Review Article

Metagenomic next-generation sequencing (mNGS) for diagnosis of invasive fungal infectious diseases: a narrative review

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Abstract: Invasive fungal diseases (IFDs) remain important causes of high morbidity and mortality in many immunosuppressed patients. Accurate etiology diagnosis coupled with subsequent initiation of effective treatment is crucial for severe or critical IFDs. As conventional diagnostic approaches require the successful culture of pathogenic organisms or suspicion of certain pathogens before testing, the implementation of non-targeted metagenomic next-generation sequencing (mNGS) is rapidly increasing in IFDs. mNGS is a high-throughput sequencing technology that provides direct information on nucleic acids from various types of specimens without relying on culture and hypothesis. It can detect all potential pathogens in theory and is especially useful for identifying unknown, rare, newly emerging or mixed infections. Currently, the clinical application of mNGS in the diagnosis of IFDs may be in the most difficult cases to diagnose or for patients who are intolerant to invasive operations, immunocompromised, or seriously ill. Studies have shown that the sensitivity and specificity of mNGS in fungal diagnosis are better than culture and histopathology. However, some hurdles to mNGS testing in the diagnosis of IFDs remain to be addressed, including high costs, influence of human host background, exogenous microbial contamination, poor detection efficiency of thick-wall fungi, etc. With strategies developed to overcome these obstacles, mNGS is expected to be a routine diagnostic method of IFDs in clinical practice.

Keywords: Metagenomic next-generation sequencing (mNGS); diagnosis; invasive fungal infections; *Pneumocystis jirovecii*; *Cryptococcus* spp

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Introduction

Invasive infections caused by opportunistic fungi have high morbidity and mortality, especially in immunocompromised patients. It is estimated that more than 1.6 million people die of fungal diseases annually (1). Therefore, accurate and timely identification of pathogenic fungi is essential for patient management and can significantly improve the prognosis. Conventional diagnostic tests include microscopy, culture (through biochemical phenotyping, matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry analysis, or nucleic acid probes), antigen and/or antibody immunology, and specific polymerase chain reaction (PCR) testing microbial nucleic acid (2,3). Among them, the molecular diagnostic analysis provides a fairly efficient and quick (usually less than 2 hours) way to diagnose the most common fungal infections (4,5). However, almost all traditional microbiological tests currently in use are able to

detect only one or a limited number of pathogens at a time or require a successful culture of microorganisms from clinical specimens.

In recent years, metagenomic next-generation sequencing technology (mNGS) has been increasingly used by clinicians as a culture-independent and hypothesis-free method to diagnose pathogens (6,7). It can directly obtain the microbial nucleic acid from various samples, including sputum, bronchoalveolar lavage fluid (BALF), blood, cerebrospinal fluid (CSF), pleural fluid, ascites, pus, and tissue samples (5,8,9). All potential pathogens with known genomic sequences, such as bacteria, viruses, fungi, parasites, mycoplasma and leptospira, are unbiasedly detected in theory (6,10). At present, mNGS could release results on an average of 24 hours after sampling, generally no longer than 48 hours (11). The workflow of mNGS consists of two components including experimental operations (sample preprocessing, nucleic acid extraction, library preparation and sequencing) and bioinformatic analysis (database comparison, report generation and result interpretation), which demands a high level of the technical platform and personnel quality (7). Although this new technology greatly facilitates the clinical identification of pathogens, it remains a second-line option because of lengthy procedures and microbial contamination introduced during experimental operations. A recent advance has been achieved in mNGS, for example, shorter turnaround time, reduced exogenous contamination, improved workflow and sensitivity (12-15). The potency of the mNGS test using cell-free DNA from of 182 body fluids was evaluated using two sequencing platforms. The test sensitivity and specificity of identification were 91% and 89% for fungi, respectively, using Illumina sequencing assigned to the second-generation sequencing; and 91% and 100% for fungi, respectively, based on nanopore sequencing attributed to the third-generation sequencing (16). Moreover, an outstanding advantage of mNGS lies in that its detection rate is less likely to be affected by prior antimicrobial treatment than by traditional methods (17,18). Thus, it has a high potential value in accurately diagnosing and treating clinical infectious diseases. We present the following article in accordance with the Narrative Review reporting checklist (available at https://dx.doi.org/10.21037/jlpm-21-25).

