The Prevalence of Human Cytomegalovirus Infection in Children Leukemia by Chip Digital PCR

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Research

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Abstract

Background

To establish a method for detecting HCMV viral load to guide clinical treatment by chip digital PCR (cdPCR).

Methods

5.67×10^6 TCID₅₀/ml of HCMV AD169 was serially diluted to evaluate sensitive of cdPCR, HSV-1, HSV-2, VZV, EBV, HHV-6 and HHV-7 were used to evaluate the specificity of HCMV cdPCR. HCMV infection were analyzed in 110 children leukemia whole blood by RT-qPCR and cdPCR.

Results

The sensitive of HCMV cdPCR was up to 71 ± 32 copies/ml, which is higher than that of RT-qPCR. HCMV cdPCR did not cross react with other herpesviruses. The cdPCR effectively detected 7 HCMV positive samples, making the laboratory diagnosis rate of HCMV increased by 6.36% (7/110) for children leukemia patients. And the prevalence of HCMV infection increased from 28.18–34.54% in 110 children leukemia patients by cdPCR.

Conclusion

cdPCR is more sensitive to detect viral load than RT-qPCR. The cdPCR may be used to evaluate relationship between viral load and progression of HCMV infection in patients.

1 Background

Human cytomegalovirus (HCMV), a ubiquitous β -herpesvirus, infects as high as 90% of the human population worldwide in developing countries[1]. HCMV infection remains largely asymptomatic for healthy, immunocompetent individuals, persistent infection or recurrent infection often occur immunocompromised individuals[2–4]. Like all herpesvirus, HCMV establishes latency in haematopoietic cells and persists for lifelong of the host after primary infection[5, 6]. HCMV infection may result in significant morbidity and mortality in immunodeficiency patients, especially transplant patients including allogeneic stem cell transplantation(allo-SCT)[7–9]. HCMV infection is also one of the most leading cause of infant birth defects[5, 10].

Diagnostic testing to confirm HCMV and to monitor viral loads and immune responses among solid-organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients is crucial to effective patient care. Laboratory tests that directly detect HCMV are recommended for surveillance, diagnosis, and monitoring, while assays of immune status are relied upon for HCMV risk assessment and stratification of risk factors. Virus isolation is highly specific for the diagnosis of HCMV infection. Culture can also be used

to assess phenotypic antiviral drug testing. The most common approach for direct virus detection is commercial quantitative nucleic acid amplification tests (Real-time fluorescent quantitative PCR, RT-qPCR), which are higher sensitive and offer rapid turn-around times. Trends in viral loads over time (viral load kinetics) directly correlate with the likelihood of severe HCMV disease[10–12]. The higher viral loads or more rapid viral load increase correlate with both development of HCMV disease and a higher risk for severe HCMV disease[10–13]. The quantitative nature allows for assessment of the degree of HCMV replication, which is expressed as the absolute viral load value.

However, because the quantify of RT-qPCR relies on the standard curve, the diversity of RT-qPCR result in significant variability of the reported quantitative and qualitative data among different laboratories[14–16]. The divergences in qualitative data may result in misjudge development and severity of disease, and initiation or termination of antiviral therapies.

In this study, we established a new method for detection HCMV by Chip Digital PCR (cdPCR), which is a new potential generation digital PCR technology. The reliability of cdPCR in quantifying HCMV was evaluated and it was used in HCMV diagnosis in children leukemia.

2 Materials And Methods

2.1 Virus

HCMV AD169 strain (5.67×10^6 TCID₅₀/ml), HCMV virus supernatant was 10-fold diluted: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , and herpes simplex virus 1 (HSV-1, KOS strain) (1.5×10^5 copies/ml), herpes simplex virus 2 (HSV-2, G strain) (3.9×10^5 copies/ml), varicella zoster virus (VZV, Ellen strain) (5.7×10^5 copies/ml), Epstein-Barr Virus (EBV, B95-8 strain) (3.4×10^5 copies/ml), human herpesvirus 6A (HHV-6A, GS strain) (HHV-6 Foundation) (4.8×10^5 copies/ml), human herpesvirus 6B (HHV-6B, Z29 strain) (HHV-6 Foundation) (3.7×10^5 copies/ml), human herpesvirus 7 (HHV-7, JI strain) (HHV-6 Foundation) (4.0×10^5 copies/ml) stored at -80°C .

