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Research article

Comparison of plasma and blood cell samples in metagenomic next-generation sequencing for identification of the causative pathogens of fever

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ABSTRACT

Background: Metagenomic next-generation sequencing (mNGS) of plasma DNA has become an attractive diagnostic method for infectious diseases; however, the rate of false-positive results is high. This study aims to evaluate the diagnostic accuracy of mNGS in plasma versus blood cell samples for immunocompromised children with febrile diseases.

Methods: The results of conventional microbiological test (CMT) and mNGS using plasma and blood cells in 106 patients with 128 episodes of febrile diseases from the Department of Hematology/Oncology were analyzed and described.

Results: The positivity rates for CMT and mNGS of plasma and blood cells were 35.9 %, 84.4 % and 46.9 %, respectively ($P < 0.001$). Notably, mNGS identified multiple pathogens in a single specimen in 68.5 % of plasma samples and 38.3 % of blood cell samples ($P < 0.001$). Furthermore, plasma and blood cell mNGS identified causative pathogens in 58 and 46 cases, accounting for 53.7 % and 76.7 % of the mNGS-positive cases for each sample type, respectively ($P = 0.002$). By integrating results from both plasma and blood cell samples, causative pathogens were identified in 77 cases (60.2 %), enhancing sensitivity to 87.5 % but reducing specificity to 15.0 %, compared to plasma (65.9 % sensitivity and 20.0 % specificity) and blood cell samples (52.3 % sensitivity and 80.0 % specificity).

Conclusions: mNGS of plasma is sensitive but has a high false-positive rate, while mNGS of blood cells has low sensitivity but higher specificity.

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Abbreviations

mNGS	Metagenomic next-generation sequencing
CMT	Conventional microbiological test
cfDNA	Cell-free DNA
HSCT	Hematopoietic stem cell transplantation
RPTM	Reads per ten million
PPV	Positive predictive value
NPV	Negative predictive value
RPM	Reads per million
BSI	Bloodstream infection

1. Introduction

Metagenomic next-generation sequencing (mNGS) represents a high-throughput technology capable of deeply sequencing all genetic material within a biological sample [1,2]. This technology has seen extensive use in diagnosing clinical infections, following its inaugural success in identifying pathogens within central nervous system infections [3]. Distinct from targeted approaches, mNGS enables the concurrent detection of virtually all known pathogens present in clinical samples.

Thus, metagenomic Next Generation Sequencing (mNGS) proves to be an invaluable tool for the detection of novel or unanticipated pathogens. Evidence from prior research highlights that mNGS efficiently pinpoints clinically significant pathogens in patients with compromised immune systems [4–8]. Our preceding study revealed that plasma mNGS conferred benefits on 27.9 % of pediatric patients with hematologic malignancies suffering from fevers of unknown origins; their feverish conditions were resolved following the adjustment of antimicrobial regimens based on mNGS findings [9]. Additionally, a retrospective analysis by Niles and colleagues indicated that antimicrobial treatment plans were modified in 26 % of cases upon the identification of additional organisms through mNGS [10]. Given the uncertainty of pathogens and sites of infection in one-third of patients with hematology/oncology diseases, peripheral blood remains the most commonly used specimen for NGS [11]. A recent review of 22 studies involving 2,325 patients with hematologic diseases found that 9 studies (40.9 %) solely analyzed blood samples through mNGS, achieving an overall positivity rate of 71.64 % (619/864). This sensitivity is lower than that of the BALF group at 89.86 % (195/217), despite similar findings that NGS of blood samples improves the positivity rates for bacteria, fungi, and viruses compared to conventional microbiological tests (CMT) [12]. However, the sensitivity remains less than satisfactory. The majority of these previous studies have used plasma as the specimen for NGS. However, several limitations accompany this approach. Firstly, the brief half-life of cell-free DNA (cfDNA) in plasma can lead to false-negative outcomes [13]. Secondly, in immunocompromised patients, a breakdown of mucosal barriers results in an elevated presence of cfDNA from commensal organisms, complicating the interpretation of these findings [14]. Thirdly, given that cfDNA in plasma is fragmented and lacks a complete genome sequence, crucial information about resistance may be overlooked [15].

The pitfalls of mNGS using plasma raise the question of whether mNGS of blood fraction will improve accuracy. Recent research conducted by Wu and colleagues in cohorts of patients with sepsis has embarked on a similar study, demonstrating that whole-blood metagenomic next-generation sequencing (mNGS), combining both blood cells and plasma, exhibits a higher sensitivity compared to traditional blood cultures or mNGS using plasma alone. This approach shows great potential in the diagnosis of bloodstream infections (BSI) [16]; however, to date, such studies remain exceedingly limited, and the value of utilizing different blood components for mNGS testing warrants further exploration. Our study was conducted among pediatric patients with febrile illnesses in hematology/oncology, who differ from previous study cohorts by being immunocompromised with a higher incidence of opportunistic infections. Through the analysis of our results, we further substantiate the value of employing blood cells in mNGS testing.

2. Patients and methods

2.1. Patients

This study was approved by the Children's Hospital of Zhejiang University School of Medicine Institutional Review Board (IRB number 2021-IRB-004), and the participants' legal guardians provided written informed consent to participate. Patients who experienced febrile disease after chemotherapy or during hematopoietic stem cell transplantation (HSCT) were enrolled at the Department of Hematology/Oncology between May 2021 and September 2022. Patients were excluded if drug-related fever or immune disorders related were suspected. Fever was defined as one ear temperature reading of >38.5 °C or at least two measurements in the range of 38.0–38.4 °C at an interval of more than 1 h. For patients with more than one febrile episode, the no-fever interval between the two episodes should be longer than seven days with normal C-reactive protein levels.

