

Clinical applications of real-time PCR for diagnosis and treatment of human cytomegalovirus infection in children

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There are many methods of detecting human cytomegalovirus (HCMV) infection. So far, the quantitative polymerase chain reaction (PCR) has been very useful not only in aiding in the diagnosis of HCMV but also in determining the severity and predicting HCMV infection. However, it is time-consuming and labor intensive. Real-time PCR (RT-PCR) is an exception, for it allows rapid quantification of HCMV DNA load. Our group used this method for detecting and monitoring HCMV and compared it with the diagnostic criterion recommended by the Pediatric Branch of Chinese Medical Association, in 45 children suspected of having HCMV infection. The response to two types of antiviral treatment on HCMV DNA load was also monitored in HCMV hepatitis cases. RT-PCR was positive in 30 cases while the diagnostic criterion, which includes enzyme-linked immunosorbent assay (ELISA) and/or conventional PCR, was positive in 32 cases. The decrease in the HCMV DNA load was achieved earlier in the modified treatment group compared with the conventional treatment group. A 10^3 copies/ml of HCMV DNA load of is a useful cut-off value in predicting patients who will have symptoms of the disease. RT-PCR can be used not only in detecting HCMV but also in monitoring response to antiviral treatment and risk of having symptoms of the disease.

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Human cytomegalovirus (HCMV) is a major pathogen of congenital and perinatal infections; hence, children are the most common group affected for primary infection (1, 2). HCMV infective rate in children increased significantly in recent years with the increasing practice of bone marrow transplantation and immunocompromised status after chemotherapy in children with malignancy. For this reason, there is a need for early detection of HCMV so that proper management may be provided immediately. At present, viral culture, serologic assays such as enzyme-linked immunoabsorbent assay (ELISA), antigenemia assay and conventional polymerase chain reaction (PCR) are being used for detection of the viral agent. However, these methods would only indicate the presence or absence of HCMV and/or would not necessarily indicate active infection and clinically relevant disease.

Quantitative PCR, which includes competitive PCR assays and hybridization of PCR products to probes in a microwell format, has been shown to be helpful in determining the severity of an infection and predicting the risk of a patient of developing HCMV disease (6). Although, these tests are labor-intensive and require post-PCR handling. In this paper, real-time PCR (RT-PCR) which is more rapid and convenient, (7, 9) was used to detect HCMV copy numbers in sera and its value in the diagnosis and treatment of HCMV infection was evaluated.

Materials and methods

Patients

Forty-five children of < 5 years old suspected of having HCMV infection seen in both outpatient

and inpatient department of the Third Affiliated Hospital of Sun Yat-sen University (SU) from November 1998 to November 2000 were included in the study. HCMV infection suspect was defined as having one or more of the following signs and symptoms: prolonged fever for 2 weeks, jaundice, hepatosplenomegaly, and unresolved cough for more than 1 month, purpura and anemia. Sera from all 45 HCMV suspects were isolated and used for ELISA, conventional PCR, and RT-PCR. Ten normal healthy children with age <1 year were selected as a RT-PCR normal control group. This age group was chosen to reduce the false-positive results of HCMV DNA since children older than this age group is most likely to have had asymptomatic HCMV and the patients with HCMV hepatitis were within this age group. The study were approved by the ethics committee of our hospital and the parents gave informed consent.

Diagnostic criterion

Definitive diagnosis of HCMV infection was based on the proposal of the Pediatric Branch of Chinese Medical Association which is defined as clinical symptoms, such as prolonged fever for 2 weeks, jaundice, hepatosplenomegaly, unresolved cough for more than 1 month, purpura and anemia, with positive HCMV-IgM by ELISA and/or positive for HCMV DNA by conventional PCR, and other diseases being excluded (8).

HCMV real-time PCR assay

The upstream and downstream primers were: 5'-GATAAGGTTTCATGAGCCTTTCG-3' and 5'-GCATTGAGGCAAGTTCTGTAAC-3'; fluorogenic probe was 5'-CTTGTATTAAGGAAC TCTATAATGTGACC-3'. HCMV DNA was extracted from sera and performed according to the manufacturer's recommendations. PCR reaction was performed as described previously (7, 11). Briefly, either 10 μ l of calibrated plasmid dilution or DNA was added to the PCR mixture. All PCR reaction mixture contained 1X PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 5 mM MgCl₂, 0.2 mM dNTP (Da An Gene Diagnostic Center, GZ, China). The 0.2 mM each primer and 1 U of platinan *Taq* DNA polymerase (Da An Gene Diagnostic Center). The reaction, data acquisition, and analysis were performed using the ABI 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Cycling include an initial denaturation for 2 min at 93°C, followed by 40 cycles of denaturation at 93°C, and annealing at 55°C for 130 s.

Conventional PCR assay

The upstream and downstream primers were 5'-TGGGCTAACTATGCAGAGCA-3'; and 5'-AGCCATTGGTGGTCTTAGGG-3' respectively. The amplification of the 244 bp was carried out in a GenAmp 9600 Thermal Cycler (Perkin-Elmer) upon the recommendation of the manufacturer. Thirty-five cycles of amplification (93°C for 45 s, 56.3°C for 45 s and 72°C for 60 s) and an extension for 15 min at 72°C was performed at the end of the reaction. The amplified PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and results were photographed. Detection of false-positive results caused by contamination were performed and excluded. Likewise, positive and negative controls were used in all the tests.

HCMV ELISA

The IgM antibodies to HCMV were measured using a qualitative ELISA (Sorin Biomedical, Milan, Italy). A positive result is defined as the presence of HCMV-specific IgM antibody response.

