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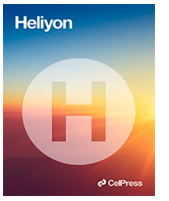

Research article

Predictive performance of Metagenomic Next Generation Sequencing in early detection of post-liver transplantation infections

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A R T I C L E I N F O

Keywords:
Metagenomic next-generation sequencing
Bronchoalveolar lavage fluid
Pathogenic identification
Liver transplantation
Pulmonary infection
Prognosis

A B S T R A C T

Objective: To evaluate the predictive performance of metagenomic next-generation sequencing (mNGS) in identifying and predicting pulmonary infections following liver transplantation and to investigate its association with patient outcomes within the initial four-week post-transplantation period.

Methods: We retrospectively analyzed 41 liver transplant patients with suspected pulmonary infections from August 2022 to May 2023. Bronchoalveolar lavage fluid (BALF) samples were collected on the first postoperative day for metagenomic next generation sequencing (mNGS) and culture. The predictive capability of mNGS for subsequent infections was assessed by monitoring inflammatory biomarkers and comparing the detection rates with culture methods. Real-time Polymerase Chain Reaction (Rt-PCR) was used to monitor Human betaherpesvirus 5 (CMV) and Human parvovirus B19 (B19) weekly during a four-week postoperative period. Inflammatory biomarkers and blood coagulation function were evaluated on specific days throughout the first, third, fifth, and during four weeks following surgery. The study was conducted until August 2023 to evaluate the patients' prognostic survival outcome, classifying them into groups based on the mortality and survival.

Results: The analysis included a total of 41 patients, comprising 32 males and 9 females, with an average age of 52 (47, 63) years. Within one week after liver transplantation, there were 7 cases of bacterial infections, 5 cases of fungal infections, 19 cases of mixed infections, 8 cases without any infection, and 2 cases with unidentified pathogen-associated infections. mNGS successfully predicted 39 (72 %) strains of pathogens, while culture-based methods only detected 28 (52 %) strains. Among the 8 patients diagnosed as non-infected, culture methods identified positive results in 4 cases (50 %), whereas mNGS yielded positive results in 7 cases (87.5 %). The detection rates of CMV and B19 by Rt-PCR within 4 weeks after liver transplantation were 61 % and 17 %, respectively (25/41, 7/41) among the patients. During the study period, a total of 9 patients

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succumbed while 32 patients survived. The death group and the survival group exhibited significant differences in CRP, HGB, and INR levels at specific monitoring time points. The proportion of CMV detection in blood was significantly higher in the death group compared to the surviving group. Elevated CRP level was identified as a prognostic risk factor.
Conclusions: Despite the presence of false positives, mNGS still presents a potential advantage in predicting pulmonary infection pathogens following liver transplantation. Furthermore, the levels of CRP and CMV carrier status within four weeks post-surgery exhibit significant associations with patient survival and prognosis.

1. Introduction

Liver transplantation is a critical treatment modality for end-stage liver disease, yet postoperative infections pose a significant threat to patient outcomes [1]. Postoperative infection not only prolongs hospitalization but also leads to increased medical expenses and is the primary cause of mortality in liver transplant recipients [2,3]. Infections were responsible for the majority of deaths within the first month following liver transplantation (39 %), and continued to be the leading cause thereafter [4]. The lungs are a common site for postoperative infections, especially in the early postoperative phase [5,6]. The incidence of pulmonary infections after liver transplantation varies from 8 % to 23 %, with a concerning mortality rate that can reach 50 % [7]. The early diagnosis of these infections is paramount, as it enables timely intervention, potentially reducing morbidity and mortality rates.

Traditional diagnostic methods, while valuable, may be limited by their sensitivity and specificity, particularly in the context of diverse and rapidly evolving microbial communities [8]. Metagenomic next-generation sequencing (mNGS) has emerged as a powerful tool with the potential to revolutionize the field of infectious disease diagnostics [9]. Importantly, the predictive capacity of mNGS extends beyond mere identification, enabling the prognostic assessment of infection risk. In the realm of liver transplantation, mNGS holds particular promise for preemptive management strategies [10]. By predicting the onset of infections, clinicians can tailor prophylactic measures and adjust immunosuppressive regimens to balance the delicate immune response necessary for graft acceptance while mitigating the risk of infectious complications. Furthermore, mNGS can facilitate the prompt initiation of targeted antimicrobial therapies, personalized to the specific pathogen profiles identified, thereby improving treatment efficacy and potentially averting the escalation of infection severity [11].