2.2. Study design

Peripheral blood samples were collected from the enrolled patients for microbiological culture and the assessment of inflammatory biomarkers within 6 h following the onset of fever. Additional conventional microbiological tests (CMTs) were conducted if the febrile

condition persisted beyond 48 h, as detailed in Table 1. For each instance where an organism was identified via metagenomic next-generation sequencing (mNGS), a corresponding CMT was undertaken to confirm the presence of that specific organism. The peripheral blood samples were subjected to mNGS analysis subsequent to obtaining informed consent from the patient's legal guardian. In cases where the organism detected by mNGS was deemed the causative agent of the febrile illness (i.e., the microbiological etiology) and had not been targeted by prior antimicrobial treatment, the treatment regimen was modified in accordance with the mNGS findings.

2.3. mNGS analysis

The process of mNGS comprised library preparation, metagenomic sequencing, and bioinformatics pipeline analysis, as shown in Fig. 1. The details are listed in the following:

Whole blood samples (2–5 mL) were collected in anticoagulation tubes and transported at 4 °C after collection. After centrifugation at 1900×*g* for 10 min at 4 °C, nucleic acid was extracted from plasma and blood cells for further sequencing. For blood cell detection, host depletion was performed by the differential lysis method before DNA extraction [17,18]. Libraries for NGS were prepared from cfDNA and genomic DNA according to the manufacturer's protocol. Libraries were sequenced on a NextSeq™ 550Dx (Illumina) instrument, and at least 20 million sequencing reads were acquired per sample. The pathogenic microorganism database was established on the basis of a previous study [19]. Reference genomes for microorganisms (bacteria, fungi, viruses, parasites, archaea and other pathogenic microorganisms) were obtained from NCBI GenBank. During database establishment, sequence similarities among microorganism references were inspected to identify taxonomic mislabelling and sequence contamination. As part of the selection process, NCBI BioSample, GenBank, RefSeq and FDA-ARGOS data were used to ensure the inclusion of reference genomes from both clinical and nonclinical isolates.

The bioinformatics analysis processes were as follows. The raw FASTQ-format data obtained by sequencing were subjected to Trimmomatic v0.40 for quality control and evaluation, whereby low-quality or undetected sequences, sequences contaminated by splices, high-coverage repeats, and short-read-length sequences were filtered out [20]. High-quality sequencing data were compared with the human reference genome GRCh37 (hg19) using Bowtie2 v2.4.3 [21], enabling the removal of human host sequences. The remaining sequences were aligned with the constructed reference database using Kraken2 v2.1.0 to identify pathogens present in the sample [22]. Pathogen positivity was determined using the reads per ten million (RPTM) value. Viruses were needed to meet an RPTM threshold $\geq$3 to be considered positive; for bacteria and fungi, this threshold was $\geq$8. Special pathogens (including *Cryptococcus* and *Mycobacterium*) were identified as positive when the RPTM values were $\geq$1.

The assay was quality-controlled using external controls in every batch. A no-template control containing buffer instead of blood was processed in parallel to monitor background microbial DNA signals at the time of batch processing. Positive control samples containing *Klebsiella pneumoniae, Escherichia coli,* and *Staphylococcus epidermidis* were included in every batch.

2.4. Evaluation of mNGS results

Two investigators independently evaluated the data to determine whether the organisms identified by mNGS were the causative pathogens of the febrile conditions. In instances of disagreement, the case was submitted to a group meeting for further discussion. The results were categorized into the following four levels: (1) Definite: when the organism detected by mNGS matched that identified by conventional microbiological tests (CMTs) within seven days of the mNGS sample collection; (2) Probable: when the identified organism was deemed pathogenic, consistent with clinical, radiological, or laboratory indicators, not included in the spectrum of previous antimicrobial treatments, and the patient's condition resolved after the antimicrobial therapy was adjusted based on mNGS findings; (3) Possible: when the organism had potential pathogenicity in line with the clinical signs, but was likely included in the spectrum of the prior antimicrobial regimen or the condition did not improve despite the adjustment of the antimicrobial therapy based on mNGS insights; (4) Unlikely: when the organism was determined to be nonpathogenic, not aligned with the clinical signs, required no intervention, or did not respond to the adjusted antimicrobial therapy as informed by mNGS results. An organism was deemed the causative pathogen of the fever if it met the "definite" or "probable" criteria.

Table 1
Conventional microbiological tests performed in this study.

Types of pathogens	Conventional microbiology tests (CMTs)
Bacterium	Blood culture for bacteria and fungi and bacterial culture with other specimens such as sputum and urine
Virus	Real-time PCR for Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV) and human parvovirus B19 (HP–B19); serum antibody test for EBV, CMV, HSV and HP-B19 and common respiratory viruses; antigen tests for common respiratory viruses
Fungus	Microbiological culture of blood and BALF; IgM and IgG antibody test for *Aspergillus Fumigatus*
Others	Serum antibody test and antigen test for *Mycoplasma Pneumonia* and *Legionella*, T-spot assay for *Mycobacterium Tuberculosis*

BALF, broncho alveolar lavage fluid.

