncommon [3]. Hence, we believe that the presence of fungal conidia without fruiting bodies or hyphae in FFPE tissue is mostly the endospores released from those broken spherules. For A. fumigatus, conidia can bind to lung type II alveolar epithelial cells and invade the cells by inducing their own internalization, and gliotoxin might be responsible for modulating A. fumigatus internalization into epithelial cells through phospholipase D (PLD) activation and actin cytoskeleton rearrangement. Consequently, A. fumigatus conidia survive and disseminate within these normally nonphagocytic host cells upon evasion of host defense by phagocytes, and then colonize the respiratory tissue and initiate vascular diffusion [54–56]. Previous studies showed that 70% of severe asthma in adult patients was caused by fungal sensitization [57]. The predominant etiologic agents, including the species of Aspergillus, Alternaria, Cladosporium, Penicillium, and Fusarium were also identified in FFPE tissue from this patient, which could cause asthma in this patient for up to 20 years since these fungi were speculated colonizing the respiratory tract and triggering IgE-mediated allergic reactions [57, 58].

A total of 47 reported cases of coccidioidomycosis in China were collected. Ten cases were reported from 1958 to 2000, 9 cases were reported from 2000 to 2009, and 28 cases were reported from 2010 to
| Year | References | Age/Sex | Provinces | Job | Underlying disease | Site of infection | Travel history | Method of identification | Treatment | Outcome |
|------|------------|---------|------------|-----|--------------------|------------------|---------------|-------------------------|------------|---------|
| 1958 | [16]       | 35/male | Guangdong | Pilots | None               | Skin             | California     | Biopsy/Culture          | Surgery    | Cured   |
| 1963 | [17]       | 43/male | Shandong   | Merchant | None               | Disseminated(Lung/Skin) | None          | Culture                | Antibiotics | Died    |
| 1985 | [18]       | 31/male | Jiangxi   | Auto worker | None           | Lung             | None          | Culture                | CTZ        | Remission |
| 1985 | [18]       | 32/female | Jiangxi | Auto worker | None           | Lung             | None          | Culture                | CTZ        | Remission |
| 1988 | [19]       | 26/female | Shanghai | Weaver | None               | Disseminated(Lung/CNS/Vertebrae/Spleen/Liver) | None        | Biopsy                 | KTZ        | Cured   |
| 1991 | [20]       | 28/male | Taiwan    | NM | NM                 | Disseminated(Lung/Skin/Lymph node) | Arizona    | Biopsy                 | KTZ        | Cured   |
| 1995 | [21]       | 45/female | Guangdong | NM | NM                 | cornea           | None          | Biopsy                 | AMB        | Cured   |
| 1998 | [22]       | 32/male | Hong Kong | NM | NM                 | Disseminated(Lung/Skin/Lymph node/Bone) | California | Biopsy/Culture/AMF/FLU | Remission  |         |
| 1998 | [23]       | 52/male | Guangdong | NM | HIV                | Skin             | None          | Biopsy                 | NM         | NM      |
| 1999 | [24]       | 37/male | Jiangsu   | prisoner | Drug abuse      | Disseminated(Lung/CNS/Spleen) | None        | Biopsy                 | NM         | Died    |
| 2001 | [25]       | 44/female | Hubei | Worker | NM | Lung             | None          | Biopsy                 | Surgery     | NM      |
| 2002 | [26]       | 49/female | Zhejiang | Farmer | None               | Bone             | None          | Biopsy                 | NM         | NM      |
| 2008 | [27]       | 5/male | Hunan    | NM | None               | Disseminated(Lung/CNS/Spleen/Skin) | NM          | Biopsy                 | None       | Died    |
| 2008 | [28]       | 38/male | Shandong | NM | None               | Skin             | USA           | Biopsy                 | Surgery     | NM      |
| 2008 | [29]       | 24/male | Hubei    | Student | None               | Lung             | NM            | Biopsy                 | AMB        | Remission |
| 2009 | [30]       | 75/male | Zhejiang | NM | None               | Lung             | Arizona       | Biopsy                 | Surgery     | NM      |
