

Metagenomic Next-Generation Sequencing for the Diagnosis of Suspected Opportunistic Infections in People Living with HIV

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Objective: The diagnosis of suspected opportunistic infections in HIV patients is challenging due to the wide range of potential causes. This study used mNGS to analyse specimens of suspected opportunistic infections in HIV patients from a single centre to explore this method's applicability as a diagnostic tool compared to that of CMTs.

Methods: We retrospectively investigated 46 suspected opportunistic infections in people living with HIV (PLWH) hospitalized at Hangzhou Xixi hospital from January 2020 to August 2021. In total, we collected 49 samples (3 patients provided 2 samples) and sent them out for mNGS.

Results: mNGS had a better detection rate for fungi and nontuberculous mycobacteria than that of CMTs. Specifically, the diagnostic detection rate of fungi (11 vs 19, $P < 0.05$) and nontuberculous mycobacteria (1 vs 6, $p < 0.05$) was significantly higher; there was no difference in detection rate for other pathogens (bacteria, Mycobacterium tuberculosis, or viruses). The sensitivity of mNGS was 90.91%, 50%, 0%, 100%, and 100% for detecting fungi, bacteria, Mycobacterium tuberculosis, nontuberculous mycobacteria, and viruses, respectively; the corresponding specificities were 74.29%, 97.73%, 86.36%, 86.67%, and 91.11%.

Conclusion: mNGS technology provides an alternative and promising method of identifying suspected opportunistic infections in PLWH. Thus, the best diagnosis strategy may be using a combination of mNGS and CMTs.

Keywords: metagenomic next-generation sequencing, infection, aetiological diagnosis, HIV, opportunistic infections

Introduction

AIDS remains a major global health threat. In 2020, 1.5 million people were newly infected with HIV, 37.6 million people were living with HIV, and over 690,000 people died of AIDS-related illnesses worldwide.¹ Opportunistic infections are one of the main causes of morbidity and mortality in HIV patients; thus, rapid and early diagnosis of pathogens is essential for optimizing treatment strategies and improving patient prognosis. Conventional methods of detecting pathogenic microorganisms have many limitations, such as long culture times, low positivity rates, susceptibility to interference from antibiotics and difficulty in detecting certain pathogens. Therefore, the diagnosis of opportunistic infections in HIV patients is challenging.

In the past decade, next-generation sequencing (NGS) technology has rapidly developed, gaining utility in scientific research as well as clinical research; in recent years, this technology has gradually expanded to the field of Infectious Diseases due to its swift identification of pathogens.² In particular, metagenomics next-generation sequencing (mNGS) technology offers high throughput, rapid processing, and high accuracy,^{3–5} and is thus revolutionizing traditional microbial detection. However, due to the high cost, its application in people living with HIV (PLWH) is still relatively limited. The

purpose of this study was to evaluate the potential of mNGS as a first-line diagnostic method in HIV patients with suspected opportunistic infections compared with the utility of comprehensive conventional microbiological tests (CMTs).

Materials and Methods

Study Design and Patient Population

We retrospectively investigated 46 suspected opportunistic infections in PLWH hospitalized at Hangzhou Xixi Hospital from January 2020 to August 2021 and collected 49 total samples (3 patients provided 2 samples). The samples were then sent out for mNGS; the results were compared with those of CMTs. Hangzhou Xixi Hospital is a class 3 infectious disease specialist hospital in Hangzhou. Its AIDS Department includes the Hangzhou AIDS Diagnosis and Treatment Center and the Hangzhou AIDS Research Institute. This hospital treats a large number of HIV infections in East China. Our study was approved by the ethics committee of this institution. Due to the retrospective nature of the study, written informed consent from the patients was not required. This study was conducted in accordance with the Declaration of Helsinki.

Inclusion Criteria

1. Patients >18 years old;
2. Patients infected with HIV (confirmed as HIV-positive by the laboratory's preliminary HIV screening test and the CDC's HIV antibody test);
3. Patients admitted to hospital for suspected opportunistic infections and meeting one of the following two criteria:
 - (a) New-onset fever patients given empirical antibiotic therapy but who experienced little effect of treatment;
 - (b) Patients without fever whom imaging suggested had an opportunistic infection.

