

Comparison of Droplet Digital PCR and Metagenomic Next-Generation Sequencing Methods for the Detection of Human Herpesvirus 6B Infection Using Cell-Free DNA from Patients Receiving CAR-T and Hematopoietic Stem Cell Transplantation

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Purpose: The aim of this study was to examine and compare the differences between droplet digital PCR (ddPCR) and metagenomic next-generation sequencing (mNGS) in the detection of human herpesvirus 6B (HHV-6B). Long-term monitoring of HHV-6B viral load in patients receiving chimeric antigen receptor-modified T-cell (CAR-T) therapy and hematopoietic stem cell transplantation (HSCT) can be used to identify immune effector cell-associated neurotoxicity syndrome (ICANS) and guide drug therapy.

Methods: Twenty-seven patients with suspected HHV-6B infection who had both mNGS and ddPCR test results were analyzed retrospectively, including 19 patients who received CAR T-cell therapy and 8 who received HSCT. The HHV-6B probe and primers were designed, and the performance of the ddPCR assay was evaluated. Subsequently, ddPCR was performed utilizing blood and urine. Data on clinical information and mNGS investigations were collected.

Results: The ddPCR test results correlated significantly with the mNGS test results ($P < 0.001$, $R^2 = 0.672$). Of the 27 time-paired samples, ddPCR showed positive HHV-6B detection in 20 samples, while mNGS alone showed positive HHV-6B detection in 12 samples. ddPCR detected additional HHV-6B infections in 8 samples that would have been missed if only mNGS were used. In addition, the first HHV-6B infection event was detected at a median of 14 days after CAR T-cell infusion (range, 8 to 19 days). Longitudinal monitoring of HHV-6B by ddPCR was performed to assess the effectiveness of antiviral therapy. The data showed that with antiviral treatment HHV-6B viral load gradually decreased.

Conclusion: Our results indicated that ddPCR improved the HHV-6B positive detection ratio and was an effective adjunct to mNGS methods. Furthermore, the longitudinal detection and quantification of HHV-6B viral load in patients undergoing CAR T-cell therapy and HSCT may serve as a guide for drug treatment.

Keywords: droplet digital PCR, CAR T-cell therapy, HSCT, infection, HHV-6B, mNGS

Introduction

In recent years, chimeric antigen receptor-modified T-cell (CAR T) therapy and hematopoietic stem cell transplantation (HSCT), including sequential CAR T-cell immunotherapy following autologous stem cell transplantation (ASCT), have

achieved dramatic improvements in the treatment of patients with refractory/relapsed hematologic malignancies.^{1–3} HSCT and CAR T recipients are very susceptible to infections due to the immune deficiency caused by B-cell aplasia and pretreatment with chemotherapy. Recent studies have highlighted the importance of human herpesvirus 6 (HHV-6) in driving infection.^{4,5} Studies have shown that nearly 40% and occasionally up to 70% of HSCT recipients develop HHV-6 infection.^{6,7} However, the time of onset and the incidence of HHV-6 after CAR T-cell infusion have not been adequately studied.

HHV-6 is a β -herpesvirus first isolated from patients with hematologic malignancy.⁸ HHV-6 shows broad cell tropism *in vivo* and induces lifelong latent infection in humans.⁹ Patients with active HHV-6 infections often remain totally asymptomatic, while HSCT patients with HHV-6 encephalitis have variable outcomes, ranging from full recovery with no residual neurologic deficits to permanent disability and death.^{7,10} Previous analyses have shown that mortality rates attributable to HHV-6 encephalitis are high.^{11,12} HHV-6B reactivation can occur in severely immunocompromised patients and is well described in the HSCT population, but data for patients undergoing CAR T-cell therapy are lacking.

CAR T recipients are highly susceptible to infections due to the immunodeficiency caused by prior chemotherapy treatment.¹³ Two major toxicities resulting from CAR T-cell therapy are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS).¹⁴ Patients undergoing CAR T-cell therapy may also be at risk for HHV-6B-associated encephalitis, which can be difficult to distinguish from ICANS. Beyond these factors, the management of HHV-6B infection and CRS/ICANS is different. HHV-6B infection requires immediate initiation of antiviral therapy, while CRS/ICANS can be successfully improved with interleukin (IL)-6 receptor inhibitors and corticosteroids.^{15,16} Therefore, it is necessary to distinguish between HHV-6B infection and CRS/ICANS to conduct appropriate treatment during CAR T-cell therapy.