| 2009 | [31]       | 33/male | Jiangsu  | Worker | None               | Disseminated(CNS/Lymph node) | NM          | Biopsy/Fluotherapy     | FLU        | Cured   |
| 2009 | [32]       | 50/male | Hong Kong | NM | Lung               | Disseminated(Lung/Ribs/Vertebrae) | California | Biopsy                 | Culture     | FL/AMB   |
| 2010 | Mean 43     |          | Fujian | NM | None               | Lung             | None          | Biopsy                 | AMB/FLU/ITR | Remission (7) |
| 2010 | [33]       |          | Male/female | 7(7) | HIV(1)       | Lung             | None          | Biopsy                 | AMB        | Cured   |
| 2010 | [34]       | 74/male | Jiangsu | NM | Hypertension       | Lung             | None          | Biopsy                 | FLU + VRC   | Cured   |
| 2010 | [35]       | 14/male | Hubei   | NM | None               | Lung             | NM            | Biopsy                 | FLU/AMB     | Cured   |
| 2010 | [36]       | 58/female | NM | NM | NM | Lung             | NM            | Biopsy                 | FLU        | Remission |
| 2010 | [37]       | 81/male | Zhejiang| Farmer | Chronic bronchitis | Lung             | None          | Biopsy                 | FLU        | Remission |
| 2011 | [38]       | 71/male | Zhejiang| NM | Hypertension/Coronary heart disease | Lung             | Arizona       | Biopsy                 | Surgery     | Cured   |
| 2011 | [39]       | 43/male | Hong Kong| Driver | NM | Lung             | None          | Biopsy/Fluotherapy     | FLU        | Cured   |
| 2016 | [40]       | 24/female | Beijing | Student | None | Disseminated(Lung/Lymph node) | USA          | Biopsy                 | Antifungal  | Remission |
| 2016 | [41]       | 51/female | Guangdong | NM | NM | Stomach         | None          | Biopsy                 | FLU        | Remission |
| 2016 | [42]       | 49/female | Shandong | NM | Nephrotic Syndrome | Lung             | None          | Biopsy                 | Surgery     | Cured   |
| 2016 | [43]       | 61/male | Zhejiang | NM | Hypertension      | Lung             | Arizona       | Biopsy                 | FLU        | Remission |
| 2017 | [44]       | 24/female | Beijing | NM | None               | Disseminated(Lung/Lymph node/Bone) | USA          | Biopsy                 | AMB/ITC/DEX | Remission |
| 2017 | [45]       | 27/male | Jiangsu | NM | NM | Disseminated(Lung/Lymph node/Bone/CNS) | Arizona     | Biopsy/Culture          | IT2/FLU    | Remission |
| 2017 | [46]       | 28/male | Fujian   | pilot | None               | Disseminated(Lung/Lymph node/Bone/CNS/Vertebrae) | USA          | Biopsy/Culture          | IT2        | Remission |
| 2018 | [47]       | 29/male | Jiangsu | Student | None | Lung             | Arizona       | Culture/PCR            | VOR        | Remission |
| 2018 | [48]       | 55/male | Guangdong | NM | NM | Lung             | California    | Biopsy                 | AMB/ITC     | Cured   |
| 2018 | [49]       | 22/female | Jiangsu | Student | None | Lung             | California    | Biopsy                 | FLU        | Cured   |
2019, showing the increased incidence of coccidioidomycosis in China. In endemic areas, individuals with AIDS, diabetes mellitus, and those who have received organ transplantation are believed to be high-risk groups [1]. Farmers and construction workers who are in frequent contact with soil also exhibit a high incidence rate [1]. However, 26 patients (55.3%) had no underlying diseases among 47 Chinese patients. Furthermore, disseminated infection mainly occurred in previously healthy young individuals in China, unlike those who are more likely occurrence in immunosuppressed patients reported in endemic areas, and the high incidence rate was 53.4% during the past 5 years [1, 3]. Therefore, more epidemiological studies on the risk factors and underlying diseases for coccidioidomycosis need to be further investigated in China. Additionally, 22 (46.8%) patients have no history of exposure to endemic areas or contact with patients or individuals returned from the endemic regions, suggesting that native Coccidioides may exist in China.

