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ORIGINAL ARTICLE

Clinical usefulness of metagenomic next-generation sequencing for Talaromyces marneffei diagnosis in China: a retrospective study

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Abstract

Purpose Metagenomic next-generation sequencing (mNGS) has been widely used in the diagnosis of infectious diseases. However, studies on *Talaromyces marneffei* detection using mNGS remain scarce. Therefore, this study aimed to explore the diagnostic performance of mNGS in *T. marneffei*.

Methods Between March 2021 and June 2023, patients who were discharged with a final diagnosis of talaromycosis, or confirmed *T. marneffei* infection by mNGS, culture or pathological examination were included in the study. Culture and mNGS were performed simultaneously for all patients. Clinical data were retrieved for analysis.

Results A total of 78 patients were enrolled, with 40 in the talaromycosis group and 38 in the suspected-talaromycosis group. In the talaromycosis group, mNGS showed a higher positivity rate(40/40, 100.0%) compared to culture(34/40, 85.0%) ($P=0.111$). All patients in the suspected-talaromycosis group tested negative via culture, while mNGS yielded positive results. The *T. marneffei* reads in the talaromycosis group were significantly higher than in the suspected-talaromycosis group (4399 vs. 28, $P<0.001$). In the suspected-talaromycosis group, of the four patients with low reads who did not receive antifungal therapy, one died and one lung lesion progressed; most patients(31/34, 91.2%) recovered after receiving appropriate antifungal therapy.

Conclusion mNGS proves to be a rapid and highly sensitive method for detecting *T. marneffei*. Higher reads of *T. marneffei* correspond to a higher likelihood of infection. However, cases with low reads necessitate a comprehensive approach, integrating clinical manifestations, laboratory tests, and imaging examinations to confirm *T. marneffei* infection.

Keywords Diagnosis · Metagenomic next-generation sequencing · Talaromyces marneffei · Talaromycosis

Introduction

Talaromycosis, an invasive fungal infection caused by *Talaromyces marneffei*, prevails predominantly in tropical and subtropical regions of Asia [1]. It exhibits high endemicity in specific areas, notably in northern Thailand, Vietnam, Myanmar, Hong Kong, Taiwan, southern China, and northeastern India [2]. Talaromycosis primarily affects individuals with underlying primary or secondary immunocompromising conditions [3], with human immunodeficiency virus (HIV) being a significant risk factor [4]. Its prevalence among HIV-infected individuals ranges from 0.1 to 19.6%, with an overall pooled prevalence of 3.6%, varying across geographic regions and countries [5]. Moreover, *T. marneffei* stands as the second leading cause of HIV-associated bloodstream infections and related fatalities in Vietnam and southern China, boasting a mortality rate of up to 28% [6].

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This fungal infection can affect multiple systems, involving the respiratory tract, liver, spleen, gastrointestinal tract, skin, bone marrow, lymph nodes, and bloodstream. However, the clinical manifestations lack specificity, making laboratory assays the primary diagnostic method. Although culture serves as the gold standard for diagnosing talaromycosis, it requires up to 14 days for fungal growth and may miss up to 50% of infections, leading to delayed diagnosis and treatment initiation [1, 6]. Microscopic examination of skin lesions, lymph nodes, or bone marrow, as well as histopathological examination of tissue sections, can provide a presumptive diagnosis but are invasive, time-consuming, and impractical for patients lacking local lesions. Previous studies have highlighted a doubling in talaromycosis mortality from 24 to 50% with delayed diagnosis and reaching 100% mortality with missed diagnosis, underscoring the critical need for rapid and accurate detection methods [7].

Metagenomic next-generation sequencing (mNGS) represents an innovative genetic testing method proven effective in diagnosing various infectious diseases clinically [8–10]. However, studies on *T. marneffei* detection using mNGS remain scarce [11, 12]. Hence, this retrospective study aims to assess diagnostic performance of mNGS in identifying *T. marneffei* compared to conventional fungal culture methods.