Recently, droplet digital PCR (ddPCR) has been used to quantify nucleic acids and detect pathogens.^{17–19} A wide range of human samples, including blood, plasma, serum, cerebrospinal fluid, urine, saliva and bronchoalveolar lavage fluid, can be utilized in this method.²⁰ Diagnosis of HHV-6B infection relies on the quantification of viral load in body fluids, and ddPCR is known to have a higher sensitivity (< 0.1%) than that achieved with currently used PCR strategies (0.5–5%).^{21,22} mNGS is a high-throughput or massively parallel sequencing method capable of detecting a wide variety of pathogens ranging from viruses to bacteria to fungi and parasites.²³ It is also commonly used to detect HHV-6 infection. Compared to traditional blood cultures, mNGS significantly increases the diagnostic sensitivity of the patient's pathogens even after antibiotic treatment, while blood cultures are often negative.^{24,25} Allnutt's research team has used both mNGS and ddPCR to detect HHV6 in samples from three independent repositories. Another study compared the differences in the detection of MYD88 p.(L265P) in cerebrospinal fluid by mNGS and ddPCR methods.²⁶

The aim of this study was to compare ddPCR and mNGS for the detection of HHV-6B in patients treated with HSCT and CAR T-cell therapy. To our knowledge, this application has not yet been investigated. In this study, considering the sensitivity limitations of mNGS and the time required, we applied ddPCR for longitudinal testing and quantification of HHV-6B viral load in patients receiving CAR T-cell therapy and HSCT, which may help distinguish HHV-6B encephalitis from ICANS and guide drug therapy.

Materials and Methods

Study Design and Participants

In this study, the general workflow for detecting HHV-6B included mNGS and ddPCR. First, mNGS has been widely used in the clinical microbiological diagnosis of patients with hematological malignancies. If the patient exhibited a confirmed HHV-6B infection, dynamic monitoring of HHV-6B viral load was followed by ddPCR. Participants presented in this retrospective study were highly suspected of having HHV-6B infections (N = 27) and had both mNGS and ddPCR testing performed with informed consent between November 2019, and February 2022 at Tongji Hospital of Huazhong University of Science and Technology in Wuhan, China.

Specimens and Clinical Data Collection

Ten milliliter peripheral blood (PB) samples were collected upon receipt of informed consent and loaded into EDTA-K2 anticoagulant tubes. Once the sample was taken, the tube was inverted to mix the blood with EDTA in the collection tube

to prevent clotting. Five milliliter urine samples were loaded into urine collection tubes. The collected samples were stored at 4° and transferred to -80° within 8 hours. Clinical data, including data regarding age, sex, treatment history, neutropenia, CRS and therapeutic responses, were extracted from the medical records. Dynamic changes in serum IL-6 and HHV-6B were recorded by clinical monitoring.

Cell-Free DNA Extraction

cfDNA was extracted from samples with the QIAamp Circulating Nucleic Acid Kit (Qiagen), and carrier RNA was added before lysis. After extraction, cfDNA was quantified using a Qubit fluorometer 3.0 and a highly sensitive DNA detection kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). The extracted cfDNA was stored at 20°C until ddPCR.

Design of Primers and Probes

Two sets of specific primers and probes targeting the conserved regions of the target gene *HHV-6B* and the internal reference gene sequence of the human nuclear RNase P protein *POP4* were designed using Primer Express 3.0.1 (Thermo Fisher Scientific, MA, USA). The TaqMan minor groove binding probes were labeled with FAM fluorophores and VIC fluorophores, respectively (Applied Biosystems, Foster City, CA, USA). Then, they were synthesized by Shanghai Biological Engineering Corporation Ltd. (Shanghai, China). The sequences of the primers and probes used in the ddPCR are given in (Table 1).

Generation of the Plasmid and Verification of Primers and Probes

The plasmid was generated by joining the HHV-6B target sequence to the pUC-57 plasmid vector, and then the product was amplified. The restriction enzymes BamHI (catalog number R0136S; NEB, USA) and HindIII (catalog number R0104S; NEB, USA) were used to linearize the plasmid to obtain the standard while retaining the intact HHV-6B target sequence. The plasmid standard was then diluted, and 5-fold serial dilutions ranging from approximately 10,000 copies/ μ L to 3.2 copies/ μ L were made for ddPCR validations. Standards at each concentration were tested in duplicate under identical conditions to test quantitative linearity.

Detection of HHV-6B by Droplet Digital PCR

The total volume of the ddPCR mixture was 20 μ L, and ddPCR was performed using a QX200 Droplet Digital PCR System (Bio-Rad). Droplets were then analyzed on the QX200 Droplet Reader (Bio-Rad) and analyzed using QuantaSoft software version 1.7.4 with a user-defined threshold. Thresholds were determined manually for each experiment according to negative controls that included twelve healthy samples. Droplet positivity was determined by the fluorescence intensity; only droplets above a minimum amplitude threshold were counted as positive. The copy number of the cfDNA in samples was reported as copies/ μ L DNA and then converted into copies/ μ g DNA.