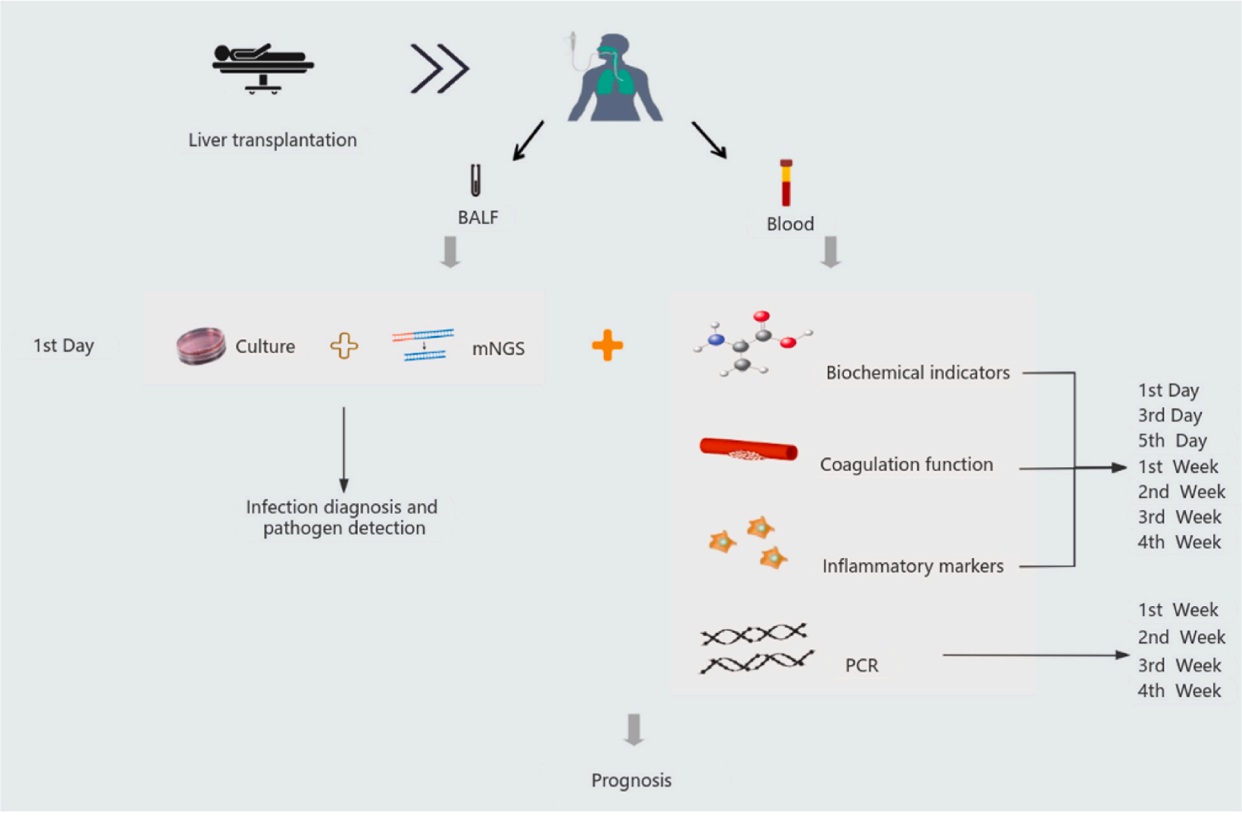

Fig. 1. Schematic diagram of study workflow.

In this study, mNGS was employed to detect pathogens in bronchoalveolar lavage fluid (BALF) samples obtained from liver transplant recipients on the first postoperative day. Over a four-week period, we monitored BALF culture results, inflammatory markers, viral nucleic acid results, and coagulation function while also tracking the patients' prognosis. Ultimately, we evaluated the predictive performance of mNGS in identifying and predicting pulmonary infection pathogens and assessed the correlation between infection severity and prognosis.

2. Materials and methods

2.1. Study population and design

We conducted a retrospective analysis on patients who underwent liver transplantation at Shulan (Hangzhou) Hospital from August 2022 to May 2023, and included 41 patients with suspected pulmonary infection in the analysis. On the first postoperative day, BALF samples were subjected to mNGS and culture. The presence of Human betaherpesvirus 5 (CMV) and Human parvovirus B19 (B19) was monitored weekly using Real-time Polymerase Chain Reaction (Rt-PCR) during a postoperative period lasting four weeks. The levels of inflammatory biomarkers, including procalcitonin (PCT) and C-reactive protein (CRP), as well as blood coagulation function assessed by international normalized ratio (INR), were evaluated on postoperative day 1, day 3, day 5, during the first week, second week, third week, and fourth week following surgery. The study conducted a median follow-up time of 152 (95, 219) days until August 2023 to evaluate the prognostic survival outcomes of patients, who were categorized into either the survival or death group (Fig. 1).

Inclusion criteria: I) Age over 18 years; II) Liver transplantation performed during hospitalization; III) Sufficient sample availability for laboratory tests and regular follow-up; IV) Informed consent form signed by the patient or authorized representative; V) Patients presenting with fever, cough, or other symptoms suggestive of pulmonary infection or imaging evidence of inflammatory infiltration.

Exclusion criteria: I) Incomplete follow-up information; II) Unavailability of mNGS results from BALF on the first postoperative day; III) Patients declined to participate.

The study was approved by the Research Ethics Committee of Shulan (Hangzhou) Hospital under the reference number KY2022052.

