Review

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Clinical applications and challenges of metagenomic next-generation sequencing in the diagnosis of pediatric infectious disease

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Abstract: Infectious diseases seriously threaten the lives of children. Timely and accurate detection of pathogenic microorganisms and targeted medication are the keys to the diagnosing and treatment of infectious diseases in children. The next-generation metagenomic sequencing technology has attracted great attention in infectious diseases because of its characteristics such as no culture, high throughput, short detection cycle, wide coverage, and a good application prospect. In this paper, we review the studies of metagenomic next-generation sequencing in pediatric infectious diseases and analyze the challenges of its application in pediatric diseases.

Keywords: next-generation sequencing; infectious diseases; pediatric

Introduction

Infectious diseases pose a certain threat to the life and health of children. According to studies, 6.3 million children under 5 years of age died in 2013, of which 51.8 % (3,257,000) died of infectious diseases. Pneumonia, diarrhea, and measles were responsible for half of these deaths [1]. In China, infectious diseases were the leading cause of death in children under 5 years of age from 2010 to 2016, with pneumonia and sepsis accounting for up to 19 % of the causes of child mortality in 2016 [2]. Among newborns, preterm and very low birth weight infants are highly susceptible to infections because of their low immune function, resulting in high neonatal mortality [3].

Identification of early pathogenic microorganisms is particularly important for the diagnosis and treatment of infectious diseases, and studies have shown that mortality increases significantly with the increase in the duration of treatment administration [4]. The current traditional culture methods can easily be affected by antibiotics which result in long culture cycles. Moreover, many pathogenic microorganisms in the environment and humans are difficult to culture. Additionally, PCR detection techniques are limited to amplifying the gene sequences of known pathogenic microorganisms and cannot detect and diagnose new and rare pathogens. The detection of pathogens, whether based on bacterial culture or targeted molecular methods, relies on the clinical judgment of symptoms of infectious diseases and the empirical assessment of suspected pathogens in a clinical context. Therefore, there is a clinical need for more sensitive and accurate experimental techniques to complement traditional methods and to improve the detection rate of pathogenic pathogens for early and accurate drug administration.

Metagenomic next-generation sequencing (mNGS) is an unbiased, culture-free, high-throughput method that directly extracts deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) from clinical samples for high-throughput sequencing, and after comparison with pathogenic microbial databases and raw letter analysis, all pathogens such as bacteria, viruses, fungi, and parasites can be detected in clinical samples at one time, theoretically. It is especially suitable for the diagnosis of infectious diseases caused by complex, rare, atypical, and emerging pathogens [5] and has unique technical advantages and wide application prospects. Table 1 summarizes and compares the advantages and disadvantages of different laboratory diagnostic techniques. This review focuses on the clinical application of mNGS in different pediatric infectious diseases, emphasizing its indispensable role and value in the diagnosis and treatment of pediatric infectious diseases. Furthermore, it analyzes the current limitations of mNGS in clinical applications to provide guidelines for pediatricians to use this technology in the clinic.

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The etiology of respiratory infections in children is complex and diverse, encompassing viruses, bacteria, atypical pathogens, fungi, etc. [6]. In recent years, emerging and re-emerging respiratory pathogens such as novel coronaviruses and avian influenza viruses have posed serious threats to the health and lives of children. Accurate identification of the infecting pathogen is instrumental for the rational use of antimicrobial agents and for the early detection, isolation, reporting, and treatment of respiratory infectious diseases. Therefore, rapid and accurate etiological diagnosis is of significant importance for the clinical management and control of common respiratory infectious diseases.

**Application of mNGS in pediatric respiratory infections**

The etiology of respiratory infections in children is complex and diverse, encompassing viruses, bacteria, atypical pathogens, fungi, etc. [6]. In recent years, emerging and re-emerging respiratory pathogens such as novel coronaviruses and avian influenza viruses have posed serious threats to the health and lives of children. Accurate identification of the infecting pathogen is instrumental for the rational use of antimicrobial agents and for the early detection, isolation, reporting, and treatment of respiratory infectious diseases. Therefore, rapid and accurate etiological diagnosis is of significant importance for the clinical management and control of common respiratory infectious diseases.