Methods

To obtain information on mNGS testing for the diagnosis of fungal infections, we searched PubMed, Embase, CNKI and Wanfang, using the search terms ("fungi" OR "fungus" OR "fungal infection*" OR "fungal disease*") AND ("mNGS" OR "metagenomic next-generation sequencing" OR "metagenomic NGS" OR ("cell free" AND ("NGS" OR "next-generation sequencing"))) without date (up to June 16, 2021). After carefully examining the title, abstract, and full text, we found a few articles related to this subject. Most evidence is primarily derived from case reports or small-scale retrospective studies (*Table 1* and Table S1). In the diagnosis of IFDs, mNGS has been used for severe or difficult infections mainly caused by *Pneumocystis jirovecii*, *Cryptococcus* spp., *Histoplasma capsulatum*, *Aspergillus* spp. and *Candida* spp. Here, we reviewed the literature to explore the role of mNGS in the diagnosis of fungal infections by highlighting the species most commonly isolated in these studies.

mNGS for diagnosis of Pneumocystis jirovecii

Pneumocystis jirovecii is an opportunistic pathogen that can cause fatal pneumocystis pneumonia (PCP). The incidence of PCP in patients with organ transplantation, autoimmune diseases, tumors, etc. is on the rise (35). Therefore, to reduce mortality, there is an urgent need for timely diagnosis and prompt PCP-specific treatment (36). The traditional diagnostic method for PCP detection is based on microscopic identification of cysts or trophozoites of *Pneumocystis jirovecii* in stained samples collected from the respiratory tract of patients. This method has low sensitivity and is affected by many factors, such as staining protocol, specimen collection, and pathogen load (37,38). Meanwhile, although the serum 1,3-β-D-glucan (BDG) test has a certain sensitivity to *Pneumocystis jirovecii*, it lacks specificity (39).

There are studies taking advantage of mNGS to find *Pneumocystis jirovecii* in samples obtained from the respiratory tract (19,40-42). However, as *Pneumocystis jirovecii* colonizes the surface of human type I alveolar cells, it is difficult for mNGS to determine an appropriate threshold between infection and colonization when confronted with samples from the respiratory tract (40,43). Excitingly, Zhang *et al.* combined the sequencing order of *Pneumocystis jirovecii* (ranking top 15) with the relative sequencing proportion in fungi (higher than 85%) and speculated a promising cutoff value for mNGS in the diagnosis of PCP (20). More samples need to be collected to verify the validity and clinical significance of this value.

In addition, mNGS was used to detect *Pneumocystis jirovecii* in the peripheral blood samples of patients with

Table 1 Case series of IFDs reported using mNGS

Year	Species [No. of cases]	Samples Types	mNGS platform	Traditional methods	Results	Most common co-infecting pathogens	Ref.
2019	37 fungal species [17]	Contamination-formalin-fixed, paraffin-embedded tissue samples	Illumina v3	Specific PCR, panfungal PCR	mNGS analysis showed low sensitivity compared to specific PCR (which needs to suspect the pathogen in advance)	n/a	(5)
2019	5 fungal species [7]	Plasma	Illumina NextSeq® 500	Culture, 18SrDNA, serum GM and BG test	6/7 proven samples were positive for mNGS	None	(9)
2020	*Pneumocystis jirovecii* [9]	BALF	BGISEQ-50	Culture, smear, PCR	Compared with the culture method, the diagnostic sensitivity and specificity of mNGS were 88.89% and 74.07%, respectively. Compared with smear method and PCR, the diagnostic sensitivity of mNGS was 77.78% and the specificity was 70.00%	CMV, HSV1, *Pseudomonas aeruginosa*	(19)
	Candida albicans [1]					*Candida glabrata*	
	Aspergillus niger [1]					None	
	Chlamydia psittaci [2]					None	
	Pichia kudriavzevii [1]					None	
	Neosartorya fischeri [1]					None	
2019	*Pneumocystis jirovecii* [13]	Blood, BALF, lung tissue, sputum	n/a	Culture, microscopy of Wright-Giemsa stained smear, G test	mNGS show satisfying PCP detection (100%) than conventional methods (microscopic stained samples from 4/8 BALF and 1/13 sputum samples were positive)	HHV5, HHV4, HSV1	(20)
2020	*Pneumocystis jiroveciii* [37]	Blood	BGISEQ-500/50	G test combined with lactate dehydrogenase detection	The sensitivity and specificity of mNGS were 94.5% and 100%, respectively; while G test combined with lactate dehydrogenase detection were 89.19% and 56.0%	CMV, EBV, HSV1, *Parvovirus*	(21)
2020	*Pneumocystis jirovecii* [1]	Blood	Illumina NextSeq 500	*Pneumocystis jirovecii* BAL PCR, Pan-Aspergillus PCR in BAL	The mNGS and BAL PCR were positive	None	(22)
	Aspergillus oryzae [1]				The mNGS and Pan-Aspergillus PCR were positive	None	
2020	*Pneumocystis jirovecii* [1]	Blood, BALF	BGISEQ-100	Culture, PCR, pathology	Pathology of Methenamine silver staining and mNGS was positive	None	(23)
	Candida albicans [1]				BALF culture and mNGS were positive	*Candida glabrata*	