2.2. Metagenomic sequencing

The cell wall was disrupted by subjecting 2 ml of BALF to FastPrep-24™ 5G. Nucleic acid extraction was performed using the Microsample Genomic DNA Extraction Kit (DP316, Tiangen). Sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs Inc.), and sequencing was conducted on a Nextseq 550 DX platform (75 bp single-end reads).

Low-quality reads, including duplicated reads and short reads (length $<$50 bp), were eliminated from the dataset. The Burrows-Wheeler Alignment tool was employed for mapping to the GRCh38 human reference genome in order to exclude any human sequence data. Subsequently, SNAP was utilized for aligning the remaining sequencing data against the NCBI nt database. The mapped data underwent advanced analysis using custom scripts developed in-house, encompassing taxonomy annotation, calculation of genome coverage/depth, and abundance estimation. Further details can be found in our previous publication [12].

2.3. Identification of pathogens

To ensure the accurate identification of true pathogens and to differentiate them from contaminants or colonized organisms, we implemented a multi-faceted approach encompassing microbiological, clinical, and radiological assessments.

Microbiological assessment: The reporting guidelines for mNGS were established based on the study conducted by Miao et al. [13]. Bacteria (excluding mycobacteria), viruses, and parasites were deemed significant if their coverage exceeded that of any other microorganism by at least 10-fold. Clinical significance should be attributed to the detection of one or more mycobacteria. A positive report for a fungus required its detected coverage being more than 5 times higher that of other fungal species.

(1–3) - β - D - glucan (G test) and galactomannan (GM test) assays: The results from the G and GM tests will be interpreted alongside the mNGS data to provide a comprehensive assessment of fungal infections. A positive G test result, indicative of fungal cell wall components, was considered supportive of a fungal infection, whereas a positive GM test was suggestive of Aspergillus species. Discordant results between mNGS and the G/GM tests will be further investigated, and additional clinical and microbiological data (such as culture) will be considered for a conclusive diagnosis.

Clinical assessment: Clinical symptoms indicative of infection were meticulously documented, including fever, leukocytosis, or specific signs related to the suspected pathogen. The clinical response to targeted antimicrobial therapy was also monitored, with improvement being a supportive criterion for the presence of a true pathogen.

Radiological assessment: Chest computed tomography (CT) scans were evaluated for the presence of infiltrates or other abnormalities suggestive of infection. Radiological findings were correlated with microbiological data to reinforce the diagnosis of true infection.

A multidisciplinary team of infectious disease specialists, radiologists, and transplant surgeons reviewed all data, including mNGS results, clinical presentations, radiological images, and test results from the G and GM assays. This comprehensive review allowed us to reach a consensus on the diagnosis of true infection versus colonization or contamination.

2.4. Detection of CMV and B19 in blood

The ABI 7300 instrument (ABI, USA) and the human parvovirus B19 nucleic acid assay kit from Zhijiang (Shanghai) were employed for the detection of B19 virus. The ABI 7500 instrument (ABI, USA) along with the CMV assay kit from Tianlong (Suzhou) were utilized for CMV detection. All procedures were conducted in accordance with the respective instrument manuals.

2.5. Statistical analysis

The data analysis was performed using SPSS 22.0, while Graphpad Prism 8 was utilized for generating graphics. Normally distributed data were presented as mean ± standard deviation, whereas non-normally distributed data were expressed as quartiles using the non-parametric Mann-Whitney *U* test to compare between groups. Counting data were reported as the number of cases (percentage) [n (%)], and comparisons between groups were conducted using either chi-square test or Fisher's exact test. A two-tailed *P*-value <0.05 indicated statistical significance.

3. Results

3.1. Demographic characteristics of study population

The analysis included a total of 41 patients, comprising 32 males and 9 females, with an average age of 52 (47, 63) years. Among the patients, 29 (70.7 %) were diagnosed with hepatitis B, while hepatitis E was observed in only one patient (2.4 %) **(Table 1).**

3.2. Infection diagnosis and pathogen detection

Within the first week following the operation, 30 cases exhibited symptoms and imaging indicative of pulmonary infection. This was further followed by an additional three cases reporting similar symptoms by the end of the second week post-surgery (Fig. 2).

The mNGS identified a total of 24 bacterial species and 14 fungal species, with *Enterococcus faecium (E. faecium)*, *Klebsiella pneumoniae (K. pneumoniae)*, and *Streptococcus pneumoniae (S. pneumoniae)* being the most prevalent bacteria, and *Candida albicans (C. albicans)* and *Pneumocystis jirovecii (P. jirovecii)* as the dominant fungi. Additionally, *Human betaherpesvirus 5* (CMV) was found in 17 % (7/41) of patients (Fig. 3). The culture method detected a total of 10 bacterial species, with *Acinetobacter baumannii (A. baumannii)*, *Stenotrophomonas maltophilia (S. maltophilia)*, and *Pseudomonas aeruginosa (P. aeruginosa)* as the predominant three. Among the fungal species identified through the culture method (8 in total), *C. albicans* and *Candida glabrata (C. glabrata)* were found to be the most prevalent.

