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Original Article

Human cytomegalovirus glycoprotein B genotypic distributions and viral load in symptomatic infants

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Abstract

Introduction: *HCMV* infection is widespread in humans. This retrospective study aimed to explore the relationship between human cytomegalovirus (*HCMV*) glycoprotein B (gB) genotype distribution, viral load, and the demographic and clinical features of symptomatic infants. The detection rate of *HCMV* in blood and urine samples was also compared.

Methodology: Retrospective data from 265 infants who underwent urine *HCMV* DNA testing were analyzed. The viral load and gB genotype were detected in 91 *HCMV* positive infants by quantitative fluorescence polymerase chain reaction (PCR) and DNA sequencing, respectively.

Results: The positive rate of *HCMV* infection was 46.04% (122/265) in all infants, and increased rapidly with age. Among the 91 infants investigated, liver function abnormality was the most common diagnosis (34/91, 37.36%), followed by pneumonia (21/91, 23.07%). Sequence analysis of gB yielded two genetic subtypes: the most prevalent gB3 (47/91, 51.65%), followed by gB1 (44/91, 48.35%). The gB3 *HCMV* infection was more prevalent in infants aged 0-2 months than in infants aged 3-12 months ($\chi^2 = 4.38$, $p = 0.0364$). The data showed that ALT and AST levels were significantly higher in the anti-*HCMV* IgM^+IgG^- group than in the anti-*HCMV* IgM^+IgG^+ and IgM^-IgG^+ groups. In addition, this study showed that the detection rate of *HCMV* DNA in the blood was significantly lower than that in the urine ($\chi^2 = 6.7131$, $p = 0.0096$).

Conclusions: This study presents the *HCMV* infection status of infants and its relationship with their demographic characteristics and clinical manifestations. In addition, this study suggests that urinary PCR is the most appropriate assay for detecting *HCMV* infections.

Key words: Human cytomegalovirus; glycoprotein B; genotype; viral load.

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Introduction

Human cytomegalovirus (*HCMV*), also known as human herpesvirus type 5, belongs to the β subfamily of the herpesvirus family [1]. *HCMV* infection is widespread in humans with a worldwide seroprevalence of 60-90% [2]. *HCMV* can infect virtually all organ systems. *HCMV* congenital infection is the most frequent non-genetic cause of neurodevelopmental abnormalities [3] and sensorineural hearing loss in infants worldwide [4,5]. Nevertheless, the most common clinical diagnoses of *HCMV* infection are hepatitis and pneumonia [6,7]. The clinical diagnosis and fatality of *HCMV* pneumonia may be underestimated, particularly in immunocompromised patients. An autopsy study of 121 children showed that *HCMV* pneumonia accounted for 7% of pathological diagnosis [8]. In another study of post-mortem histopathological examination in 39 cases, *HCMV* DNA was found positive in 48.7% (19/39) lung [9].

HCMV is a linear, double-stranded DNA virus comprising approximately 236kb pairs. Sequence analysis revealed that the *HCMV* genome had over 751 translated open reading frames (ORFs) encoding about 178 polypeptides [10]. The *HCMV* glycoprotein B (gB) is encoded by the ORF UL55 gene and binds to heparan sulfate on the cell surface. Glycoprotein B is the most abundant envelope glycoprotein of *HCMV*, and is critically involved in the recognition, adsorption, penetration, and induction of host immune response by cell membrane receptors [11]. *HCMV* can be classified into four genotypes (gB1 to gB4) based on the sequence variations in the N-terminus of the gB gene. These gB genotypes may be associated with different clinical symptoms and outcomes [12,13].

The diagnosis of *HCMV* infection is traditionally based on virus isolation. Owing to its time-consuming nature, this assay as a routine test method has limitations. Detection of anti-*HCMV* IgM or *HCMV*

pp65 antigen in the blood is a common clinical diagnostic test, which suggests current infection. However, detection of *HCMV* IgM or pp65 antigen alone appears insufficient because of the presence of a considerable number of false-negative cases [14,15]. Demonstration of *HCMV* DNA has emerged as the diagnostic criteria for *HCMV* infection [16], for PCR assays' high sensitivity and specificity. In particular, quantification of the *HCMV* DNA copies is a powerful means for assaying viral activity, evaluating antiviral efficacy, and determining the severity of infection [13,17]. Blood, urine, and saliva can be used as samples for PCR screening of *HCMV* DNA, but their detection rates are different [14].

