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Review Article

Metagenomic Next-generation Sequencing: Application in Infectious Diseases

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Abstract

Infectious diseases have always been a difficult clinical problem, especially severe infections and those infections of unknown origin. The invention of a new detection method is particularly important and urgent. Metagenomic next-generation sequencing (mNGS) is a high-throughput sequencing method that sequences microbial DNA and RNAs from fluid or solid tissue samples in hours. All important sequence data that is present in a specimen can be used for pathogen identification after a series of bioinformatics analysis. Recently, mNGS has been used in preclinical trials to find and identify pathogens from the respiratory system, central nervous system, bloodstream, and other infections. mNGS technology has advantages of being faster, accurate, and detection of unknown pathogens over conventional laboratory methods for microbial identification and detection of antimicrobial resistance and virulence markers. However, mNGS has limitations that include human source DNA and RNA removal, intracellular bacteria extraction difficulties, and background pollution. mNGS technologies are innovative methods, especially when a bacterial culture is negative; however, a comprehensive collection of clinical evidence is required before they move from research into clinical laboratories.

Introduction

Infectious diseases involve all systems in the body and have a high incidence in all clinical departments, which lead to sepsis, infectious shock, and even death in serious cases.[1,2] The ability to quickly identify the focus of the infection and pathogenic bacteria in patients and use targeted antibiotics is essential in treatment. Bacterial culture, serological immunology, and nucleic acid examination are widely used in clinical pathogenic microbiological identification; however, they all have different limitations, such as sensitivity, specificity, and detection cycle. In some hospitals, rare and unknown microorganisms are often not identified or cannot be identified quickly and accurately. Recently, metagenomic next-generation sequencing (mNGS) has been developing rapidly and has gradually been applied in medical practice. Compared with the first generation of sequencing technology, mNGS, which is a high-throughput technology, directly measures the millions of nucleic acid DNA's sequences in a variety of specimens (Fig. 1).[3,4] According to the sequences obtained, which are compared with database information, the pathogenic bacteria can be identified. Therefore, mNGS is a faster and more accurate method than traditional laboratory microbial detection methods that provide greater assistance in the clinical diagnosis and treatment of patients with infectious diseases. The use of genetics and genomics to promote the study of infectious diseases is an important part of precision medicine, which could be the direction of development in the future; therefore, diseases could be studied at the genetic level.

mNGS

Fleischmann *et al.*[5] completed the first full sequence determination of the genome of *Haemophilus influenzae* that used gene sequencing technology in the 1990s. In 2008, three Australian transplant patients who received different organs from the same donor died successively from encephalopathy-related diseases from the do-

Keywords: Next-generation sequencing; Metagenomic next-generation sequencing; Infection.

Abbreviations: mNGS, metagenomic next-generation sequencing; BALF, bronchoalveolar lavage fluid; CPF, cystic pulmonary fibrosis; FTLS, fever, thrombocytopenia and hyperleukocytosis syndrome; HNF virus, Henan fever virus; PCR, polymerase chain reaction.