3.3. Evaluation of predictive performance

Among the 31 patients diagnosed with infection, the mNGS successfully predicted 39 (72 %) strains of pathogens, while culture-based methods only detected 28 (52 %) strains (Fig. 4). Among the 8 patients diagnosed as non-infected, culture methods identified positive results in 4 cases (50 %), whereas mNGS yielded positive results in 7 cases (87.5 %), including the detection of bacteria and fungi. The comparison of viral infections was not conducted due to the limitations of culture methods in pathogen detection.

Table 1
Baseline data of the patients.

Characteristics	41 patients
Age, years, median (Q1, Q3)	52 (47,63)
Gender, female, n (%)	9 (22.0)
Underlie disease, n (%)	
Hepatitis B	29 (70.7)
Liver failure	26 (63.4)
Hypertension	7 (14.1)
Diabetes	10 (24.4)
Hepatic encephalopathy	18 (43.9)
Liver cirrhosis	13 (31.7)
Hepatorenal syndrome	3 (7.3)
Peritonitis	2 (4.9)
Coronary heart disease	2 (4.9)
Liver cancer	9 (22.0)
Colon cancer	1 (2.4)
Hepatitis E	1 (2.4)
Cholangiocarcinoma	1 (2.4)
Infection in other sites	
Abdominal infection	14 (34.1)
Bloodstream infection	5 (12.2)

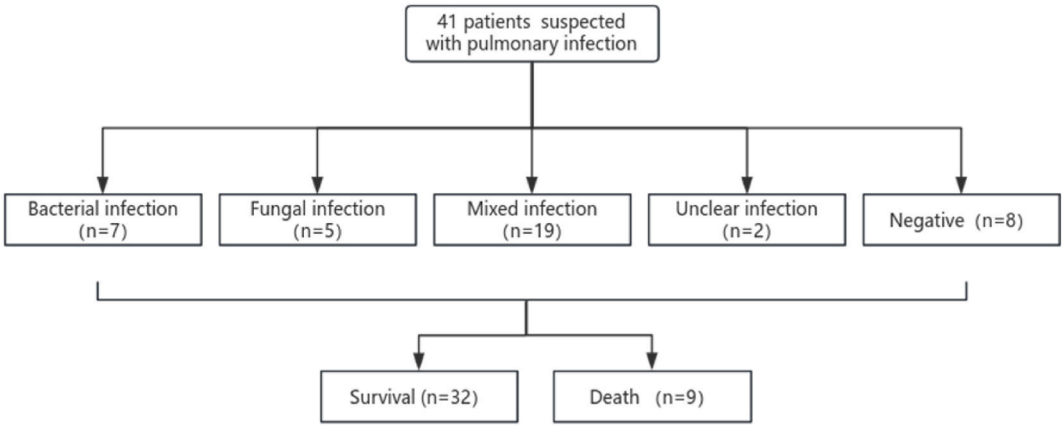

Fig. 2. The distribution of infection diagnosis.

3.4. The monitoring of CMV and B19 viruses in the bloodstream

The detection rates of CMV and B19 by Rt-PCR within four weeks after liver transplantation were 61 % and 17 %, respectively (25/41, 7/41) among the patients. Notably, the incidence of CMV infection peaked at 29 % during the second week post-surgery (Fig. 5).

3.5. Reactive fluctuations of inflammation markers

The trends observed in the WBC count, neutrophil count, and CRP were consistent. The eosinophil count exhibited a gradual increase starting from one week post-surgery, whereas the lymphocyte count demonstrated a delayed increase beginning at two weeks post-surgery. PCT levels significantly decreased until the fifth day after surgery and subsequently stabilized (Fig. 6).

3.6. Prognostic comparison

During the study period, a total of 9 patients succumbed while 32 patients survived. All patients, including the 9 who eventually passed away, were monitored and had data collected from the time of liver transplantation up to the 4-week postoperative mark. The range of survival time for these patients was 31–187 days, with a median of 180 days. There were no statistically significant differences observed in the WBC count, neutrophil count, lymphocyte count, and PCT levels between the survival and death groups at the monitored time points ($P > 0.05$). The comparative analysis of CRP, HGB, and INR between the two groups at different time intervals was illustrated in Fig. 7.

The proportion of CMV detection was significantly higher in the death group compared to the survival group **(Table 2)**. The single factor logistic regression analysis revealed that the postoperative CRP level emerged as a significant prognostic factor for mortality **(Table 3)**.

4. Discussion

In-hospital mortality after liver transplantation is primarily attributed to infection, with pulmonary fungal infection identified as the leading infectious cause of death [14]. The application of mNGS in our study has unveiled its profound potential in the early and accurate prediction of post-transplant infections. Our findings underscore the superior sensitivity of mNGS in detecting a broad spectrum of pathogens, including those that are often missed by conventional diagnostic methods. On the other hand, the findings revealed a higher CMV positive rate in the death group compared to the survival group and established a correlation between postoperative CRP levels and prognosis.