**Use of mNGS technology in the diagnosis of pediatric respiratory infections has increased sensitivity for pathogen detection compared with traditional methods**

Wang et al. tested 32 alveolar lavage fluids from children with severe pneumonia, and no pathogens were detected in 15 of the traditional tests, whereas all 32 tests using mNGS detected pathogens, including *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *cytomegalovirus*, and *bocavirus* [7]. Takeuchi et al. showed that mNGS could be used for the diagnosis and epidemiological surveillance of pathogens involved in respiratory tract infections. The investigators enrolled 10 children with respiratory failure in the study, and their alveolar lavage fluid specimens were used for pathogen detection. In eight cases, pathogens with significant read numbers were detected, including *Stenotrophomonas maltophilia*, *M. catarrhalis*, and *human metapneumovirus*. In three cases, the pathogens were not detected using conventional methods, whereas a case of *human rhinovirus* and two cases of *enteric EV-D68 virus* were detected using mNGS [8]. In addition, the team’s study in 2020 found that viral and bacterial infections are common triggers for sudden deterioration of cardiopulmonary function in pediatric patients and that mNGS can help determine the etiology of sudden critical illness in pediatric patients. A total of 16 pediatric patients with sudden cardiopulmonary failure were enrolled; at least one bacterial or viral infection was detected in 10 patients, and *coxackievirus A6*, *Chlamydia sp.*, and *rhinovirus A49* were detected in four patients using mNGS, while conventional culture and viral antigen test results were negative [9]. Compared with other molecular diagnostic techniques, mNGS has several advantages. Slinporn et al. found that the advantage of mNGS in the diagnosis of respiratory viruses in children compared with real-time quantitative PCR technology is the immediate availability of viral typing information, which

**Table 1: The advantages and disadvantages of mNGS and traditional microbiological detection methods in pathogen diagnostics.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopic examination</td>
<td>1. Quick and convenient</td>
<td>1. Sensitivity depends on the pathogen load in the sample</td>
</tr>
<tr>
<td></td>
<td>2. Inexpensive</td>
<td>2. Cannot identify species</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Unable to detect certain pathogens like mycoplasma and chlamydia</td>
</tr>
<tr>
<td>Conventional culture</td>
<td>1. Moderate price</td>
<td>1. Affected by patient’s prior antibiotic use</td>
</tr>
<tr>
<td></td>
<td>2. Considered as the gold standard</td>
<td>2. Time-consuming</td>
</tr>
<tr>
<td></td>
<td>3. Antimicrobial susceptibility testing</td>
<td>3. Different pathogens require specific culture media and methods</td>
</tr>
<tr>
<td>Immunological</td>
<td>Moderate price, easy to test</td>
<td>4. Some pathogens are difficult to culture</td>
</tr>
<tr>
<td>Molecular testing (PCR)</td>
<td>1. Moderate price, easy to test</td>
<td>1. Antigen detection may have low sensitivity</td>
</tr>
<tr>
<td></td>
<td>2. Can be automated</td>
<td>2. Early infection may result in false negatives of antibody</td>
</tr>
<tr>
<td></td>
<td>3. Quantitative detection</td>
<td>3. Limited range of detectable microorganisms</td>
</tr>
<tr>
<td></td>
<td>4. High sensitivity and specificity</td>
<td></td>
</tr>
<tr>
<td>mNGS</td>
<td>No need to preselect target pathogens, can</td>
<td>1. Clinical interpretation of results can be challenging</td>
</tr>
<tr>
<td></td>
<td>detect new, unknown, and rare pathogens</td>
<td>2. Susceptible to interference from human DNA and commensal microorganisms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Complex workflow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Expensive</td>
</tr>
</tbody>
</table>

Guo and Zhang: Clinical applications and challenges of mNGS
can also be used as an additional complementary method when real-time quantitative PCR screening results are negative [10].