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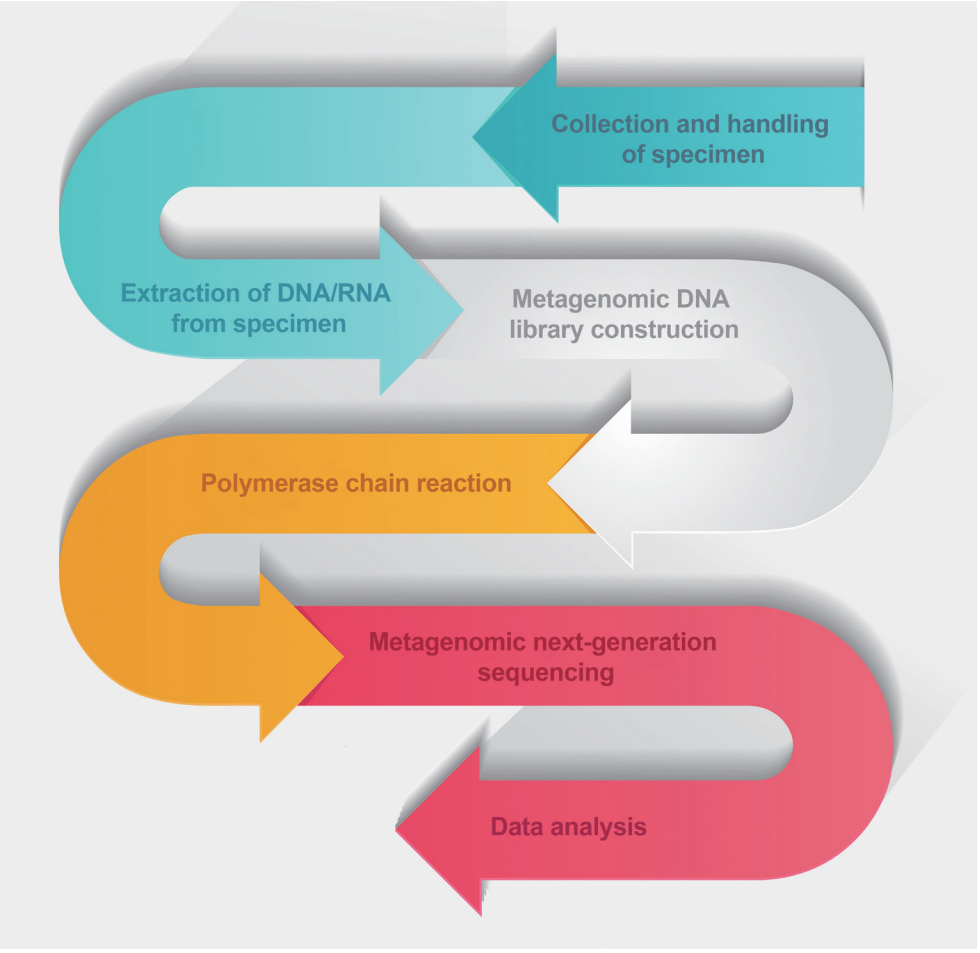

Fig. 1. Flow chart for mNGS. This flowchart describes the basic steps of mNGS. mNGS: metagenomic next-generation sequencing.

nor. The pathogens were not detected by conventional pathogenic, immunological, or molecular biological means. Partial tissue samples from the donor and recipient were analyzed by next-generation sequencing (NGS) technology, both suggested the presence of an arenavirus infection, which was later confirmed by other means.[6] In 2010, a whole-genome sequence analysis based on NGS data from the cholera outbreak in the Haiti earthquake showed that the *Vibrio cholera* that triggered the outbreak was not of local origin, but was more closely related to a strain isolated from Asia and later confirmed by single nucleotide polymorphism analysis to be of Nepalese origin.[7,8] In addition, NGS provides strong evidence to control the spread of pathogenic bacteria, guiding the use of antimicrobials, and analyzing the drug resistance and virulence characteristics of bacteria.[9–14] Recently, due to the continuous technological development of NGS (Table 1), it has shown unique advantages and broad application prospects in infectious agent detection, pathogen biological characterization, and molecular epidemiological analysis.[15,16]

Application of metagenomic NGS in infectious diseases

Infection of the respiratory system

Respiratory system infections are extremely prevalent, and through chest imaging, bacterial culture, and smears, and many patients remain with unclear infection pathogens and insensitivity to the selected antibiotics. Some patients with the rapidly progressive disease develop life-threatening respiratory failure and infectious shock. mNGS detects pathogens from specimens such as bronchoalveolar lavage fluid (BALF), lung puncture/mediastinal lymph node puncture/tracheal biopsy tissue, pleural effusion, and sputum.

