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*Original Article*

# Clinical features of patients presenting with fever of unknown origin caused by non-tuberculous mycobacterium infection

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## Abstract

Introduction: Although non-tuberculous mycobacterium (NTM) infection accounts for only a small proportion of fever of unknown origin (FUO) cases, it has become a more common etiology in recent years. Therefore, we reviewed FUO patients with underlying NTM infection to better understand its clinical features.

Methodology: The medical records of patients presenting with FUO and diagnosed with NTM infection admitted to Peking Union Medical College Hospital between January 2016 and June 2021 were reviewed. The clinical information of patients whose follow-up data were available were summarized. Specimens submitted for pathogenic identification were processed by mycobacterial culture, acid-fast staining, and mycobacterial nucleic acid detection. IBM SPSS Statistics v22.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis.

Results: Fifty-five FUO patients were diagnosed with NTM infection (55/785; 7.0% of FUO cases). Patients were mostly middle-aged men and had a relatively long disease course. Seven, 29, and 54 patients had previously no respondence to glucocorticoids, immunosuppressants, and multiple antibiotics, respectively; their inflammatory indexes were significantly increased; and there was no obvious risk of immunosuppression in this group, who were likely to be T.SPOT-TB negative (33/41; 80.5%). The most commonly identified NTM was *Mycobacterium intracellulare* followed by *Mycobacterium chelonae/abscessus*, *Mycobacterium kansasii*, and *Mycobacterium avium*.

Conclusions: Microbiological investigations including culture, acid-fast staining, NTM nucleic acid examination, and next-generation sequencing were performed to confirm the diagnosis of NTM in FUO patients. FUO patients should screen for NTM infections so that this important etiology can be recognized, targeted treatments administered early, and outcomes improved.

**Key words:** Fever of unknown origin; non-tuberculous mycobacterium infection; diagnosis; clinical characteristics.

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## Introduction

As early as 1907, Cabot reported that patients with persistent fever had unique differential diagnoses [1]. In 1961, Petersdorf and Beeson refined the concept of this phenomenon, proposing the now widely accepted definition of fever of unknown origin (FUO): a temperature of 38.3 °C or higher for at least three weeks without a diagnosis despite one week of inpatient investigations [2]. FUO is not a single disease but describes a group of diseases with numerous etiologies. Although more patients with FUO have been diagnosed with autoimmune or inflammatory diseases in recent years [3], infection remains the leading cause of FUO in low- and middle-income countries [4], accounting for up to 40% of FUO cases [5].

As a group of environmental saprophytes and opportunistic pathogens, nontuberculous mycobacteria (NTM) infections account for a small but uncertain proportion of total FUO cases. However, since patients with NTM often share the same clinical manifestations as those with tuberculosis (TB), NTM infection may often be misdiagnosed as multi-drug resistant *Mycobacterium tuberculosis* because the drugs administered appear to lack efficacy. Moreover, as the treatment duration for NTM may be much longer than that for TB and the therapeutic options are quite different, a misdiagnosis could seriously affect the prognosis [6]. It is therefore necessary to explore the clinical characteristics of patients infected with NTM and FUO to highlight the most important diagnostic features to guide therapeutic decision-making and to

ensure that this important diagnosis is not delayed or missed.

## Methodology

*Study design and participants*

This was a retrospective study of patients admitted to the Peking Union Medical College Hospital (PUMCH), a 2000-bed university-affiliated tertiary hospital in Beijing, China. PUMCH is a national tertiary referral center for the diagnosis and treatment of difficult and serious diseases. To comprehensively investigate the clinical features of patients admitted with FUO caused by NTM infections, we found in total 785 patients were admitted to PUMCH between January 2016 and June 2021 with the diagnosis of FUO, which met the diagnostic criteria of FUO established in 1961 [2], and among them, 173 patients were diagnosed with NTM infection. To improve our understanding of the features of FUO caused by NTM infection in patients without human immunodeficiency virus (HIV) infection and to benefit future clinical practice, two experienced physicians reviewed all 173 medical records of NTM infected patients and then focused on 55 of them, the other 118 patients were excluded due to lack of specific pathogenic information (74 patients), not meeting criteria for NTM infection (43 patients) [7], and concurrent HIV infection (1 patient).

