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ORIGINAL RESEARCH

Metagenomic Next-Generation Sequencing in the Diagnosis of Infectious Fever During Myelosuppression Among Pediatric Patients with Hematological and Neoplastic Diseases

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Purpose: To analyze the contribution of metagenomic next-generation sequencing (mNGS) in the guidance of clinical treatment and outcomes of infection during myelosuppression among children with hematological and neoplastic diseases.

Patients and Methods: The clinical data and results of mNGS assay of febrile patients suspected of infection were retrospectively collected. The characteristics of pathogenic microorganisms and clinical course of myelosuppressed children with hematological diseases were summarized.

Results: Our study included 70 patients (45 males) with a median age of 5 years (range: 0.5 to 13 y). During the study period, there were 96 events of suspected infection. According to comprehensive clinical diagnosis, 73 blood infections, 43 pneumonia and 2 urinary tract infections occurred. The positive rate of mNGS was significantly higher than that of traditional microbial detection (83.3% vs 17.7%). The main pathogens detected by mNGS were *Pseudomonas aeruginosa, Acinetobacter*, human herpesvirus, *Candida* and *Aspergillus*. The average duration of fever was 4.9 days and 11.6 days ($P < 0.05$), and the average cost of anti-infection treatment was RMB ¥28,077 and 39,898 ($P < 0.05$) among children received mNGS within 48 hours and more than 48 hours after the onset of infection symptoms.

Conclusion: mNGS contributes to clinical management of children with infection during myelosuppression, especially among patients with negative traditional microbial detection. Early implementation of mNGS in children with symptoms has a tendency to reduce the time of infection, fever and the cost of treatment.

Keywords: metagene, pathogenic microorganism, child, hematology and oncology

Introduction

Infection due to myelosuppression after chemotherapy for malignancies is a common complication and cause of treatment-related death.[1,2] In clinical practice, the traditional identification of pathogens relies heavily on laboratory capacity to detect common pathogenic microorganisms with discrete methods, such as assays for pathogen-specific antibodies, nucleic acids and antigens, and pathogen culture. Although the early diagnosis and correct anti-infection treatment are imperative for patients with myelosuppression, the traditional methods are limited by its low throughput and narrow coverage of pathogen spectrum. It has been shown that more than 60% of the patients with infection cannot receive a pathogen diagnosis.[3–5] This results in a wide practicing of empirical broad-spectrum antimicrobial therapy, which not only increases the risk of antibiotic resistance but also brings related toxic and side effects.[6]

Metagenomic next-generation sequencing (mNGS) is an emerging technology for comprehensive analysis of microbial genetic components in patients' samples. In recent years, mNGS has been successfully applied in the detection of

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pathogens in blood, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL), urine, and other samples.[7–9] Although mNGS is superior over traditional methods due to its wide coverage, its use is also limited by high cost and low specificity. Recent studies suggested that the efficacy of mNGS in the pathogenic diagnosis of infection is limited in immunocompetent patients.[10,11] However, apparent in immunocompromised patients, especially in the diagnosis of complex, severe infection, mNGS shows advantages over conventional assays in many aspects, including turnaround time, sensitivity, throughput, and mNGS is less affected by prior antibiotic exposure.[12–14]

The current study retrospectively reviewed the pathogens identified by mNGS among children clinically diagnosed with infection during myelosuppression. The purpose of this study was to analyze the contribution of mNGS to the guidance of clinical treatment and outcomes in children with hematological and neoplastic disease.

Materials and Methods

Patients

The study involves a retrospective patient cohort of consecutive children who presented fever and subjected to mNGS assay in the Department of Hematology, Children's Hospital of Fudan University from July 2019 to March 2021. None of the patients had infection-related symptoms before receiving chemotherapy or immunosuppressive therapy in hospital. Health records of children who completed the mNGS assay were retrospectively reviewed. Only patients diagnosed with hematological and neoplastic diseases were included (Figure 1).