DNA Library Construction, Sequencing, and Bioinformatic Analysis

mNGS is used to detect pathogenic microorganisms. More than 3 mL of peripheral blood or more than 1.2 mL of cerebrospinal fluid was collected, loaded in collection tubes, and then stored at room temperature. Plasma was isolated for nucleic acid extraction. DNA libraries were constructed through DNA fragmentation, end-repair, adapter-ligation and

Table 1 The Sequences of the Primers and Probes Used in ddPCR

| Genes | Primers/Probes | Sequences |
|--------|----------------|---------------------------------|
| HHV-6B | Forward primer | 5'-TCCATTGTTTGATTGATTTCCGTAT-3' |
| | Reverse primer | 5'-AACGCGGGATGTTCTATTGG-3' |
| | Probe | 5'-FAM-CTTGAGCTTGTAGATAAT-3' |
| POP4 | Forward primer | 5'-GGCGGTGGTCCTGGAGTACT-3' |
| | Reverse primer | 5'-AGAGGCCTTTGGCTTTCTTCTT-3' |
| | Probe | 5'-VIC-ACCCGCCACAAGC-3' |

PCR amplification. Agilent 2100 (Agilent Technologies, Palo Alto, California, USA) was applied for quality control of the DNA libraries. Qualified libraries were pooled. DNA nanoball was made and sequenced by BGISEQ-50 /MGISEQ-2000 platform (BGI, Shenzhen, Guangdong, China).²⁷

For bioinformatic analysis, computational subtraction of human host sequences mapped to the human reference genome (hg19) using the Burrows-Wheeler Alignment,²⁸ then removed low-quality reads and generated high-quality sequencing data. The remaining data were classified by simultaneously aligning to Pathogens Metagenomics Database, consisting of bacteria, fungi, viruses, and parasites. The classification reference databases were downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>). RefSeq contains 4945 whole-genome sequences of viral taxa associated with human diseases.

Criteria for Positive Detection of HHV-6B by mNGS

The blood of healthy volunteers was tested in the same batch and used as a negative control. The background microbiology database is an internal database containing microorganisms that are present in more than 50% of the samples that appear in our laboratories. The criteria for positive microbial detection used in this study were as previously described.²⁹ HHV-6 detection was considered positive if the read number was among the top 10 and the fold change ≥ 10 times.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 8.0.2, IBM SPSS Statistics for Windows version 26.0, and Adobe Illustrator CS6 was employed for figure editing. Pearson's chi-square test and Kappa concordance test were used for statistical analysis, with results with *P* values of < 0.05 considered to be statistically significant.

Results

Evaluation of ddPCR Assay Performance

HHV-6B plasmids standards were used to evaluate the accuracy and the limit of detection (LOD) of ddPCR detection. The designed HHV-6B-specific probe and primers showed high specificity in ddPCR ([Supplemental Figure S1](#)). HHV-6B plasmid standards were prepared by fivefold serial dilution at concentrations ranging from 10,000 copies/ μL to 3.2 copies/ μL , and each concentration was measured in triplicate. As expected, the viral copy of HHV-6B measured by ddPCR agreed well with the expected concentration. The results showed that ddPCR had good linearity in the detection range ($R^2 = 0.998$, $P < 0.001$), indicating that ddPCR using the probe-primer set for HHV-6B could accurately quantify HHV-6B copies ([Figure 1A](#)). LOD of ddPCR was defined as the lowest concentration at which 95% of the samples exhibited positive results that could be reliably detected. A twofold serial dilution was used to generate the following concentrations: 80, 40, 20, 10, 5, 2.5, 1.25, and 0.625 copies/ μL , and each standard sample was tested 3 times. From a concentration of 80 copies/ μL to 2.5 copies/ μL , the results were positive in 100% of the samples. The concentration of 1.25 copies/ μL resulted negative results in 60% of samples, and the concentration of 0.625 copies/ μL resulted negative results in 100% of samples in the ddPCR ([Figure 1B](#)). The LOD in the ddPCR was thus five copies per reaction.

Characteristics of Patients

Twenty-seven patients with suspected HHV-6B infection who had both mNGS and ddPCR testing performed were analyzed retrospectively. The detailed clinical characteristics of the patients are shown in ([Table 2](#)). The median age was 43 years (range: 20–67 years), and 55.56% (15/27) of patients were male. It is worth noting that 48.15% (13/27) exhibited neurological symptoms, which indicates they should be carefully monitored for HHV-6B encephalitis. A total of 19 patients received CAR T-cell therapy, of whom 9 patients received HSCT followed by CAR T-cell therapy, and 8 patients received HSCT.