Materials and methods

Study design and participants

This study conducted a retrospective analysis of patients admitted to the First Affiliated Hospital, School of Medicine, Zhejiang University, between March 2021 and June 2023. Inclusion criteria encompassed patients discharged with a conclusive diagnosis of talaromycosis or confirmed *T. marneffei* infection via mNGS, culture, or pathological examination. Additionally, patients underwent routine microbiological examination and simultaneous mNGS. Exclusion criteria comprised patients with incomplete clinical data or samples not concurrently subjected to culture or mNGS. Subsequently, enrolled patients were segregated into the talaromycosis group and suspected-talaromycosis group. The talaromycosis group comprised patients with positive *T. marneffei* results via culture or pathological examination, while those exhibiting solely positive mNGS results were placed in the suspected-talaromycosis group.

Data extraction involved the retrieval of information from the hospital's electronic patient record system. Parameters such as demographic characteristics (age and gender), comorbidities, laboratory findings (inclusive of white blood cell count, C-reactive protein, CD4 + T cell count, mNGS, and culture results), history of intensive care unit treatment, and prognosis were collected for analysis.

Workflow of mNGS

The mNGS assay was conducted within our clinical laboratory. For blood samples, we utilized a microbial cell-free nucleic acid detection scheme (plasma cfNA mNGS testing) [13]. Other samples like sputum, bronchoalveolar lavage fluid (BALF) and tissue were mainly processed for nucleic acids or genomes released after microbial cell lysis. The procedures for all schemes encompassed nucleic acid extraction, library construction, sequencing, bioinformatics analysis, and result interpretation. Detailed experimental parameters and quality controls for each process are extensively described in our previously published studies [13–16]. Specifically, in the nucleic acid extraction step, we primarily relied on the QIAamp® nucleic acid extraction kits, extracting free nucleic acids from 200 µL plasma and microbial genomic nucleic acids from 300 µL of other sample types. The library construction employed the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA). The pooled library was sequenced on the Illumina Nextseq CN500 sequencer for 75 bp or 50 cycles of single-end sequencing (SE-75 or SE-50), producing roughly 20 million reads per sample. After removal of low-quality reads from the sequencing data, human sequences were filtered out using a defined bioinformatics pipeline, followed by alignment to a dedicated microbial database for species identification. Regarding result interpretation, similar logic and rules were applied across all sample types [13]. For *T. marneffei*, if the detected specific non-repeating reads were greater than 5, they were reported as detected; if fewer than 5, repeat testing was required. If the repeat testing positive for *T. marneffei* as well, it was also reported as detected. While if the retesting resulted in negativity, a determination would be made based on the patient's medical history and the clinical judgment of the physician. The reported unit for detected pathogens is SMRN (the number of reads per pathogen at a sequencing depth of 20 million reads) [13].

Statistical analysis

Continuous variables conforming to a normal distribution were presented as mean ± standard deviation (SD). For continuous variables deviating from a normal distribution, they were expressed as median (interquartile range). The Mann–Whitney U-test was applied to compare continuous variables between two groups, while the Kruskal-Wallis H test was utilized for comparisons involving three groups. Categorical data were compared using the $\chi 2$ test. A significance level of $P < 0.05$ was considered statistically significant.

Statistical analyses were conducted using SPSS software version 26.0 (IBM, Armonk, NY, USA), and figures were generated using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

Results

Clinical characteristics and laboratory findings

In our investigation, 109 patients were initially considered for inclusion based on discharge diagnosis of talaromycosis or findings suggestive of *T. marneffei* infection via mNGS, culture, or pathological examination. However, 31 patients had to be excluded due to the absence of culture or mNGS results. Ultimately, our study comprised 78 enrolled patients, with 40 cases categorized in the talaromycosis group and 38 cases in the suspected-talaromycosis group.

Table 1 illustrates the characteristics of both the talaromycosis and suspected-talaromycosis groups. Significant disparities were observed in the count of immunocompromised patients, CD4 + T cell counts, and C-reactive protein levels between the two groups ($P = 0.034$, 0.017, and 0.017, respectively). However, no noteworthy differences were found in other parameters.