Definition and Causes of Fever

Fever is defined by elevated oral temperature (>38.3°C or >38.0°C for more than 1 hour). The diagnosis and management of suspected infection in patients were conducted according to the guidelines of the Infectious Disease Society of America (IDSA) for the treatment of patients with fever and neutropenia.[15] All patients were given prophylactic sulfamethoxazole against Pneumocystis jirovecii infection during chemotherapy. The differential diagnosis for causes of fever among infection, drug, and neoplasm was made based on the review of traditional microbial detection methods, mNGS, laboratory tests, medical imaging, response to empirical antibiotic treatment, the possibility of febrile coincides with drugs that were frequently associated with fever (eg, large dose of cytarabine),[16] and the exclusion of other causes of fever in the patient.[17] The diagnosis was made by two investigators (YF and XZ) and discrepancies resolved by discussion with a third reviewer (HW).

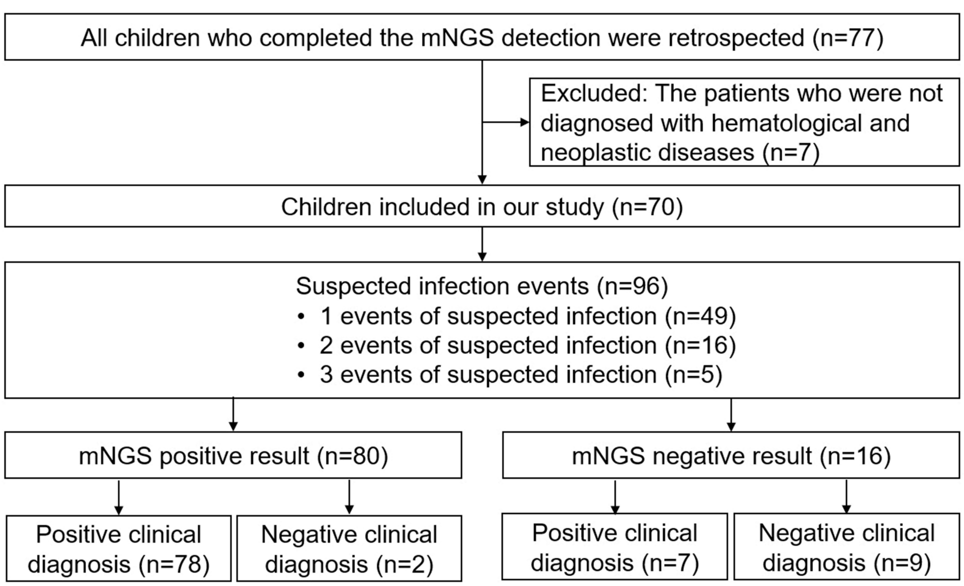

Figure 1 Flow diagram of participants through study.

Traditional Microbial Detection Methods, Laboratory Tests and Medical Imaging

All patients received microbial culture using patients' samples (blood, sputum, cerebrospinal fluid, urine, feces, swabs, etc.). In addition, we selected other traditional microbiological tests based on the clinical characteristics of different patients according to the recommendations of the IDSA, including serology [influenza A, influenza B, syncytial virus, adenovirus, cytomegalovirus (CMV), Epstein Barr virus (EBV)], and antigen assays [mycoplasma pneumoniae, rotavirus, norovirus antigen, (1,3)-β-D-glucan assay (G test) and galactomannan (GM) test].[15] At the same time, complete blood count (CBC), C-reactive protein (CRP), blood biochemical, inflammatory indices including procalcitonin (PCT) and interleukin-6 (IL-6), chest CT were measured to support the clinical diagnosis of infection. All the laboratory test results are defined as positive or negative according to the pre-specified reference value. The traditional microbial detections of all patients in this study were conducted before the empirical broad-spectrum antimicrobial therapy.