Table 1 (*continued*)

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Year	Species [No. of cases]	Samples Types	mNGS platform	Traditional methods	Results	Most common co-infecting pathogens	Ref.
2019	*Pneumocystis jirovecii* [13]	Pulmonary biopsy, BALF	Torrent Proton	Smear, culture, GM test, pathology, Xpert MTB, Sputum T-spot, CMV nucleic acid, CrAg test	The sensitivity of mNGS in diagnosing mixed lung infections was significantly higher than that of routine testing (97.2% *vs.* 13.9%; P<0.01)	CMV	(24)
	Aspergillus fumigatus [7]					CMV	
	Aspergillus niger [3]					CMV	
	Aspergillus oryzae [3]					CMV	
	Rhizopus oryzae [1]					*Klebsiella pneumoniae*	
	Rhizopus delemar [1]					*Haemophilus parainflfluenzae*	
	Rhizomucor pusillus [1]					*Klebsiella pneumoniae*	
	Cryptococcus neoformans [3]					CMV	
	Candida tropicalis [1]					*Haemophilus parainflfluenzae*	
2018	*7 fungal species* [9]	Plasma	Illumina NextSeq® 500	Culture, galactomannan or Specific PCR	7/9 samples were positive for mNGS	*Staphylococcus epidermidis*, CMV, *Pseudomonas aeruginosa*	(25)
2019	*Cryptococcus neoformans* [5]	CSF	BGISEQ-100	CSF India ink stain, culture, CrAg test	5/6 India ink stain, 2/6 fungal culture, 4/6 antigen and 6/6 mNGS were positive, respectively	None	(26)
	Cryptococcus gattii [1]					None	
2019	*Cryptococcus neoformans* [1]	CSF	Illumina HiSeq 4000	Serum and CSF cryptococcal antigen test, culture	The antigen test, culture and mNGS were positive.	None	(27)
	Aspergillus oryzae [1]			Culture, GM test, 18s rRNA PCR	The GM test and mNGS were positive, a CSF 18s rRNA PCR result showed *Aspergillus* species.	None	
	Histoplasma capsulatum [1]			Culture, 18s rRNA PCR	Culture, 18s rRNA PCR and mNGS revealed *H capsulatum*	None	
	Candida dubliniensis [1]			Pathology, 18s rRNA and 16s rRNA PCR tests, G test	The CSF G test and the second CSF mNGS was positive	None	

Table 1 (*continued*)