Results of culture and mNGS on T. marneffei detection

In the talaromycosis group, we collected whole blood samples from 20 patients, BALF samples from 14 patients, and local tissue samples from 6 patients. Among them, 34 patients (85%) exhibited positive culture results, while 40 patients (100%) showed positive results via mNGS. Although the positivity rate of mNGS was higher than that of culture, the difference did not reach statistical significance

Table 1 The clinical characteristics of the talaromycosis group and suspected-talaromycosis group

characteristic	talaromycosis group $n = 40$	suspected-talaromycosis group $n = 38$	*P* value
Gender(n,%)			0.357
Female	8(20.0%)	11(28.9%)	
Male	32(80.0%)	27(71.1%)	
Age, years	46.2 ± 13.4	51.5 ± 18.2	0.133
Immunocompromised patients(n, %)	36(90.0%)	27(71.1%)	0.034
HIV	26(65.0%)	12(31.6%)	0.003
Organ transplantation[a]	8(20.0%)	7(18.4%)	0.860
Hematologic malignancy[b]	0(0.0%)	6(15.8%)	0.028
Autoimmune disease[c]	2(5.0%)	3(7.9%)	0.953
Clinical manifestations(n, %)			
Fever	34(85.0%)	28(73.7%)	0.216
Respiratory symptoms	24(60.0%)	25(65.8%)	0.597
Digestive tract symptom	6(15.0%)	4(10.5%)	> 0.999
Skin lesions	5(12.5%)	1(2.6%)	0.226
Lymphadenopathy	7(17.5%)	2(5.3%)	0.181
Laboratory findings			
white blood cell (×109/L)	3.6(3.5)	5.1(4.8)	0.101
C-reactive protein (mg/L)	54.3(69.7)	33.1(44.1)	0.017
β-1,3-D-glucan test(n, %)	37(92.5%)	24(63.2%)	0.113
< 10pg/ml	17(45.9%)	16(66.7%)	
≥ 10pg/ml	20(54.1%)	8(33.3%)	
CD4 + T cell (n, %)	37(92.5%)	25(65.8%)	0.017
< 200 cells/ul	33(89.2%)	16(64.0%)	
≥ 200 cells/ul	4(10.8%)	9(36.0%)	
Antifungal agents(n, %)	40(100.0%)	34(89.5%)	0.067
Amphotericin B + itraconazole	20(50.0%)	11(32.4%)	
Amphotericin B + voriconazole	14(35.0%)	10(29.4%)	
Voriconazole	6(15.0%)	13(38.2%)	
ICU treatment (n,%)	6(15.0%)	4(10.5%)	0.801
28-day all-cause mortality(n, %)	5(12.5%)	4(10.5%)	> 0.999

[a] included liver transplantiation and kidney transplantation; [b] received chemotherapy or immunotherapy recently; [c] received immunosuppressan therapy

HIV, human immunodeficiency virus; ICU, intensive care unit

Table 2 Summary of the primary disease and the results of mNGS and culture in different clinical specimens from the talaromycosis group

	Whole blood $n=20$	BALF $n=14$	Tissue $n=6$	*P* value
mNGS (No. of reads, median)	1535 (6570)	9034.5 (144425.5)	1037241.5 (3873350.75)	0.009
Positivity of Culture (n, %)	16(80.0%)	13(92.9%)	5(83.3%)	0.582
Immunocompromised patients (n, %)	19(95.0%)	11(78.6%)	6(100.0%)	0.197
HIV	14(70.0%)	7(50.0%)	5(83.3%)	0.288
Kidney transplantation	4(20.0%)	3(21.4%)	1(16.7%)	0.971
Autoimmune disease[a]	1(5.0%)	1(7.1%)	0(0.0%)	0.798