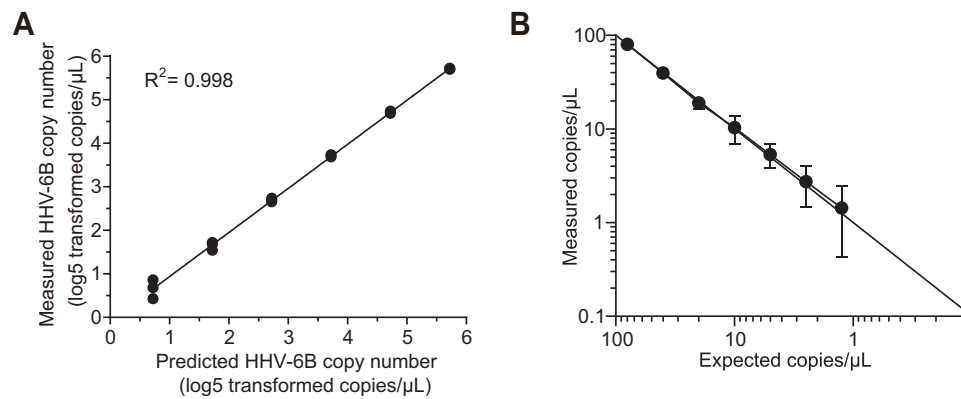


Figure 1 Evaluation of the HHV-6B probe primer set using plasmid standards. **(A)** Dilution curve of plasmid standard quantification by ddPCR. The correlation between the expected and observed copy numbers is shown. Each black dot represents a single replicate well of the dilution experiment, while the regression line is based on the average concentration at each dilution. **(B)** The lower detection limit of droplet digital PCR. The results are presented as the means \pm 1 SD.

Comparison Between ddPCR and mNGS Assessments of HHV-6B Detection

In this study, we combined mNGS with ddPCR to examine HHV-6B infection in CAR T and HSCT recipients. A total of 164 samples, including peripheral blood, urine, cerebrospinal fluid and anal swabs, were collected from 27 patients. Of the 164 samples, 81 samples (69 in peripheral blood samples and 12 in urine samples) were tested with ddPCR and 83 samples (80 in peripheral blood samples, 2 in cerebrospinal fluid samples and 1 in anal swab sample) with mNGS. The measurements for the individual samples were shown in (Supplemental Table S1-S2). Overall, a total of 27 time-paired samples were comparable. The correlation between ddPCR and mNGS detection of HHV-6B is shown in (Figure 2A). There was a significantly positive correlation between ddPCR and mNGS results ($R^2 = 0.672$). Of the 27 time-paired samples, 20 were ddPCR positive (detection ratio 74.07%), and 12 were mNGS positive (detection ratio 44.44%). ddPCR detected HHV-6B infection in 8 additional samples. Thus, 40% (8/20) of patients would have been missed if only mNGS

Table 2 Characteristics of Infection in Patients with HHV-6B

| Study Population | | N (%) |
|-----------------------|----------------|------------|
| Age (Years) | Median (range) | 43 (20~67) |
| Sex | Female | 12 (44.44) |
| | Male | 15 (55.56) |
| Diagnosis | NHL | 19 (70.37) |
| | HL | 1 (3.70) |
| | B-ALL | 2 (7.41) |
| | ANKL | 1 (3.70) |
| | AA | 4 (14.82) |
| Neurological symptoms | Yes | 13 (48.15) |
| | No | 14 (51.85) |
| Treatment | CAR T+HSCT | 9 (33.33) |
| | CAR T | 10 (37.04) |
| | HSCT | 8 (29.63) |

Abbreviations: NHL, non-Hodgkin's lymphoma; HL, Hodgkin's lymphoma; B-ALL, B cell-acute lymphoblastic leukemia; ANKL, aggressive NK cell leukemia; AA, aplastic anemia.