2.2 Patients selection

110 children leukemia were enrolled in this study. The subjects were divided into 2 groups: leukemia group of 51 cases (M/F: 29/22, the median age is 7.5). Hematopoietic Stem Cell transplantation (HCT) group of 59 cases (M/F: 39/20, the median age is 7.5). Children patients were collected from May 2016 to May 2019 in Beijing Capital Institute of Pediatrics' Children's Hospital. All patients were confirmed according to diagnostic criteria. The subject was approved by Ethics Committee of National Institute for Viral Disease Control and Prevention. Whole blood samples from 110 patients were tested by RT-qPCR and cdPCR.

2.3 Primers and Probe

In the experiment, the primers and probe of cdPCR and RT-qPCR were designed according to the gene sequence (MK425187.1) as follows.

HCMV-F 5'-GCAGCCACGGGATCGTACT-3'

HCMV-R 5'-GGCTTTTACCTCACACGAGCATT-3'

HCMV-Probe 5'-FAM-CGCGAGACCGTGGAAGTGGC-BHQ - 3'.

2.4 RT-qPCR

Diluted virus supernatant and clinical whole blood samples were collected, extracted according to the QIAamp Viral DNA Blood Mini Kit (Qiagen, Germany), and stored at -80°C for use. RT-qPCR reactions were assembled using 10µL of Premix Ex Taq™ (TaKaRa, Japan), 0.5µL of primers(10µM) and probe(10µM), 2µL DNA template of each sample, and ddH₂O up to 20µL. The cycling procedure as follows: 95°C, 30s; and 40 cycles of 95°C for 5s, 60°C for 30s (CFX96, Bio-Rad, USA).

2.5 Chip digital PCR

2µL DNA were added in the cdPCR reaction system containing ToughMix buffer (Stilla, France), 2.5µL Fluorescein (1µM) (PEXBIO, China), 0.5µL primers(10µM) and probe(10µM). Cycling procedure as follows: 95°C, 10 min; followed by 40 cycles of 94°C, 5s, 60°C, 30s. cdPCR run in Naica™ Crystal Digital PCR system(Stilla, France). Data were analyzed using CrystalMiner. 59°C, 59.5°C, 60°C, 60.5°C, and 61°C was chosen to determine the optimal annealing temperature of cdPCR.

2.6 Nest PCR and sequencing

Four primers were used to amplify the IE of HCMV [17]. The first was carried out in a 50µL PCR reaction containing 25µL PCR StarMix (Genstar, China), 1.5µL primers(10µM) and 4µL template. The cycling profile was 94, 5min; 35 cycles of 94°C, 30s, 58°C, 30s, 72°C, 60s; followed by 72°C, 5min. Subsequently, 2µL of first PCR products was subjected to the second PCR in 25µL mixture, including 12.5µL PCR StarMix (Genstar, China), 1µL primers(10µM). After 94 for 5min, 30 cycles of amplification were performed 94°C, 30s, 58°C, 30s, 72°C, 50s and a final step at 72°C for 5min. The PCR products were analyzed on 2% agarose gel and confirmed by sequencing.

2.7 Statistical analyses

SPSS20.0 software was used to analyze data. Measurements were expressed by mean ± standard deviation (SD). Counting data were compared by the X²-test; measurement data were compared by the T-test, with statistically significant difference at P < 0.05.

3 Results

3.1 Determination of optimal annealing temperature of chip digital PCR

To look for optimal annealing temperature of cdPCR, the temperature was set to increase progressively from 59°C to 61°C with an interval of 0.5°C. After viral DNA was extracted from 140 µL HCMV AD169 strain (5.67×10⁶ TCID₅₀/ml) according to Kit's instruction, 2µL DNA was used in each PCR reaction. The results showed that virus copies/25µL were 287.20 ± 36.85, 280.73 ± 11.71, 296.43 ± 3.11, 262.00 ± 28.16, 280.00 ±

13.72 at each temperature. The largest average copies was amplified at 60°C of the annealing temperature. Thus, 60°C was determined as the optimal annealing temperature of cdPCR.