[a] received immunosuppressan therapy

BALF, bronchoalveolar lavage fluid; HIV, human immunodeficiency virus

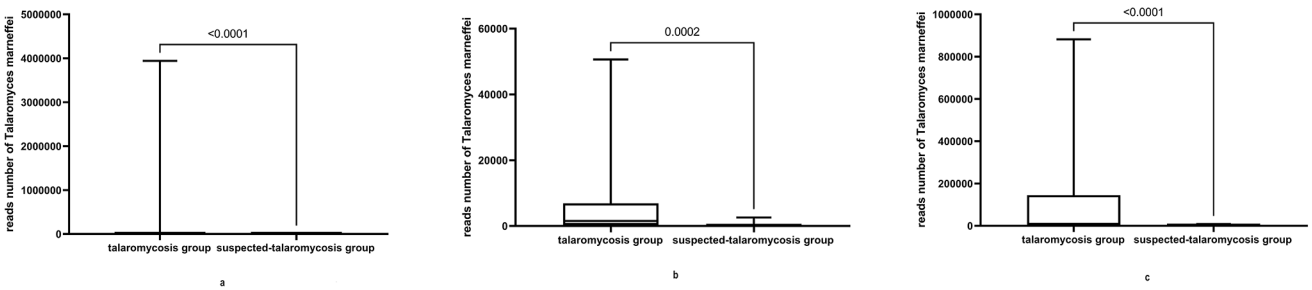

Fig. 1 Comparison of *Talaromyces marneffei* reads assessed by metagenomic next-generation sequencing in the talaromycosis group and suspected-talaromycosis group revealed compelling findings. Figure a demonstrates that the reads count in the talaromycosis group, considering all samples, significantly surpassed that in the suspected-talaromycosis group ($P<0.001$). Figure b and c further elucidate that within the talaromycosis group, the reads count in whole blood (Figure b) and bronchoalveolar lavage fluid (Figure c) samples were notably higher compared to the suspected-talaromycosis group ($P<0.001$ for both comparisons)

($P=0.111$). Notably, the medians of reads detected in whole blood, BALF, and tissue samples in the talaromycosis group were 1535 (6570), 9034.5 (144425.5), and 1037241.5 (3873350.75), respectively, demonstrating statistically significant differences ($P=0.009$)(Table 2).

Within the suspected-talaromycosis group, we collected whole blood samples from 16 patients, BALF samples from 20 patients, and sputum samples from 2 patients. Despite all patients testing negative via culture, mNGS results were positive. The medians of reads identified in whole blood, BALF, and sputum samples in the suspected-talaromycosis group were 33 (99.75), 19.5 (198.75), and 4.5, respectively, without statistically significant differences ($P=0.353$).

However, the number of reads related to *T. marneffei* in the talaromycosis group significantly exceeded those in the suspected-talaromycosis group [4399 (45171.25) vs. 28 (177.25), $P<0.001$] (Fig. 1a). Moreover, the reads identified in the talaromycosis group using whole blood and BALF were also notably higher than those in the suspected-talaromycosis group ($P<0.001$) (Fig. 1b and c).

mNGS in suspected-talaromycosis group

In the suspected-talaromycosis group, 34 patients (89.5%) underwent effective antifungal therapy, leading to improvement in the condition of 31 patients (91.2%). However, four patients did not receive adequate antifungal therapy, and their *T. marneffei* reads counts were 1, 1, 2, and 8, respectively. Unfortunately, the patient with a reads count of 8 eventually succumbed, another patients experienced exacerbated lung lesions. It's noteworthy that patients who received effective antifungal therapy exhibited significantly higher reads counts than those who did not receive treatment [28.5 (174.25) vs. 1.5 (5.5), $P=0.014$] (Fig. 2).

Clinical mNGS testing for other pathogens

In both the talaromycosis group and suspected-talaromycosis group, alongside *T. marneffei*, our investigation using mNGS revealed numerous other pathogenic infections, as illustrated in Fig. 3. The distribution of these pathogens is visually represented in Fig. 3.

Cytomegalovirus (CMV) was found in 19 cases, *Epstein-Barr virus* (EBV) in 14 cases, and *Pneumocystis carinii* in 6 cases, emerging as the three most prevalent pathogens identified within the talaromycosis group. Similarly, in the suspected-talaromycosis group, the three most common pathogens detected were CMV (15 cases), EBV (15 cases), and *Pneumocystis carinii* (8 cases).