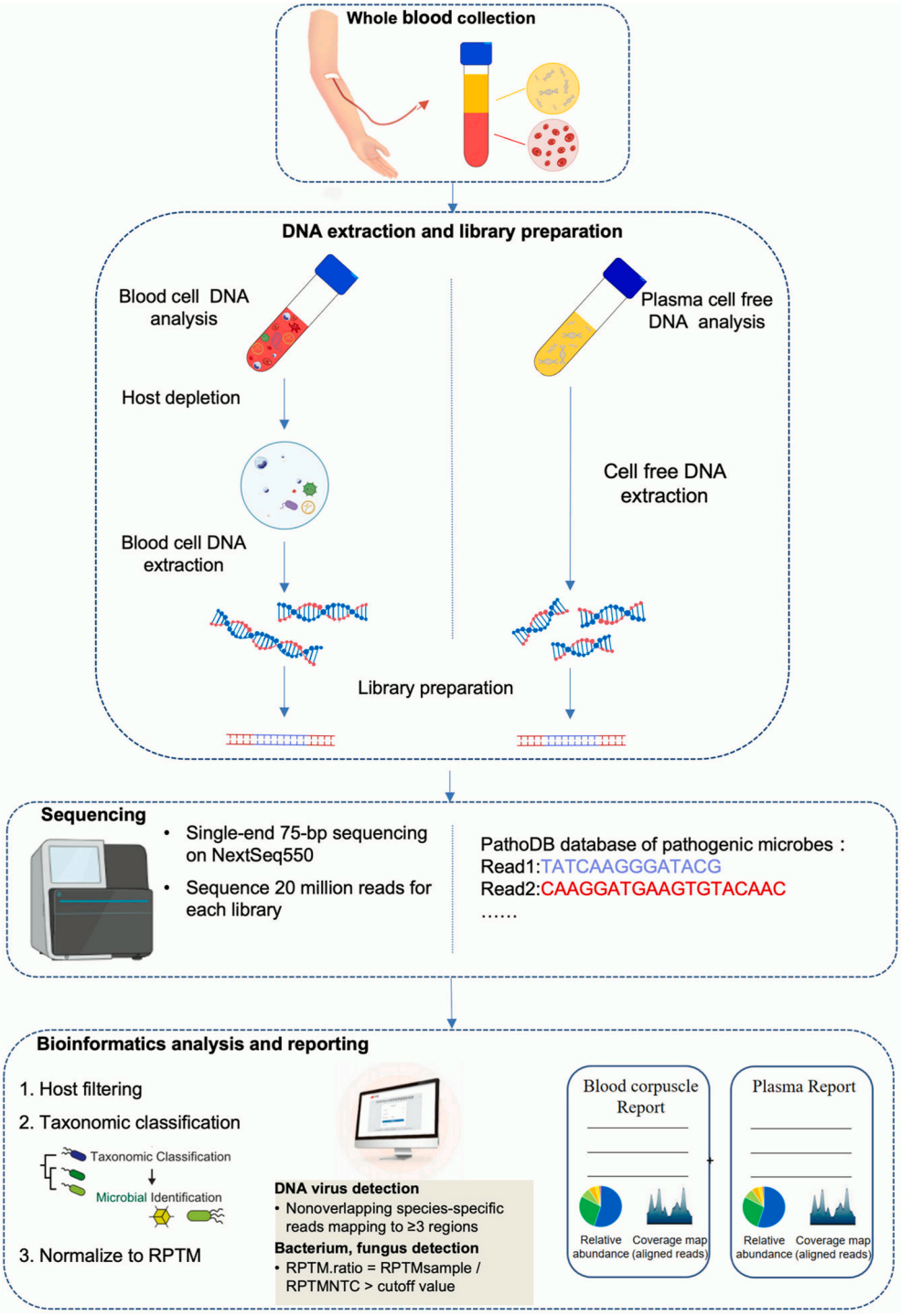

Fig. 1. Schematic workflow of plasma and blood-cell mNGS. Whole-blood samples were collected in anticoagulation tubes. After centrifugation, nucleic acid was extracted from the plasma and blood cells for further sequencing. The mNGS procedure included library preparation, metagenomic sequencing, bioinformatics analysis, and reporting.

2.5. Statistical analysis

Comparative analyses were conducted using a chi-squared test; Fisher's exact test was used for categorical variables. For diagnostic analytics, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and their corresponding 95 % CIs

were calculated by exact (Clopper-Pearson) methods. The clinical concordance rate refers to the ratio of cases with causative pathogens identified to those with positive mNGS results. All statistical analyses were performed using GraphPad Prism 6.03 (GraphPad Software Inc.), SPSS 20.0 (IBM, Armonk, NY, USA), and R software (4.2.2). A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

In this study, we enrolled 106 patients who presented with 128 febrile episodes, of which 13 experienced two episodes, three had