This retrospective study aims to investigate the gB genotype distribution and viral load in the infant < 5 years old with *HCMV* infection, and explore its correlation with the demographic and clinical characteristics. We also compared the detection rate of *HCMV* DNA in blood and urine samples.

Methodology

Patients and samples

This retrospective study was performed in Wuxi People's Hospital of Nanjing Medical University (Jiangsu, China), between August 2019 and January 2021. We retrieved data in the Laboratory Information System (LIS) based upon the query condition that the subject had undergone *HCMV* DNA of urine test and was not older than five years. A total of 265 eligible infants were included in the study. Medical records of 265 infants were also reviewed to obtain demographic and clinical information, including age, sex, clinical diagnosis, anti-*HCMV* serological status, serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and qualitative results of *HCMV* DNA in blood and urine. The urine specimens used for this retrospective study were the remaining samples after clinical testing and were stored at -80 °C before this study.

This study was approved by the Ethics Committee of the Wuxi People's Hospital of Nanjing Medical University, and all protocols conformed to the ethical guidelines of the 1975 Helsinki Declaration. The medical records of all infants in this study were obtained through the hospital's electronic medical record system and be carefully analyzed by the researchers.

Extraction of HCMV nucleic acid

HCMV nucleic acid were extracted from clinical urine samples by magnetic bead method using a nucleic acid extraction kit (Ex-DNA/RNA virus, Tianlong Biotechnology Co., Ltd. Shanxi, China) according to the manufacturer's instructions.

Detection of HCMV DNA with real-time fluorescence PCR

Quantitation of *HCMV* DNA in urine samples was performed using a quantitative *HCMV* nucleic acid assay kit (Shengxiang Biotech Co., Ltd., Hunan, China) on ABI QuantStudio™ Dx PCR system according to the manufacturer's instructions.

Detection of HCMV gB genotype using sequencing method

The gB region of *HCMV* was amplified by nested PCR with external primers (sense: 5'-GGA TCT GGT GCC TGG TAG TC-3', antisense: 5'-CCT ATG ATA TGC CAC GAA AAC-3') and internal primers (sense: 5'-GGC ATC AAG CAA AAA TCT-3', antisense: 5'-CAG TTG ACC GTA CTG CAC-3'). Briefly, a 25 μL PCR reaction system contains 600nM sense and antisense primers (0.15 μL each), 5.0 μL amplification reaction solution, 5μL extracted DNA, 0.2 μL *Taq* DNA polymerase (Shuoying Bio Technology Co., Ltd, Shanghai, China). PCR was performed on ABI 9700 PCR instrument with the following PCR amplification program: pre-denaturation at 95 °C for 6 minutes; 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 56°C for 40 seconds, and extension at 72 °C for 40 seconds; extension at 72°C for 10 minutes. The first-round PCR product (5μL) was used as a template for the second round of PCR with annealing at 58 °C for 35 cycles. PCR products were sequenced on ABI 3730xl by Shanghai Jierui Bioengineering Co., Ltd. The sequences obtained were visualized using Chromas v1.68. The nucleotide sequences of gB gene were checked, edited, and analyzed by DNAMAN v9.0. A full alignment method was used for the multiple sequence alignment. Our sequences were compared with published sequences of C327A (GenBank M60929, gB1), AD196 (X04606, gB2), CO76A (M85228, gB3), and C194A (M60926, gB4) prototype strains.

Statistical Analysis

Enumeration data were analyzed by Chi-square test or Fisher's exact test. Quantitative data of *HCMV* DNA were logarithmically transformed and were assessed by the Shapiro-Wilk test for normality distribution. The statistical significance of *HCMV* load was estimated using Student's test. The p-value < 0.05 was considered as a statistically significant difference.