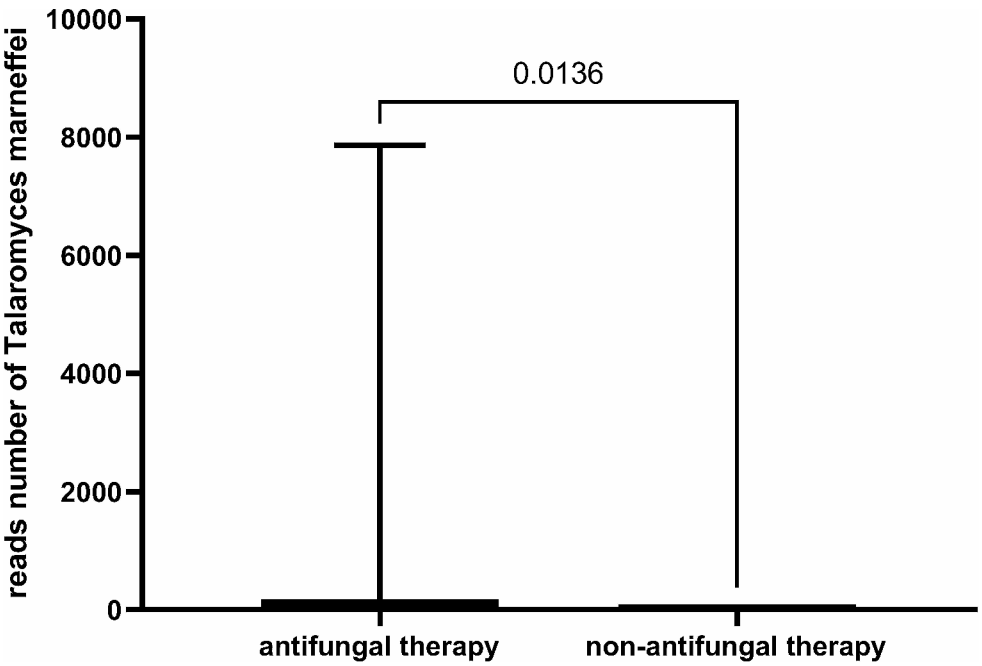

Fig. 2 Comparison of *Talaromyces marneffei* reads detected via metagenomic next-generation sequencing between patients who received antifungal therapy and those who did not in the suspected-talaromycosis group revealed a notable distinction. The reads count among patients undergoing antifungal therapy was significantly higher compared to untreated patients ($P = 0.014$)

Discussion

In our study, we investigated the utility of mNGS for detecting *T. marneffei* in various samples, including whole blood, BALF, tissue, and sputum. The diagnosis in the talaromycosis group primarily relied on culture results, aligning with the established recognition of culture as the gold standard for diagnosing talaromycosis. However, it became apparent that relying solely on culture results may not suffice for diagnosis. Although the culture positivity rate reached 85% in the talaromycosis group, all patients in the suspected-talaromycosis group tested negative via culture. Despite this, mNGS results were positive in both groups. Furthermore, a significant majority of suspected-talaromycosis patients exhibited improvement following effective antifungal therapy. Consequently, our study revealed that mNGS demonstrates a higher positivity rate than culture in detecting *T. marneffei*. Moreover, in our experience, mNGS typically required 1–2 days, whereas culture took approximately 1–2 week, indicating a clear advantage of mNGS in terms of time efficiency. Prior studies [7, 17] have highlighted the substantial mortality risk associated with delayed diagnosis and ineffective treatment of talaromycosis. Hence, considering these findings, we regard mNGS as highly promising for the detection of *T. marneffei*.