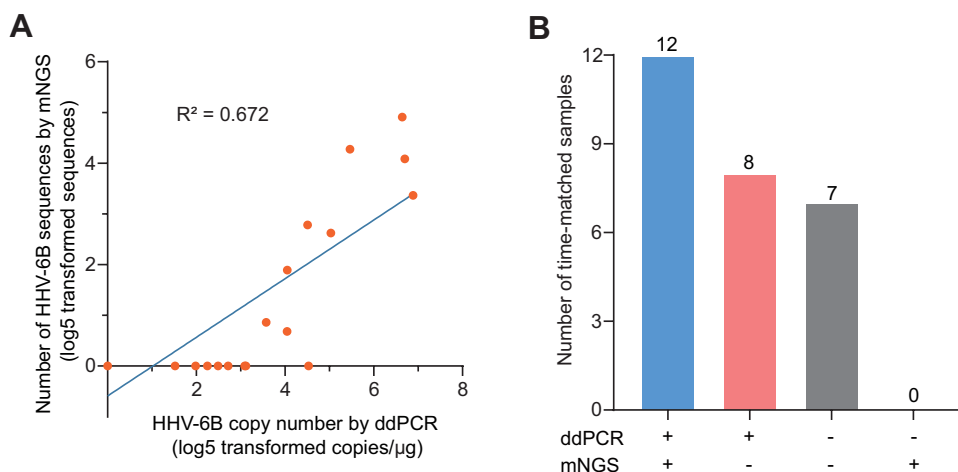


Figure 2 Comparison between ddPCR and mNGS for HHV-6B detection. **(A)** Scatter plot and correlation of ddPCR and mNGS results in HHV-6B detection. **(B)** Comparison of ddPCR and mNGS results for HHV-6B detection. The blue bar shows that both ddPCR and mNGS detected HHV-6B positivity; the burgundy bar shows that ddPCR detected HHV-6B positivity, while mNGS results were negative; the gray bar indicates that neither ddPCR nor mNGS detected HHV-6B virus.

were applied (Figure 2B). In addition, 12 time-paired samples were double positive, and 7 time-paired samples were double negative. Therefore, the agreement rate was 70.4% and the kappa statistic showed a moderate agreement (kappa value = 0.438, $P = 0.006$).

Time of Onset and Levels of HHV-6B After CAR T-Cell Therapy

We further analyzed 10 patients who were continuously monitored by ddPCR for HHV-6B infection after CAR T-cell therapy. The clinical characteristics of the patients are described in (Table 3). The first HHV-6B infection event was detected at a median of 14 days after CAR T-cell infusion (range, 8 to 19 days). CRS events were detected within 7 days (range, 1 to 7 days). CRS events occurred earlier than HHV-6B infection after CAR T-cell infusion; three patients developed HHV-6B infection after the end of CRS, and seven developed HHV-6B infection during the presence of CRS (Figure 3A). After HHV-6B infection was detected, all patients received antiviral therapy. Subsequently, HHV-6B was serially monitored by ddPCR to assess the efficacy of antiviral therapy. Longitudinal analyses demonstrated the rapid clearance of HHV-6B cfDNA from responding patients (Figure 3B).

Table 3 Clinical Characteristics of Patients Treated with CAR T for Continuous HHV-6B Monitoring

| Patient ID | Sex | Age | Disease | ddPCR Sample Type | Neurological Symptoms | CRS Grade | ICANS Grade | Time to First mNGS Test After CAR-T Treatment |
|------------|--------|-----|---------|-------------------|-----------------------|-----------|-------------|---|
| Pt.1 | Female | 24 | DLBCL | PB | Yes | 4 | 3 | 14 |
| Pt.2 | Male | 43 | PCNSL | PBIU | Yes | 2 | 0 | 8 |
| Pt.3 | Male | 33 | DLBCL | PB | No | 1 | 0 | 18 |
| Pt.4 | Female | 25 | PTCL | PB | Yes | 2 | 0 | 19 |
| Pt.5 | Female | 44 | DLBCL | PBIU | No | 1 | 0 | 12 |
| Pt.6 | Female | 40 | DLBCL | PB | No | 2 | 0 | 15 |
| Pt.7 | Male | 39 | PMBCL | PB | No | 2 | 0 | 11 |
| Pt.8 | Female | 48 | DLBCL | PB | Yes | 3 | 0 | 22 |
| Pt.9 | Female | 52 | HL | PB | No | 1 | 0 | 16 |
| Pt.10 | Female | 50 | DLBCL | PBIU | Yes | 3 | 0 | 17 |

Abbreviations: DLBCL, diffuse large B cell lymphoma; PCNSL, primary central nervous system lymphoma; PTCL-NOS, peripheral T cell lymphoma, not otherwise specified; PMBCL, primary mediastinal large B-cell lymphoma; HL, Hodgkin's lymphoma; PB, peripheral blood; U, urine.