3.2 The sensitive and specificity of chip digital PCR

5.67×10⁶TCID₅₀/ml of HCMV AD169 was 10-fold serially diluted from 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ to 10⁻⁸. Viral DNA of each dilution was extracted described above. 2μL DNA of each dilution was detected by RT-qPCR and cdPCR, respectively. Results of RT-qPCR showed that the Ct/well of each dilution were 23.51 ± 0.10, 25.30 ± 1.05, 29.15 ± 0.14, 32.84 ± 0.32, 35.91 ± 0.53, 38.07 ± 0.70, respectively. Viral DNA of 10⁻⁷ and 10⁻⁸ dilution was not detected by RT-qPCR. Results of cdPCR showed that the copies/ml of each dilution were 147931 ± 5533, 29694 ± 4102, 6979 ± 2040, 2077 ± 740, 565 ± 94, 300 ± 73, 169 ± 8, 71 ± 32, respectively. Virus DNA of 10⁻⁸ dilution could be detected through cdPCR. The results showed that the sensitives of cdPCR was better than that of RT-qPCR.

To determine the specificity of cdPCR, 8 herpesviruses, including HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6A, HHV-6B, HHV-7 were detected by HCMV cdPCR, respectively. Our results showed that DNA of 7 herpesvirus were not detected but DNA of HCMV. The results showed that HCMV cdPCR method were specificity with no cross-reaction with other herpesviruses.

3.3 Clinical samples validation of chip digital PCR

To understand whether cdPCR is suitable for sample detection, 110 whole blood samples from children leukemia were conducted by RT-qPCR and cdPCR, respectively. 31 samples were positive by RT-qPCR and cdPCR. The copy number was from 98 copies/ml to 3208 copies/ml. 79 samples were negative by RT-qPCR. However, seven out of 79 RT-qPCR negative samples were positive by cdPCR. The copy number of 7 samples was from 15 copies/ml to 73 copies/ml. The 7 positive samples were confirmed further by nest PCR and sequencing. The results showed that the method of cdPCR was more sensitive than RT-qPCR for HCMV detection (Table 1).

Table 1 Comparisons between RT-qPCR and cdPCR				
RT-qPCR	Chip digital PCR		Total	<i>P</i>
	Positive	Negative		
Positive	31	0	31	0.05
Negative	7	72	79	
Total	38	72	110	

Table legends: 110 samples from children leukemia were conducted by RT-qPCR and cdPCR. 31 samples were positive by RT-qPCR and cdPCR. 79 samples were negative by RT-qPCR. Meanwhile, seven out of 79 RT-qPCR negative samples were positive by cdPCR. As revealed by X²-test, statistical differences were observed between the two methods (*P* < 0.05). And the results showed that cdPCR was more sensitive than RT-qPCR for HCMV detection.

3.4 Prevalence of HCMV in Children leukemia

Subsequently, we analyzed prevalence of HCMV in the children leukemia by cdPCR. There were male 69 and female 41 among above 110 patients including 59 HCT patients and 51 Leukemia patients. There were male 40 and females 19 among 59 HCT patients; there were 29 male and 22 females among 51 Leukemia patients. The prevalence of HCMV infection was 34.54% (38/110) in 110 patients. For HCT patients, the prevalence of HCMV infection was 38.98% (23/59) (Table 2). The range of copy number is from 15 copies/ml- 2896 copies/ml. For leukemia patients, the prevalence of HCMV infection was 29.41% (15/51) (Table 3), the range of copy number is from 179 copies/ml -3208 copies/ml. The prevalence of HCMV infection were 40.00% in the HCT patients aged 0–6 years old. And the prevalence of HCMV infection were 36.84% in the patients aged 7–12 years old (Table 2). For leukemia patients, the prevalence of HCMV infection were 34.78% in the leukemia patients aged 0–6 years old and 26.09% in the patients aged 7–12 years old (Table 3).