mNGS

The informed consent of the guardian was obtained before specimens were collected for mNGS. The collected samples included blood, urine, sputum, CSF, BAL and tissue samples according to the clinical infection symptoms. All clinical samples were store at 4°C for less than 24 hours before the assay. For sputum and tissue samples, total DNA was extracted using QIAamp cador pathogen mini kit (Qiagen, Valencia, USA) according to the manufacturer's instruction; For BAL, CSF, urine and blood, cells were removed through centrifugation to minimize host background. Then, 400 μL sample was separated into a new 1.5mL microcentrifuge tube and underwent total nucleic acid extraction using the QIAamp circulating nucleic acid kit. The extracted DNA specimens were used to the construction of DNA libraries. The DNA underwent library construction through DNA-fragmentation (150 bp), end-repair, adapter-ligation, and unbiased PCR amplification. The quality of the DNA libraries was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) to measure the adapters before sequencing. High throughput sequencing was performed using the 75-bp paired-end protocol on an Illumina NextSeq550Dx platform. On average, 2.5 million reads (75 bp) were obtained from each sample after sequencing. Reads that mapped to a human reference genome were removed by using Burrows-Wheeler alignment. The remaining data by removal of low-complexity reads were classified by simultaneously aligning to four Microbial Genome Databases retrieved from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genome), consisting of viruses, bacteria, fungi, and parasites. The database used for this study contained 6030 bacteria, 3551 viruses, 185 fungi, and 87 parasites. The quantity for each microbe identified was expressed as the normalized number (NN) of DNA sequencing reads in terms of Langelier's study.[18] Species with NN less than three were removed, as three NNs of nucleotide reads are approximately equivalent to two copies/mL or 35 qPCR Ct value, and two or less NNs are highly suspected to be false positive. Species with NN greater than ten were reported.[19] The Basic Local Alignment Search Tool for nucleotide was implemented in the nucleotide database to verify the identification accuracy of species with NN between three and ten, and the verified species were reported.[20] All the mNGS results are defined as positive or negative according to the pre-specified reference value, and the relative abundance of microorganisms was reported. The analysis and report were performed by experienced laboratory doctors who were blinded to all patient information, including the traditional microbial detection report. Finally, the results of mNGS will be reported within 24 hours after specimen collection.

Clinical Relevance of mNGS Results

For a positive mNGS result, clinicians will judge whether it is applicable according to the relative abundance of microorganisms reported in the laboratory, and comprehensively consider the clinical significance of microorganisms and whether they match the clinical characteristics of patients. The following conditions will also help clinicians make a judgment that the positive mNGS result was applicable: (1) It detected the same pathogens as reported by traditional microbial detection; (2) The mNGS tests are inconsistent with traditional microbial detection results, but the patient clinically improved within 3 days after adjusting antibiotics based on mNGS, or mNGS reported pathogens that have already been covered by prior antibiotics and the patient clinically improved within 3 days.

A negative mNGS result was considered in combination with the clinical manifestation, laboratory tests and medical imaging and categorized as (1) True negative: the symptoms are not related to pathogenic microorganism (drug-induced fever, neoplastic fever, etc.); (2) False negative: confirmed infection diagnosed by other clinical information.

Discrepancies between mNGS and traditional microbial detection were resolved by three clinicians independently according to the clinical course of the patient.

Evaluation Index of mNGS Detection or Traditional Microbial Detection

Based on the clinical diagnosis of infection, we conducted mNGS detection and traditional microbial detection as diagnostic tests. Sensitivity refers to the proportion of pathogen correctly identified by mNGS or traditional microbial detection according to the clinical diagnosis of infection. Specificity is defined as the proportion of clinically non-infected patients whose pathogen was not identified by mNGS detection or traditional microbial detection. Positive predictive value refers to the proportion of patients whose infection was clinically diagnosed among the patients received positive results from mNGS detection or traditional microbial detection. Negative predictive value refers to the proportion of patients free of clinical infection among patients negative for mNGS or traditional microbial detection.

Statistical Analysis

The statistical analysis was conducted using Stata, version 16.0 (Stata Corp., Texas, TX). The independent sample *t*-test or Wilcoxon rank-sum test was used for continuous data as applicable, and the difference in frequencies of events between groups was compared using the χ^2 test.

Results

Patient Characteristics

Our study included 70 patients (45 males) with a median age of 5 years (range: 0.5 to 13 y). Among these patients, there were 29 diagnosed with acute lymphoblastic leukemia (ALL), 15 acute myeloid leukemia (AML), 20 non-Hodgkin's lymphoma (NHL), three Langerhans cell histiocytosis (LCH), one aplastic anemia, one retinoblastoma (RB), and one patient with Evans syndrome.

mNGS, Traditional Microbial Detection and Clinical Characteristics

During the study period, there were 96 events of suspected infection (Figure 1). The mNGS assay showed positive results in 80 events and traditional microbial detection was positive in 17 events (9 blood culture, 4 fecal culture, 2 urine culture and 2 serological test). There were no indeterminate or missing data in all patients. The overall positive rate of mNGS was significantly higher than that of conventional culture (83.3% vs 17.7%, $P < 0.05$). CBC found that 52 infection events appeared in children with agranulocytosis and 43 chest CT showed pulmonary infection. According to comprehensive clinical diagnosis, 73 blood infections, 43 pneumonia and 2 urinary tract infections occurred (Table 1).