*Data collection*

The medical records of the included patients were reviewed to extract (i) demographic data; (ii) past medical history, medication history, and contact history; (iii) laboratory findings; and (iv) therapies administered prior to admission and following diagnosis. All data were collected and summarized using a standard form. The PUMCH Ethics Committee approved the study.

*Pathogen detection and identification*

The specimens submitted for pathogenic detection in this study included sputum, peripheral blood, bronchoalveolar lavage fluid (BALF), pus, urine, wound secretions, pleural effusion, ascites, subcutaneous nodules, skin tissue, lymph node biopsy, subcutaneous tissue, and bone marrow. Pathogen diagnosis and identification were carried out in one of three ways.

(1) Mycobacterial culture: The fully automatic BACTEC MGIT 960 mycobacterial culture system (BD, Franklin Lakes, NJ) was used to culture mycobacteria. If the specimens contained mycobacteria, the result was usually reported within 2-4 weeks. For culture-positive specimens, smear microscopy was performed to confirm the positive results and real-time PCR was used to differentiate TB from NTM. If there was no positive result after 42 days of culture, the result was reported as negative.

(2) Acid-fast staining: Specimens were first smeared and then stained with concentrated petrolatum compound red under heating, decolorized with hydrochloric acid alcohol, and then counterstained with methylene blue solution. Acid-fast mycobacteria stained red. Although a preliminary distinction between TB or NTM may sometimes be made through morphological analysis with microscopy by an experienced observer, it is not a reliable method to make final diagnosis, and further molecular biological examinations are still needed to confirm the diagnosis.

(3) Mycobacterial nucleic acid detection and NTM strain identification: First, mycobacterial nucleic acids were detected through a combination of dual polymerase chain reaction (PCR) and TaqMan probe technology. Nucleic acid detection kits were obtained from BioCrystal Biotechnology Co, Ltd. (Chengdu, China). Primers and probes were designed to detect distinct *Mycobacterium tuberculosis* complex and NTM sequences, and the probes were labeled with different fluorescent markers.

DNA microarrays were then used for specimens positive for NTM to obtain specific strain identification. Specific PCR amplification primers and oligonucleotide fragment probes were first designed for 17 species or groups of common *Mycobacterium* species. The ends of each primer were labeled with fluorescent probes, and the DNA hybridizing to primers containing a specific fluorescent marker were amplified and the DNA amplification products subsequently allowed to hybridize to the corresponding microarray probe. In this way, the sequence-matched PCR amplification product formed a stable secondary structure with the specific probe and the fluorescent signal was used to identify the NTM.

*T-SPOT-TB assays*

The T.SPOT-TB test is an interferon (IFN)-γ release assay that detects IFN-γ secreted from *M. tuberculosis*-specific T cells stimulated by *Mycobacterium*-specific antigens. Four milliliters of peripheral blood were obtained from each patient. T-SPOT-TB (Oxford Immunotec, Abingdon, UK) was used to detect specific T cell responses to region of difference 1 (RD1) encoded antigens, and tests were performed within 6 hours of sample collection by laboratory personnel blinded to patients' clinical data.

AIM-V (GIBCO™ AIM V Medium liquid, Invitrogen, Carlsbad, CA) was used as a negative control, PHA as positive control, and ESAT-6 and CFP-10 as specific antigens.

Briefly, each subject's peripheral blood mononuclear cells (PBMCs) were collected and plated ($2.5\times10^5$ cells per well) on a plate pre-treated with an anti-interferon antibody. Plates were incubated for 16-18 hours at 37 °C in 5% $CO_2$. Cells were incubated in wells containing an enzyme substrate and a conjugate directed against the employed antibody. Using an automated ELISpot scanner (AID-iSpot, Strassberg, Germany), spot-forming cells (SFCs) were enumerated. Each SFC signified an antigen-specific T cell secreting interferon. When the antigen well had at least six spots and twice as many spots as the negative control well, the reaction was deemed positive. For negative controls, less than 10 spots should make up the majority of the spots in the negative control well.