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Year	Species [No. of cases]	Samples Types	mNGS platform	Traditional methods	Results	Most common co-infecting pathogens	Ref.
2019	*Cryptococcus neoformans* [3]	CSF	Illumina HiSeq	CSF culture, CrAg test, CSF 1,3-β-D-glucan, CSF fungal 28S rRNA and ITS PCR	3/4 CSF culture were positive while 4/4 mNGS were positive	None	(28)
	Candida tropicalis [1]					None	
2020	*Histoplasma capsulatum* [1]	Bone marrow, blood	n/a	Culture, smear	Bone marrow cultures and mNGS results were positive	None	(29)
	Talaromyces marneffei [1]					None	
2020	*Aspergillus fumigatus* [78]	Sputum, BALF, lung tissue homogenate	BGISEQ-500	Culture, serum *Aspergillus fumigatus*-specific antibody IgG	The sensitivity of mNGS, serum *Aspergillus fumigatus*-specific antibody IgG and culture were 65.7%, 48.6% and 28.6%, respectively; and the specificity were 86.0%, 90.0% and 93%, respectively	None	(30)
2019	*Aspergillus fumigatus* [3]	BALF	BGISEQ-100	Smear, culture	14.7% of smear and culture methods were positive, while 97.1% of mNGS results were positive	*human adenovirus type 7*	(31)
2020	10 fungal species [30]	The epineurium of the facial nerve	NextSeq 550Dx	None	HHV-7 and *Aspergillus* were first identified in the epineurium of facial nerve in BP patients by mNGS technique	n/a	(32)
2018	10 most abundant fungal species [20]	Lung biopsy tissues	BGISEQ-500	Smear, culture, histopathology	Compared with pathology, mNGS showed the highest spcificity (100%) and PPV (100%) for fungi	n/a	(33)
2019	*Aspergillus spp*. [14]	lower respiratory specimens	Illumina HiSeq 4000	Culture, GM test	14 Aspergillus RNA levels were detecble by mNGS, of which only one case was positive for both culture and GM test	n/a	(34)

n/a, not available; BALF, bronchoalveolar lavage fluid; CSF, cerebrospinal fluid; WES, Whole Exome Sequencing; CrAg, cryptococcal-antigen; HHV, *human herpes virus*; HSV1, *human simple virus 1*; CMV, *cytomegalovirus*; EBV, *Epstein-Barr virus*; HH7, *human betaherpes virus 7*; PPV, *positive predicted value*; BG, 1,3-beta-D-glucan; GM, galactomannan.

renal disease receiving immunosuppressive therapy, associated with the clinical manifestations and radiological features to determine the final diagnosis of PCP (21). Although studies have shown that blood mNGS is less sensitive than BALF mNGS in the detection of bacteria and fungi, it has unique advantages in certain circumstances (44,45). It is not only non-invasive, simple, and fast, but also free from the influence of *Pneumocystis jirovecii* colonizing the respiratory tract. Sensitivity and specificity are higher than those of the fungal G test in combination with the detection of lactate dehydrogenase detection (94.59% *vs.* 89.19% and 100% *vs.* 56.0%) (21). Interestingly, of two mild PCP patients whose peripheral blood samples were initially negative, one became positive after a week, which indicated that *Pneumocystis jirovecii* could penetrate the local infection site into the peripheral blood when the immune system is damaged (20,21). This suggests that for seriously ill patients who cannot tolerate invasive procedures, future high-throughput sequencing of peripheral blood samples may be an alternative and, to some degree, a marker for disease severity (22,23,46). Apart from simply identifying fungi, the application of mNGS also revealed mixed pulmonary infections in a single assay, and co-pathogens, such as the new coronavirus SARS-CoV-2, *Mycobacterium tuberculosis*, and *Human cytomegalovirus* were previously detected with one or more fungal species by mNGS (24,25,47,48). Mover, the *Human Cytomegalovirus* is the most common co-infected pathogens of *Pneumocystis jirovecii* infections (19,24). Therefore, mNGS is a promising technology to detect co-pathogens of mixed infection and provides information to improve culture conditions and develop a reasonable antifungal plan.

mNGS for diagnosis of Cryptococcus spp.

Cryptococcal meningitis (CM) occurs primarily in immunocompromised individuals, particularly those infected with HIV. There are over one million new cases of CM every year globally, among which about 600,000 people die within three months of infection (49). The *Cryptococcus neoformans/Cryptococcus gattii* species complexes dominate, leading to over 90% of human cryptococcosis cases. However, significant differences between the two are found in epidemiology, clinical manifestations, progression, and treatment strategies. In HIV-negative patients, *C. gattii* s.l. has a higher proportion of neurological complications, low response to antifungals, long-term therapies, and more surgical interventions (50,51). At present, traditional laboratory diagnostic methods, including India ink staining and cryptococcal antigen (CrAg) detection in CSF, cannot distinguish the two. Although l-Canavanine glycine bromothymol blue (CGB) agar and MALDI-TOF-MS can be used to differentiate *C. neoformans* and *C. gattii*, they must be based on a successful culture (52). The mNGS has been confirmed to be effective in identifying *C. neoformans* and *C. gattii*, which could reduce the misdiagnosis of CM in immunocompetent patients, promote the accurate treatment of central nervous system (CNS) infections, and considerably reduce the abuse of antifungal agents and resistance to fungi (26,53).