Use of mNGS in pediatric respiratory infectious diseases improves the odds of detecting rare pathogens and diagnosing mixed pulmonary infections

Min et al. describe a child infected with tuberculosis after being wounded. Two months after surgery, his wound was still swollen and painful. Secretions from the wound were sent for mNGS, which revealed three reads related to the Mycobacterium tuberculosis complex group (MTBC) [11]. The wound disappeared after anti-TB drugs were administered. This indicates that mNGS has a certain sensitivity in detecting M. tuberculosis infections. Besides, mNGS technology was used to identify rare pathogens, including Ureaplasma parvum and bocavirus, in 397 pediatric patients on mechanical ventilation. Moreover, 144 patients (52 %) had mixed infections with viral and bacterial pathogens [12]. Wang et al. showed that the sensitivity of mNGS in diagnosing mixed lung infections was 97.2 % in 55 pediatric patients with lung infections, compared with 13.9 % for conventional diagnostic methods [13], suggesting the advantages of mNGS in detecting mixed lung infection pathogens.

Potential antimicrobial stewardship advantages of using mNGS

Farnaes et al. identified 15 hospitalized children with community-acquired pneumonia without other underlying diseases and extracted patient plasma for mNGS, showing that the use of sequencing technology led to the detection of the relevant causative pathogen in 13 of 15 children (86 %), whereas the use of standard culture and PCR methods led to the identification of pathogens of interest in 47 % of the children. Seven out of 15 children (47 %) changed their antibiotic medication management as a result of the sequencing results [14]. In addition, data from mNGS can be used to further analyze respiratory diseases. Zhang et al. used mNGS to analyze more than 4,000 clinical specimens of children with acute respiratory infections and found that susceptible and polyvalent children had high rates of respiratory viral infections and high viral diversity, indicating higher respiratory susceptibility to viruses. In addition, plaque abundance of Propionibacterium spp. and serum levels of tissue inhibitor of metalloproteinases-1 and recombinant human platelet-derived growth factor-BB were strongly associated with multiple acute respiratory infections [15].

Application of mNGS in pediatric central nervous system infections

Central nervous system infections (CNSIs) are caused by multiple pathogens that result in central nervous system damage. In pediatric populations, due to the differences among various pathogens, central nervous system infections can present a range of symptoms, making pathogen identification crucial for guiding clinical treatment and characterizing prognosis. Currently, over 40 % of encephalitis patients remain undiagnosed for specific pathogens following routine clinical testing [16]. In bacterial meningitis, pre-treatment with antibiotics often leads to negative culture results. Although PCR testing is fast and highly sensitive, it depends on clinical presumptions. mNGS enables extensive screening of pathogens from a single sample, reducing the duration of the diagnostic process.

mNGS can be used to detect pathogens that are impossible or difficult to detect using traditional methods in the diagnosis of CNSIs