Among the 47 patients, the most common clinical features were cough and fever, which were nonspecific and consistent with those reported in endemic areas. A majority of domestic cases were initially misdiagnosed as tumours or tuberculosis. Therefore, more attention should be paid to the precise diagnosis of coccidioidomycosis. The pathogenic species were only identified in 4 patients using a combination of culture and subsequent molecular diagnostic techniques such as PCR, NGS, or MALDI-TOF MS. In our patient, Coccidioides spp. was identified in FFPE tissue by LCM combined with nested PCR and Real-time PCR based on histopathology. Currently, the LCM technique can isolate precise cell populations or microorganisms from complex clinical FFPE samples, avoiding the cross-contamination of surrounding heterogeneous tissues in the specimen [6]. This method and subsequent molecular techniques have been used to isolate pure tumour tissues and

Table 1. Continued.

| Year | Reference | Age (Sex) | Provinces | Job | Underlying disease | Year | Reference | Age (Sex) | Provinces | Job | Underlying disease | Site of infection | Treatment | Method of identification | Outcome | Travel history | Site of infection | Underlying disease | Travel history | Site of infection | Underlying disease | Method of identification | Outcome |
|------|-----------|-----------|------------|-----|-------------------|------|-----------|-----------|------------|-----|-------------------|-----------------|-----------|-----------------------|---------|---------------|------------------|----------------|---------------|------------------|----------------|----------------|------------------|-----------------|-----------|
| 2019 | [50]      | 61/male   | Shanghai   | NM   | None              |       | 2019      | 61/male   | Shanghai   | NM   | None              | Disseminated(Lung/Lymph node) | USA       | Culture/MALDI-TOF MS | Remission | FLU            | None             | Travel history   | Site of infection | Underlying disease | Method of identification | Outcome |
| 2019 | [51]      | 55/male   | Zhejiang   | University Professor | None | 2019      | 55/male   | Zhejiang   | University Professor | None | None              | Disseminated(Lung/Lymph node) | Arizona | Culture/MALDI-TOF MS | Remission | VOR            | FLU+NGS          | Travel history   | Site of infection | Underlying disease | Method of identification | Outcome |
| 2019 | [52]      | 24/male   | Guangxi    | Student | None | 2019      | 24/male   | Guangxi    | Student | None | None              | Disseminated(Lung/Lymph node) | Los Angeles | Culture/MALDI-TOF MS | Remission | FLU+NGS          | VOR/ITC         | Travel history   | Site of infection | Underlying disease | Method of identification | Outcome |
| 2019 | [53]      | 63/male   | Hebei      | Miner | Asthma           | 2019  | 63/male   | Hebei      | Miner | Asthma           | Disseminated(Lung/Lymph node) | US       | Culture/MALDI-TOF MS | Remission | DEX            | CNS               | Travel history   | Site of infection | Underlying disease | Method of identification | Outcome |

Table 2. Summary of diagnostic methods of 48 cases of coccidioidomycosis.

| Diagnostic methods | Number | Fungal species |
|--------------------|--------|----------------|
| Histopathology alone | 33 | Coccidioides spp. |
| Fungal culture alone | 14 | Coccidioides spp. |
| Histopathology + Fungal culture | 1 | Coccidioides spp. |
| Histopathology + Serology | 2 | Coccidioides spp. |
| Fungal culture + PCR | 1 | C. posadasii |
| Fungal culture + MALDI-TOF MS + NGS | 1 | C. posadasii |
| Fungal culture + MALDI-TOF MS | 1 | C. immitis |
| Histopathology + Fungal culture + NGS | 1 | C. posadasii |
| Histopathology + LCM + PCR sequencing (Here) | 1 | Coccidioides spp. |

MALDI-TOF MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry; NGS: next-generation sequencing; LCM: laser capture microdissection.
detect the expression of target genes, which was initially applied for analyzing cancers [6]. In a previous study, Trichoderma DNA in FFPE specimens was detected using the combined LCM and PCR technique, resulting in the clinical diagnosis of rare Trichoderma infections [8]. Whereas the formalin fixation and paraffin embedding process can cause damage to nucleic acids such as DNA fragmentation, degradation, and chemical modification of the bases [59]. Therefore, it is important to improve DNA extraction yields. Nested PCR and Real-time PCR techniques have been proven to increase the sensitivity and specificity of detecting microorganism nucleic acids in FFPE tissues. For example, Schofield et al. [59] reported that the sensitivity of nested PCR was 1,000 times higher than that of conventional PCR in the detection of Candida albicans, and Binnicker et al. [13] reported that the specificity of Real-time PCR was 100% in the detection of Coccidioides spp. in FFPE tissues. Therefore, we used a combination of LCM, PCR and Real-time PCR to detect and identify Coccidioides spp. in the limited number of FFPE samples, which improved the specificity and sensitivity of detection comparing with the conventional method of DNA extraction from FFPE tissues.