In the past few years, there has been a significant increase in the use of mNGS for diagnosing infectious diseases. Zhao et al. [15] conducted a study comparing the effectiveness of mNGS and traditional culture methods in diagnosing pathogens after liver transplantation. Their research showed that mNGS testing greatly improved clinical decision-making and helped in selecting appropriate treatments. The high detection rate of mNGS, which stood at 72 %, significantly outperformed the 52 % rate achieved by culture-based methods. This disparity in detection rates not only highlights the enhanced sensitivity of mNGS but also its predictive accuracy in identifying infections prior to the manifestation of clinical symptoms. However, the discrepancy in positive rates between mNGS and culture methods in non-infected patients is noteworthy. The higher positivity rate observed with mNGS could be attributed to its high sensitivity in detecting low levels of microbial nucleic acids, which may not necessarily indicate active infection but could be remnants from previous exposures or colonization [16]. The findings underscore the importance of considering mNGS as a complementary tool to culture methods, particularly in the early stages of infection or in cases where clinical symptoms are ambiguous. The use of mNGS may provide additional insights but should be interpreted in conjunction with clinical judgment and other diagnostic information.

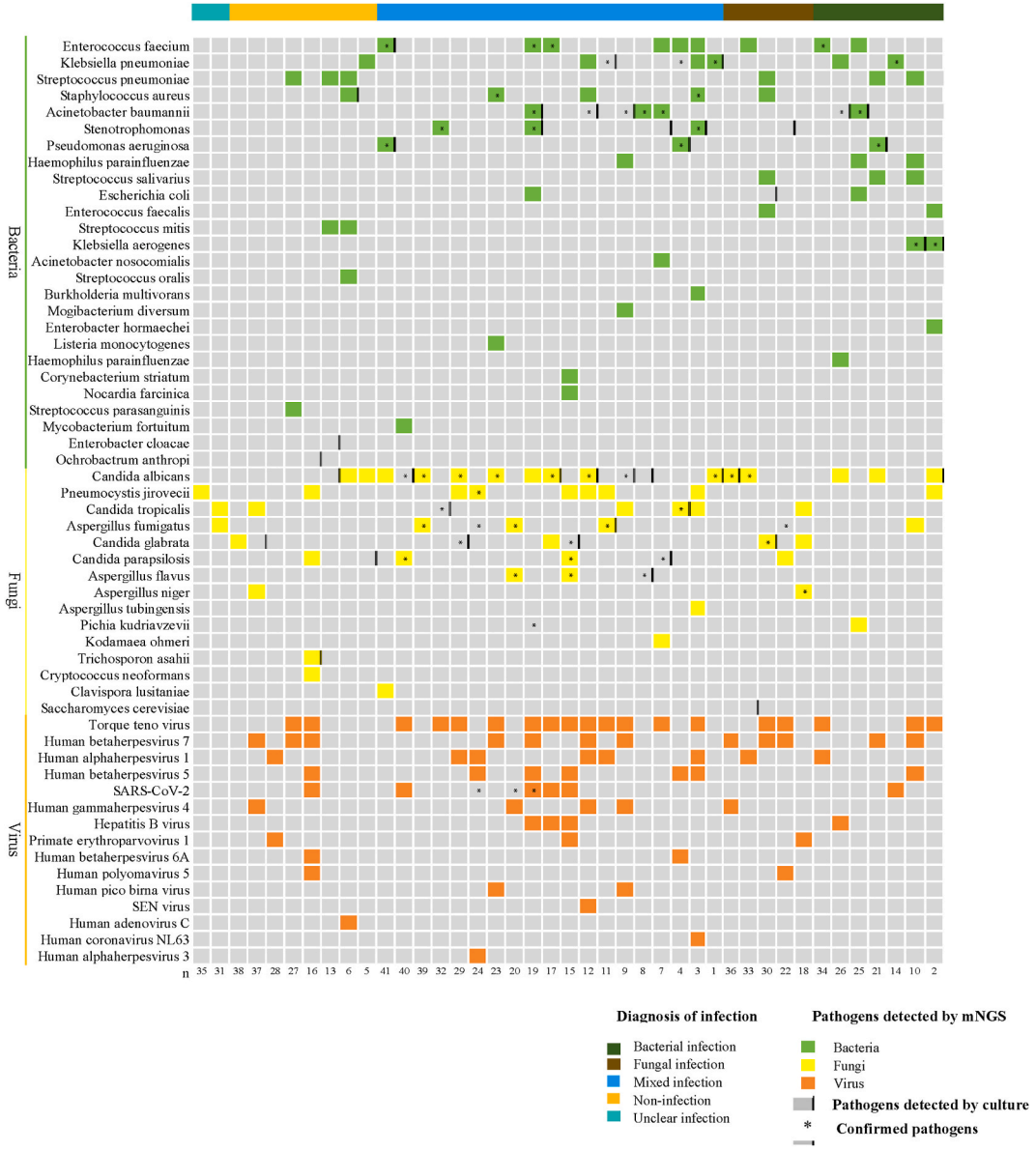

Fig. 3. Distribution mapping of infection diagnosis and pathogen detection.