In 2014, Wilson reported a case of a 14-year-old child with bacterial meningitis and combined immunodeficiency who was treated after using mNGS. Sequencing and analysis of the patient’s cerebrospinal fluid revealed 475 Leptospira DNA fragments within 48 h, and the patient eventually recovered after prompt administration of penicillin treatment and was discharged from the hospital successfully [17]. This case initiated the application of mNGS in CNSIs. Maria et al. reported a case of a 13-year-old child with encephalitis in whom mNGS testing of the cerebrospinal fluid resulted in the detection of infection with Psychrobacter, a rare opportunistic pathogen in humans; it was the first report of Psychrobacter-associated meningitis, in which the investigators also performed whole-genome sequencing and identified associated virulence genes [18]. In a study by Wilson et al., Histoplasma capsulatum was detected using mNGS after repeated treatment failure in a 10-year-old patient with chronic meningitis, and after 2 months of treatment, he was able to walk independently; however, he had sequelae, such as partial hearing loss [19]. Kawada et al. studied 18 pediatric patients with encephalitis of unknown etiology and detected a large number of coxsackievirus and mumps virus sequences in the cerebrospinal fluid of three patients. They used mNGS, which was validated by PCR and detected a thin-ring virus in the serum of another...
patient, suggesting that mNGS is useful in detecting pathogenic viruses in pediatric patients with encephalitis/encephalopathy [20]. This indicates that mNGS can play an important role in the diagnosis of CNS diseases caused by rare pathogens. In addition to the detection of viruses or bacteria by mNGS, parasites in children with CNSIs have also been reported in the literature: Wu et al. [21], Greninger et al. [22], and Yang et al. [23] detected *Leishmania* spp. in cerebrospinal fluid specimens from patients using mNGS. Chen et al. detected *Angiostrongylus cantonensis* using mNGS. mNGS had a sensitivity of 73.1% for the diagnosis of *S. pneumoniae* meningitis, and the specificity was 88.1%. Moreover, the study also found a statistically significant difference between the read numbers of cerebrospinal fluid specimens within 14 days after onset and the read numbers of *S. pneumoniae* sequences within 14 days after onset [27]. This indicated that mNGS has a high sensitivity for *S. pneumoniae* identification and that the number of reads for *S. pneumoniae* detection correlated with the time of cerebrospinal fluid specimen collection. Ge et al. performed a single-center prospective study in neonates with CNSIs, which resulted in a significantly higher diagnostic power of mNGS samples (19.8%) than the conventional method (4.95%). In the empirically treated group, the detection rate of mNGS was significantly higher than that of the conventional methods (27 vs. 6.3%). It has also been shown that mNGS combined with whole-exome sequencing can significantly improve the etiological diagnosis rate and effectively guide clinical strategies in patients with suspected CNSIs [28]. In a recent study, Wang et al. showed that cerebrospinal fluid from 48 children was sent for both macro genomic mNGS testing and culture, and the pathogen detection rate of macro genomic mNGS was higher than that of culture methods (27 vs. 6.3%). The concordance between the results of cerebrospinal fluid macro sequencing and culture testing was 79.2% (38/48), in which the same pathogen was detected in 11 children who were positive at the same time. Using cerebrospinal fluid culture results as the gold standard, the sensitivity of macro genomic mNGS in the diagnosis of bacterial meningitis was 91.7%, and the specificity was 75.0% [29].

### Further application of mNGS in CNSI in children

In a prospective study of children hospitalized with meningitis, Liao et al. used mNGS technology for analysis and found differences between the microbiome characteristics of patients with meningitis, undiagnosed children, and healthy individuals. Changes in microbiome composition were associated with C-reactive protein levels in the blood and the percentage of neutrophils in the cerebrospinal fluid. In addition, patients with predominant *Klebsiella pneumoniae* subgroups were clinically monitored using blood samples instead of cerebrospinal fluid samples [30]. These results provide an important reference for

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**Table 2:** Application of next-generation sequencing technology to detect rare pathogens in pediatric with CNSIs.

<table>
<thead>
<tr>
<th>Author</th>
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<tr>
<td>Wilson [18]</td>
<td>Miseq</td>
<td>CSF</td>
<td><em>Leptospira</em></td>
<td>475</td>
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<td>Maria [19]</td>
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<td>1640</td>
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CNSI, central nervous system infection.

In children with CNSIs, mNGS has increased sensitivity for detecting pathogens compared with conventional methods