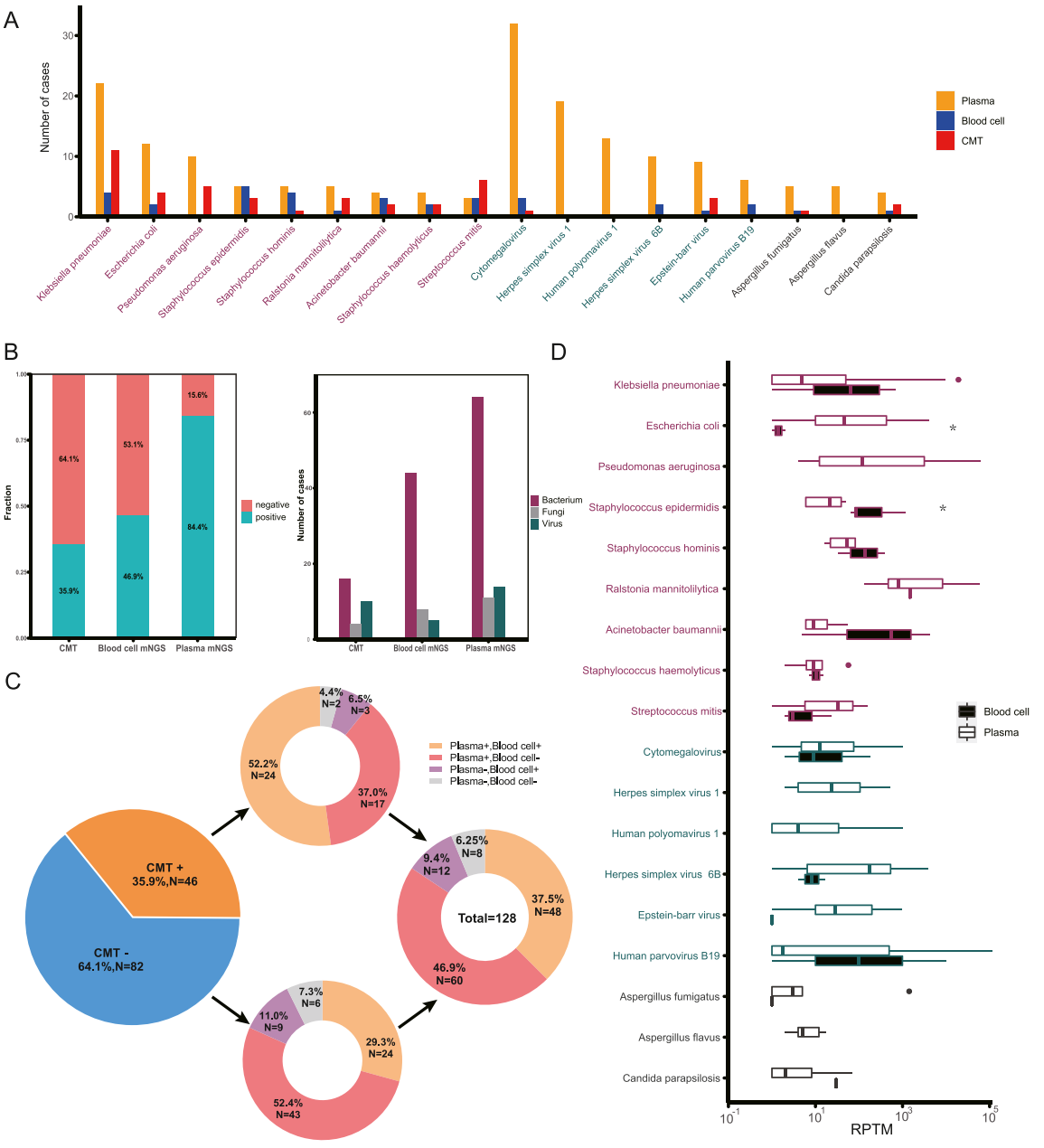

Fig. 2. Comparison of the conventional microbiological test (CMT) and mNGS. (A) Distribution of the main pathogens (bacteria, viruses, and fungi) identified by CMT, plasma mNGS and blood-cell mNGS. (B) Comparison of positivity rates and the numbers of bacteria, viruses, and fungi detected by CMT, plasma mNGS and blood-cell mNGS. (C) Pie chart demonstrating the positivity distribution with CMT and mNGS. (D) Read abundance of the main organisms identified by plasma mNGS and blood-cell mNGS. *, $P < 0.05$.

three episodes, and one encountered four episodes. The median age of the participants was 6.55 years, with an age range of 1.1–17.3 years, and the male-to-female ratio stood at 1.37:1. The predominant underlying conditions among the patients included acute lymphoblastic leukemia (n = 73), acute myeloid leukemia (n = 29), lymphoma (n = 5), and hematopoietic stem cell transplantation (HSCT, n = 17). A significant proportion, 91 (71.1 %) of the patients, developed febrile neutropenia, characterized by fever and an absolute neutrophil count below 500/mm^3. The median time from the onset of the febrile condition to the collection of mNGS specimens was 4.0 days. In terms of infection sites, diagnoses included bloodstream infections in 58 patients, respiratory tract infections or pneumonia in 31, gastrointestinal infections in nine, viremia in six, infections of the oral cavity in four, soft tissue infections in four, central nervous system infections in three, urinary tract infections in one, and fever of unknown origin in 19 patients. Multiple-site infections were observed in five patients. Moreover, the febrile illnesses of 19 patients were resolved following adjustments to antimicrobial regimens based on mNGS findings, with 11 cases of bacterial infections, three of viral infections, and five of fungal