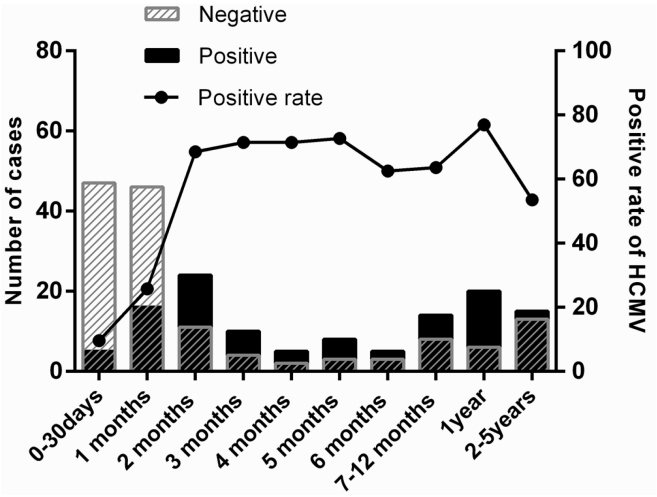
Results

Demographic and clinical characteristics of the infants

The 265 study subjects consisted of 181 hospitalized and 84 outpatient patients, including 161 males and 104 females. Among 265 infants, 122 were positive for *HCMV* DNA in urine, with a positive rate of 46.04% (122/265). When analyzing the positive rate of *HCMV* infection in infants of different ages, as shown in Figure 1, it rapidly increased with age, reached a plateau at two months of age, subsequently fluctuated, and peaked at one year of age. Data showed significant differences in *HCMV* infection positivity between 0-30 days and one month, one month, and two months of age ($\chi^2 = 3.91$, $p < 0.05$; $\chi^2 = 16.89$, $p < 0.01$, respectively). However, the positive rate of *HCMV* infection did not differ between other age groups older than two months.

Among the 122 *HCMV*-DNA positive infants, 91 infants with adequate urine specimens for subsequent testing were further investigated. The 91 infants comprised 57 males and 34 females, ranging in age from one day to 60 months (Table 1). Among them, the number of infants aged 0-2 months was the highest (36/91, 39.56%), followed by infants aged 3-12 months (31/91, 34.07%), and infants aged 1-5 years had the lowest number (24/91, 26.37%). Liver function abnormality was the most common diagnosis (34/91, 37.36%), followed by pneumonia (21/91, 23.07%). All infants diagnosed with liver function abnormality were excluded from hepatitis virus infection. Laboratory data, including the count of leukocytes and its subtypes as well as procalcitonin (PCT), were examined to determine that the infectious agent in infants diagnosed with pneumonia was not bacterial.

Figure 1. Positive rate of HCMV infection in 265 infants at different ages. The positive rate of HCMV infection in infants aged one month was significantly higher than that in infants aged 0-30 days ($\chi2 = 3.91$, $p < 0.05$). It was also significantly higher in infants aged two months than that in infants aged one month ($\chi2 = 16.89$, $p < 0.01$). The positive rate of HCMV infection plateaued at two months of age and did not differ between groups older than two months.



Distribution of gB1 and gB3 genotypes in infants with various clinical symptoms

All the sequences derived from the present study have been submitted to Science Data Bank (https://www.scidb.cn). Accession number of the submitted sequences is 10.57760/sciencedb.08397. Sequence analysis was conducted to detect the genetic subtypes of gB. This study revealed the presence of both gB1 and gB3 subtypes. No gB2, gB4 genotypes were detected, and co-infection with multiple gB genotypes was not found. Figure 2 shows the alignment of part of the samples gB sequences with the prototypes. Table 2 shows the gB genotypes distribution in 91 *HCMV* infected infants. The data showed the *HCMV* gB3 genotype predominated (51.65%, 47/91), followed by gB1 (48.35%, 44/91). Infants with different organ or system impairments did not differ significantly in gB genotype distribution.