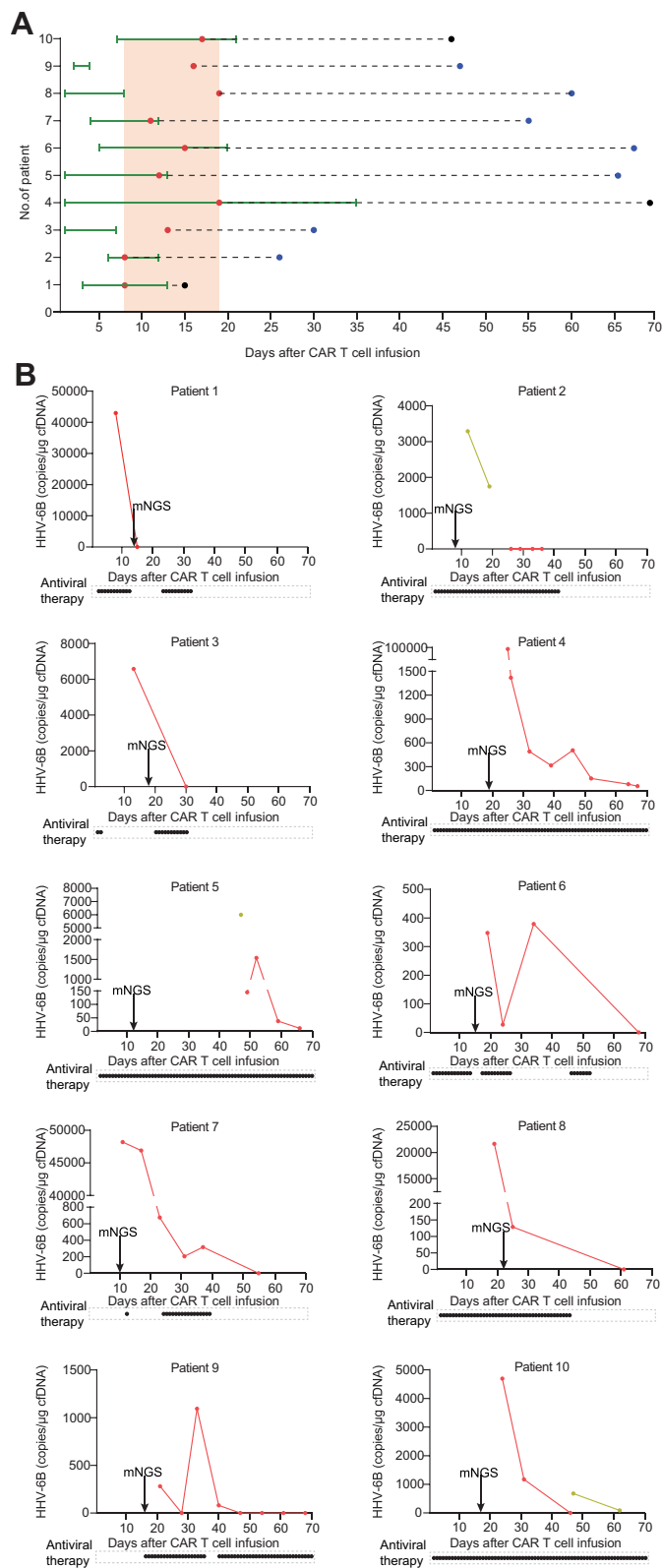


Figure 3 Time of onset and levels of HHV-6B after CAR T-cell therapy. **(A)** Time of onset of HHV-6B and CRS in 10 patients after CAR T-cell infusion. Red dots represent the onset of HHV-6B infection; blue dots indicate the end of HHV-6B infection; black dots represent HHV-6B levels that were greatly reduced but that did not drop to 0; green solid lines indicate ranges of CRS (days); black dotted lines indicate ranges of HHV-6B infection (days). **(B)** HHV-6B copy number levels after CAR T-cell therapy. Arrows represent the first mNGS monitoring time point after CAR T-cell infusion; red dots/red solid lines indicate HHV-6B detection in a peripheral blood sample; dark green dots/dark green solid lines represent HHV-6B detection in a urine specimen; black dots represent antiviral therapy. A-J each represent a lymphoma patient treated with CAR T-cell therapy.

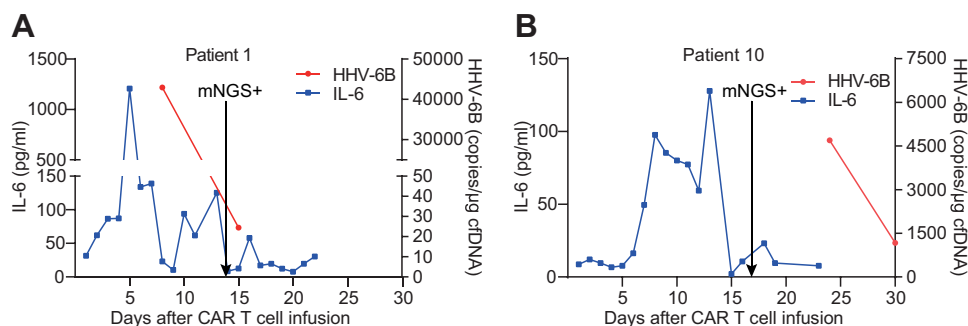


Figure 4 Differentiation of viral encephalitis caused by HHV-6B activation and ICANS after CAR T-cell therapy. **(A)** One case of HHV-6B encephalitis after CAR T-cell therapy. **(B)** Another case of ICANS after CAR T-cell therapy. Dynamic changes in IL-6 levels and HHV-6B copy number in two patients after CAR T-cell infusion. The arrows represent the first mNGS monitoring time point after CAR T infusion.