Table 2
HCMV infection rate by cdPCR in HCT Patients

Age	No.	HCM Positive	Positive Rate (%)	Male		Female			
				Positive	Negative	Positive Rate (%)	Positive	Negative	Positive Rate (%)
0–6	35	14	40.00	8	13	38.10	6	8	42.86
7–12	19	7	36.84	7	8	46.67	0	4	0.00
≥ 12	5	2	40.00	1	3	25.00	1	0	100.00
Total	59	23	38.98	16	24	40.00	7	12	36.84

Table legends: For HCT patients, the prevalence of HCMV infection was 38.98% (23/59), and infection rate in male is 40.00% (16/16 + 24), in female is 36.84% (7/7 + 12). HCMV infection were 40.00% (14/35) in aged 0–6 years old, and infection rate in male is 38.10% (8/8 + 13), in female is 42.86% (6/6 + 8). The infection of HCMV in aged 7–12 years old is 36.84% (9/17), and infection rate in male is 46.67% (7/7 + 8), in female is 0.00% (0/0 + 4). For patients over 12 years old, HCMV infection was 40.00% (2/5), the infection rate in male is 25% (1/1 + 3), in female is 100% (1/1 + 0). And this phenomenon needs to be further confirmed by large samples.

Table 3
HCMV infection rate by cdPCR in Leukemia Patients

Age	No.	HCMV Positive	Positive Rate (%)	Male		Female			
				Positive	Negative	Positive Rate (%)	Positive	Negative	Positive Rate (%)
0–6	23	8	34.78	3	11	21.43	5	4	55.55
7– 12	23	6	26.09	1	12	7.69	5	5	50.00
≥ 12	5	1	20.00	0	2	0.00	1	2	33.33
Total	51	15	29.41	4	25	13.79	11	11	50.00

Table legends: For leukemia patients, the prevalence of HCMV infection was 29.41% (15/51), and infection rate in male is 13.79% (4/4 + 25), in female is 50.00% (11/11 + 11). The infection of HCMV were 34.78% (8/23) in aged 0–6 years old, and in male the infection rate is 21.43% (3/3 + 11), in female is 55.55% (5/5 + 4). HCMV infection in aged 7–12 years old is 26.09% (6/23), and in male is 7.69% (1/1 + 12), in female is 50.00% (5/5 + 5). For patients over 12 years old, the prevalence of HCMV infection was 20.00% (1/5), the infection rate in male is 0.00% (0/0 + 2), in female is 33.33% (1/1 + 2).

4 Discussion

It has been confirmed that the HCMV viral load and growth in clinical samples can predict the risk of disease in patients[11]. But the lack of well-established viral load thresholds limits HCMV RT-qPCR in clinical application. Due to the results of RT-qPCR cannot be directly compared, the results of HCMV viral load cannot be used after change hospital[14]. No viral load value can be used to initiate preemptive therapy for patients infected HCMV without consensus standardization of HCMV RT-qPCR[10, 14]. There is widely variability of viral load in 33 laboratories using different HCMV RT-qPCR in a study[18]. Though a World Health Organization (WHO) provide an international standard for calibration of HCMV RT-qPCR, viral load variability remains because of assay performance (limits of detection and quantification), sample type, method for nucleic acid extraction, gene target, and amplicon size, even the type of patients[19–21].

Digital PCR solves this problem well. Droplet digital PCR (ddPCR) and cdPCR are two types of commercial digital PCR platforms. Other studies have shown that the sensitive of digital PCR is significantly higher than that of RT-qPCR[22, 23]. Our results also gave a similar conclusion. ddPCR mainly forms water-in-oil droplets, and each droplet is an independent PCR reaction system. Furthermore, the sensitivity of ddPCR for HCMV is 100 copies/ml[24]. And cdPCR limits detection of HCMV viral load is 15 copies/ml in clinical sample. cdPCR complete PCR reaction through 2D array of microchamber, and cdPCR can realize three-color multiplexing amplification[25, 26]. Due to simplified steps, cdPCR effectively reduce risk of contamination. Thus, we established a HCMV cdPCR method to evaluate HCMV infection in this study.