Liu et al. examined the cerebrospinal fluid of 99 children with bacterial meningitis and showed that the detection rate of pathogens was increased from 55.6 to 68.7% using the mNGS technique compared with the conventional method of culture plus antigen detection, wherein *Streptococcus agalactiae* was detected in 15 cases using the mNGS technique, whereas only 11 cases were detected using the conventional method [26]. In their study of the significance of mNGS in the diagnosis of bacterial meningitis caused by *S. pneumoniae* in children, Zhang et al. found that a total of 26 (19.3%) of 43 patients with jointly confirmed *S. pneumoniae* infections were detected using conventional methods, and 32 (23.7%) patients were detected using mNGS. mNGS had a sensitivity of 73.1% for the diagnosis of *S. pneumoniae* meningitis, and the specificity was 88.1%. Moreover, the study also found a statistically significant difference between the read numbers of cerebrospinal fluid specimens within 14 days before sudden onset and the read numbers of *S. pneumoniae* sequences within 14 days after onset [27]. This indicated that mNGS has a high sensitivity for *S. pneumoniae* identification and that the number of reads for *S. pneumoniae* detection correlated with the time of cerebrospinal fluid specimen collection. Ge et al. performed a single-center prospective study in neonates with CNSIs, which resulted in a significantly higher diagnostic power of mNGS samples (19.8%) than the conventional method (4.95%). In the empirically treated group, the detection rate of mNGS was significantly higher than that of the conventional methods (27 vs. 6.3%). It has also been shown that mNGS combined with whole-exome sequencing can significantly improve the etiological diagnosis rate and effectively guide clinical strategies in patients with suspected CNSIs [28]. In a recent study, Wang et al. showed that cerebrospinal fluid from 48 children was sent for both macro genomic mNGS testing and culture, and the pathogen detection rate of macro genomic mNGS was higher than that of culture methods (27 vs. 6.3%). The concordance between the results of cerebrospinal fluid macro sequencing and culture testing was 79.2% (38/48), in which the same pathogen was detected in 11 children who were positive at the same time. Using cerebrospinal fluid culture results as the gold standard, the sensitivity of macro genomic mNGS in the diagnosis of bacterial meningitis was 91.7%, and the specificity was 75.0% [29].

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CNSI, central nervous system infection.
the clinical monitoring and antibiotic therapy of patients with meningitis. Liu et al. reported the detection of *Human herpes virus type 1* using mNGS in an 18-month-old child with encephalitis and the possibility of semi-quantitative observation of therapeutic efficacy using mNGS [31]. A prospective multicenter study was conducted to investigate the value of mNGS in the identification of cerebrospinal fluid pathogens and host transcription analysis by including 71 children with CNSIs. The positive predictive concordance of mNGS was 80 %, and the negative predictive concordance was 91.1 %. The team then performed bioinformatics transcriptome analysis to conclude that interleukin-1β was effective in identifying patients with bacterial infections, and chemokine (C-C motif) ligand eight was effective in identifying patients with viral infections [32].

**Application of mNGS in pediatric suspected bloodstream infection**

Bloodstream infection (BSI) is a process in which pathogens enter the human bloodstream and multiply to release a variety of metabolites and toxins, resulting in symptoms such as infectious toxicity and severe inflammatory metaphasia. In critically ill patients, BSI is a life-threatening complication. In children, especially neonatal patients, because of the imperfect development of the autoimmune function, it can rapidly spread to multiple organs once the disease develops, and the clinical mortality rate is high [33]. Therefore, early and rapid diagnosis and selection of sensitive antimicrobial drugs are crucial to reducing the mortality rate associated with BSIs in children. Blood culture is the gold standard method for the diagnosis of BSI in clinical laboratories; however, the process of blood culture takes a long time, and some slow-growing bacteria require a culture time of >7 days, which may delay the early treatment of children. The use of mNGS significantly shortens the treatment time because it does not require bacterial culture, reduces the volume of blood drawn from children by using plasma-free DNA as the specimen, and compensates for the disadvantage that some caustic bacteria cannot be cultured owing to their rigorous culture conditions; thus, mNGS provides a new treatment method for children with clinical BSIs.