In the 96 suspected infections, there were 85 events diagnosed as clinically infection, while the symptoms in other 11 were not related to pathogenic microorganism (drug-induced fever, neoplastic fever, etc.). The data are shown in Figure 2 and Table 2. The sensitivity, specificity, positive predictive value and negative predictive value of mNGS were 91.8 ± 5.5%, 81.8 ± 7.7%, 97.5 ± 3.1%, 56.3% ± 9.9%, respectively. The sensitivity, specificity, positive predictive value and negative predictive value of traditional microbial detection methods were 17.7 ± 7.6%, 81.8 ± 7.7%, 88.2 ± 6.5%, 11.4 ± 6.4%, respectively.

Pathogen Sequencing Results

During the 96 suspected infections, 127 samples were sent for mNGS assay, of which 105 samples were positive, including 87/107 blood samples, 5/6 cerebrospinal fluid samples, 1/2 bronchoalveolar lavage fluid samples, 3/3 sputum samples, 7/7 urine samples and 2/2 tissue samples. The spectrum of pathogens identified by mNGS is shown in Figure 3. In general, *Pseudomonas aeruginosa* (20.5%, 26/127) and *Klebsiella pneumoniae* (8.7%, 11/127) were most commonly detected bacteria, CMV (21.3%, 27/127) was most common in viruses, and *Candida* (12.6%, 16/127) was the most

Table 1 Test Data of the 96 Suspected Infections

Variable	Number of Suspected Infections (n)	Ratio (%) n = 96
mNGS		
Positive	80	83.3
Negative	16	16.7
Traditional microbial detection		
Positive blood culture	9	9.4
Positive fecal culture	4	4.2
Positive urine culture	2	2.1
Positive serological test	2	2.1
Negative	79	82.2
Neutropenia		
Agranulocytosis	52	54.2
Normal	44	45.8
Chest CT		
Abnormal	43	44.8
Normal	53	55.2
Clinical diagnosis		
Bloodstream infection	73	76.0
Pneumonia	43	44.8
Urinary infection	2	2.1

Abbreviation: mNGS, Metagenomic next-generation sequencing.

common fungus infection. In 96 suspected infections, there were 35 events positive for multiple bacteria, 17 positives for both bacteria and virus, 9 bacterial and fungal infections and 7 were triple positive for bacteria, virus and fungus.

The Influence of Detection Time of mNGS on Treatment

In 96 suspected infections, aside from routinely administered anti-fungal treatment among high-risk children,[21] all patients were empirically treated with broad-spectrum antibiotics including meropenem, cefepime, cefoperazone sulbactam, vancomycin, linezolid and voriconazole. After mNGS, antibiotics were adjusted according to the type of pathogens and antibiotic resistance in 65 courses of treatment. Among these treatment courses, 62 resulted in good prognosis, but death occurred in 3 patients. Among them, 2 cases were diagnosed as AML and died of septic shock (1 case was caused by *Moraxella ostilus* and *Staphylococcus kocheri*, another case was caused by *Enterococcus avium* and *Candida tropicalis*), one case was diagnosed as NHL and died of cerebral hernia (intracranial recurrence of anaplastic large cell lymphoma).

We also found that 49 samples were collected within 48 hours after the onset of infection symptoms, and the other 47 samples were collected more than 48 hours. In these two groups of children, the average duration of fever with timely sampling group (<48h) and longer sampling time (≥48h) group was 4.9 days and 11.6 days ($P < 0.05$); In addition, the average cost of anti-infection treatment was RMB ¥ 28,077 and 39,898 ($P < 0.05$), and the average total hospitalization cost was RMB ¥ 97,236 and 128,475 ($P < 0.05$, Table 3) in children with early mNGS (<48 h) and late mNGS (≥48 h).