Since C. posadasii had not been split from C. immitis until 2002 [60], the C. immitis-specific primers described in 2000 [11] were in fact able to amplify both C. immitis and C. posadasii DNA. So, the result of the nested PCR that was being performed with the primers according to this literature [11] just suggested the presence of Coccidioides spp. in FFPE tissue. We hence speculated that the pathogen may be C. immitis, or C. posadasii, or the mixture of these two species. In addition, a Real-time PCR assay using specific TaqMan probes labelled with VIC and FAM fluorescence was reported to identify and distinguish the clinical isolates of C. immitis and C. posadasii by Castañón-Olivares et al. in 2007 [14]. However, this method has not been used to detect Coccidioides spp. from FFPE tissue, yet. And because of lacking the strains stock of Coccidioides spp. in our laboratory, we were unable to evaluate the specificity and sensitivity of this Real-time PCR method in detecting C. immitis and C. posadasii in this study. Therefore, we detected Coccidioides spp. directly by performing the Real-time PCR assay as described previously [14] with the very limited amount of DNA that being extracted from the FFPE tissue. And the result showed there were two amplification curves, indicating the two species of C. immitis and C. posadasii may be existed in FFPE tissue. Taken together, the diagnosis of coccidioidomycosis for this patient has been confirmed by histopathological examination combined with the detection of Coccidioides spp. in FFPE tissue with further LCM and PCR sequencing strategies.

In summary, we identified a case of imported coccidioidomycosis and analyzed all 47 cases of coccidioidomycosis reported in China since 1958. The incidence rates of coccidioidomycosis in China have been increasing in recent years. In 47 reported cases, Coccidioides spp. was unable to be identified in the clinical specimens that unable to be cultured or culture negative. This patient described here is the first study to identify Coccidioides spp. using LCM in combination with PCR sequencing strategies based on histopathological examination, which have been proven being with good safety and sensitivity in clinical application. We believe that this method will be used for the identification of Coccidioides spp. directly in FFPE tissues, allowing for the timely diagnosis of coccidioidomycosis and the appropriate initiation of antifungal therapy.

Acknowledgments

We are grateful to the patient for participation in this study. Contribution: Xinyu Yang and Yinggai Song mainly completed the molecular experiments and analyzed the clinical cases. Wei Liu and Yinggai Song designed all the experiments of the study. Xinyu Yang, Yinggai Song, TianYu Liang, QiQi Wang, Ruoyu Li, and Wei Liu contributed to writing and reviewing the manuscript. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by National Natural Science Foundation of China (grant number 81861148028, 81971912, 81671990) and National Science and Technology Major Project (grant number 2017ZX09304028009, 2018ZX10712001).

References

[1] Stockamp NW. Thompson GR 3rd. coccidioidomycosis. Infect Dis Clin North Am. 2016;30(1):229–246.
[2] Liang G, Shen Y, Lv G, et al. Coccidioidomycosis: imported and possible domestic cases in China: A case report and review, 1958-2017. Mycoses. 2018;61(7):506–513.
[3] Malo J, Luraschi-Monjagatta C, Wolk DM, et al. Update on the diagnosis of pulmonary coccidioidomycosis. Ann Am Thorac Soc. 2014;11(2):243–253.
[4] Canteros CE, Vélez HA, Toranzo AI, et al. Molecular identification of Coccidioides immitis in formalin-fixed, paraffin-embedded (FFPE) tissues from a Colombian patient. Med Mycol. 2015;53(5):520–527.
[5] Sheff KW, York ER, Driebe EM, et al. Development of a rapid, cost-effective TaqMan real-time PCR assay for identification and differentiation of Coccidioides immitis and Coccidioides posadasii. Med Mycol. 2010;48(3):466–469.
[6] Zhou YB, Zhang GJ, Song YG, et al. Application of laser capture microdissection and polymerase chain reaction in the diagnosis of Trichoderma longibrachium infection: a promising diagnostic tool for ‘fungal contaminants’ infection. Med Mycol. 2020;58 (3):315–321.

[7] Selva E, Hofman V, Berto F, et al. The value of polymerase chain reaction detection of Mycobacterium tuberculosis in granulomas isolated by laser capture microdissection. Pathology. 2004;36(1):77–81.