Cryptococcal osteomyelitis is very rare. Since X-rays are not specific and serum CrAg show low sensitivity, the diagnosis usually depends on culture or biopsy histopathology. However, negative culture results are not uncommon in clinical practice (54). Zhang *et al.* reported that an HIV-negative patient with an intact immune system, initially wrongly diagnosed as a soft tissue tumor of ribs, was eventually identified as a case of cryptococcal osteomyelitis via mNGS (55). Given that mNGS is based on the unknown to the known screening process, it often provides significant diagnostic information when faced with an atypical clinical presentation. Yet, it is simply used as an auxiliary means for traditional pathogenic diagnosis at present, due to limited reporting of mNGS applications in cryptococcosis and small sample sizes (27,28,53,56).

mNGS for diagnosis of Histoplasma capsulatum

Histoplasmosis is an endemic disease that mainly occurs in North America (especially the Midwest and Southeastern United States). It is generally asymptomatic or self-limiting, but may also cause severe symptomatic disease. For example, disseminated histoplasmosis is a progressive extrapulmonary disease that can be life-threatening if not treated. Therefore, a rapid diagnosis will make it possible to detect and curb infectious outbreaks at an earlier stage, saving lives and reducing medical costs (57). However, when the disease occurs in non-endemic areas, clinicians are often unaware of it, leaving them neglected and misdiagnosed (58).

Although the microscopy and culture are still recognized as the golden standards for the diagnosis of histoplasmosis, the morphology of *H. capsulatum* is similar to that of pathogens such as *Talaromyces marneffei* and *Leishmania* under a microscope, which frequently confuses inexperienced lab technicians (59). The detection of galactomannan antigen in body fluids provides a rapid and

sensitive method for the diagnosis of histoplasmosis and was included in the second edition of the WHO Basic in Vitro Diagnostic List in 2019 (60). The rate of antigen detection in patients with progressive disseminated histoplasmosis or acute pulmonary histoplasmosis is as high as 80% to 95%, but cross-reactions occur most commonly in patients with blastomycosis (90%), penicilliosis marneffei (80%), coccidioidomycosis (60%), and aspergillosis (10%) (61).

The mNGS is a valuable tool for detecting pathogens in non-endemic areas of endemic diseases such as histoplasmosis, and reducing the misdiagnosis and missed cases, especially in cases where clinicians are not initially aware of the disease. For example, mNGS was applied to BALF specimens from an individual originally diagnosed with tuberculosis in China, and ultimately identified as *H. capsulatum* (62). Recently, Zhang *et al.* performed mNGS on blood and bone marrow specimens from five patients infected with *H. capsulatum*, *Leishmania*, or *T. marneffei* but presenting similar clinical symptoms. They found that mNGS had 100% diagnostic accuracy, remarkably higher than that of traditional methods including bone marrow smear, microscopy, and culture (29). As well, direct pathogen identification from blood samples makes mNGS a less invasive option for patients with contraindications to bone marrow puncture. Moreover, during the follow-up period, it was detected in three patients that the decrease in the abundance of sequencing reads was consistent with the clinical recovery stage. In other words, mNGS would be expected to monitor disease progression and assess therapeutic effectiveness, a common practice in clinical work, despite limited data and the need for a lot of time to confirm (18,63,64).

mNGS for diagnosis of Aspergillus spp.