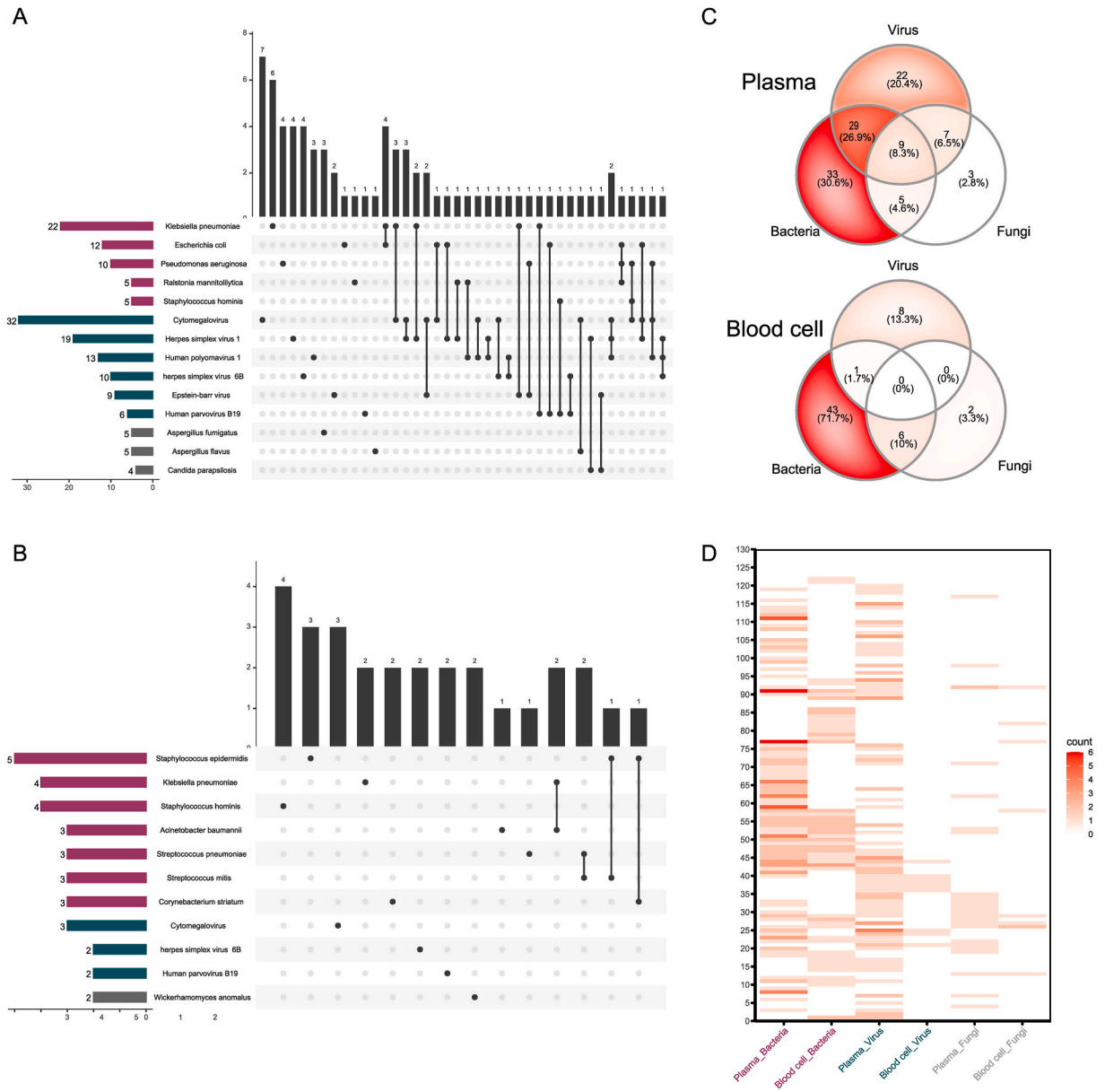

Fig. 3. Distribution of microorganisms detected by mNGS. (A) Upset diagram for plasma mNGS. In the upset diagram, each row represents one organism, and each column represents the sample number of each pathogen and infection or coinfection type identified by mNGS. The black dots connected by a line indicate multiple organisms detected in one sample. Bacteria reported ≥5 times, viruses reported ≥5 times, and fungi reported ≥4 times were included. (B) Upset diagram for blood-cell mNGS. Bacteria reported ≥3 times, viruses reported ≥3 times, and fungi reported ≥2 times were included. (C) Venn diagram representing the coexistence of different microbiological species in each plasma sample and blood cell sample. (D) Heatmap represents the numbers of bacteria, viruses, and fungi detected in each specimen. The left y-axis indicates the case number.

infections.

3.2. Comparison of the performance of CMT and mNGS

The detection rate for conventional microbiological tests (CMT) was 35.9 % (46 out of 128), identifying 14 types of bacteria, eight viruses, and five fungi. In contrast, the positivity rate for metagenomic next-generation sequencing (mNGS) of plasma samples reached 84.4 % (108 out of 128), with 64 bacterial species, 14 viruses, and 11 fungi identified. mNGS analysis of blood cells yielded a positivity rate of 46.9 % (60 out of 128), detecting 44 bacterial species, five viruses, and eight fungi. Fig. 2A displays the most commonly identified microorganisms. Plasma-based mNGS identified significantly higher numbers of bacteria ($P = 0.001$), viruses ($P < 0.001$), and fungi ($P = 0.002$) compared to mNGS of blood cells, as illustrated in Fig. 2B.

Fig. 2C illustrates that among the 46 patients who received positive results from conventional microbiological tests (CMT), 24 (52.2 %) also had concordant positive findings in both plasma and blood-cell mNGS assays. Conversely, in the group of 82 patients with negative CMT outcomes, 24 (29.3 %) were found to have positive results in both plasma and blood-cell mNGS analyses. Overall, in 48 (37.5 %) of the cases, both plasma and blood-cell mNGS tests yielded positive results; whereas, in 120 (93.8 %) of the cases, positive findings were obtained through either plasma or blood-cell mNGS.

Overall, the reads per ten million (RPTM) values derived from plasma mNGS were on par with those obtained from blood-cell mNGS, showing medians of 11 and 15, respectively ($P = 0.828$). Fig. 2D displays the mNGS RPTM values for various organisms. Across the spectrum of detected organisms, RPTM values were generally consistent, with the notable exceptions of *Escherichia coli* and *Staphylococcus epidermidis*, where disparities were observed.

3.3. Distribution of microorganisms detected by mNGS

In 68.5 % of febrile illness cases (74 out of 108), plasma mNGS identified two or more organisms simultaneously within a single