While mNGS presents clear advantages in diagnosing infectious diseases, interpreting its results in clinical practice remains challenging. Prior studies focusing on using mNGS to diagnose *T. marneffei* were largely limited to case reports [18]. For instance, Zhang W et al. reported a lung infection case where cultures from BALF and endobronchial biopsied tissue identified *T. marneffei*, with a reads count of 566 [19]. Similarly, Ba H et al. reported a case confirmed through blood culture, detecting *T. marneffei* with a reads count of 248 using mNGS [20]. Additionally, Chen Q et al. reported mNGS reads ranging from 5 to 414 in five talaromycosis cases [11]. However, no uniform standard exists for defining the number of reads indicating a positive or infected case of *T. marneffei*. Given that *T. marneffei* is an exogenous pathogenic fungus that doesn't typically colonize the lungs or blood, reads ≥ 1 were considered positive for mNGS in our study. Our findings demonstrated significantly higher reads in the talaromycosis group compared to the suspected-talaromycosis group ($P < 0.001$), particularly in whole blood and BALF ($P < 0.001$). Consequently, we postulate that higher reads count of *T. marneffei* indicates a greater likelihood of infection.

It's noteworthy that within the talaromycosis group, the reads count was highest in tissue samples, followed by BALF, and lastly, whole blood. This difference held statistical significance ($P < 0.001$). This implies that when considering the application of mNGS for detecting *T. marneffei*, sampling from a localized lesion might be a more suitable choice. However, in cases without any discernible lesion, conducting the test using whole blood could be considered.

In the talaromycosis group, only one case had a read count of 1 (blood sampled after 2 weeks of voriconazole as diagnostic therapy for suspected fungal lung infection), while the rest displayed counts higher than 20. Among the suspected-talaromycosis group, four cases did not undergo antifungal therapy, three of which exhibited reads counts less than 3. Following subsequent observation, one patient