HCMV load in 91 infants

The quantitative real-time PCR assays were performed to detect the *HCMV* DNA copy number in

Table 1. Demographic and clinical characteristics of 91 infants with *HCMV* infection.

Characteristics	n (%)
Age (months)	
0-2	36 (39.56)
3-4	9 (9.89)
5-6	11 (12.09)
7-8	6 (6.59)
9-10	5 (5.49)
11-12	0 (0)
13-24	13 (14.29)
25-60	11 (12.09)
Gender	
Female	34 (37.36)
Male	57 (62.64)
Symptoms	
Liver function abnormality	34 (37.36)
Pneumonia	21 (23.08)
Hyperbilirubinemia	6 (6.59)
Upper respiratory tract infection	4 (4.40)
Diarrhea	3 (3.30)
Nephrotic syndrome	2 (2.20)
Infectious mononucleosis	2 (2.20)
Epilepsy	2 (2.20)
Others	17 (18.68)

Table 2. Distribution of gB1 and gB3 genotypes in 91 infants with various clinical symptoms.

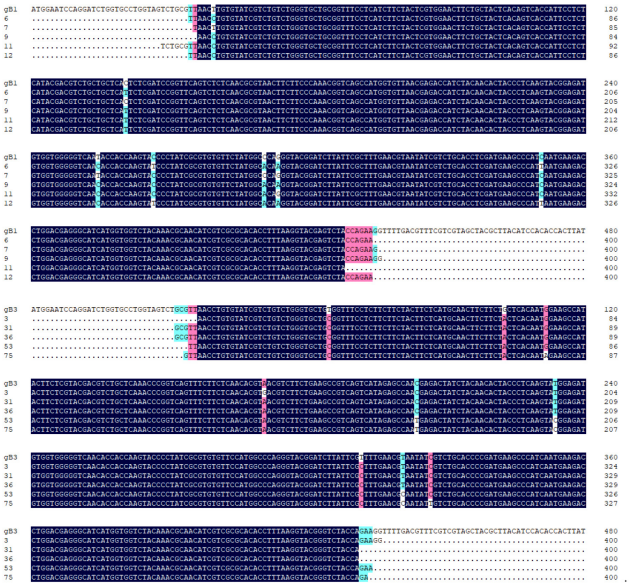
Clinical symptoms	gB1 (n, %)	gB3 (n, %)	Total (n, %)
Liver function abnormality	18 (52.94)	16 (47.06)	34 (37.36)
Pneumonia	11 (52.38)	10 (47.62)	21 (23.08)
Hyperbilirubinemia	1 (16.67)	5 (83.33)	6 (6.59)
Upper respiratory tract infection	4 (100)	0 (0)	4 (4.40)
Diarrhea	1 (33.33)	2 (66.67)	3 (3.30)
Nephrotic syndrome	1 (50.00)	1 (50.00)	2 (2.20)
Infectious mononucleosis	0 (0)	2 (100)	2 (2.20)
Epilepsy	1 (50.00)	1 (50.00)	2 (2.20)
Others	7 (41.18)	10 (58.82)	17 (18.69)

gB1: Glycoprotein B1; gB3: Glycoprotein B3.

the urine of 91 infants. The data showed the median copy number was 1.05×10^4 copies/ml (ranging from 4.82×10^1 to 1.77×10^8 copies/ml). We also analyzed the *HCMV* load in infants with liver function abnormality and pneumonia, the data showed no significant difference (median copy number 7.13×10^3 and 8.5×10^3, respectively).

We further investigated the relationship between viral load and gB genotype. The mean copy number of gB1 genotype *HCMV* is 1.27×10^4 copies/mL (ranging from 4.8×10^1 to 1.77×10^8 copies/mL); for gB3 genotype *HCMV*, the mean copy number is 8.62×10^3 copies/mL (ranging from 1.16×10^2 to 2.53×10^6 copies/mL). Infants infected with gB3 did not differ significantly in viral load from those infected with gB1 *HCMV*.

Figure 2. Alignment of representative gB sequences with the prototypes (only part of the samples is shown). The sequences are listed with the unique study subject identifiers. The nucleotide sequences were aligned with the gB prototypes using DNAMAN V9.0.