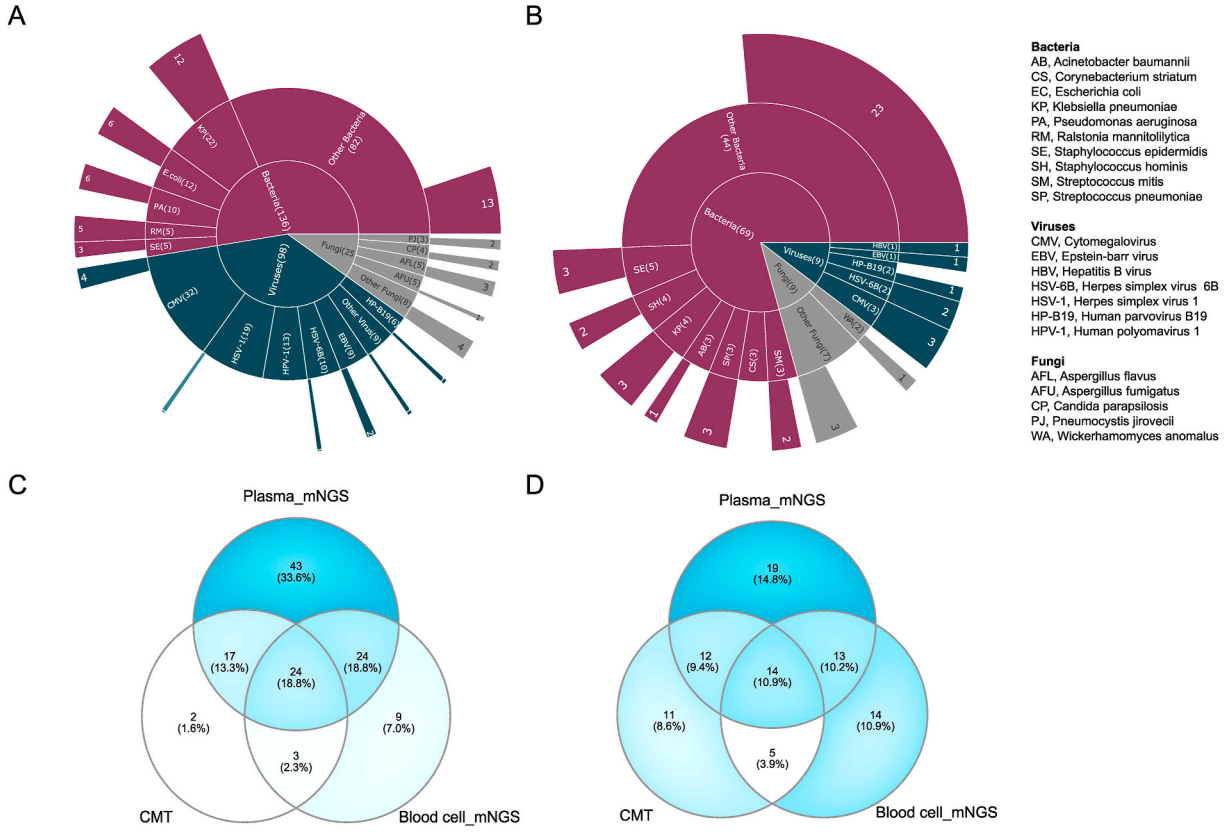

Fig. 4. Performance of mNGS in identifying causative pathogens. (A) Summary of the total numbers of bacteria, viruses, and fungi detected by plasma mNGS and the numbers of causative pathogens. (B) Summary of the total numbers of bacteria, viruses and fungi detected by blood-cell mNGS and the numbers of causative pathogens. (C) Venn diagram showing the numbers and percentages of patients with positive results tested by conventional microbiological test (CMT), plasma mNGS and blood-cell mNGS among 128 cases of febrile illness. (D) Venn diagram showing the numbers and percentages of patients with causative pathogens identified by CMT, plasma mNGS and blood-cell mNGS among 128 cases of febrile illness.

specimen, whereas blood-cell mNGS did so in 38.3 % of cases (23 out of 60), demonstrating a significant difference ($P < 0.001$). Fig. 3A and B illustrate the variety and co-occurrence of the primary organisms frequently detected by both plasma and blood-cell mNGS. Specifically, plasma mNGS detected one, two, or three types of organisms (bacteria, viruses, or fungi) in 53.7 %, 38.0 %, and 8.3 % of the tests, respectively. In contrast, blood-cell mNGS identified one, two, or three organism types in 88.3 %, 11.7 %, and 0 % of the tests, respectively, as shown in Fig. 3C. The detection of multiple organisms in a single test was notably more common with plasma mNGS than with blood-cell mNGS, as highlighted in Fig. 3D.

3.4. *Performance of mNGS in identifying causative pathogens*

Our investigation focused on determining whether the organisms reported by metagenomic next-generation sequencing (mNGS)