Invasive pulmonary aspergillosis (IPA) is a potentially fatal opportunistic infection that usually occurs in patients with hematological malignancies. The 2016 American Society of Infectious Diseases Diagnostic and Management Practice Guidelines for Aspergillosis stated that for certain adults and children (hematological malignancies, HSCT) patients, it is recommended to detect galactomannan (GM) in serum and bronchoalveolar lavage (BAL), as an accurate marker for the diagnosis of IPA (65). Recently, the number of cases of IPA in non-neutropenia patients, especially those with chronic obstructive pulmonary disease (COPD), has been increasing (66). Unfortunately, for this group of people, the diagnosis of IPA is usually more difficult, because circulating biomarkers show a relatively low sensitivity (67). Three cases of severe pneumonia, two of which had a history of COPD and asthma, were reported and identified as *Aspergillus fumigatus* by mNGS in BALF samples, further emphasizing the diagnostic role of mNGS in non-neutropenic IPA (68). Interestingly, Ge *et al.* described a patient whose clinal and radiological characteristics overlapped with the IPA, serum β-D-glucan was positive, and sputum culture mimicked *Aspergillus fumigatus*, but who was eventually identified as *Nocardia Gelsenkirchen* in both the bronchoalveolar lavage culture and mNGS results (69). It has been shown that mNGS can sometimes assist in differentially diagnosing IPA when the results of traditional tools appear confusing (30,31,70).

In addition, mNGS has important advantages in identifying unknown, rare, and atypical pathogenic microorganisms, which facilitates the discovery of novel disease-causing fungi in humans. For the first time, mNGS helped Dai *et al.* to save a 36-year-old woman by the timely detection of *Aspergillus flavus* leading to a rare but lethal fungal endocarditis (71). Wilson and his colleagues successfully resolved seven cases with diagnostic challenges for chronic meningitis with the help of mNGS and reported the first case of CNS vasculitis resulting from *Aspergillus oryzae* (27). Furthermore, in thirty Bell's palsy (BP) cases, Chang *et al.* discovered human herpesvirus 7 (HHV-7) and *Aspergillus* through analyzing the results generated by mNGS in samples obtained from the facial nerve epithelium, suggesting that more attention be paid to both pathogens in the pathogenesis of BP (32).

mNGS for diagnosis of Candida spp.

Invasive candidiasis is the most common fungal disease in intensive care unit (ICU), accounting for 70–90% (72). Once considering fungal infections, they often use specific diagnostic methods to screen *Candida* species at first. Moreover, the rapid development of diagnostic technology based on the genetic sequence of known pathogens often works well. Consequently, the need for mNGS in candidiasis may be less urgent. *Candida* spp. are often reported as strains of colonization or mixed infection strains occurring in the preliminary report generated by mNGS, which requires further interpretation by clinicians. When conventional diagnostic tools fail to identify the pathogen, clinicians can turn to mNGS for help in diagnosis. For instance, when confronted with a negative culture result in a chronic disseminated candidiasis case, Jin *et al.*

successfully identified the pathogen-*Candida tropicalis* via mNGS and it was further confirmed by calcofluor white staining (73). Additionally, Wilson *et al.* reported the fourth case of meningitis caused by *Candida dubliniensis* using mNGS technology, whereas the two 18S rRNA and 16S rRNA PCR tests in cerebrospinal fluid were negative (27). Importantly, mNGS is written into the Chinese consensus on the diagnosis and management of adult candidiasis, providing a basis for the etiological diagnosis of difficult or rare infectious diseases (74).

Challenges for mNGS in the diagnosis of IFDs

While many studies and case reports have confirmed that the success of mNGS in improving the diagnosis of IFDs, mNGS has not been included in the revised EORTC/MSG definitions (75,76) because of its limited standardization and validation. Several challenges remain in the routine implementation of mNGS technology in IFDs. Firstly, mNGS assays cost several hundreds to thousands of dollars per analytical sample, more than that for many other clinical tests, which is one of the main factors limiting their widespread use in the clinic (4). Secondly, although it has been reported that mNGS has superior fungal sensitivity and specificity to culture and histopathology (33,41,77), the sensitivity of mNGS depends heavily on the background and decreases in the high background, mostly from the human host or microbiome (4,67). In human samples, pathogen sequencing reads account for only a small portion of all mNGS results, while over 95% of sequencing results indicate human reads (11,78). As a result, removing human DNA sequences to enrich pathogen reads is a major direction in mNGS for microbial diagnosis (79). Ji *et al.* proposed an approach to effectively reduce host DNA contamination in CSF samples by collecting saponin-treated supernatant for DNA extraction, which has greatly improved the unique mNGS reads of *Cryptococcus* ($P<0.01$) (80). Meanwhile, the specificity of mNGS is commonly limited by contamination with DNA fragments from various microorganisms on the surfaces of reagents and consumables (10,12,48). A more stringent reporting threshold would be appropriate to increase specificity. Scoring algorithms such as Z-SCORE or SIQ-SCORE were designed in an attempt to separate sequences from the pathogen from those of environmental microbial sequences, which greatly simplifies the data interpretation (27,81,82). Thirdly, mNGS showed poor efficiency in extracting nucleic acid from pathogenic microorganisms with thicker cell walls, such as fungi (83). The efficient extraction method is a key step in achieving truly impartial sequencing of a sample because fungi need significant disruption of the cell walls to efficiently lyse the organisms for nucleic acid release. Some researchers have optimized the extraction conditions of *Aspergillus* RNA, while preserving the detection of bacterial and viral nucleic acid by mNGS (34). Lee *et al.* reported a simple and reproducible method extracting high molecular weight (~20 kb) genomic DNA from filamentous fungi for use in next-generation sequencing (NGS) (84). Last but not least, detection of nucleic acids by the mNGS itself does not prove that an identified microorganism is responsible for the disease, and results should be interpreted in the clinical context (4,7,77). When using the mNGS test, it is best to consider the findings in conjunction with other diagnostic tests and clinical feature.