**Improved detection of pathogens in patients with BSI using plasma-free DNA for mNGS**

Goggin et al. used mNGS to predict the occurrence of BSI in children with recurrent or refractory tumors. The results revealed that circulating microbial cell-free DNA (mcf-DNA) had a sensitivity of 75 % for predicting BSI in children 3 days before infection and a sensitivity of 80 % for predicting bacterial BSI. It was confirmed that clinically relevant pathogens can be identified using mcf-DNA sequencing during the days before the onset of most BSI, which may make preventive treatment possible [34]. In eight of 12 pediatric patients with confirmed BSI, Horiba et al. detected dominant organisms using NGS, consistent with blood culture results. In addition, pathogenic bacteria were detected in the plasma of two patients with catheter-associated BSI 7 days before onset, and pathogens, including adenovirus, were identified in three patients with suspected BSI. The investigators concluded that mNGS is a new method for identifying pathogenic microorganisms in BSI and can predict the occurrence of BSI in some patients [35]. Bo et al. enrolled 25 children with clinically suspected BSI, and their blood specimens were retained for blood culture and mNGS pathogen detection. The blood culture result was positive in one case, whereas mNGS yielded positive results in a total of 13 cases, 10 of which were consistent with clinical symptoms; they all recovered and were discharged after adjustment of treatment. This indicates that macro genic mNGS can help in the pathogenic diagnosis of BSI in febrile children [36]. Gangfeng et al. performed mcf-DNA mNGS in 34 children in the pediatric intensive care unit (PICU) and showed the presence of potential eukaryotic microbial pathogens in children’s blood samples. Among them, the abundance of *Pneumocystis jirovecii* was positively correlated with a reduced total leukocyte count and immunodeficiency. Patients with hospital-acquired pneumonia had significantly increased blood bacterial species abundance compared with children with community-acquired pneumonia. Bloodstream bacterial abundance was positively correlated with serum calcitonin levels. Microbial genome sequences of potential pathogens were detected in the blood of children with suspected sepsis in the PICU, suggesting the presence of BSI in these children [37].

**Application of mNGS in other infectious diseases**

**Application in children with immunodeficiency**

Infections are a major cause of morbidity and mortality in patients with immunodeficiency diseases. A timely and accurate microbiological diagnosis is particularly important for these patients. Studies have suggested that mNGS can identify infections, especially fungal infections, in immunodeficient children on time.
mNGS plays an active role in the treatment of infectious diseases in children with immunodeficiency. Armstrong et al. showed that after hematologic stem cell transplantation in children with hematologic neoplastic diseases, analysis of patients’ plasma-free DNA using mNGS led to the detection of fungal pathogens, including *Aspergillus fumigatus*, *Candida albicans*, and mucor. A subsequent follow-up of 1–2 months after treatment with antifungal therapy revealed negative results [38]. Fei Feng et al. reported a case of fever and respiratory symptoms in a child with acute lymphoblastic leukemia (ALL) after chemotherapy, which was analyzed using mNGS of peripheral blood DNA sequences; the causative agent was identified as *Cunninghamhamella sp.*, and antifungal therapy was shown to be effective [39]. Sun et al. reported three cases of children with ALL combined with mucor infection diagnosed using mNGS and suggested that ALL combined with mucor mostly occurs during the induction chemotherapy phase and lacks specificity in clinical presentation [40]. Early mNGS can assist in the identification of the causative pathogen and provide a basis for definitive diagnosis. Zinter et al. showed that the use of optimized mNGS technology in immunodeficient children allowed the analysis of bacterial, viral, and fungal microbiomes in the lower respiratory tract and identified potential pathogens based on the absolute and relative abundance in clinically negative samples [41], providing a possible line for early implementation of targeted therapies and potentially improving clinical outcomes in immunocompromised children. Heping Shen [42], Dao Wang [43], and Fang Guo [44], by performing mNGS testing in children with hematologic disorders, suggested that this technology boosts the existing ability to detect pathogens and positively influences the clinical management of suspected infections by differential diagnosis, allowing for the exclusion of infectious diseases and the adjustment or reduction of empirical antibiotic use.

**Fever of unknown origin**

Ramesh et al. analyzed 90 serum specimens of febrile children in Uganda using mNGS, among which the presence of at least one microorganism was detected in 66.7% of the serum specimens, and 37 malaria parasites were detected using mNGS, while only 12 were detected using blood cultures. In addition, the investigators used mNGS to identify three new genotypes of viruses through an exploratory retrospective analysis of local febrile children in Uganda [45], suggesting that mNGS may be a beneficial tool in the analysis of the microbiome of childhood infectious diseases.

**Challenges in the application of mNGS to clinical pediatric infectious diseases**

The application of mNGS has brought new ideas to the diagnosis and treatment of infectious diseases, especially in the treatment of children with unexplained infections and severe multiple infections. However, many challenges remain in the clinical application of mNGS owing to its complex operation procedure and numerous variables.