Discussion

With the rapid development of diagnosis and treatment technology for children's hematological tumors, the prognosis of children's hematological malignancies has been greatly improved in the past 20 years. However, various treatment-related complications caused by chemotherapy are inevitable, especially the complicated infections caused by bone marrow suppression after chemotherapy, severe microbial infection, if not identified or delayed, will lead to prolonged hospital stay and increased mortality.[22] Faster and more effective identification of pathogens to guide antibiotic treatment

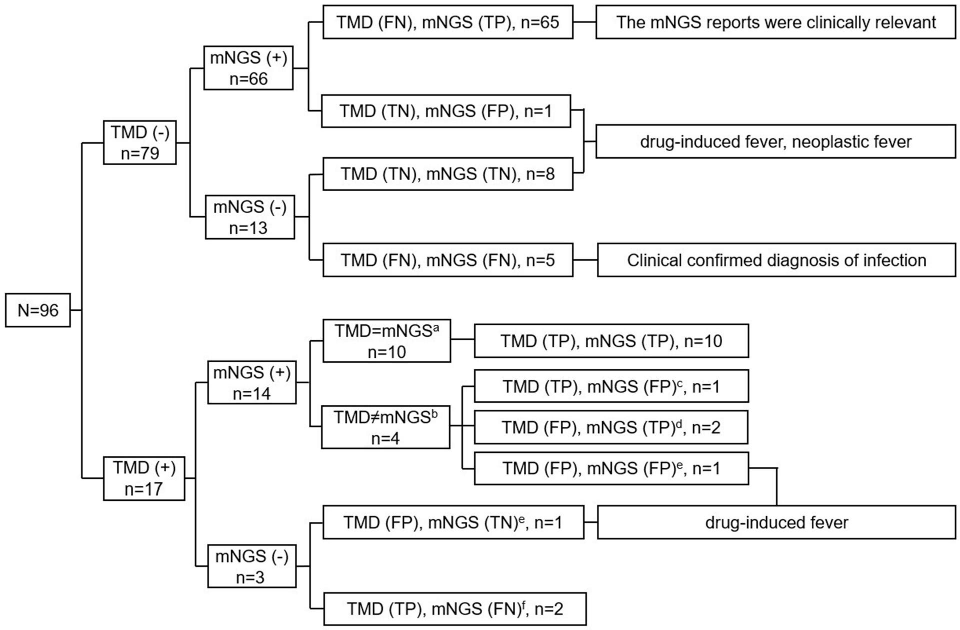

Figure 2 Microbial detection results of the 96 suspected infections.

Notes: [a]TMD = mNGS, TMD detected the same pathogens as reported by mNGS. [b]TMD ≠ mNGS, TMD detected the different pathogens as reported by mNGS; [c]TMD (*Escherichia coli*, blood culture), mNGS (*Pseudomonas aeruginosa, Acinetobacter haemolyticus, Acinetobacter baumannii*). After comprehensive consideration, the TMD report was clinically relevant; [d]TMD (*Staphylococcus epidermidis*, blood culture), mNGS (*Klebsiella pneumoniae*). The patients of the two suspected infections were different, but the test results are the same. After comprehensive consideration, the mNGS report was clinically relevant. The TMD reports were considered to be specimen contamination during percutaneous puncture; [e](1) TMD (*Escherichia coli*, fecal culture), mNGS (*Mycobacterium abscessus*); (2) TMD (*Escherichia coli*, fecal culture), mNGS negative. Results of two independent suspected infection courses in a single patient. The final diagnosis was drug-induced fever. When the patient had no suspected infection symptoms in the past, the fecal culture also reported *Escherichia coli*. The TMD reports were considered as microbial colonization; [f](1) TMD (*Achromobacter xylosoxidans*, blood culture), mNGS negative; (2) TMD (*Enterobacter cloacae, Serratia marcescens*, blood culture), mNGS negative. The TMD report was clinically relevant.

Abbreviations: TMD, traditional microbial detection; mNGS, metagenomic next-generation sequencing; TP, true positive; FP, false positive; TN, true negative; FN, false negative.

are undoubtedly important.[23] Traditional microbial detection methods often have great limitations, such as low positive rate of pathogenic culture, long detection time, impact of the empirical broad-spectrum antimicrobial therapy, and generally, the positive rate of blood culture is less than 10%.[24] In this study, we found that the positive rate of pathogen identification using traditional methods was similar to previous reports, which is much lower than that by mNGS. The average turnaround time of traditional methods was 5 days and significantly longer than mNGS (<24 hours).