Summary

mNGS can be used for samples from multiple sources, identifying unknown, rare, and newly emerging pathogens, distinguishing mixed pathogenic microorganisms, and subsequently narrowing them down to a certain level of genus. It could help guide treatment decisions for a group of people who are intolerant to invasive operations, immunosuppressed, or critically ill. An ideal diagnostic method should include the following characteristics: the use of non-invasive biological specimens, high precision, good reproducibility, and short processing time. Furthermore, the related technology should be easily available and low-cost, thus it can be promoted in many places. However, mNGS has certain limitations in terms of the diagnosis of IFDs, so it is only used as an auxiliary diagnostic tool for traditional detection methods. In the near future, with the gradual determination of unified standard procedures or interpretation standards, improved nucleic acid extraction schemes, reduced turnaround time, and substantial cost reductions, mNGS is expected to become a routine diagnostic method of fungal infections.

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Footnote

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Supplementary

Table S1 Single case of IFDs reported using mNGS

Year	Species	Samples Types	mNGS platform	Traditional methods	Results	No. of reads, median	Most common co-infecting pathogens	Ref.
2020	*Pneumocystis jirovecii*	BALF	n/a	Blood and sputum culture, nasopharyngeal aspirate respiratory virus antigen and PCR detection	Only mNGS was positive	1,007	None	(40)
2020	*Pneumocystis jirovecii*	BALF	BGISEQ-50	Smear, culture, GM test, G test	Only mNGS was positive	66	*Aspergillus fumigatus, Rhizopus oryzae*	(41)
2020	*Pneumocystis jirovecii*	BALF	MGISeq 2000	Microscopy of stained sputum, BALF smear specimens, various antibody and culture	The mNGS and Gomori methe-namine-sliver staining of the lung biopsy were positive	1,665,693	None	(42)
2019	*Cryptococcus neoformans*	Biopsy of the rib	n/a	G test and GM test, T-SPOT, HIV test, culture, Gram staining and acid-fast staining smear, CrAg detection, pathology with staining	CrAg detection and pathology were conducted after the positive mNGS results	47	None	(55)
2020	*Histoplasma capsulatum*	Epiglottis tissues, BALF	n/a	Culture, microscopy, serology tests for HIV and *Mycobacterium tuberculosis*, T-SPOT.TB, Xpert MTB/RIF assay	Only mNGS was positive	n/a	None	(62)
2021	*Aspergillus fumigatus*	Serum	n/a	MRI, blood cultue, CT, serum GM, CrAg detection	m NGS was the first positive	n/a	None	(70)
2021	*Aspergillus flavus*	Blood	Nextseq550 platform	Culture, histopathology	mNGS and culture were positive	n/a	n/a	(71)
2021	*Candida tropicalis*	Liver puncture	n/a	G test, culture, histopathology, calcofluor white staining	G test, calcofluor white staining and mNGS were positive	50	None	(73)

IFDs, invasive fungal diseases; n/a, not available; BALF, bronchoalveolar lavage fluid; CrAg, cryptococcal-antigen; G, 1,3-beta-D-glucan; GM, galactomannan.

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