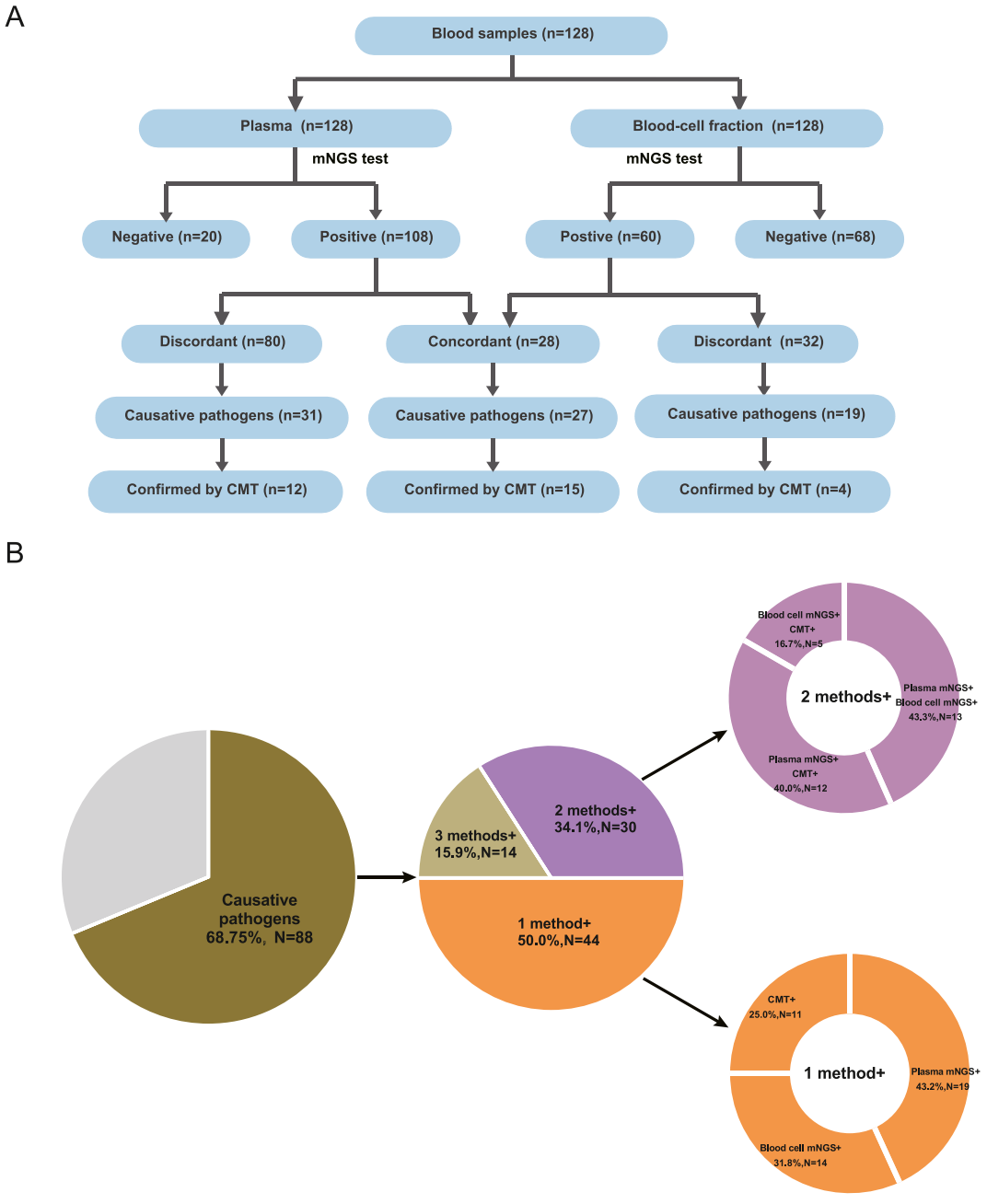

Fig. 5. Clinical evaluation of the efficiency of mNGS. (A) Schematic workflow and comparison of plasma mNGS and blood-cell mNGS. (B) Pie chart demonstrating the distribution of cases with confirmed causative pathogens according to different methods.

were the actual causative pathogens of febrile illnesses. In the plasma mNGS group, microorganisms were detected in 108 cases, with 58 being identified as causative pathogens—20 as "definite" and 38 as "probable." Conversely, in the blood-cell mNGS group, microorganisms were found in 60 cases, with causative pathogens recognized in 46–16 classified as "definite" and 30 as "probable." Notably, the clinical concordance rate for blood cell samples (76.7 %, 46/60) was significantly higher than that for plasma samples (53.7 %, 58/108), with a P-value of 0.003 indicating statistical significance.

In terms of the pathogens detected, the plasma mNGS group reported 136 bacteria, 98 viruses, and 25 fungi, but only 45 bacteria (33.1 %), 10 viruses (10.2 %), and 12 fungi (48.0 %) were deemed causative pathogens, as illustrated in Fig. 4A. Meanwhile, the blood-cell mNGS group reported 69 bacteria, 9 viruses, and 9 fungi, with 37 bacteria (53.6 %), 8 viruses (88.9 %), and 4 fungi (44.4 %) identified as causative pathogens, as depicted in Fig. 4B.

3.5. Integration of plasma and blood-cell mNGS results improves the sensitivity and accuracy

Next, we investigated the potential enhancement in sensitivity and accuracy afforded by the concurrent analysis of organisms in both plasma and blood cells via mNGS. By incorporating results from blood cells, the sensitivity of plasma mNGS was notably improved, with the positivity rate climbing from 84.4 % to 93.8 % within this cohort ($P = 0.016$), as depicted in Fig. 4C. In terms of detecting causative pathogens, the amalgamation of plasma and blood-cell mNGS findings led to the identification of causative pathogens in 77 cases of febrile illness, marking a substantial improvement compared to using plasma mNGS alone (60.2 % vs. 45.3 %, $P = 0.017$), as shown in Fig. 4D. Notably, causative pathogens were confirmed in 9 out of the 12 cases that presented positive results in blood cell mNGS but negative in plasma mNGS.

3.6. Assessment of mNGS diagnostic efficacy

We evaluated the diagnostic capabilities of conventional microbiological tests (CMT) and metagenomic next-generation sequencing (mNGS). In total, causative pathogens were identified in 88 cases of febrile illness, as independently verified by the clinical team and depicted in Fig. 5A. Among these, bacteria were confirmed in 65 cases, viruses in 17, fungi in 18, and other pathogens in four. Concordance between plasma and blood-cell mNGS results was observed in 28 cases, with causative pathogens confirmed in 27 of these patients.

As illustrated in Fig. 5B, within the 88 cases with verified causative pathogens, 14 pathogens were concurrently identified by all three methods, 30 by two methods, and 44 by one method alone. The integration of plasma and blood-cell mNGS results captured 87.5 % (77/88) of the causative pathogens. Considering the 88 cases with established microbiological causes as positive reference standards, and the remaining 40 cases without identified causative pathogens as negative reference standards, the sensitivity and specificity were calculated as 65.9 % (95 % CI, 55.5%–75.0 %) and 20.0 % (95 % CI, 10.5%–34.8 %) for plasma mNGS, 52.3 % (95 % CI, 42.0%–62.4 %) and 80.0 % (95 % CI, 65.2%–89.5 %) for blood-cell mNGS, and 87.5 % (95 % CI, 79.0%–92.9 %) and 15.0 % (95 % CI, 7.1%–29.1 %) for the combined approach, respectively.