First of all, the challenge is to resolve the high host background to improve the sensitivity of mNGS. By consulting the literature, we found that the specimens applied to childhood infectious diseases are the same as those applied to adult infections, most of which are human samples such as blood, cerebrospinal fluid, nasopharyngeal swabs, and alveolar lavage fluid. The content of microorganisms and genomes in these samples is extremely low compared with that of the host cells, and the host genetic information is often >90% by sequencing [46]. It remains debated whether it is necessary to perform de-hosting in experiments and whether the removal of human background or enrichment of microorganisms can increase the percentage of pathogens in the whole genome and thus improve the detection rate [47, 48]. However, it is possible that the pathogens of interest are removed at the same time as de-hosting, and the nucleic acid concentration of the samples is reduced, which may not meet the requirements for library construction.

The second challenge is to overcome the interference of reagents and background laboratory bacteria. Some unrelated microorganisms are widely present in clinical samples and reagents. Wilson et al. investigated the use of hybridization probes to remove redundant sequences to detect pathogenic sequences preferentially. In this approach, only 20 pg of purified RNA was added to the cerebrospinal fluid sample to suppress most of the non-human reads from water and reagents, thus reducing the number of reads from non-target microorganisms [19]. The nucleic acid extraction process is an important factor contributing to the variability of mNGS results. Currently, commercial pathogen nucleic acid extraction kits mainly employ two methods: magnetic bead-based and column-based extraction. Typically, during the development process, optimization is focused on extracting nucleic acids from specific types or categories of pathogens. Therefore, it is crucial to validate the selected pathogen nucleic acid extraction kits, including verifying their efficiency in extracting nucleic acids from Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, parasites, and other pathogens, in order to avoid any potential pathogen detection.
failures. Meanwhile, before handling clinical specimens, it is necessary to conduct performance verification for the entire experimental process, including verifying the detection limit, inclusivity, anti-interference ability, precision, carryover and cross-contamination, stability, etc., for the “representative” species commonly associated with infectious diseases. Due to the complex, multi-step nature of mNGS testing, multiple quality control indicators are necessary, including sample controls, external controls, internal controls, library quality metrics, sequencing quality metrics, and contamination controls. As laboratory-developed tests, laboratories can adopt acceptable standards to define each parameter, ensuring sample quality and operational quality, and preventing errors during sample processing.

In addition, the establishment of laboratory standards for detecting pathogenic bacteria, laboratory-specific background pathogen libraries, and interpretation of reports have yet to be addressed and standardized. A survey at the Chinese Academy of Health and Food Inspection showed significant differences in detection capacity among laboratories, and differences in detection performance were mainly reflected in the ability to detect false positives, with large differences in the detection capacity for fungi and RNA viruses [49]. Therefore, each laboratory should confirm the performance of this experimental technology before clinical specimen testing and determine the threshold value for each pathogenic bacterium. In addition, the interpretation of mNGS results is relatively complex, with large sequence data, complex parameters, and high requirements for confidence analysts and report review and clinical interpretation personnel. Multiple factors influence the threshold for pathogen diagnosis in mNGS, including sequencing platform, sequencing process, specimen type, pathogen species, and patient condition. Currently, there is no universally recognized threshold, so it is recommended that each laboratory establish its threshold and validate it in clinical practice. Zhang et al. conducted a performance confirmation of the pathogenic macro genomic project before hospital localization from four aspects of clinical intended use, methodology establishment, performance confirmation, and standard operation workbook, which were proposed as specific implementation plans for clinical laboratories [50]. Currently, with the development of macro genomic mNGS, expert consensus on mNGS in clinical applications has been successively released in China [51–57] for clinical medical personnel and laboratory workers to learn from.

The impact of different read lengths of different pathogenic microorganisms on result interpretation is as follows: under the same conditions (same microorganism, same specimen), a higher number of reads detected for a particular microorganism suggests a higher possibility of it being a pathogenic microorganism. However, different pathogenic microorganisms have varying genome sizes, and there are differences in nucleic acid extraction efficiency. Moreover, the pathogenicity of different microorganisms also varies significantly [53]. Therefore, it is not sufficient to rely solely on the number of reads to determine infection status. It is recommended to consider both the differences in pathogenic microorganism species and their pathogenic characteristics.