*Statistical analysis*

IBM SPSS Statistics v22.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Descriptive statistics were used to characterize the study population. For continuous variables, data are presented as mean ± standard deviation or median and range, depending on the distribution. Categorical data are presented as numbers and percentages.

*Ethics approval and consent to participate*

The ethics committee of Peking Union Medical College Hospital approved the study (K2045), and all data collected were de-identified.

## Results

*Demographic characteristics and pre-admission medical conditions*

Fifty-five patients were admitted with FUO and diagnosed with NTM infection (55/785; 7.0% of FUO cases). The demographic characteristics of the 55 included patients are detailed in Table 1. Their median age was 45.7 years (range 16–74 years); only 10 patients were older than 60 years. Twenty-nine patients were male (52.7%) and 26 (47.3%) were female. Most were from the northern and inland regions as defined in [8], where the climate is relatively colder and drier than in southern and coastal areas. Only six patients were in good health before disease onset; respiratory diseases (27.3%) such as bronchiectasis or diffuse parenchymal lung diseases, autoimmune diseases (27.3%), cancer (14.5%), TB infection (10.9%), and diabetes mellitus (5.5%) were relatively common in the medical histories of affected individuals. Three patients (5.5%) were immunodeficient: one with MonoMAC syndrome and two with anti-interferon antibody syndrome.

Nearly all patients had been prescribed antibiotics prior to admission, over half had been medicated with oral or intravenous corticosteroids without effect, and seven patients had received immunosuppressants before admission. Some patients had a history of smoking tobacco and alcohol consumption. A number of patients also had specific environmental exposures prior to disease onset such as exposure to minerals, animals, tuberculosis patients, and others.

*Clinical features*

Details of the clinical characteristics of the study cohort are shown in Table 2. The median fever duration prior to admission was 14.7 (range 0.75-144) months.

**Table 1.** Basic conditions of 55 patients with NTM infection and with FUO as the predominant presentation.

| Variable | n (%) |
|---|---|
| **Age**, median (range) | 45.7 (16–74) years |
| **Gender** | |
| Male | 29 (52.7) |
| Female | 26 (47.3) |
| **Geographic areas** | |
| Northern region | 38 (69.1) |
| Southern region | 17 (30.9) |
| Inland region | 41 (74.5) |
| Coastal region | 14 (25.5) |
| **Past medical history** | |
| Autoimmune disease | 15 (27.3) |
| Respiratory diseases | 15 (27.3) |
| Oncological disease | 8 (14.5) |
| TB infection | 6 (10.9) |
| Lymph nodes | 3 (5.5) |
| Lungs | 1 (1.8) |
| Skin | 1 (1.8) |
| Tuberculosis pleurisy | 1 (1.8) |
| Surgical history | 6 (10.9) |
| Immunodeficiency diseases | 3 (5.5) |
| Diabetes mellitus | 3 (5.5) |
| Others | 14 (25.5) |
| **Past medications** | |
| Glucocorticoids | 29 (52.7) |
| Immunosuppressants | 7 (12.7) |
| Antibiotics | 54 (98.1) |
| **Exposure history** | |
| Not mentioned | 35 (63.6) |
| Smoking history | 20 (36.4) |
| Alcohol history | 17 (30.9) |
| Animals | 9 (16.3) |
| Minerals | 4 (7.3) |
| Tuberculosis patients | 4 (7.3) |
| Others | 3 (5.5) |

NTM: non-tuberculous mycobacterium; FUO: fever of unknown origin; TB: tuberculosis.

More than half of patients suffered from respiratory symptoms. Other common clinical features were muscle, bone, or joint pain; palpitations; edema; lymphadenectasis; fatigue and night sweats; neurological symptoms; rash; and black stools. Twenty-seven patients had multi-system involvement. The respiratory system was most commonly involved followed by the lymph nodes, bones, blood, skin, and soft tissues.