Bacterial infection is the most common type of infection following liver transplantation, involving a wide range of pathogens. Previous research has consistently identified *A. baumannii*, *K. pneumoniae*, *E. faecium*, and *S. maltophilia* as the main culprits behind bacterial infections after liver transplantation [17,18]. However, it is worth noting that two decades ago, *P. aeruginosa* was highly prevalent among the strains isolated from lower respiratory tract samples following liver transplantation [19,20]. These findings highlight the temporal variations in pathogens responsible for hospital-acquired infections after liver transplantation, underscoring the importance of regular surveillance of nosocomial pathogen profiles.

The prevalence of invasive fungal infection (IFI) in liver transplant recipients is remarkably high, reaching 55 %, and it is significantly associated with a mortality rate of 64 % [21]. The majority of IFI cases are mainly caused by Candida species (81 %) and Aspergillus species (16 %) [22]. *C. albicans* is the main pathogen responsible for pulmonary IFI following liver transplantation. In this study, 24 % (10/41) of the patients were diagnosed with *C. albicans*, consistent with the incidence of fungal infection following liver transplantation reported by Yang et al. [23]. The high incidence of Candida infection may be attributed to donor colonization or potential organ contamination during transportation and storage [24]. According to reports [25], patients showed an overall mortality rate of 26 % after a 90-day observation period following invasive Candida infection. The findings of this study indicate that *Aspergillus fumigatus*, following *C. albicans*, emerged as the second most frequently identified invasive fungus. Aspergillus is a rarely encountered pathogen in fungal infections originating from donors. It is important to note that the use of antifungal prophylaxis has not shown a

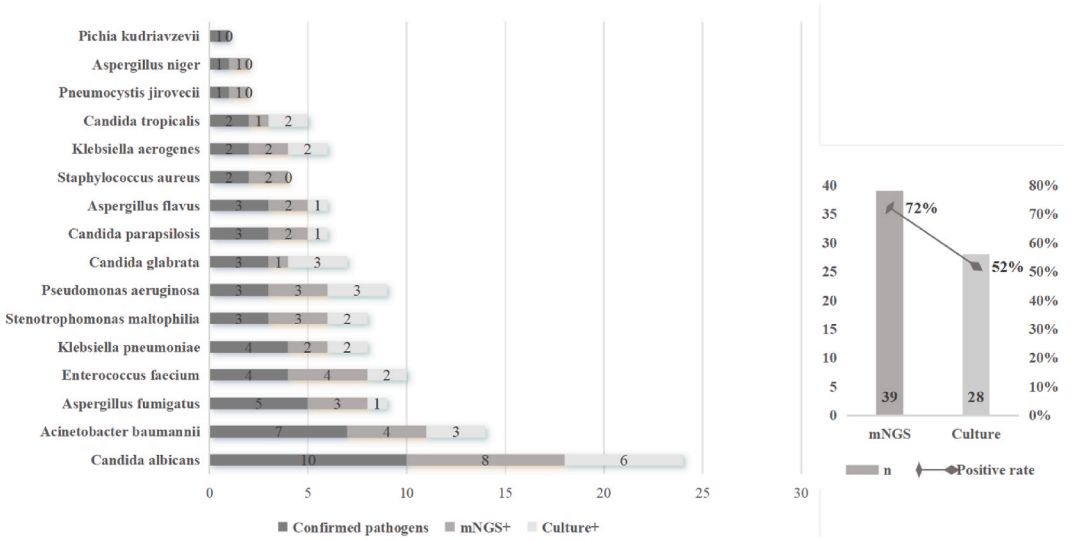

Fig. 4. The distribution of confirmed pathogens and the positive rates of mNGS and culture.