[8] Kaserer K, Knezevic V, Pichlhöfer B, et al. Construction of cDNA libraries from microdissected benign and malignant thyroid tissue. Lab Invest. 2002;82(12):1707–1714.

[9] Espina V, Heiby M, Pierobon M, et al. Laser capture microdissection technology. Expert Rev Mol Diagn. 2007;7(5):647–657.

[10] Korabecná M, Liska V, Fajfrlík K. Primers ITS1, ITS2 and ITS4 detect the intraspecies variability in the 28S rRNA gene region in clinical isolates of fungi. Folia Microbiol. 2003;48(2):233–238.

[11] Greene DR, Koenig G, Fisher MC, et al. Soil isolation and molecular identification of Coccidioides immitis. Mycologia. 2000;92(3):406–410.

[12] O’Shaughnessy JB, Chan M, Clark K, et al. Primer design for automated DNA sequencing in a core facility. Biotechniques. 2003;35(1):112–121.

[13] Binnicker MJ, Buckwalter SP, Eisberner JJ, et al. Detection of Coccidioides species in clinical specimens by real-time PCR. J Clin Microbiol. 2007;45(1):173–178.

[14] Castañón-Olivares LR, Güereña-Elizalde D, González-Martínez MR, et al. Molecular identification of Coccidioides isolates from Mexican patients. Ann N Y Acad Sci. 2007;1111:326–335.

[15] Arya M, Shergill IS, Williamson M, et al. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn. 2005;5(2):209–219.

[16] Gan D, Guo ML, He YG. First case of primary cutaneous coccidioidomycosis. Chin J Pathol. 1958;4 (2):107–114. (in Chinese).

[17] Wu XC, Xu AR. Coccidioidomycosis (report of two cases). J Gannan Med Coll. 1985;2:34. (in Chinese).

[18] Li YP, Yi YZ, Feng GG, et al. One case report of coccidioidomycosis. Chin J Ophthalmol. 1995;31(5):362. (in Chinese).

[19] Koehler AP, Cheng AF, Chu KC, et al. Successful treatment of disseminated coccidioidomycosis with amphotericin B lipid complex. J Infect. 1998;36 (1):113–115.

[20] Buller A, Ly JJ, Liu P, et al. One case of skin coccidioidomycosis complicating with acute meningitis. J Forensic Med. 1998;15(2):114–115. (in Chinese).

[21] Ceng XJ, Hu RC, Ren JL. One case of lung coccidioidomycosis misdiagnosed as lung cancer. Med J. 2002;12(2):112. (in Chinese).

[22] Mo YH, Yu YY, Chen PH, et al. One case of primary bone coccidioidomycosis. Chin J Pathol. 2003;32 (2):182. (in Chinese).

[23] Ouyang WX, You JY, Zhu YM, et al. A case of infantile coccidioidomycosis. Chin J Pediatr. 2008;46(12):934–935. (in Chinese).

[24] Gao W, Zhang SL, Zhang XM, et al. Case report of a primary cutaneous coccidioidomycosis and literature review. Pract Med. 2008;24(7):1262–1263. (in Chinese).

[25] Liu GX. A case of coccidioidomycosis. Pract Med. 2008;24(11):1870. (in Chinese).

[26] Xu QY, Wang YH, Jin M, et al. A case of pulmonary coccidioidomycosis. Chin Med J. 2009;89(28):1998. (in Chinese).

[27] Li YP, Yi YZ, Feng GG, et al. One case report of coccidioidomycosis. Chin J Pathol. 2001;11(3):740. (in Chinese).

[28] Kwok HK, Chan JW, Li IW, et al. Coccidioidomycosis as a rare cause of pneumonia in non-endemic areas: a short exposure history should not be ignored. Respir Med. 2009;14(4):617–620.

[29] Coccidioides immitis. Chin J Tuberc Respir. 2010;33(4):225–226. (in Chinese).

[30] Wang CB, Zhao Y, Nan PF. A case of pulmonary coccidioidomycosis. Chin J Pathol. 2010;33(3):227–228. (in Chinese).

[31] Wang ZY, Wen SL, Ying KJ. A study case of imported pulmonary coccidioidomycosis. J Zhejiang Univ Sci B. 2011;12(4):298–302.

[32] Tan TH, Tsang OT. Images in clinical medicine. Fungal infection from sweeping in the wrong place. N Engl J Med. 2011;364(2):e3.