Data Collection

The data collected from patients included the following:

1. General patient information: sex, age, onset of highly active antiretroviral therapy (HAART), whether patients presented with a fever, and outcome (improvement or lack thereof).
2. Samples collected according to the site of the suspected opportunistic infection: bronchoalveolar lavage fluid (BALF) was obtained from a bronchoscopy; whole blood was obtained from a peripheral vessel; cerebrospinal fluid was obtained from a lumbar puncture; lymph node tissue was obtained from a lymph node biopsy; pus was obtained from a neck mass puncture; pleural effusion was obtained from a thoracic puncture; bone marrow was obtained from a bone marrow puncture; and sputum was obtained from a deep sputum expectoration.
3. mNGS and comprehensive CMTs results for the detection of pathogenic bacteria. CMTs included at least one smear and culture of the bacteria, fungi, and mycobacteria; hexamine silver staining and acid-fast staining; detection of serum cryptococcal capsular polysaccharide antigen, viral quantitative polymerase chain reaction; and T-spot and GeneXpert MTB/RIF testing to detect *Mycobacterium tuberculosis* RNA (TB-RNA) and thus diagnosis tuberculosis.
5. Routine blood and inflammatory markers evaluated within 24 hours of mNGS sample collection (before or after), including the number of white blood cells (WBCs), neutrophil ratio (N%), C-reactive protein (CRP) levels, and procalcitonin (PCT) levels.
6. HIV RNA testing via RT-PCR and CD4+ T lymphocyte counts by cytometry.

mNGS Data Collection

The mNGS methods included the following three steps, which are described in detail below: data storage, in-depth sequencing, and data processing.

1. Data storage: After the sample was obtained, all nucleic acid was extracted and stored at -80°C .
2. In-depth sequencing: The specimen DNA was cold chain transported to the gene company for PMseq™ library construction; the BGISEQ-50/MGISEQ-2000 platform was used for high-throughput metagenomic sequencing.
3. Data processing: After the sequencing data were provided, low-quality sequences were removed to restrict analyses to high-quality data. BWA (<http://bioinformatics.sourceforge.net/>) was employed to screen out human reference genome

sequence data from the high-quality data.⁶ The remaining sequences were further filtered to eliminate background microbial interference; the final data were then compared with the microbial genome database to acquire information on the type and relative abundance of the suspected pathogenic microorganisms.

mNGS Interpretation

Given the lack of standard methods for interpreting the results of mNGS and the diversity of reporting parameters between different sequencing platforms, this study used the following criteria, which are based on the interpretation of mNGS results on the BGISEQ platform, to define clinically significant microbes (CSMs).^{7,8}

1. Bacteria (excluding mycobacteria), fungi (excluding moulds), viruses, and parasites: If the relative abundance of a microorganism at the species level exceeded 30% and if that microorganism was previously found to be pathogenic, it was classified as a CSM;

2. Mycobacteria: Due to the low likelihood of mycobacterial contamination and low nucleic acid acquisition rate, if the number of strictly aligned sequences at the species level was ≥ 1 , the microorganism was classified as a CSM;

3. If the CMTs and mNGS detected the same pathogen, and there were more than 50 reads of that pathogen according to mNGS, the pathogen was classified as a CSM;

4. If only mNGS identified the pathogen, the clinical manifestations of patients were used to determine if it was considered a CSM.

Clinical Composite Diagnosis as the Reference Standard

Two doctors with years of professional experience in HIV diagnosis and treatment independently reviewed the medical records of all patients, as well as the results of the routine microbiological tests (CMTs) and mNGS. First, they determined whether the cause of the patient's opportunistic infection was infectious or non-infectious. Then, the pathogen was identified based on clinical manifestations, laboratory examinations, imaging of relevant areas, microbiological examinations (including routine microbiological testing and mNGS), and treatment response. Any disagreements between the two doctors were resolved through in-depth discussions, and if a consensus could not be reached, another senior expert was consulted.

Statistical Analysis

Count data are represented by the median and interquartile range (IQR); categorical variables are represented by frequency and percentage. Comprehensive clinical diagnosis and determination of microbial aetiology were used as reference standards. The McNemar test was used to compare the diagnostic performance of CMTs and mNGS. All tests were two-tailed, and a P value of <0.05 was considered statistically significant.

Results

Distribution of Specimen Types

A total of 49 samples were collected, including 17 cases of BALF, 14 cases of cerebrospinal fluid, 10 cases of whole blood, 4 cases of lymph node tissue, 1 case of pus, 1 case of pleural fluid, 1 case of bone marrow, and 1 case of sputum (Figure 1).