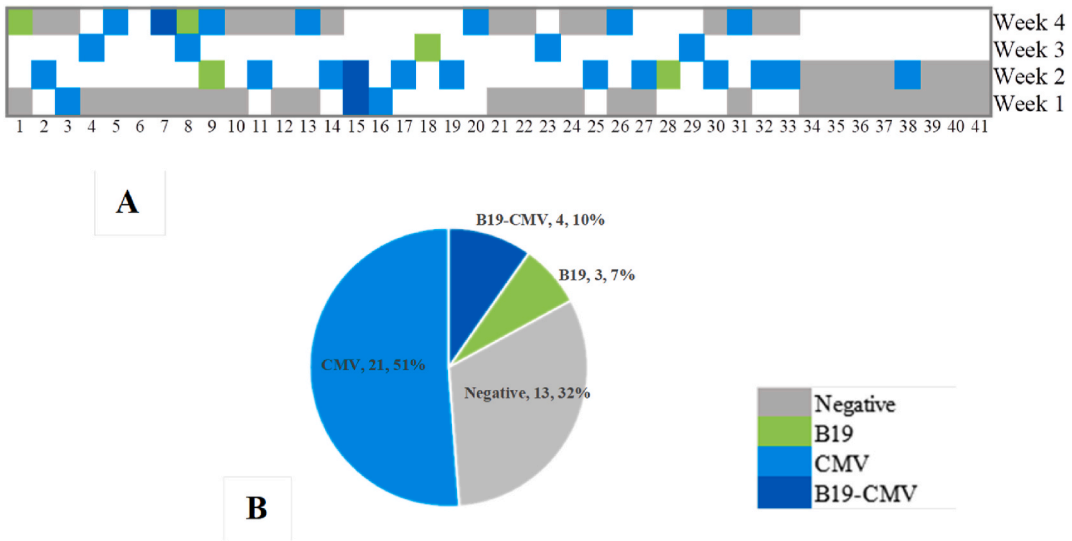

Fig. 5. Distribution of virus detection in bloodand positive rate of CMV and B19. A, Heat map illustrating the detection of viruses through Rt-PCR. **B,** Detection of CMV and B19 exhibited positive rates within four weeks post liver transplantation.

reduction in the incidence of Aspergillus invasive fungal infections [26]. Additionally, Aspergillus was identified as the predominant species detected in the liver transplant recipient autopsy records during a comprehensive analysis of solid organ transplant recipients conducted in Japan [27]. The early and accurate prediction of infections using mNGS carries significant clinical implications. It enables a more personalized approach to antimicrobial stewardship, reducing the unnecessary use of broad-spectrum antibiotics and facilitating the selection of appropriate treatment. Moreover, the predictive capabilities of mNGS can guide clinicians in making informed decisions regarding the immunosuppressive regimens for transplant recipients, thus balancing the need to prevent rejection while minimizing infection risks [28].

On one hand, CMV serves as a predisposing factor for invasive aspergillosis infection, and early-stage prevention of CMV infection can effectively mitigate patient mortality [29]. Additionally, CMV upregulates alloreactive T cells and has the potential to induce allograft rejection [30]. Furthermore, reactivation of CMV may be linked to the clinical outcome of decompensated cirrhosis [31], which could explain the significantly higher prevalence of CMV in death individuals compared to survivors in this study. In a retrospective analysis of CMV infection within 90 days following living donor liver transplantation, patients with CMV infection demonstrated a significantly higher incidence of acute rejection, an extended duration of stay in the intensive care unit (ICU), and increased mortality within 90 days. Furthermore, the group with CMV infection exhibited a significantly elevated occurrence of

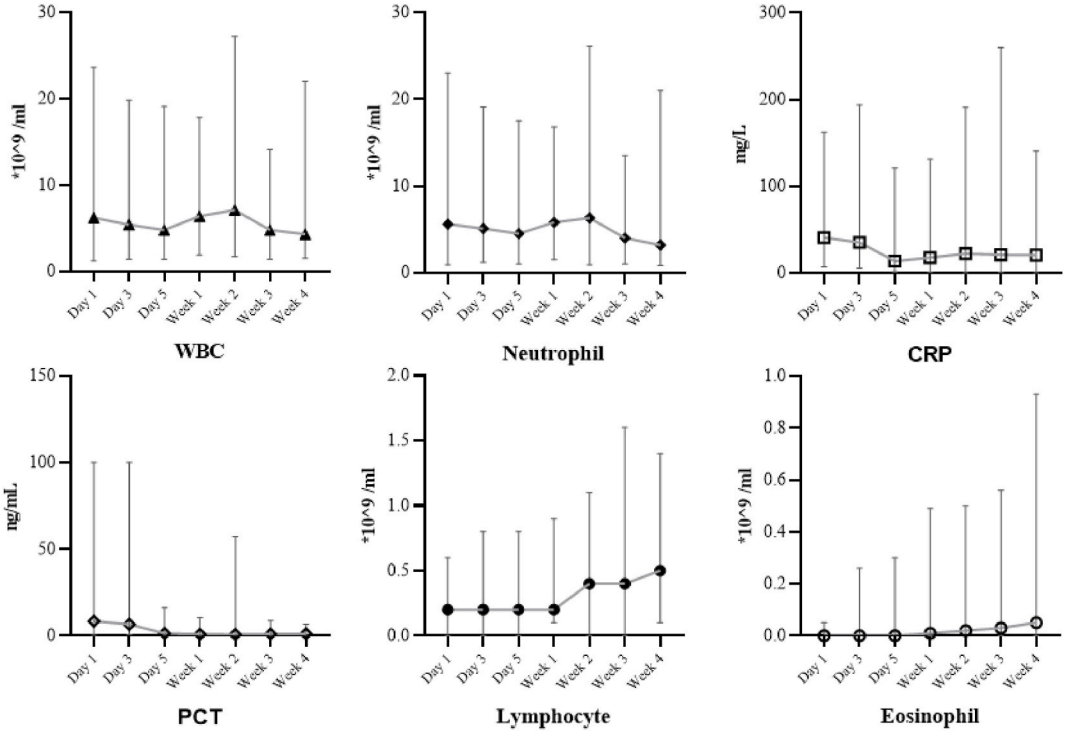

Fig. 6. Monitoring of inflammatory markers post liver transplantation.