In this study, compared with traditional methods, mNGS technology detected 24 additional pathogens, which increased the clinical diagnosis rate by 73.0%. Although we calculated the sensitivity and specificity of mNGS and traditional methods according to clinical diagnosis, the results' precision is limited by the retrospective design and limited number of observations. While the traditional methods of pathogen diagnosis are widely accepted and practiced, our study suggests that mNGS can be used as a supplement to traditional methods. For example, in addition to the common bacteria and fungi in traditional microbial detection methods, a variety of viruses were detected in mNGS, including human parvovirus B19, microcyclic virus, JC virus, WU polyomavirus and human herpesvirus (HSV-1, EBV,

Table 2 Relationship Between mNGS Detection and Clinical Diagnosis

		Clinical Diagnosis	
		Positive (n)	**Negative (n)**
mNGS	Positive (n)	78	2
	Negative (n)	7	9
Traditional microbial detection	Positive (n)	15	2
	Negative (n)	70	9

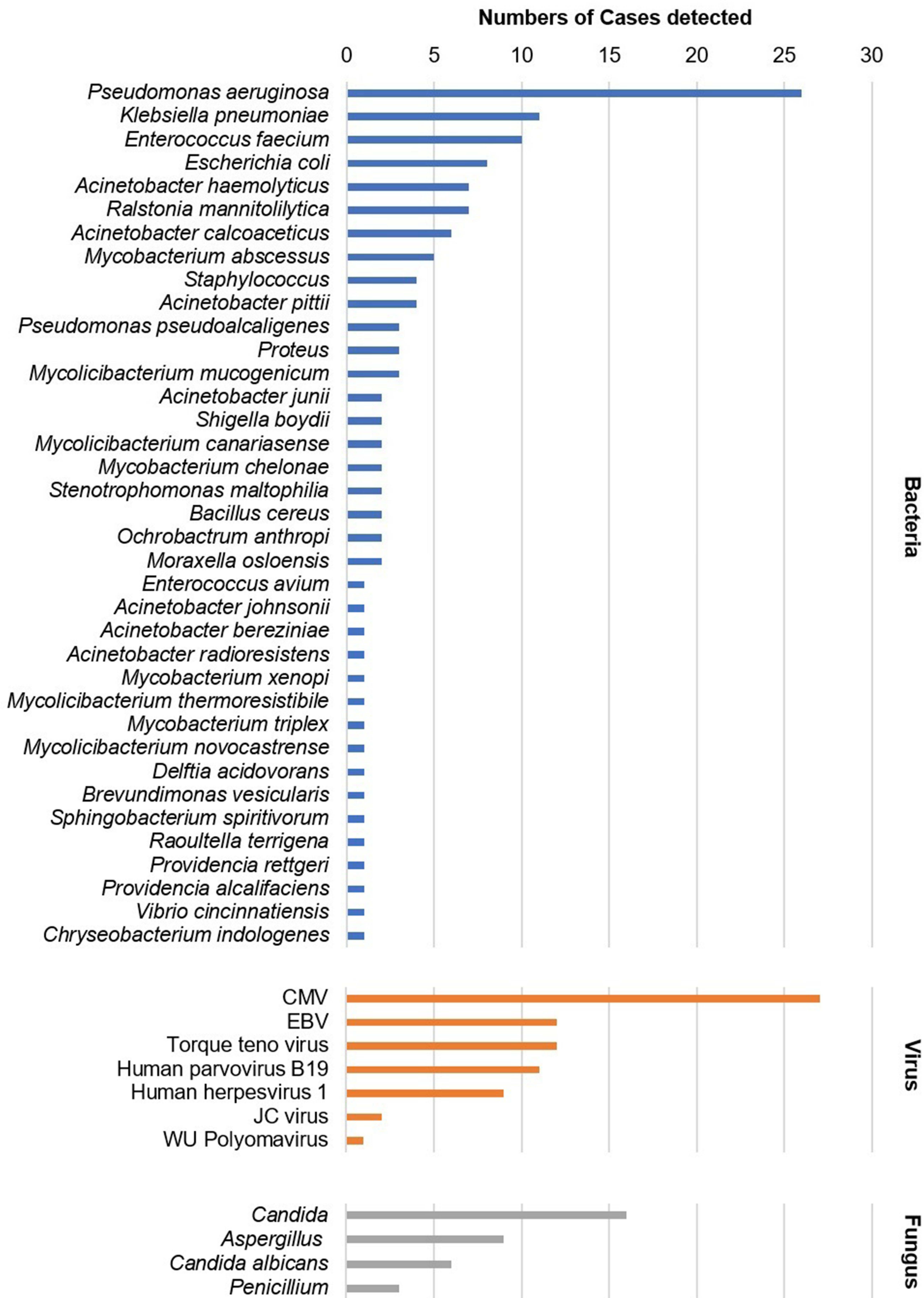

Figure 3 Results of pathogenic microorganisms in mNGS.

Table 3 Relationship Between Sampling Time of mNGS and Fever Time and Treatment Cost

	Sampling Time of mNGS		P value
	< 48 h	≥ 48 h	
Average duration of fever (h)	4.9	11.6	0.0001
Average anti infection treatment (RMB ¥)	28,077	39,898	0.0194
Average total hospitalization cost (RMB ¥)	97,236	128,475	0.0384

CMV). In general, compared with antibiotics or antifungal drugs, antiviral drugs are rarely considered in empiric anti-infective treatment regimes and mNGS detection can help clinicians better consider the possibility of viral infection and decide whether antiviral drugs need to be used. However, when nucleotide acid abundance was identified in multiple pathogens, the interpretation of mNGS results can still be a challenging task. First, there are viral infection lacking disease-specific clinical manifestations. Second, there are limited indicators that can effectively distinguish colonization from infection, especially among immune-compromised patients. There are still a large proportion of pathogens detected by mNGS with unclear clinical significance. Therefore, the diagnostic efficacy of pathogen by mNGS alone is still limited, and clinicians still need to consider the relative abundance and clinical significance of microorganisms, laboratory tests, medical imaging, clinical manifestations, and treatment response to determine the causal pathogen. At the same time, it is noted in our study that the results reported by mNGS do not have extraordinary specificity in the diagnosis of pathogen during patient's infection regardless of other clinical information (laboratory tests, medical imaging and treatment response). Future studies with comparative design are required to better understand whether there is true superiority of mNGS over traditional methods in the diagnosis of pathogen in the myelosuppressed children.