Patient Characteristics

During the study period, a total of 46 HIV patients (median age: 39 years) including 42 men (91.3%) met the inclusion criteria. A total of 49 samples were collected and included in the final analysis. Ten patients (21.7%) were fever-free. Based on a retrospective review of the clinical manifestations and the results of CMTs and mNGS, 33 samples (67.3%) from 33 patients (71.7%) were confirmed to have opportunistic infections: 18 samples identified by mNGS alone, 11 cases of pathogens in samples identified by mNGS and CMTs, and 4 samples identified by CMTs alone. Of these, 32 patients (97.0%) improved after treatment, and 1 patient (3.0%) was discharged automatically without improvement after treatment. Three samples (6.1%) from the other three patients were considered to have non-infectious causes, including

Specimen types

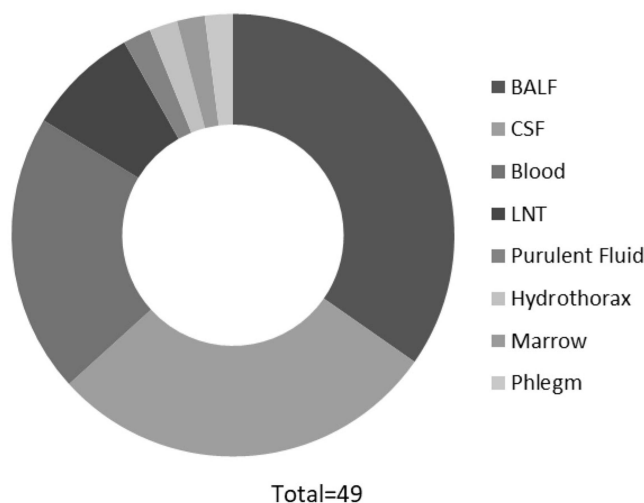


Figure 1 Specimen types of 49 cases of suspected opportunistic infections in PLWH.

one case each of Kaposi's sarcoma, autoimmune encephalitis, and eosinophilic gastroenteritis. Of these 3 cases, the patient diagnosed with Kaposi's sarcoma from a lymph node biopsy also had herpesvirus type 8 according to the mNGS of the lymph node tissue, which prompted the diagnosis. This patient showed no improvement and was eventually discharged automatically. No pathogen was found in 8 patients (17.4%) and 10 samples (20.4%), and these patients improved after empirical treatment. Three samples (6.1%) of 2 patients (4.3%) did not have an identifiable pathogen; these patients were discharged after no improvement from treatment. In summary, a total of 42 patients (91.3%) improved and were discharged from the hospital, and a total of 4 patients (8.7%) worsened and finally decided to stop treatment. Detailed clinical characteristics are shown in [Table 1](#).

Distribution of Pathogens

After excluding the 3 patients with non-infectious aetiology, there were 46 samples from the remaining 43 patients. Of these samples, 33 had clear pathogens, 21 had single infections and 12 had mixed infections (11 cases of 2 pathogens, 1 case of ≥ 2 pathogens). Of the 12 cases of mixed infections, 8 cases were diagnosed by mNGS, 3 cases were diagnosed by mNGS and comprehensive CMTs, and 1 case was diagnosed by comprehensive CMTs. Combined treatment measures, all improved and discharged. The CD4⁺ T cell count of patients with clear pathogens was significantly lower than that of patients with unclear pathogens (68 (16–168.5) vs 165.5 (61–294.25) $P=0.043$, [Figure 2](#)). The CD4⁺ T cell count of patients with mixed infections was significantly lower than that of patients with a single infection (140 (42–208) vs 22 (7.5–62.25) $P=0.010$, [Figure 3](#)). Of the 3 patients with non-infectious aetiology, one that was diagnosed with Kaposi's sarcoma according to a lymph node biopsy also had herpesvirus-8 in the lymph node tissue according to mNGS, which greatly influenced the pathological diagnosis. The distribution of pathogens is shown in [Table 2](#). NGS Sequence number results (defined as pathogens) is shown in [Supplementary Table 1](#).

1. Seven kinds of pathogens were detected by both mNGS and CMTs: bacteria (*Staphylococcus aureus*), viruses (cytomegalovirus), and fungi (*Cyanobacterium marneffeii*, *Pneumocystis jirovecii*, *Cryptococcus neoformans*), nontuberculous mycobacteria (mycobacterium avium complex), and *Mycobacterium tuberculosis*.