Differentiation of HHV-6B Infection and ICANS After CAR T-Cell Therapy

ddPCR is currently the most sensitive method for detecting viral load and may be used to distinguish viral encephalitis caused by HHV-6B activation from ICANS. In this study, there was one case of HHV-6B encephalitis after CAR T-cell therapy and another case of ICANS after CAR T-cell therapy. Here, we describe the 2 cases in more detail.

Patient 1 was a 24-year-old female diagnosed with relapsed diffuse large B-cell lymphoma (DLBCL) who received CAR19/22 T-cell cocktail therapy (day 0) at our center. After the CAR T-cell infusion, she had grade 4 CRS with elevated IL-6 levels and grade 3 ICANS. Data regarding the IL-6 levels are presented in (Figure 4A). On day 8, she was delirious and agitated. At that time, the IL-6 level was 23.23 pg/mL (reference range: < 7 pg/mL). Levels at this time were significantly lower than those observed at the previous time point. After excluding the possibility of other causes of neurological symptoms, HHV-6B reactivation was suspected. At this time point, HHV-6B levels were determined to be 42,968.75 copies/μg by ddPCR. Then, the patient received antiviral therapy with foscarnet sodium and methylprednisolone pulse therapy. A decrease in the number of HHV-6B copies was observed, and her neurological symptoms improved. However, on day 13 after CAR T treatment, the patient reappeared with neurological symptoms, such as disturbances in consciousness and epilepsy. Given that the viral copy numbers of HHV-6B decreased to 24.44, the HHV-6B infection was considered to be under control. Since she was experiencing neurological symptoms again, we considered the possibility that the disturbances in consciousness and epilepsy could be caused by ICANS after CAR T-cell infusion. The IL-6 level was 125.4 pg/mL at that time, and it was significantly higher on day 13 than on day 8.

Patient 10 was a 50-year-old female diagnosed with refractory DLBCL who received CAR20/22 T-cell cocktail therapy (day 0) at our center. After CAR T-cell infusions, she had grade 3 CRS with elevated IL-6 levels and without ICANS. The IL-6 levels gradually returned to baseline values 3 weeks after CAR T therapy (Figure 4B). However, on day 15 after CAR T-cell infusion, she experienced disorientation regarding time and location. The IL-6 level at this time was 10.65 pg/mL. The IL-6 level was monitored continuously for one week, and the levels were mostly less than 10 pg/mL. Therefore, the possibility of ICANS was excluded after CAR T-cell infusion. After excluding the possibility of other factors, the patient's neurological symptoms were suspected to be due to HHV-6B activation after CAR T-cell treatment. Blood tests and cerebrospinal fluid tests were positive for HHV-6B at the same time point, and the levels of HHV-6B were 48,582 copies/μg and 127,928 copies/μg, respectively. The number of HHV-6B copies were significantly reduced after one month of antiviral foscarnet therapy, and the patient's main symptoms completely disappeared. This result further confirmed that HHV-6B activation causes viral encephalitis, which triggers neurological symptoms in patients receiving CAR T-cell treatment.

Discussion

HSCT and CAR T-cell immunotherapy bring unprecedented therapeutic effects in patients with refractory/relapsed hematologic malignancies. However, HSCT and CAR T recipients are highly susceptible to infections due to the

immunodeficiency caused by prior chemotherapy, prior intensive therapy, hypogammaglobulinemia, and cytopenia. The most common infectious events are viral, bacterial and invasive fungal infections. Viral infections are particularly common in late infections in CAR T recipients.^{13,30,31} HHV-6B infection is a common type of viral infection after HSCT.⁷ However, evidence regarding HHV-6 infection in CAR T recipients is limited to case reports and lacks sufficient clinical data.^{10,32} This study is the first to investigate HHV-6B infection by detecting cfDNA from patient samples using ddPCR in patients undergoing CAR T-cell therapy or HSCT.

HHV-6 infection can cause serious complications after HSCT. These patients are at high risk of developing reactivation within the first 4 weeks after cell transfer and subsequently developing life-threatening illnesses of the central nervous system (CNS) and/or bone marrow, two well-known sites of HHV-6 latency.³³ Previous analysis has shown that the mortality rate attributable to HHV-6 encephalitis is high, and many survivors exhibit cognitive sequelae.^{34,35} HHV-6 reactivation was found to be associated with poor outcomes following allo-HSCT.^{36,37} The possibility of treating the infection with antiviral drugs active against HHV-6 must be considered. Decreasing the HHV-6B copy number through antiviral treatment. However, patients 6 and 9 showed a significant re-elevation of HHV-6B copy number. Since patient 6 was discharged on day 24 of CART treatment in stable condition with no neurological symptoms and no fever, antiviral medication was discontinued. HHV-6B was rechecked on day 34 after CART and HHV-6B copy number was again elevated, with re-administration of antiviral drugs HHV-6B copy number gradually decreased to 0. Patient 9 was initially treated with foscarnet for 12 days and the HHV-6B copy number was reduced to 0. Curiously, HHV-6B copy number increased again with antiviral treatment. Considering the possibility of foscarnet resistance, foscarnet was discontinued and replaced by ganciclovir, and the patient's HHV-6B copy number decreased again. The antiviral compounds ganciclovir, foscarnet and cidofovir are effective in active HHV-6 infection,^{38,39} but the indications for treatment and the conditions of use have not yet been officially approved.⁹ More research is needed on therapy for HHV-6. Consequently, diagnostic procedures for monitoring infection should be implemented after initial diagnosis and therapy, if initiated. This process includes the serial quantification of virus replication and the detection of putative resistance to antivirals in case of therapeutic failure.