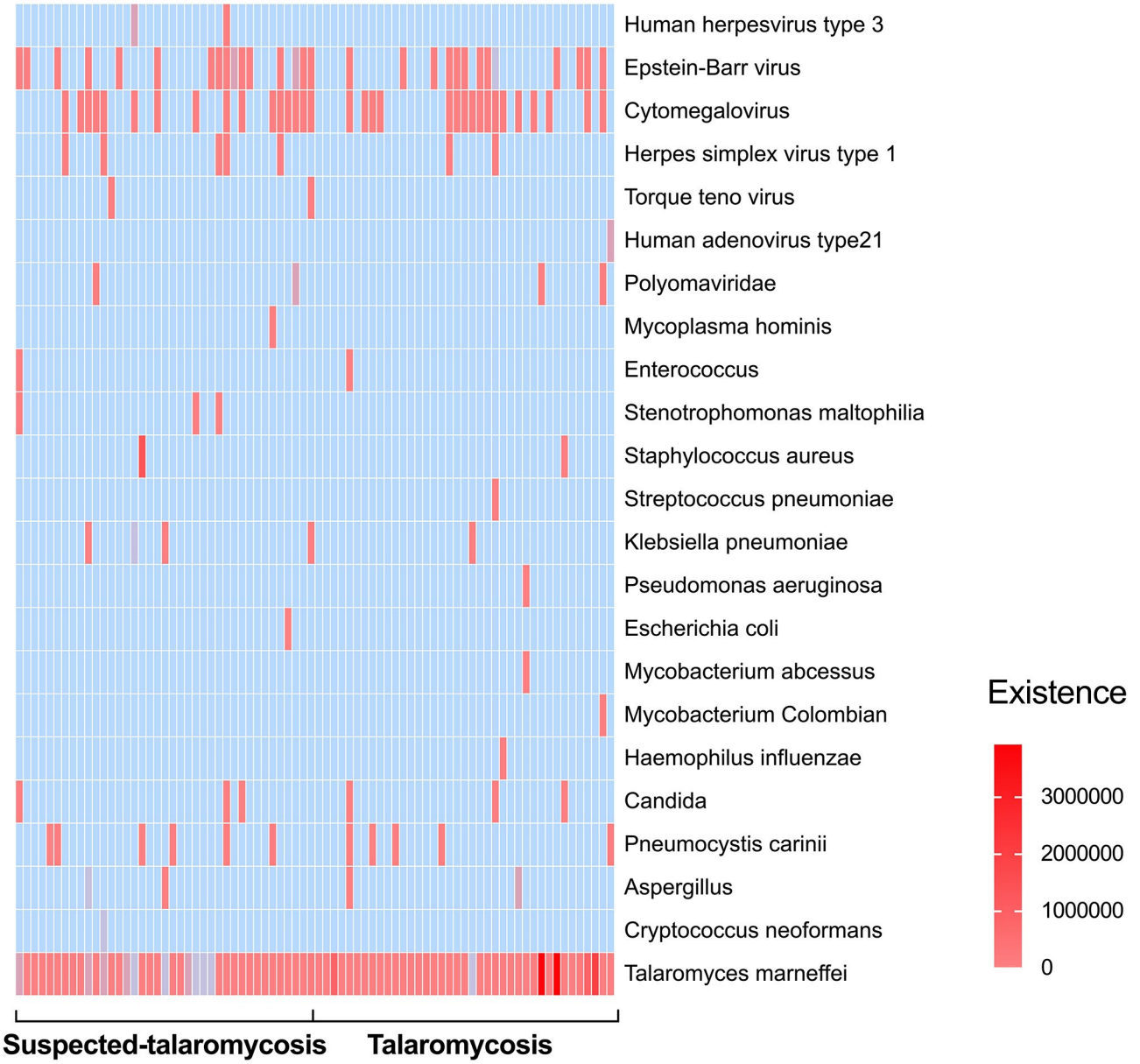

Fig. 3 Overview of the mNGS analysis of pathogens in talaromycosis group and suspected-talaromycosis group. The heatmap demonstrates the reads of pathogens. Each column represents a patient. Red indicates positive results; blue indicates negative results

passed away, another experienced exacerbated lung lesions, and the remaining two patients did not display progression. The possibility of contamination during sampling and testing procedures, or mistake in bioinformatics analyses could result in the detection of *T. marneffei* with low mNGS reads, potentially leading to false-positive outcomes. Consequently, for cases exhibiting low reads, it remains imperative to complement the findings with clinical manifestations and imaging examinations to ascertain the existence of *T. marneffei* infection.

A previous study suggested that approximately 50% of individuals co-infected with *T. marneffei*/HIV simultaneously experience other opportunistic infections [7]. Similarly, in our study utilizing mNGS, we also identified several co-infections. CMV, EBV, and *Pneumocystis carinii* emerged as the three most prevalent pathogens in both the talaromycosis and suspected-talaromycosis groups, aligning with findings from previous research [12].

Despite these insights, our research has limitations. Firstly, this study was a single-center retrospective analysis conducted with a relatively small sample size. Secondly,

in the suspected-talaromycosis group, we did not employ polymerase chain reaction or other methods to confirm the presence of *T. marneffei* infection. Additionally, the influence of antifungal agents on the detection of *T. marneffei* by mNGS remains unclear. Consequently, there is a clear need for a large-scale multicenter prospective study to further clarify the diagnostic efficacy of mNGS in talaromycosis.

Conclusion

mNGS proves to be a swift and highly sensitive approach in detecting *T. marneffei*. A higher number of reads related to *T. marneffei* signifies a heightened probability of infection. However, in instances where the reads are low, it becomes essential to integrate clinical manifestations, laboratory tests, and imaging examinations to ascertain the presence of *T. marneffei* infection. Additionally, mNGS demonstrates a clear advantage in detecting mixed infections.

Author contributions Data collection was performed by Xuan Zhang, Xinfei Yao and Meifang Yang. The study conception, design and data interpretation were porformed by Xuan Zhang and Dongsheng Han. The first draft of the manuscript was written by Xuan Zhang, Huixin Chen and Dongsheng Han. All authors commented on previous versions of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethics committee of the First Affiliated Hospital of Zhejiang University.

Consent to participate This was a retrospective observational study and informed consent to participate was waived in accordance with the ethics approval.

Consent to publish Not applicable.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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