[33] Yang D, Ji HY, Lin MG. A case of coccidioidomycosis misdiagnosed as lymph node tuberculosis. Chin J Antitubere. 2016;38(3):235–237. (in Chinese).

[34] Liao Y, Bai L. A case of primary gastric coccidioidomycosis. Chin J Dig. 2016;36(3):204–205. (in Chinese).

[35] Wang CB, Zhao Y, Nan PF. A case of pulmonary coccidioidomycosis. J Diag Pathol. 2016;23(12):968–971. (in Chinese).

[36] Gu YC. Pulmonary coccidioidomycosis: one case report and literature review [master’s thesis]. Hangzhou (N): Zhejiang University; 2016.

[37] Pan H, Liang RY, Yu Y, et al. A case of disseminated coccidioidomycosis. Chin J Infect Dis. 2017;35 (7):434–436. (in Chinese).

[38] Zhang S, Wang RY, Zhang HZ, et al. A case of disseminated coccidioidomycosis. Chin J Infect Dis. 2017;35(6):374. (in Chinese).

[39] Gao J, Zhao XJ, Dai YT, et al. A case of pilot disseminated coccidioidomycosis. J Civil Aviat Med. 2017;27 (1):42–43. (in Chinese).
[47] Wang RJ, Wang XW, Shibuya K, et al. Image gallery: cervical lymphadenitis as an unusual presentation of disseminated coccidioidomycosis. Br J Dermatol. 2018;178(6):e394.

[48] Zhou W, Xiao W, Xue LJ, et al. A case of misdiagnosis and mistreatment of pulmonary coccidioidomycosis. Chin J Antiuber. 2018;40(11):1240–1243. (in Chinese).

[49] Ma Y, Chen YC, Zhao X, et al. A case of primary pulmonary coccidioidomycosis and literature review. Int J Respir. 2018;38(24):1863–1867. (in Chinese).

[50] Wang MR, Jin WT, Zhang X, et al. Clinical analysis and treatment of imported coccidioidal lung infection. Chin J Nosocomiol. 2019;29(13):1975–1979, 2068 (in Chinese).

[51] Shen Q, Zheng J, Zhou JY. The 468th case of fever-lung effusion associated with lymph node enlargement - blood acidophilic granulocyte and elevated IgE. Natl Med J China. 2019;99(24):1917–1920. (in Chinese).

[52] Liang H, Lin ZM, Liu YY, et al. A case of non-immune deficient disseminated coccidioidomycosis. Chin J Infect Dis. 2019;37(9):566–569. (in Chinese).

[53] Luna-Isaac JA, Muñiz-Salazar R, Baptista-Rosas RC, et al. Genetic analysis of the endemic fungal pathogens Coccidioides posadasi and Coccidioides immitis in Mexico. Med Mycol. 2014;52(2):156–166.

[54] Zhang CJ, Chen FY, Liu XY, et al. Gliotoxin induces cofilin phosphorylation to promote actin cytoskeleton dynamics and internalization of Aspergillus fumigatus into Type II human pneumocyte cells. Front Microbiol. 2019;10:1345.

[55] Han X, Yu R, Zhen D, et al. β-1,3-Glucan-induced host phospholipase D activation is involved in Aspergillus fumigatus internalization into type II human pneumocyte A549 cells. PLoS One. 2011;6(7):e21468.

[56] Jia X, Chen F, Pan W, et al. Gliotoxin promotes Aspergillus fumigatus internalization into type II human pneumocyte A549 cells by inducing host phospholipase D activation. Microbes Infect. 2014;16 (6):491–501.

[57] Rick EM, Woolnough K, Pashley CH, et al. Allergic fungal airway disease. J Investig Allergol Clin Immunol. 2016;26(6):344–354.

[58] Richardson M, Bowyer P, Sabino R. The human lung and Aspergillus: you are what you breathe in? Med Mycol. 2019;57(Supplement_2):S145–S154.

[59] Schofield DA, Westwater C, Paulling EE, et al. Detection of Candida albicans mRNA from formalin-fixed, paraffin-embedded mouse tissues by nested reverse transcription-PCR. J Clin Microbiol. 2003;41(2):831–834.

[60] Fisher MC, Koenig GL, White TJ, et al. Molecular and phenotypic description of Coccidioides posadasi sp. nov. previously recognized as the non-California population of Coccidioides immitis. Mycologia. 2002;94(1):73–84.