2. Ten pathogens were only detected by mNGS: bacteria (*Tropheryma whipplei*), viruses (JC virus, herpesvirus-8), and nontuberculous mycobacteria (*Mycobacterium haemophilum*, *Mycobacterium Columbia*, *Mycobacterium intracellulare*, *Mycobacterium vulneris*, *Mycobacterium parascrofulaceum*, *Mycobacterium abscessus*, and *Mycobacterium kansasii*).

Table 1 Clinical and Laboratory Characteristics of 46 PLWH with Suspected Opportunistic Infections

Characteristic	Value
Male, n(%)	42(91.3%)
Age(year), median(IQR)	39(29–50)
Duration of ART	
ART native, n(%)	22(47.8%)
≤6 months, n(%)	17(37.0%)
>6 months, n(%)	7(15.2%)
Laboratory findings	
White blood cell count($10^9/L$), median(IQR)	5.58(3.80–6.93)
Neutrophil ratio(%), median(IQR)	71.80(57.18–79.48)
C reactive protein(mg/dl), median(IQR)	18.12(5.00–54.75)
Procalcitonin(ng/mL), median(IQR)	0.11(0.06–0.24)
HIV RNA<100IU/mL, n(%)	7(15.56%)
CD4 ⁺ T cell count(cells/ul), media(IQR)	112.5(26–222.25)
Patient outcomes	
Improved, n(%)	42(91.3%)
Deteriorated, n(%)	4(8.7%)

Abbreviation: ART, antiretroviral treatment.

3. One pathogen was found only in the culture of ordinary cerebrospinal fluid. Clinical treatment of *Staphylococcus hominis* was effective. It was assumed to be caused by pathogenic bacteria rather than pollution.

4. mNGS was better at detecting fungi and nontuberculous mycobacteria. Specifically, the diagnostic detection rate of mNGS was significantly higher than that of CMTs for fungi (11 vs 19, $P<0.05$) and nontuberculous mycobacteria (1 vs 6, $p<0.05$). The detection rate of other pathogens (bacteria, *Mycobacterium tuberculosis*, viruses) did not significantly differ between the two diagnostic methods.

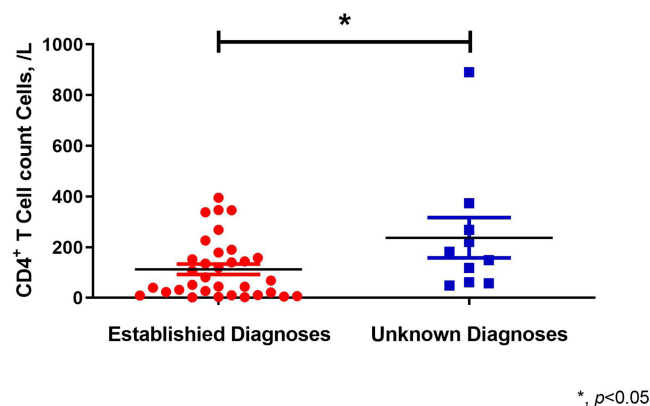


Figure 2 The CD4⁺ T cell count of patients with diagnosed pathogens was significantly lower than that of patients not diagnosed with pathogens (68 (16–168.5) vs 165.5 (61–294.25) $P=0.043$). * Indicates that there is a significant difference between the two groups.

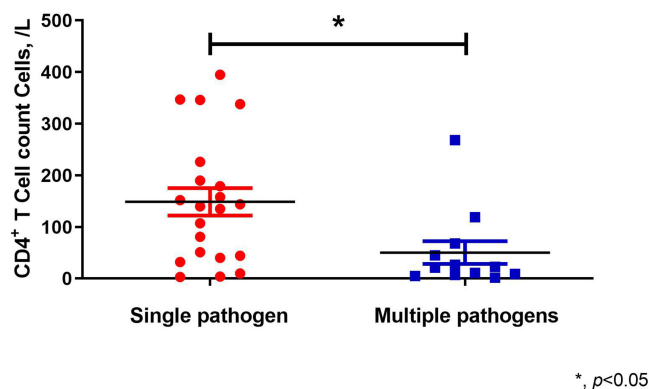


Figure 3 The CD4⁺ T cell count of patients with mixed infections was significantly lower than that of patients with a single infection (140 (42–208) vs 22 (7.5–62.25) P=0.010). *Indicates that there is a significant difference between the two groups.