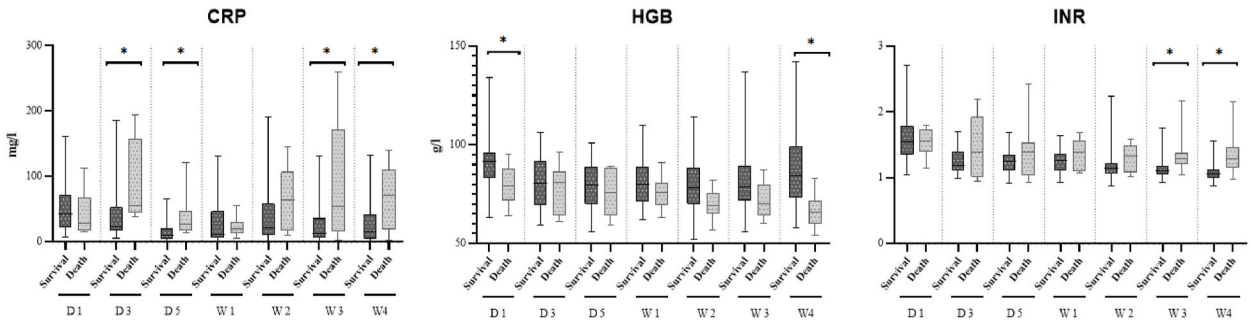

Fig. 7. Comparison of inflammatory index monitoring between patients in the survival and death groups.

Table 2
Comparison of blood virus distribution between the two groups.

Virus	Survival group	Death group	*P*
CMV	16 (50 %)	9 (100 %)	0.007
B19	7 (22 %)	0	0.315

Table 3
Single factor logistic regression analysis of CRP at various time points.

	B	OR	*P*
3rd Day	0.018	1.018	0.018
5th Day	0.053	1.054	0.032
3rd week	0.019	1.02	0.02
4th week	0.021	1.022	0.027

bacteremia and fungal diseases compared to the non-CMV infection group [32]. In recent years, there have been reported cases of refractory anemia following liver transplantation, primarily associated with incidents of parvovirus B19 infection [33,34]. The co-infection of B19 and HBV may lead to the development of more severe hepatitis-related liver disease in individuals with HBV [35]. However, no statistically significant difference was observed in the distribution of B19 between the death and surviving groups. It is noteworthy that a study investigating B19 infection following liver transplantation revealed a gradual decline in hemoglobin levels among all patients within the first two months post-transplantation [36].

Jafarpour Z. et al. developed a risk score for predicting bacterial infections, taking into account factors such as gender, length of hospital stay, and abdominal reoperation. However, they did not consider the monitoring of inflammatory indicators [6]. In our study, we found that postoperative inflammatory indicators, particularly CRP levels, also exhibited a certain predictive value in determining patient prognosis. The impact of CRP on prognosis exhibited notable significance during the 3rd day, 5th day, as well as the 3rd and 4th week following surgery. The INR index of coagulation function also reflects liver functionality. Insufficient production of coagulation factors and the disruption in vivo may contribute to the elevated INR levels observed in the death group. Coincidentally, there were significant differences observed in CRP, HGB, and INR levels between the death group and the surviving group during the fourth week. The elevated level of PCT post-surgery may be attributed to surgical complications, and it exhibited a gradual decline during the monitoring period.

Our study also has certain limitations. Firstly, we did not observe pre-transplantation infections, which may potentially impact postoperative infection rates and patient prognosis. Additionally, the small sample size of our study may constrain the broader applicability of our findings. The limited number of participants could affect the statistical power to detect subtle differences or to generalize our results to the wider population of liver transplant recipients. Therefore, future research should focus on conducting large-scale prospective studies to evaluate the prognosis of patients after liver transplantation.

5. Conclusions

In conclusion, our study initialy affirms the predictive accuracy of mNGS in identifying post-transplant infections and emphasizes its role in early risk identification. Furthermore, the levels of CRP and CMV carrier status within four weeks post-surgery exhibit potential associations with patient survival and prognosis.

Data availability statement

The datasets supporting the findings of this study are publicly available and have been deposited in a reputable online repository. The repository's name and the accession number are as follows: [https://ngdc.cncb.ac.cn/omix/, OMIX006685] (https://ngdc.cncb.ac.cn/omix/, OMIX006685). For any further information or clarification, the corresponding authors can be contacted.

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Ethics statement

The study was approved by Research Ethics Committee of Shulan (Hangzhou) Hospital (KY2022052). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

CRediT authorship contribution statement

Li Zhuang: Writing – original draft. **Chi Zhu:** Writing – original draft. **Jincheng Ma:** Data curation. **Dan Zhu:** Data curation. **Hengkai Zhu:** Data curation. **Siyi Zhong:** Formal analysis. **Xiangyan Liu:** Software. **Zhuoyi Wang:** Data curation. **Zhe Yang:** Resources. **Wu Zhang:** Data curation. **Ran Ding:** Methodology. **Dongsheng Chen:** Methodology. **Shusen Zheng:** Data curation.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "Predictive performance of Metagenomic Next Generation Sequencing in early detection of post-liver transplantation infections".

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