In terms of antimicrobial drug resistance, when using mNGS for pathogenic microorganism identification, it is possible to consider detecting drug resistance genes. However, it is necessary to accurately locate the drug-resistance genes on the specific pathogen to determine their clinical value. This means that after assembling the genomic sequences obtained from mNGS, it is necessary to determine which pathogen the drug resistance gene is located on, whether it is on a plasmid or on a chromosome. However, it is important to note that the presence of resistance/virulence genes does not necessarily mean they are expressed, and there may be discrepancies between genotype and phenotype. Additionally, some mechanisms leading to resistance/pathogenicity cannot be definitively identified through gene testing alone. Current technologies for species attribution analysis of resistance/virulence genes are not yet fully developed. For normal bacterial samples, accurately matching drug-resistance genes with specific pathogenic microorganisms poses significant challenges [58].

Interpreting reports poses a significant challenge in experimental contexts. When mNGS identifies pathogens with low sequence reads, caution is necessary in determining their diagnostic value. It is imprudent to solely rely on mNGS results to guide anti-infection therapy. Instead, diagnostic decisions should be informed by analyzing the type and collection reliability of the specimen, excluding interference from technical aspects of the detection process, and considering the type of pathogens detected in conjunction with the patient’s clinical condition for a comprehensive assessment, ultimately validating its clinical significance and value. For rare pathogens, such as Cryptococcus neoformans, further validation is necessary through other laboratory tests when detected at low sequence reads by mNGS. In cases where mNGS results are negative, other auxiliary test results should be integrated for diagnosis. It has been observed in clinical practice that children with negative mNGS results can be diagnosed with infections through alternative testing methods, highlighting the influence of sample collection and experimental procedures. Qian et al. [59] suggested that compared to traditional culture and smear testing, the negative predictive value (NPV) of mNGS is 95.79 % [95 % CI, 93.8–97.8 %]. M. tuberculosis and Aspergillus are noted to be pathogens that may evade detection, potentially due to their thick, complex cell walls which
hinder nucleic acid release. Wilson et al. showed [60] that mNGS could accurately diagnose 18 cases of meningitis or encephalitis with negative cerebrospinal fluid results through serological experiments.

For positive mNGS results, if such findings align with clinical manifestations but lack other laboratory evidence, PCR verification is advised, alongside a recommendation for further conventional laboratory tests. If positive sequencing results are not supported by clinical manifestations or laboratory findings, diagnosis should not solely rely on sequencing results but rather on traditional laboratory findings as the primary clinical reference.

The heavy cost is another major challenge owing to which mNGS is currently unavailable in pediatric clinics. Viruses in pediatric respiratory infections are often caused by RNA viruses, such as respiratory syncytial virus, human rhinovirus, human metapneumovirus, influenza virus, para-influenza virus, and coronavirus [61]; therefore, DNA+RNA sequencing is often required to identify the causative pathogen in the same patient but is expensive and cannot be universally applied. Moreover, there is limited literature in our inclusion that summarizes the sensitivity of mNGS alone for virus detection. Therefore, when using mNGS, the existing consensus should be referred to, combined with clinical manifestations, to select the appropriate sequencing technology and to avoid overuse. Clinical staff should combine the mNGS results with the clinical symptoms of the child and other tests to make a joint judgment of the patient’s condition.

Conclusions

In conclusion, although there are various difficulties in the clinical use of mNGS in pediatric infectious diseases, studies have shown that mNGS can improve the sensitivity of pathogenic microbial detection compared with traditional detection methods and compensate for the shortcomings associated with traditional detection techniques. Simultaneously, doctors and scientific researchers should improve various standards for the clinical application of this technology, reflecting its advantages of high efficiency as well as fast and accurate clinical diagnosis and treatment and changing the detection and diagnosis mode of infectious diseases in the past. With the development and clinical application of high-throughput mNGS, the cost of detection will be reduced in the future, and the problems of background bacteria, host ratio, and other aspects of differential diagnosis of childhood infectious diseases will be solved.

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