5. mNGS detected three nontuberculous mycobacteria in one patient, namely, *Mycobacterium Columbia*, *Mycobacterium intracellulare*, and *Mycobacterium vulneris*; and it detected 2 nontuberculous mycobacteria, namely, *Mycobacterium abscessus* and *Mycobacterium intracellulare* in another patient.

6. One patient had *Mycobacterium avium* complex (MAC) detected by both routine microbiology (lymph node histopathological biopsy) and mNGS. mNGS also detected *Mycobacterium parascrofulaceum* in the lymph node tissue of this patient.

7. The detection sensitivity of mNGS for fungi, bacteria, *Mycobacterium tuberculosis*, nontuberculous mycobacteria, and viruses was 90.91%, 50%, 0%, 100%, and 100%, respectively; the corresponding specificities were 74.29%, 97.73%, 86.36%, 86.67%, and 91.11%.

Discussion

Opportunistic infections, including pneumocystis pneumonia, tuberculosis, nontuberculous mycobacterial infection, cytomegalovirus infection, toxoplasmic encephalitis, progressive multifocal leukoencephalopathy, and infection with *Cryptococcus neoformans* infection or *Cyanobacterium marneffeii*, are common in PLWH that have advanced immunosuppression. Although the diagnostic performance of mNGS in varying patient populations and with varying infection types has previously been studied,^{9,10} its application in HIV patients with suspected opportunistic infections requires further examination.

Although traditional pathogen detection technology that uses culture as the main detection method is still the “gold standard” for microbial pathogen detection, the in vitro culture process is cumbersome, time consuming, and has a low rate of positive culture detection; moreover, there are still many pathogens that cannot be cultivated. In this study, 10 pathogens were detected only by mNGS: *Tropheryma whipplei*, JC virus, herpesvirus-8, and nontuberculous mycobacteria (*Mycobacterium haemophilum*, *Mycobacterium Columbia*, *Mycobacterium intracellulare*, *Mycobacterium vulneris*, *Mycobacterium parascrofulaceum*, *Mycobacterium abscessus*, and *Mycobacterium kansasii*); these pathogens cannot be detected by traditional methods. Although 1 case of *Mycobacterium tuberculosis* was cultured from pleural fluid, the culture time was up to 1 month, which impedes early diagnosis; rapid and early diagnosis is essential for favourable prognoses of opportunistic infections.

Because mNGS allows unbiased detection of pathogens, it has been effective in the diagnosis of infectious diseases.^{11–14} Additionally, it can detect pathogens more quickly (mNGS report time =2.5 days on average) and more comprehensively than CMTs, which makes this method especially suitable for HIV populations that are prone to mixed infection.^{15,16} This study also showed that patients with lower CD4⁺T cellcounts are more likely to develop multiple infections. Due to the lack of ability to detect some viruses and rare pathogens using traditional detection methods, the detection of pathogens such as JC virus and *Tropheryma whipplei* by mNGS in this study has obvious advantages. The traditional culture of nontuberculous mycobacteria in our hospital is not yet available, which prevents strain identification, but this study suggests that the detection rate of mNGS for nontuberculous mycobacteria is significantly higher than

Table 2 Distribution of Pathogens

Pathogens	CMTs	mNGS	P value	Sensitivity (%)	Specificity(%)
Fungi	11	19	0.021	90.91	74.29
<i>Cyanobacterium marneffeii</i>	5	5	1.000	80	97.56
<i>Pneumocystis jiroveci</i>	2	7	0.125	50	86.36
<i>Cryptococcus neoformans</i>	6	8	0.500	100	95
Bacteria	2	2	1.000	50	97.73
<i>Staphylococcus aureus</i>	1	1	1.000	100	100
<i>Staphylococcus hominis</i>	1	0	–	0	100
<i>Tropheryma whipplei</i>	0	1	–	–	97.83
<i>Mycobacterium tuberculosis</i>	2	6	0.289	0	86.36
Nontuberculous mycobacteria	1	6	0.031	100	86.67
<i>Mycobacterium haemophilum</i>	0	1	–	–	97.83
<i>Mycobacterium avium</i> and <i>M. intracellulare</i>	1	3	0.500	100	95.56
<i>Mycobacterium columbia</i>	0	1	–	–	97.83
<i>Mycobacterium intracellulare</i>	0	1	–	–	97.83
<i>Mycobacterium vulneris</i>	0	1	–	–	97.83
<i>Mycobacterium abscessus</i>	0	1	–	–	97.83
<i>Mycobacterium kansasii</i>	0	1	–	–	97.83
<i>Mycobacterium parascrofulaceum</i>	0	1	–	–	97.83
Viruses	1	5	0.125	100	91.11
CMV virus	1	3	0.500	100	95.56
JC virus	0	1	–	–	97.83
Herpesvirus-8	0	1	–	–	97.83