Fifty-three patients received a macrolide-based antibiotic regimen, except for two patients with *Mycobacterium kansasii* infection. Over half of these patients received ethambutol and rifamycin-based drugs. Nearly half of the patients were treated with quinolones and amikacin. Other commonly used drugs included linezolid, sulfamethoxazole, isoniazid, minocycline, cephamycin, and piperacillin-tazobactam (Table 2).

**Table 2.** Clinical characteristics of 55 patients with NTM infection and with FUO as the predominant presentation.

| Variables | n (%) |
|---|---|
| **Duration of fever prior to admission (months)**, median (range) | 14.7 (0.75–144) days |
| **Accompanying symptoms** | |
| Cough | 35 (63.6) |
| Muscle bone or joint pain | 15 (27.3) |
| Lymphadenectasis | 10 (18.2) |
| Rash | 7 (12.7) |
| Hemoptysis | 7 (12.7) |
| Edema | 6 (10.9) |
| Fatigue and night sweats | 6 (10.9) |
| CNS symptoms | 5 (9.1) |
| Palpitation | 2 (3.6) |
| Black stool | 2 (3.6) |
| **Affected site** | |
| Lungs | 45 (81.8) |
| Lymph nodes | 15 (27.3) |
| Bones | 14 (25.5) |
| Blood stream | 7 (12.7) |
| Skin and soft tissue | 7 (12.7) |
| Hematopoietic system | 2 (3.6) |
| Cardiovascular system | 2 (3.6) |
| CNS | 2 (3.6) |
| Liver and spleen | 1 (1.8) |
| Kidney | 1 (1.8) |
| Digestive tract | 1 (1.8) |
| **Therapy** | |
| Macrolides | 53 (96.4) |
| Ethambutol | 40 (72.7) |
| Rifamycin | 30 (54.5) |
| Quinolones | 26 (47.2) |
| Amikacin | 23 (41.8) |
| Isoniazid | 11 (20.0) |
| Linezolid | 7 (12.7) |
| Minocycline | 6 (10.9) |
| Cephamycin | 4 (7.2) |
| Piperacillin-tazobactam | 4 (7.2) |
| Sulfamethoxazole | 3 (5.5) |
| Others | 8 (14.5) |
| **Prognosis** | |
| Recovered | 38 (69.0) |
| Relapsed | 11(20.0) |
| Died | 3 (5.5) |
| Lost to follow-up | 3 (5.5) |

NTM: non-tuberculous mycobacterium; FUO: fever of unknown origin; CNS: Central nervous system.

Three patients were not followed-up after discharge, but the remaining 52 patients were followed up for at least six months, most of whom recovered. Eleven patients were judged to have relapsed due to the reappearance of symptoms similar to those of the previous visit or a lack of relief of the previous symptoms. Three patients died due to progressive infection. Of the 14 patients who did not recover (relapsed or died), 13 had underlying diseases and over half (8/14, 57.1%) had multi-system involvement.

*Laboratory findings*

The average white blood cell count of affected patients was slightly elevated, predominantly with neutrophils. Over half of patients (33/55, 60.0%) had anemia, and the average hemoglobin concentration was 102.3 ± 31.1 g/L (normal range 110-150 g/L). Except for 34 patients suffering from hypoproteinemia, there were no significant biochemical abnormalities in these patients.

Forty-one patients had done T.SPOT-TB tests. Eight patients were T.SPOT-TB positive, three of whom had *Mycobacterium kansasii* infection; two patients had *Mycobacterium intracellulare* infection, and the other three patients were infected with *Mycobacterium fortuitum*, *Mycobacterium surga*, and *Mycobacterium abscessus*.

All 55 patients had a clear pathogenic diagnosis (Table 3). Sputum was the most common specimen, and mycobacterial culture had the highest diagnostic yield for all types of specimens, often producing a diagnosis in a third to a half of cases. The diagnostic yield for acid-fast staining and PCR were similar. Only two patients received next-generation sequencing (NGS) of peripheral blood and cerebrospinal fluid samples, respectively, and the former was positive and the latter was negative.