BALF specimens

BALF is obtained by sampling during bronchoscopy. Previously, BALF from healthy humans was considered an aseptic specimen; however, with the continuous development and improvement in the level of testing, there is a bacterial community in the lung, which becomes the microbiome, and the microbiome of diseased lungs is distinctly different from that of healthy lungs.[17] In 2018, Miao *et al*.[18] found causative pathogens in 34% of BALF that was obtained from infected or noninfected patients. Takeuchi *et al*.[19] performed mNGS on BALF from 10 children with respiratory failure and detected significant bacterial or viral sequences in 8 children, and eventually identified the pathogens in 3 children, which were difficult to identify by conventional methods. In addition, several cases and small sample studies of second-generation sequencing have successfully detected the causative pathogens, which led to

Table 1. Sequencing platforms for the detection of pathogens

Sequencing Platforms	Principle	Advantage	Disadvantage
Solid 5500 × I[15]	Detect fluorescent signals based on sequential ligation of fluorescent probes	Realization of double base correction, proofreading while reducing initial data errors	Long sequencing time, partial read length and reference sequence cannot be matched
Illumina[16]	In PCR, a DNA fragment is attached to a primer for the reaction	Short sequencing time, low cost and better uniformity	Short sequencing read length, sequencable sample size is limited
Ion Torrent	The technology is based on the use of semiconductor materials, semiconductor materials detect the release of H^+ protons when DNA synthesis	Short sequencing time (2.5–4 h), low cost	Single-end sequencing
BGI	Sequencing while connecting, combinatorial probe anchor synthesis	Low cost, large amount of data, large-scale whole-genome sequencing	Read length is short (maximum read length 50–100 bp), poor operability of gene splicing

PCR: polymerase chain reaction; BGI: Beijing Genomics Institution.

the recovery from the disease with targeted treatment.[20–22]

Lung puncture tissue specimens

Sometimes, BALF might be contaminated by pharyngeal colonizing bacteria from surgical operations and lung puncture tissue is less probable to be contaminated, which makes it more necessary and diagnostically effective, but relatively risky. Henan *et al*.[23] used mNGS to identify potential pathogens in lung puncture tissues from 15 pulmonary infections, and the results showed that the sensitivity and specificity were 100.0% and 76.5% for bacteria and 57.1% and 61.5% for fungi, respectively. mNGS compared with sputum culture, and the positive predictive values (42.9% for bacteria and 44.4% for fungi) were significantly lower than the negative predictive values (100% for bacteria and 72.7% for fungi).[23]

Sputum specimens

Sputum is the commonly used specimen that is checked for respiratory infections by smear or bacterial culture; however, sputum specimens are usually exposed to oropharyngeal colonizing bacteria and might become contaminated; therefore, mNGS is used when patients cannot tolerate bronchoscopy or other invasive procedures. In 2017, Feigelman *et al*.[24] performed mNGS on sputum specimens from 6 cases of cystic pulmonary fibrosis (CPF), which provided a profile of the pulmonary pathogens of the disease and analyzed the function, classification, and drug resistance of the dominant pathogenic bacteria.

Pleural effusion specimens

There are more etiologies of pleural effusion, including infection, tumor, hypoproteinemia, and heart failure connective tissue disease. In addition, mNGS can be used to identify the pathogens in the infectious specimens and indicate the sensitive antibiotics.

Infections of the central nervous system

Neurological infectious diseases, such as encephalitis or meningitis, often have unclear etiology in cerebrospinal fluid (CSF) and might be caused by pathogenic infections; however, there are many potential pathogens (bacteria, viruses, fungi, or parasites). Traditional testing, such as routine examination, biochemical examination, and bacterial culture often failed to detect them or miss them. Guo *et al*.[25] found that the diagnostic sensitivity of bacterial meningitis increased from 55.6% to 68.7% when mNGS was used. In a case report published by Hu *et al*.[26] a 31-year-old HIV-infected patient admitted to hospital in a critical condition with a Glasgow score of 3 was tested for *Toxoplasma gondii* by mNGS on CSF. Several cases have been reported in which the causative organism was found by mNGS of CSF.[27]