that of CMTs ($P < 0.05$); moreover, mNGS can identify the strains and provide better medication guidance. The CD4⁺T cell count of diagnosed patients was significantly lower than that of undiagnosed patients (68 (16–168.5) vs 165.5 (61–294.25) $P < 0.05$), indicating that mNGS may be more suitable for use in patients with low CD4⁺T cell counts. Furthermore, mNGS also detected non opportunistic pathogenic bacteria, such as *Staphylococcus aureus* and *Staphylococcus hominis*. In short, using this method to diagnose and inform treatment of suspected opportunistic infections in immunodeficient populations facilitates early and accurate identification of the pathogens and provides valuable information on the use of antibacterial drugs in the clinic, shortening patients' hospital stays and improving their prognosis. In addition, mNGS can reduce the likelihood of treatments that may increase the risk of adverse drug reactions in patients with unknown pathogens.

However, this method also has limitations. One mNGS test usually provides a variety of suspected pathogenic gene sequences. The detected pathogens may not be true pathogenic microorganisms but may also include contaminating and/or colonizing bacteria. Thus, clinicians are required to integrate the patient's clinical history with other test results. At present, the cost of single-use mNGS of pathogens is still relatively high, and this high cost limits its clinical promotion and application. Notably, one of the 49 samples had two pathogenic microorganisms (*Pneumocystis jiroveci* and *Cyanobacterium marneffeii*) that were detected through multiple culture methods

(whole blood culture combined with BALF culture). In this case, only one pathogen (*Pneumocystis jiroveci*) was detected by mNGS; therefore, the use of traditional detection methods in diagnosis should not be excluded. A combination of mNGS and traditional microbiological testing may be the preferred solution for diagnosing suspected opportunistic infections in PLWH in the future.

By comparing mNGS results with those of traditional microbial culture, this study confirmed that high-throughput mNGS technology was more sensitive in detecting fungi, nontuberculous mycobacteria, and viruses (sensitivities of 90.91%, 100%, and 100%, respectively). The corresponding specificities in diagnosing fungi, bacteria, *Mycobacterium tuberculosis*, nontuberculous mycobacteria, and viruses were also good (specificities of 74.29%, 97.73%, 86.36%, 86.67%, and 91.11%, respectively). The detection sensitivity was significantly higher, but this may be due to the small sample size of this study. Larger sample sizes are needed to confirm the reliability of the mNGS method.

Our research was limited to a single centre, with a small sample size and potential selection bias. Although mNGS has been widely accepted and used in HIV patients, there is no uniform standard for interpreting the test report, especially for mixed infections; relatively low-abundance viruses (such as parvovirus and herpesvirus) are arbitrarily regarded as non-pathogenic. Nonetheless, the interpretation standards in our study were derived from previous studies using similar populations, sequencing platforms, and sample sizes. Finally, we did not evaluate the impact of mNGS on clinical decision-making, nor did we evaluate its cost-effectiveness in clinical scenarios, both of which merit investigation in future studies.

Conclusion

1. Mixed infections are common in opportunistic infections in HIV patients.
2. Compared with that of conventional microbiological tests (CMTs), mNGS has a higher detection rate for fungi and nontuberculous mycobacteria. It has good sensitivity in diagnosing fungi, nontuberculous mycobacteria, and viruses, and it has good specificity in diagnosing fungi, bacteria, *Mycobacterium tuberculosis*, nontuberculous mycobacteria, and viruses.
3. mNGS technology provides an alternative and promising detection method for HIV patients with suspected opportunistic infections. The best diagnostic strategy may be a combined diagnosis using mNGS and CMTs.

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Disclosure

The authors report no conflicts of interest in this work.

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