Thirty-nine patients had complete immune cell profiling by flow cytometry. The average CD4 + T cell count was 576.3 ± 433.7/μL (normal range 561-1137/μL); the proportion of CD38 + CD8 + T cells was 69.9 ± 21.3% (normal range was 32.4%-57.4%); and the proportion of DR + CD8 + T cells was 60.3 ± 19.4% (normal range was 6.3%-23.8%), indicating an inflammatory state. Biochemical inflammatory

**Table 3.** Pathogenetic diagnosis conditions of patients with NTM infection and with FUO as the predominant presentation.

| Type of sample | Mycobacterial culture | | Acid-fast staining | | Mycobacterial nucleic acid detection | | NGS | |
|---|---|---|---|---|---|---|---|---|
| | Number of cases examined | Number of positive cases (%) | Number of cases examined | Number of positive cases (%) | Number of cases examined | Number of positive cases (%) | Number of cases examined | Number of positive cases (%) |
| Sputum | 102 | 60 (58.8) | 125 | 16 (12.8) | 87 | 21 (24.1) | NA | NA |
| Peripheral blood | 97 | 21 (21.6) | NA | NA | NA | NA | 1 | 1 (100) |
| Tissues or secretion | 30 | 15 (50.0) | 31 | 9 (29.0) | 23 | 2 (8.7) | NA | NA |
| Bronchoalveolar Lavage fluid | 35 | 20 (57.1) | 38 | 5 (13.2) | 32 | 9 (28.1) | NA | NA |
| Others | 27 | 10 (37.0) | 33 | 4 (12.1) | 26 | 7 (26.9) | 1 | 0 (0) |

NTM: non-tuberculous mycobacterium; FUO: fever of unknown origin; NGS: next-generation sequencing.

indicators were also significantly higher than normal, with average C-reactive protein values of 89.4 ± 73.6 mg/L (normal < 8 mg/L) and mean erythrocyte sedimentation rates of 79.5 ± 42.7 mm/h (normal range 0-20 mm/h). Thirty-seven patients underwent auto-antibody testing, 15 of them had positive results. Nine of these positive results were associated with known underlying autoimmune diseases, while the remaining six patients only had very low titers of antinuclear antibodies, which were considered to be of no clinical significance since these patients had neither histories nor clinical features of autoimmune diseases.

*The spectrum of NTM species*

The identified mycobacterial species are shown in Table 4. Except for one patient who had multiple NTM infections, namely *Mycobacterium avium* complex, *Mycobacterium colombiense*, and *Mycobacterium abscessus*, all other patients were single-species infections. Of the remaining patients, slow-growing mycobacteria (SGM, with an incubation time over 1 week) were identified in 36 patients and fast-growing mycobacteria (RGM, with an incubation time less than 1 week) were identified in 18 patients. Among them, *Mycobacterium intracellulare* infection was most common (21 cases). *Mycobacterium surga*, *Mycobacterium paraseoul*, *Mycobacterium lentigines*, syncytial *Mycobacterium*, and *Mycobacterium colombiense* were detected only once.

## Discussion

Decades have passed since the first definition of FUO, but it still remains a diagnostic challenge. Little attention has been paid to the proportion of NTM infections contributing to FUO, although a previous study from our hospital recorded a prevalence of 0.67% (3/449) [9]. As NTM infections account for only a small proportion of FUO cases, NTM infections have generally been ignored in the clinic. This is an oversight, since NTM infections can represent a severe progressive illness requiring complicated treatment with several anti-mycobacterial drugs for over 12 months. Most NTMs are inherently resistant to standard anti-tuberculosis drugs, and different species exhibit varying resistance phenotypes [10]. Over the last 10 years, the incidence of NTM has gradually increased worldwide [8]. Mirroring this, about 7% of our FUO cases were caused by NTMs, although this increase may also be due to increased awareness of NTM infections or improved detection. Nevertheless, when managing patients with FUO, thorough mycobacterial identification is extremely important.