Infection of the bloodstream

Infectious diseases of the blood have a high mortality rate, such as infective endocarditis, with an annual incidence of 3:100,000 to 1:10,000, and one-third of these patients died 1 year after diagnosis. The key to the treatment of infective endocarditis is the ability to rapidly identify the pathogen. Approximately 31% of patients have negative blood cultures due to early application of antibiotics or other factors, and these blood culture-negative infective endocarditis often pose multiple diagnostic and therapeutic difficulties that cause significant harm to patients.[28] In 2018, Cheng *et al*.[29] performed mNGS testing on blood specimens that were collected from seven patients with suspected infective endocarditis and confirmed that mNGS was helpful in the diagnosis and treatment of patients with infective endocarditis despite the negative blood bacterial cultures. In addition to improving the diagnostic efficacy of the 3 days routine culture, mNGS helps to explore novel and unknown pathogens. From 2007 to 2010, a large number of cases of fever, thrombocytopenia, and hyperleukocytosis syndrome (FTLS) occurred in Henan Province. Xu *et al*.[30] performed mNGS on the sera of patients and found that this was a new bunyavirus, eventually named Henan fever virus (HNF virus). However, bacterial nucleic acids have been reported in healthy volunteers tested by mNGS, which could be potential contamination of the sampling process, and therefore, requires careful screening in clinical applications.[31]

Infections of the digestive system

Acute cholecystitis, acute gastroenteritis, and liver abscess are

common infections of the digestive system. In 2018, Wu *et al*. performed mNGS on ascites and blood samples from 97 patients with acute abdominal disease; however, the bacteria analyzed by mNGS only partially matched those isolated using traditional culture methods and concluded that mNGS provided limited detection abilities of pathogens in ascites and blood specimens from patients with the acute abdominal disease.[32] In a study on acute cholecystitis, metagenomic analysis was effective for rapid the diagnosis of the causative agent of acute cholecystitis, which included the assessment of potential antibiotic susceptibility.[33] In addition, mNGS is useful for the detection of infectious diarrhea pathogens.[34]mNGS was used to study the association between gut microbial alterations with type 2 diabetes and the association between atherosclerosis with the intestinal metagenome, because of the advanced nature of this technology.[35,36]

Infections of the ophthalmology

Diagnosis of ocular infections relies on intraocular fluid culture or polymerase chain reaction (PCR). In 2012, Eleinen *et al*. subjected corneal scraping samples that were collected from 88 patients with infectious corneal ulcers to bacterial culture, fungal culture, Gram stain, potassium hydroxide wet mount, and broad-range PCR with primer pairs targeted to the 16S (bacterial) and 18S (fungal) rRNA genes. The results showed that for bacterial and fungal keratitis, the sensitivity of the culture was 57.58% and 59.09%, respectively and the PCR sensitivity was 87.88% and 90.91%, respectively.[37] In 2017, Doan *et al*. detected PCR-positive and PCR-negative specimens from intraocular infections by mNGS and concluded that the mNGS results were in good agreement with the PCR results and mNGS could be used to detect bacteria and fungi and viruses and provided information on drug resistance.[38]

Infections of the urinary system

Urinary tract infections have a high incidence and recurrence rate,[39] and diagnosis mainly relies on urine culture. The most common pathogenic bacteria is *Escherichia coli*, followed by other Enterobacteriaceae, staphylococcus, streptococcus, fungi, and some pathogens that are difficult to cultures, such as viruses and tuberculosis.[40] Moustafa *et al*.[41] found that it was difficult to identify a large number of microorganisms in urine using traditional culture methods and that mNGS provided a more comprehensive and quantitative analysis of the microbial community in urine.[41] Barraud *et al*.[42] demonstrated that mNGS provided drug resistance information in addition to the analysis of the microbiota in urine. In addition, mNGS simultaneously detects viruses, fungi, *Candida albicans*, cryptococcus, *Mycobacterium tuberculosis*, and mycoplasma in urine specimens and the virulence and drug resistance of pathogens.[43–46]

mNGS versus traditional detection methods

The cost of traditional detection methods is lower and these methods are mature and relatively simple to operate. The sensitivity and specificity of traditional detection methods are poor, and they require a long detection time.