According to the Chinese guidelines of febrile neutropenia, repeated microbiological examination within 48 hours is recommended for patients with poor therapeutic effect.[25] Some studies have shown that mNGS is different from traditional microbial detection, and the use of antibiotics has little effect on the mNGS results.[26,27] Our result suggested that the average fever duration of children whose sampling time was less than 48 hours was significantly reduced. Similarly, the time and cost of anti-infection treatment of these children were also significantly reduced. This may be related to our timely modification of treatment according to the mNGS results. Therefore, we suggest that the early introduction of mNGS may have a positive impact on the treatment cost. However, our study has a relatively small population and uncontrolled bias [such as the type of primary disease, levels of myelosuppression, pathogen of infection and patients' own characteristics (age, tumor burden)]. In addition, mNGS was more likely to be performed in children with better family economic conditions or increased severity of infection. Future studies with larger sample size and controlled design are required to further validate our results and determine the timing of mNGS in anti-infection treatment.

Conclusion

To summarize, our study suggested that mNGS technology can quickly and comprehensively detect pathogens with high sensitivity. At the same time, it increases the types of pathogens that can be detected. Combining mNGS detection with traditional microbial detection can help clinicians make more timely and accurate judgments. Early detection of mNGS may also help children reduce the duration of fever and treatment costs. Our results require confirmation from future larger studies with controlled subjects.

Ethics Approval and Consent to Participate

The study was approved by the ethics committee of the Children's Hospital of Fudan University (IRB No. 2020-270) and was conducted in accordance with the Declaration of Helsinki. General informed consent was obtained according to the local ethical committee guidelines and obtained from the parents/legal guardian of the study participants prior to study commencement.

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Disclosure

The authors report no conflicts of interest in this work.

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