4. Discussion

Recently, metagenomic next-generation sequencing (mNGS) has emerged as a promising diagnostic tool for infectious diseases. Numerous clinical studies have shown that sequencing of cell-free DNA (cfDNA) achieves a higher detection rate compared to traditional microbiological tests, with sensitivities ranging between 70.0 % and 92.9 % and specificities from 62.7 % to 88.2 % [14]. mNGS offers significant benefits over conventional microbiological testing (CMT) in identifying clinically relevant pathogens, especially in immunocompromised patients. Blood samples, frequently used in this demographic, show varying efficacy in detecting bacterial, viral, and fungal pathogens through mNGS. While mNGS is highly sensitive in identifying causative bacterial agents surpassing CMT, its effectiveness against viruses is less pronounced. Given that standard PCR and serological antibody assays can identify most viral infections, and considering the lower clinical concordance rate of mNGS for viruses, its utility for diagnosing viral infections may not be cost-effective. Conversely, although serum tests for 1,3 β-D-glucan and galactomannan provide indicators of fungal infections, they fall short of identifying specific fungi. mNGS, however, is adept at detecting fungal pathogens often overlooked by conventional testing methods, thus offering a distinct advantage for diagnosing fungal infections. Notably, in our prior research focused on pediatric hematology/oncology patients, only 58.4 % of positive mNGS findings pinpointed the causative pathogens [13]. Enhancing the sensitivity and accuracy of mNGS presents a considerable challenge in clinical settings, particularly for immunocompromised individuals.

To this point, clinical research focusing on blood-cell mNGS has been limited. Li et al. recently documented the identification of both extracellular cell-free and intracellular DNA fragments of *Legionella* using concurrent plasma and blood-cell mNGS in a severe case involving pneumonia, rhabdomyolysis, and soft tissue infection, necessitating intensive care [23]. In another study, Liu and their team demonstrated that mNGS analysis of cfDNA significantly outperformed intracellular DNA sequencing in diagnosing central nervous system infections, achieving sensitivities of 60.2 % and 32.0 %, respectively [24]. Moreover, in cases of infective endocarditis, the diagnostic efficacy of whole-blood mNGS was found to be on par with that of plasma mNGS for pathogen identification [25]. Consequently, the utility of intracellular DNA sequencing demonstrates variability, contingent upon specific conditions and patient groups.

To our knowledge, studies that systematically assess the accuracies of plasma mNGS both in isolation and in conjunction with blood-cell mNGS are exceedingly scarce. In a recent study focused on patients with bloodstream infections (BSI), Wu and colleagues

compared the detection efficiency of mNGS in plasma and blood cells. Their findings indicated that mNGS of blood cells was more effective in identifying pathogens that were positive in blood cultures (72.13 % vs 67.21 %), offering a distinct advantage in detecting culturable pathogens. However, in terms of diagnosing bloodstream infections, plasma mNGS demonstrated higher sensitivity than blood cell mNGS (69.82 % vs 53.25 %). Therefore, a combined approach of whole-blood mNGS emerges as a more advanced diagnostic method for bloodstream infections (BSI) [16]. Building upon these insights, our investigation contributes novel and previously unreported findings regarding the utility of plasma and blood-cell mNGS in pediatric patients with compromised immune systems. Although plasma mNGS identified more bacteria ($P = 0.001$), viruses ($P < 0.001$), and fungi ($P = 0.002$) compared to blood-cell mNGS, only 33.1 % of the bacteria, 10.2 % of the viruses, and 48.0 % of the fungi were considered pathogenic in these immunocompromised pediatric hematology/oncology patients. Conversely, the clinical concordance rate of blood-cell samples was significantly higher than that of plasma samples. Several factors contribute to this phenomenon: First, the positivity rate of blood-cell mNGS was much lower than that using plasma, as there are more organisms living in the plasma but not blood cells, and human DNA interference and host depletion may lead to the loss of organism information. Second, the clinical concordance rate of blood-cell mNGS was much higher than that of plasma mNGS in positive samples. Several organisms were commonly detected by plasma mNGS in single specimens, which was rare by blood-cell mNGS. Transient microbial DNA floating into the blood leads to this phenomenon and causes "false positive" results, while blood cells contain only intracellular microbes that may indicate true infection. On the other hand, using the same sequence read cut-off for blood cell mNGS as what is used for plasma may artificially reduce the "false positive" results, as human reads were included when RPM was calculated. Similarly, within our study cohort, integrating both methods also enhanced the capability of mNGS to detect a wider range of microbes, thereby increasing the likelihood of identifying pathogenic pathogens. However, the notably low specificity highlights the potential risks associated with false positive results, particularly concerning pediatric patients, who are especially susceptible to the adverse effects of unnecessary antibiotic treatment.

This study presents certain limitations. Firstly, it was a single-center investigation focusing on immunocompromised children, necessitating caution in generalizing the findings to other patient populations; multicenter trials are warranted to validate our results. Secondly, a majority of the participants received antibiotic treatments prior to undergoing mNGS testing. Given that the optimal timing for mNGS sampling is within the first 24 h following the onset of fever in neutropenic patients [26], the efficacy of plasma mNGS in detecting bacterial pathogens diminishes beyond this critical period. Nevertheless, considering the substantial cost associated with mNGS, it's common for patients to forego testing within this ideal timeframe. Consequently, the conclusions drawn may have reference value for clinicians, as this study is a real-world observation.

5. Conclusions

The current study showed that mNGS using blood cells is less sensitive than plasma mNGS in identifying causative pathogens but is also more specific with much less false-positivity. Simultaneous plasma and blood-cell mNGS enhances the overall diagnostic performance of mNGS in detecting the microbial origins of febrile illnesses. However, due to the high false-positive rate, clinicians should exercise caution when interpreting mNGS results.

Ethical approval statement

This study was approved by the Children's Hospital of Zhejiang University School of Medicine Institutional Review Board (IRB number 2021-IRB-004), and the participants' legal guardians provided written informed consent to participate.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Di Wang: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Zihan Zhang:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Heping Shen:** Writing – review & editing, Investigation, Data curation. **Fenfen Jin:** Writing – review & editing, Investigation, Data curation. **Juan Liang:** Writing – review & editing, Investigation, Data curation. **Diying Shen:** Writing – review & editing, Investigation, Data curation. **Hua Song:** Writing – review & editing, Investigation, Data curation. **Jingying Zhang:** Writing – review & editing, Investigation, Data curation. **Weiqun Xu:** Writing – review & editing, Investigation, Data curation. **Yongmin Tang:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Xiaojun Xu:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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