Hepatic impairment in infants with liver function abnormality as clinical symptoms

In the present study, we investigated hepatic impairment of infants with liver function abnormality as clinical symptoms by observing serum ALT and AST concentrations. The data showed the mean levels of ALT and AST in these infants were significantly higher than the reference values (150.69 ± 141.05 U/L and 140.88 ± 96.80 U/L, respectively). We further analyzed ALT and AST levels in infants infected with *HCMV* of different genotypes. The data showed that the mean level of ALT, and AST was higher in infants infected with gB3 *HCMV* than in those infected with gB1 *HCMV*, but there was no significant difference (Table 3).

Distribution of HCMV gB genotype in infants of different ages

When analyzing *HCMV* gB genotypes in infants of different ages, we found that the detection rate of gB3 genotype *HCMV* was significantly higher in infants aged 0-2 months (gB1 = 14, gB3 = 22) than in infants aged 3-12 months (gB1 = 20, gB3 = 11, $\chi^2 = 4.38$, $p = 0.0364$). However, there were no significant differences in the distribution of gB genotypes between infants aged 0-2 months and 1-5 years, or between infants aged 3-12 months and 1-5 years (Figure 3).

Table 3. The level of ALT and AST in infants with different gB genotype.

Index	gB1 (n = 44)	gB3 (n = 47)	*t* value	*p* value
ALT	83.51 ± 111.61	112.17 ± 137.29	1.0883	0.2794
AST	88.07 ± 80.64	106.96 ± 120.70	0.8717	0.3857

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; gB: glycoprotein B.

The level of ALT, AST, and viral load in infants with different anti-HCMV serologic status

In this study, we retrieved the data on serum anti-*HCMV* IgM and IgG antibody levels in 91 infants. The data showed that 81 subjects had IgM data (47 positive, 34 negative), 41 subjects had IgG data (29 positive, 12 negative), and 10 infants had neither IgM nor IgG data. We divided the infants into three groups (IgM^+IgG^-, IgM^+IgG^+ and IgM^-IgG^+ group) according to anti-*HCMV* serologic status. We further investigated *HCMV* load, ALT, and AST in three groups of infants. The data showed that ALT and AST levels were significantly higher in IgM^+IgG^- group than those in the IgM^+ IgG^+, and IgM^- IgG^+ group. However, there was no significant difference in *HCMV* load between the IgM^+IgG^- group and the other two groups (Table 4).

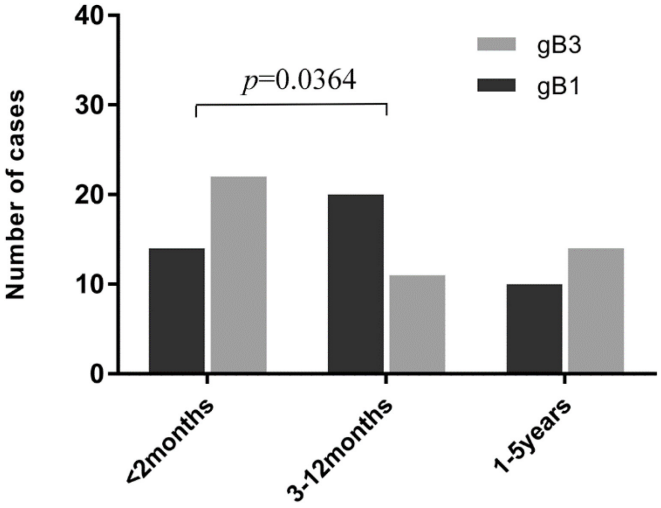
Comparison of positive rate of HCMV DNA detection in blood and urine samples

In this study, the retrospective data showed that 105 infants had undergone *HCMV* DNA test of blood and urine samples at the same time. The results showed that *HCMV* DNA was detectable in the urine of 61 (58.10%, 61/105) infants. However, *HCMV* DNA was detectable in only 6 (5.71%, 6/105) of these infants' blood samples. Moreover, six infants who were positive for *HCMV* DNA in their blood were also positive in their urine. There was a significant difference in the detection rate of *HCMV* DNA between blood and urine ($\chi^2 = 6.7131$, $p = 0.0096$).