NTMs are ubiquitous environmental saprophytes and opportunistic pathogens. Previous studies have shown that the majority of NTM patients are elderly

**Table 4.** Species distribution in 55 patients with NTM infection and with FUO as the predominant presentation.

| NTM | n (%) | |
|---|---|---|
| *Mycobacterium abscessus/chelae* | 9 (16.4) | RGM (n = 18, 32.8%) |
| *Mycobacterium abscessus* | 5 (9.1) | |
| *Mycobacterium fortuitum* | 4 (7.3) | |
| *Mycobacterium intracellulare* | 21 (38.2) | SGM (n = 36, 65.4%) |
| *Mycobacterium avium* | 3 (5.5) | |
| *Mycobacterium kansasii* | 8 (14.5) | |
| *Mycobacterium surga* | 1 (1.8) | |
| *Mycobacterium paraseoul* | 1 (1.8) | |
| *Mycobacterium lentigines* | 1 (1.8) | |
| Syncytial *Mycobacterium* | 1 (1.8) | |
| Mixed infection | 1 (1.8) | |

NTM: non-tuberculous mycobacterium; FUO: fever of unknown origin; RGM: fast-growing *Mycobacteria*; SGM: slow-growing Mycobacteria.

[11]. However, in our series, less than 20% of patients were aged over 60, so NTMs cannot be regarded as a disease of old age. Furthermore, NTM infections have long been considered opportunistic infections that are more likely to occur in immunocompromised individuals [12]. However, in our study, although the majority of patients had underlying diseases and most patients had received glucocorticoids or immunosuppressants prior to admission, the average CD4+ lymphocyte count in this group of patients was 562/μL and less than ten patients had CD4+ lymphocyte counts less than 200/μL, none suffering from neutropenia. Moreover, in this group of patients, CD8-related indicators reflecting the degree of T cell activation were significantly higher than normal [13]. In patients who are immunocompetent, NTM infection must be taken into consideration as a critical differential diagnosis. Conversely, it is important to investigate whether any individual with a confirmed diagnosis of NTM has underlying immunosuppression or immunological diseases.

One quarter of our patients had underlying pulmonary disease, six had a history of underlying tuberculosis, and several had a history of TB exposure. The respiratory tract is generally regarded the primary site of NTM infection [14], so screening for NTM infection should be prioritized in patients with respiratory diseases. Similarly, when clinicians are faced with a patient with underlying TB but a change in disease trajectory and poor responses to therapy, the possibility of NTM infection should be fully considered and active screening should be carried out. Most of our patients received T.SPOT-TB tests, but only eight were positive. T.SPOT-TB is specific for *Mycobacterium tuberculosis* infection, but cross-reactivity can result in positivity with certain NTMs including *Mycobacterium kansasii*, *Mycobacterium marinum*, and *Mycobacterium surga* [15]. Due to the similar clinical presentation and absence of discriminative serological findings, when encountering a patient with suspected TB infection but negative by T.SPOT-TB testing, a high index of suspicion for NTM infection is required and efforts should be made to find evidence of NTM infection. Likewise, a positive T.SPOT-TB result in an otherwise atypical case where TB cannot be detected should prompt further investigations for NTMs.

In terms of their geographical distribution, most of our patients were from inland and northern provinces, which is different from previous studies [10]. This may be because of where our hospital is situated. Although our hospital is a national tertiary center for the diagnosis and treatment of difficult and serious diseases, most patients hospitalized are from the northern and inland provinces; so, there is likely to be some bias from these areas. NTM infections have most often been described in patients from humid and warm climates, consistent with the climate in the southern and coastal regions of China. In previous studies and in daily clinical practice it is generally assumed that NTM infection is more common in these regions, so NTM infection is often ignored in patients originating from other areas. However, common NTM pathogens are ubiquitous in the environment. Moreover, increasing population mobility and the increased use of central air-conditioning systems [16], as well as climate change, may have also contributed to the high rate of NTM infections in the inland and northern parts of the country. NTM infections must be screened for in FUO patients regardless of their geographic origin.

The average duration of fever before presentation was 14.9 months, while the duration of fever prior to admission was only 12.9 weeks in the general FUO patients previously counted in our center [17]. While this may be due to a lack of diagnostic serological indices for NTM infection and the need for specific diagnostic approaches, it is likely that clinical awareness of NTM infections is low, so patients are undergoing multiple rounds of unnecessary treatments before a diagnosis is made. There is a need to raise awareness about NTM infections so this important diagnosis is made promptly.