It is reported that RT-qPCR alone is inadequate for the accurate diagnosis of virus infection[27]. In this study, the cdPCR effectively detected 7 HCMV positive samples with low copies nucleic acid, making the laboratory

diagnosis rate of HCMV increased by 6.36% (7/110). Our results showed that the lowest copies of detection by cdPCR was 14.58 copies/ml. The results suggested that cdPCR was suitable for low loads nucleic acid detection.

In addition, we also found that HCMV was more easily detected in whole blood than in serum. In this study, both serum and whole blood samples were collected in 44 of 110 patients (31 of 59 HCT patients and 13 of 51 leukemia patients). We found there were 3(3/31 = 9.67%) cases of HCMV positive in whole blood not in the serum from 31 HCT patients. There was 1 (1/13 = 7.69%) case of HCMV positive in whole blood not in serum from 13 leukemia patients. The results made the laboratory diagnosis rate of HCMV increased by 3.63% (4/ 110).

We still found that cdPCR was more sensitive in the whole blood samples than in serum. And in our study, the lowest copies number is 15 copies/ml in whole blood samples and 27 copies/ml in serum samples, respectively. In a word, we found that the detection rate of HCMV in whole blood was slightly higher than in serum both in leukemia and HCT patients. The reason maybe that HCMV can replicate in many cells, including epithelial cells, endothelial cells and leukocytes of peripheral blood[28–30]. Only in the presence of viremia, high levels of HCMV viral load can be detected in the serum.

As we all known, HCMV is the most common virus infection after transplantation, it is considered to be the major risk factor for transplantation. Studies have confirmed that almost all HCMV viremia after bone marrow transplantation occurs in HCMV-positive recipients, and only in few patients can be transmitted from donor[31]. In other words, it is necessary to pay attention to leukemia patients with HCMV and HCMV-infected bone marrow transplant patients. In our study, 34.54% of samples were positive for HCMV by cdPCR. More than 90% of HCMV infections occurred before the age of 12 in these patients with leukemia or HCT. Continuous monitoring and timely medication should be conducted to prevent postoperative viremia. Among HCT patients, the infection rate of HCMV in male 13.56% (8/59) is higher than that in female 10.17% (6/59). But in leukemia patients, the infection rate in female 17.65% (9/51) is nearly 3 times than that of males 5.88% (3/51). This phenomenon needs to be further confirmed by large samples.

5 Conclusion

In conclusion, the cdPCR was established for detecting HCMV viral load in this study and verified through clinical samples, thus cdPCR may be used to evaluate clinical progress of HCMV infection.

Abbreviations

HCMV: Human cytomegalovirus; cdPCR: Chip digital PCR; allo-SCT: allogenic stem cell transplantation; SOT: Solid-organ transplant; HSCT: hematopoietic stem cell transplant; RT-qPCR: Real-time fluorescent quantitative PCR; HSV-1: Herpes simplex virus 1; HSV-2: Herpes simplex virus 2; VZV: Varicella zoster virus; EBV: Epstein-Barr Virus; HHV-6A: Human herpesvirus 6A; HHV-6B: Human herpesvirus 6B; HHV-7: Human herpesvirus 7; HCT: Hematopoietic Stem Cell transplantation; SD: Standard deviation; WHO: World Health Organization; ddPCR: Droplet digital PCR.

Declarations

Acknowledgments

We thank HHV-6 Foundation for providing human herpesvirus 6A (GS strain), human herpesvirus 6B (Z29 strain), human herpesvirus 7(JI strain).

Authors' contributions

JH, HY and WW conceived and designed the experiments. WW, MF and FH performed the experiments. WW, BS and PC analyzed the data. JH, JS, QS and XD contributed reagents, materials and analysis tools. WW and JH wrote the paper. All authors read and approved the final manuscript.

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Availability of data and material

All data generated or analyzed during this study are including in this published article.

Ethics approval and consent to participate

The subject was approved by Ethics Committee of National Institute for Viral Disease Control and Prevention (Reference code: IVDC2020-014).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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