Discussion

HCMV is the most common congenitally transmitted pathogen in worldwide, affecting about one million newborns annually [18]. Although most *HCMV* infections are asymptomatic, serious disease consequences may occur following infection, including neurodevelopmental retardation, hearing impairment, and increased risk of hematological malignancy [19,20]. Prompt diagnosis and treatment of *HCMV* infection is the key to preventing its sequelae. The real-time PCR is easier to implement and standardize and has gradually replaced traditional methods for identifying *HCMV* infection. In this study, the positive rate of *HCMV* DNA in urine samples from 265 infants was 46.04% (122/265). When analyzing the positive rate of *HCMV* infection in infants at different ages, data showed that it increased rapidly after birth, reached a plateau at two months, subsequently fluctuated with age, and peaked at one year of age. A previous study reported the positive rate of *HCMV* infection peaked in infants aged from three months to one year (57.78%-58.31%) [7]. Our data showed that the positive rate of *HCMV* infection peaked earlier and was higher, which may be caused by the relatively small number of samples in this study.

Figure 3. Distribution of HCMV gB genotypes in infants at different ages. HCMV with the gB3 genotype was detected significantly more frequently in infants aged 0-2 months than in infants aged 3-12 months ($\chi2 = 4.38$, $p = 0.0364$). There was no significant difference in the distribution of gB genotypes between infants aged 0-2 months and infants in other age groups.



HCMV infection can be present in many organ systems. *HCMV* is notably prone to infect the reticuloendothelial system, particularly the liver. Our data showed that liver function abnormality was the most common diagnosis in infants infected with *HCMV* (34/91, 37.36%), followed by pneumonia (21/91, 23.08%). The most common clinical diagnosis in infants infected with *HCMV* is consistent with previous reports [7]. Our data showed significant hepatic impairment in infants infected with *HCMV*. Pneumonia can be attributed to multiple pathogens and approximately 1% of *HCMV* infections in infants result

Table 4. The level of ALT, AST and virus load in infants with different anti-HCMV serologic status.

Anti-*HCMV* serologic status	Cases (N)	ALT	AST	*HCMV* Copies (Log_{10})
IgM^+/IgG^-	11	112.64 ± 71.78	125.91 ± 70.79	3.95 ± 1.25
IgM^+/IgG^+	16	60.33 ± 51.67*	62.47 ± 37.70*	3.44 ± 0.85
IgM^-/IgG^+	13	47.45 ± 22.67*	66.91 ± 42.47	4.58 ± 0.83

*There is significant difference compared with IgM^+/IgG^- group, $p < 0.05$. *HCMV*: human cytomegalovirus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

in *HCMV* pneumonitis [21]. Another study reported an overall *HCMV* infection rate of 10.8% among infants with respiratory infections in West China [22]. Our study demonstrated a close relationship between *HCMV* and pneumonitis. Given the fatality of *HCMV* pneumonia [8,9], *HCMV* pneumonia deserves high clinical attention, particularly in immunocompromised patients.

Multiple programs have investigated whether the viral load is a reliable biomarker to predict the severity and risk of sequelae of *HCMV* infection. One study showed that *HCMV* viremia levels correlated with symptom severity [23]. Some studies have also shown that higher viral load may be associated with an increased risk of adverse sequelae of *HCMV* infection [19,24,25]. On the contrary, several studies reported that viral load has no predictive value for long-term prognosis or adverse sequelae in infants with *HCMV* infection [26,27]. The present study showed no significant difference in *HCMV* load between infants with liver function abnormality and pneumonitis. We further analyzed the data on the *HCMV* DNA copy number as well as ALT and AST concentrations. The results showed there was no significant association between *HCMV* burden and hepatic impairment.