The development of new technologies, such as ddPCR or mNGS, has greatly improved the sensitivity, specificity and precision of the detection of rare sequences.⁴⁰ In addition to the increased detection sensitivity and specificity, ddPCR and mNGS strategies have their own advantages and disadvantages in the analysis of cfDNA. mNGS can identify new genetic or epigenetic modifications and offers high multiplexing capabilities but is time-consuming and requires powerful informatics support. In contrast, ddPCR experiments are easier to set up and faster and present higher sensitivity and do not require complex informatics support for analysis. However, the use of this approach requires knowledge of the genetic or epigenetic changes to be detected and exhibits limited multiplexing skills. Immunocompromised individuals who develop fevers are routinely tested for bacterial, fungal, and viral infections. mNGS can detect a wider range of pathogens and is recommended for initial diagnosis to help patients screen for infectious agents. However, since mNGS is expensive and time-consuming, after screening the number of HHV-6B sequences, subsequent dynamic monitoring by ddPCR is more appropriate because it is cheaper, faster, and can detect low concentrations of HHV-6B cfDNA. Different methods are used to test for different periods of time, taking advantage of the respective strengths of mNGS and ddPCR to provide the best diagnosis and save money for patients. Many strategies have therefore combined the use of NGS and ddPCR for liquid biopsy analysis.^{26,41,42}

In this study, we found that the results of ddPCR detection of HHV-6B cfDNA from body fluid samples showed moderate agreement with the results of mNGS, which was consistent with the results of previous studies examining the correlation between NGS and ddPCR.⁴³ Moreover, ddPCR detected HHV-6B infections in 8 additional samples that would have been missed using mNGS methods alone. It was demonstrated that the ddPCR test detected more cases than mNGS assessment. ddPCR showed a higher sensitivity in the measurements of nucleic acids and is suitable for HHV-6B detection.

Another major strength to be highlighted is that a wide range of human samples with low concentrations of DNA, including blood, plasma, serum, cerebrospinal fluid, urine, saliva and bronchoalveolar lavage fluid, can be used in the ddPCR assay.²⁰ Previous studies have clearly demonstrated the enormous potential of ddPCR to detect minute amounts of ctDNA in plasma. Targeting known tumor mutations in plasma using the ddPCR assay in early-stage breast cancer

showed a sensitivity of 93.3%.⁴⁴ It can also be applied to tumor biopsy samples, even in formalin-fixed paraffin embedded (FFPE) tissues.⁴⁵

Patients receiving CAR T-cell therapy often exhibit a state of immunosuppression, and there is significant overlap in the symptoms associated with neurotoxicity and those associated with HHV-6 encephalitis. Further differential diagnosis is highly warranted. This study described two cases of HHV-6B reactivation after CAR T-cell therapy. One patient exhibited HHV-6 encephalitis and the other did not. We found that continuous monitoring of the HHV-6B cfDNA viral load by ddPCR was very helpful in distinguishing HHV-6B viral encephalitis from ICANS. In addition, the continuous monitoring of HHV-6B cfDNA viral load by ddPCR could guide the use of antiviral drugs to achieve a good curative effect. However, two sample sizes to distinguish HHV-6B encephalitis and ICANS has potential limitation, and we still need to expand the sample size to verify this result. Further studies are needed to describe the incidence and presentation of HHV-6 encephalitis in this patient population, as well as optimal treatment strategies and methods for assessing response to therapy.

Conclusion

Droplet digital PCR was a powerful complement to the use of mNGS methods in clinical application due to its higher rate of detection of HHV-6B positivity. Longitudinal testing and quantification of HHV-6B DNA copy number in patients receiving CAR T-cell therapy and HSCT may help distinguish HHV-6B encephalitis from ICANS and guide drug therapy. However, it should be better verified by expanding the sample size.

Institutional Review Board Statement

The studies involving human participants were reviewed and approved by Ethic Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-IRB20160310).

Informed Consent Statement

The patients provided their written informed consent to participate in this study. This study was conducted in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests.

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