mNGS has the following advantages over traditional detection methods: (1) there is no bias, for example, if a specimen has *C. albicans* and *Pneumocystis sp*., faster growth of *Pneumocystis sp*. will affect the growth of *C. albicans*. Therefore, pathogenic cultures are biased and mNGS is unbiased for pathogenesis due to its different detection mechanism; (2) for infectious diseases, clinical symptoms, signs, history, epidemiology, and risk factors are used to infer which pathogens are probably present and to select the appropriate tests, but incomplete information in any one of the tests could lead to missed pathogens. Theoretically, mNGS detects the gene sequences of all pathogens in a specimen, and therefore, has broader coverage than other testing solutions; and (3) mNGS detects multiple pathogens simultaneously and in a shorter time.

Limitations of mNGS application

Despite the increasing clinical use and continuous enhancement of mNGS technology, the limitations or drawbacks of mNGS remain a major issue that cannot be ignored. The main disadvantage of mNGS is that most nucleic acids in patient specimens predominantly originate from human host backgrounds, with the majority of detected sequences (typically >99%) coming from human hosts, which limits the overall analytical sensitivity of pathogen detection due to the relatively low number of sequenced microbial detected sequences (reads). This drawback is dealt with by targeted sequencing.[47] In addition, other disadvantages include nucleic acid contamination in reaction kits, or from colonized bacteria and laboratory procedures. Further research and optimization are required for mNGS sequencing depth, bioinformatics analysis, and clinical interpretation.[25]

The mNGS test cannot distinguish background, colonized microbes from pathogenic organisms, and requires a clinician to decide based on the patient's clinical manifestation, combined with other laboratory examination results.

Other limitation of mNGS use includes relatively low sensitivity for the detection of intracellular infected bacteria (e.g., *M. tuberculosis*, *Legionella sp*., and *Brucella sp.*), thick-walled bacteria and fungi, due to their rare release into body fluids. In addition, for pathogens with thick cell walls, such as Gram-positive bacteria, *M. tuberculosis*, fungi and parasites, the low efficiency of nucleic acid extraction results in lower positivity. RNA is susceptible to degradation and requires very low-temperature storage conditions that should be used in RNA sample transit and storage. Recently, several mNGSs have been reported for drug resistance detection, they are mainly for the study of individual pathogens, and difficulties remain when using mNGS to detect drug resistance genes in pathogenic bacteria simultaneously.

Future directions

Infectious diseases have always been a major threat to human life and health, and the continuous renewal of antibiotics has significantly reduced mortality. However, due to the development of human society, increase in human life expectancy, increase in malignant tumor prevalence, and the widespread use of glucocorticoids and immunosuppressants, infectious diseases have displayed the following characteristics: (1) an increase in the rate of severe infections; (2) an increase in rare or unknown pathogens; and (3) a significant increase in the virulence and drug resistance of pathogens. However, the widespread and early application of broad-spectrum antibiotics and because 20%–60% of human-associated microorganisms are not culturable,[48] leads to a low positive rate in traditional cultures. The failure to identify pathogens has increased

the difficulty in the diagnosis and treatment of infectious diseases, significantly prolonging the patient's treatment cycle and increasing morbidity and mortality. However, mNGS has disadvantages, such as it is expensive and might not provide a comprehensive or accurate report of antibiotic resistance, because of technical issues; however, due to the continuous improvement in the technology, mNGS remains a promising and practical means of clinical detection to help the clinical treatment of infectious diseases. The development of databases, the genetic profiling of rare pathogens, studies into the mechanisms of pathogens, and the establishment of standardized processes for mNGS require further research.

Conclusions

mNGS obtains nucleic acid sequence information on pathogens directly from the specimen without the need for bacterial culture to identify them. It is not influenced by the application of antibiotics and has higher sensitivity and specificity than traditional detection methods. In addition, because mNGS sequences millions of nucleic acids at one time it can detect bacteria, fungi, viruses, mycoplasma, chlamydia, and parasites in specimens simultaneously, which is not possible using other clinical detection methods.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

Study design (YL, QY), manuscript writing (YL), critical revision (JD). All authors have made a significant contribution to this study and have approved the final manuscript.

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