Studies of *HCMV* genotype have focused on the envelope glycoprotein gB, which play a role in virus entry and is major target for neutralizing antibody reaction [28,29]. Sequencing and genotyping allow for the identification of specific genetic subtypes of the gB protein in *HCMV*. This information can provide insight into the diversity and evolution of *HCMV*, as well as its potential impact on disease presentation and virulence. Additionally, identifying the genetic subtype of *HCMV* can be useful in designing targeted prevention and treatment strategies for specific strains. This study showed a predominance of *HCMV* gB3 genotype (51.65%, 47/91), followed by gB1 (48.35%, 44/91). The gB2, gB4 genotypes and mixed infections were not detected in this study population. The genotypes detected in this study are consistent with a previous study in Japanese infants [30], but different from studies in Indian [31], Polish [32] , and Italian [33] infants (17.64%, 15.40%, 32.50% for gB2, and 5.8, 28.8, 5.0% for gB4, respectively). Amazingly, Compared with reports from the different provinces in China, our results were similar to those of Shanghai [34], but different from those of Wuhan [35] and Zhejiang [36,37], which reported high frequencies of gB2 genotype (12.5%, 17.72% and 13.4%, respectively). These differences show that people in different regions have different susceptibilities to each type of *HCMV*, and the identification of the gB genotype helps understand the dominant epidemic strains.

Several studies have investigated the relationship between different gB genotypes and clinical manifestations and outcomes. Studies showed that patients infected with gB1 *HCMV* had a better outcome compared to those infected with gB3 *HCMV* [38,39]. Severe manifestations in *HCMV*-infected infants were also found to be associated with the gB3 genotype [30]. This may be because the average copy number of the gB3 genotype is higher than that of the gB1 genotype [37]. However, this study showed that *HCMV* load did not differ significantly between gB1 and gB3 genotype infected infants. Our study showed that infants infected with gB3 *HCMV* had a higher degree of hepatic impairment than infants infected with gB1 *HCMV*, but there was no significant difference. Furthermore, this study showed gB3 *HCMV* had a higher detection rate in infants aged 0-2 months than that in infants aged 3-12 months ($\chi 2 = 4.38$, $p = 0.0364$). All these suggest that gB3 *HCMV* may have the ability to preferentially target specific host cells and be more virulent, which needs to be confirmed at the cytological level and in clinical studies with large sample sizes.

In this study, infants infected with *HCMV* were divided into three groups (IgM^+IgG^-, IgM^+IgG^+, and IgM^-IgG^+ group) according to the anti-*HCMV* serological status. We investigated the *HCMV* load, ALT, and AST levels in the three groups. The data showed the concentration of ALT and AST in the IgM^+IgG^- group were significantly higher than those in the IgM^+ IgG^+, and IgM^-IgG^+ group. However, there was no significant difference in viral load between the IgM^+IgG^- group and the other two groups. This data indicates that there may be some relationship between anti-*HCMV* IgG antibody and hepatic impairment, which needs further in-depth study to confirm.

Both urine and blood are commonly used specimens for detecting *HCMV* DNA in infants. We compared the detection rate of *HCMV* DNA in the blood and urine of the same infant. The data showed that *HCMV* DNA was detected significantly less frequently in blood than in urine. This is consistent with previous report [40] and suggests that PCR lack sensitivity for detecting *HCMV* DNA in blood. Urinary PCR is the most appropriate assay to detect *HCMV* infection because urine is a convenient and noninvasive sample.

In summary, this study showed *HCMV* genotype distribution, viral load, and their relationship with demographic characteristics and clinical

manifestations. This study contributes to understanding the development and status of *HCMV* infection in infant populations. In addition, this study suggests urinary PCR is the most appropriate assay to detect *HCMV* infection. It must be pointed out that our findings are limited given the relatively small number of samples. Studies with larger sample sizes will contribute to a deeper understanding of the role of *HCMV* in infantile-related diseases. Another limitation of this study is its retrospective nature, which resulted in missing clinical data for some participants, such as incomplete serum anti-*HCMV* IgM and IgG data, which increased the statistical risk of a smaller sample size. In addition, this retrospective study lacked longitudinal information to determine the age at which infection occurs, and comparison of age at diagnosis may impose biological/clinical limitations.

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