The majority of patients in our study were definitively diagnosed by testing sputum, bronchoscope lavage fluid, blood cultures, and tissue biopsy specimens. Culture, acid-fast staining, and nucleic acid testing for nontuberculous mycobacteria were the most commonly used tests, with the highest number of positive results obtained by culture and a small difference in the probability of positivity between acid-fast staining and nucleic acid testing, with the latter being slightly higher. Culture is reported to be more sensitive and specific for NTM infections than other diagnostic modalities, but the diagnosis often requires long periods of culture, making it difficult to meet the clinical requirements for diagnostic timelines. Although acid-fast staining is relatively quick, the mycobacteria need to be abundant to minimize false negatives and neither of these tests identify the strain. Nucleic acid detection is rapid, can be conducted on small samples, and allows for strain identification. However, in our study, the sensitivity of nucleic acid detection was unsatisfactory.

It is a pity that NGS was not widely used during the study period. Only two patients underwent NGS, and

one of them was positive. Although NGS was not widely used in this patient cohort, as our experience with NGS has grown, we have found it to be highly sensitive and particularly suited to the detection of rare pathogens, including NTM, especially in sterile body fluids and tissues. Moreover, when there is insufficient sample for all examinations, NGS may be particularly useful given the small sample volume required.

Some of our patients were positive for immune indicators. Although some of these patients had autoimmune diseases, several patients had abnormal autoantibodies that could not be explained by the underlying disease. Therefore, in patients with underlying autoimmune disease, the diagnostic work-up in the case of recurrence or symptom exacerbation should aim to identify whether the primary disease is active or a new infection has developed so that proper clinical decisions can be made. In addition, in patients with positive atypical or non-specific autoantibodies, infectious diseases, not least NTM infection, should be excluded.

In this study, the most commonly identified NTM was *Mycobacterium intracellulare* followed by *Mycobacterium chelonae/abscessus* and then *Mycobacterium kansasii* and *Mycobacterium avium*, similar to previous results from China [10,12]. Not every laboratory can perform drug sensitivity testing for NTM, so it may be difficult for clinicians to obtain accurate drug sensitivity results after strain identification. While the time taken to culture the strain can be used to determine whether the strain is an SGM or an RGM before definitive strain identification results are available, based on past experience, RGM and SGM generally have different drug sensitivities. According to this information, it is possible to rationally administer drugs with predicted higher sensitivity (and avoid resistant drugs) even in the absence of specific drug sensitivity results, thus improving the effectiveness of empirical treatment. The patients in our study were treated empirically in this manner, and the prognosis of the majority of patients was satisfactory.

Our study has some limitations. First, this was a single-center retrospective study. As our hospital is a national tertiary referral center for the diagnosis and treatment of difficult and serious diseases, the caseload may especially lead to bias in patient selection and the conclusions may not be generalized to the whole of China or beyond. Second, NGS testing was not widely used in our clinical practice during the study period. Although it is an emerging method that has been more widely used in recent years for the diagnosis of infectious diseases, its accuracy and cost-effectiveness for diagnosing NTM in patients with fever have not been well evaluated, so we cannot currently recommend NGS as the first choice for screening. Further research is now needed to confirm the value of NGS detection in the diagnosis of patients with NTM.

## Conclusions

In conclusion, FUO patients must be considered for screening for NTM infections so that targeted treatments can be administered earlier to improve outcomes. As part of the differential diagnosis of FUO patients, patients with a long fever duration; poor previous responses to glucocorticoids, immunosuppressants, and multiple antibiotics; raised inflammatory indexes; and no obvious risk of immunosuppression and a negative T.SPOT-TB test must be considered at risk of NTM infection. In these patients, investigations including culture, acid-fast staining, and NTM nucleic acid examination as well as NGS could be performed.

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## Authors' contributions

HTL and YJ designed the study; HTL, HWF and YJ collected data and analyzed the results; HTL and YJ drafted the manuscript; TZ, YW and YJ provided supervision or mentorship; all authors revised manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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