Treatment

Twenty-five children with HCMV hepatitis were selected for monitoring the effect of treatment based on the number of HCMV DNA using RT-PCR. These patients were randomly assigned to two groups. The first group had 12 patients and received the conventional treatment (combination of prednisone (12) 1 mg/kg/day for 1–3 months, sodium glucuronate, 0.1–0.2 g/day and sodium luminal 3–5 mg/kg/day (13) for 3 months). The second group had 13 patients and received the modified treatment (combination of prednisone 1 mg/kg/day for 1–3 months, sodium glucuronate, 0.1–0.2 g/day and sodium luminal 3–5 mg/kg/day for 3 months, ganciclovir, dose: 7.5 mg/kg every 12 h $\times$ 14 days then 5 mg/kg/day three times a week for 3 months and intravenously immunoglobulin, dose: 2.5 g/day for 5 days). RT-PCR was performed on both groups at five stages: upon admission, discharge and third, sixth and ninth month after discharge.

Statistical analysis

The rank sum test (Kruskal–Wallis test) was used for comparison of the distribution of viral copy numbers while a MANOVA test was used for a comparison of viral load between the two groups in all five stages (i.e., upon admission, discharge

and third, sixth, and ninth month after discharge). For comparison of viral load between both groups, having with or without symptoms, *t*-test was used. A *p*-value of <0.05 was considered as a statistically significant result.

Results

Of the 45 HCMV infection suspects, five were HCMV-IgM-positive alone, 17 were positive for the conventional PCR alone and 10 were positive for both. Thus, based on the diagnostic criterion, 32 patients were identified as HCMV infection with clinical features tabulated in Table 1. From the 45 HCMV suspect patients, 30 were positive for RT-PCR and also satisfied the diagnostic

criterion. Since there is no gold standard for the diagnosis of HCMV infection in children, we used the diagnostic criterion as our reference and compared it with the use of RT-PCR and presence of symptoms. The sensitivity, positive predictive and negative predictive values of RT-PCR is summarized in Table 2.

Among the 30 children with positive RT-PCR results, there were 25 cases of HCMV hepatitis, two cases of interstitial pneumonia which developed after chemotherapy of acute lymphoblastic leukemia, two cases of idiopathic thrombocytopenic purpura and one case of recurrent pneumonia.

The group who received the modified treatment had 1.80×10^3 – 2.87×10^7 copies/ml of HCMV DNA while the group who received the conventional treatment had 1.40×10^3 – 2.38×10^7 copies/ml of HCMV DNA. There was no significant difference in viral copy numbers between the two groups ($p > 0.05$). Among the 10 cases in the normal control group, nine had 0 copies/ml and one had 1.2×10^2 copies/ml. There was a significant difference between the normal control group and HCMV hepatitis group ($p < 0.001$) (Table 3).

There was a decrease in copy number of HCMV DNA in both the HCMV hepatitis-treated groups. However, the decrease was more significant in the group who received the modified treatment. The drop in the copy number of HCMV DNA (10^3 copies/ml) was observed at the time of discharge. This decrease in number was only noted on the third month

Table 1. Characteristics of patients with human cytomegalovirus (HCMV) infection

Features	n (%)
Age (months)	
Range	1–48
Mean	4.9
Sex	
Male	20 (67.5)
Female	12 (37.5)
Mothers with HCMV infection	16 (50)
Symptoms	
Prolonged fever	2 (6.25)
Jaundice and hepatosplenomegaly	27 (84.4)
Cough	2 (6.25)
Purpura	2 (6.25)
Anemia	28 (87.5)

Table 2. Comparison between real-time PCR and diagnostic criterion results for 45 HCMV suspects

Number of patients				Total suspect	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Real-time PCR		Diagnostic criterion						
+	−	+	−					
30	2	32	13	45	100	13.3	93.8	100

PPV, positive predictive value; NPV, negative predictive value; PCR, polymerase chain reaction; HCMV, human cytomegalovirus.

Table 3. Distribution of HCMV DNA copy numbers

Group	Case number	Mean	s.d.	Minimum	Maximum	p-Value
Modified treatment group	13	5.139	1.435	3.26	7.48	
Conventional treatment group	12	4.968	1.449	3.15	7.28	0.7*
Cases other than HCMV hepatitis	5	5.028	1.415	3.36	6.91	0.001*
Normal control	10	0.207	0.657	0	2.08	

*Rank sum test (Kruskal–Wallis test) of the four groups after logarithmic transformation. There was a significant differences in viral copy numbers among the four groups ($p < 0.001$), but no significant difference among the human cytomegalovirus (HCMV) infected groups ($p > 0.7$).

after discharge in the group who received the conventional treatment. Also, there was a significant difference between the viral load of the two groups in the five stages based on a MANOVA test ($p < 0.001$). Details are shown in Fig. 1.

It turned out that the viral load in those who had symptoms of HCMV infection was significantly higher than that of asymptomatic patients ($t = 18.28$ and $p < 0.001$) according to the copy number of HCMV DNA from the modified treatment group and conventional treatment group at five stages and from normal control (Fig. 2). By using 10^3 copies/ml as a

symptomatic predictive critical value ($=10^3$ with symptom and $<10^3$ without symptom) to predict active HCMV infection, the test results of 135 (25 patients $\times$ 5 stages + 10 normal control) indicated that the sensitivity was 100% (46 of 46), specificity was 87.6% (78 of 89), positive predictive value was 80.7% (46 of 57) and negative predictive value was 100% (78 of 78).

In the 25 cases of HCMV hepatitis in our study, four had viral load of HCMV $> 10^7$ copies/ml. Among them, three had sequelae (one from the group given modified treatment and two from the group given conventional

Fig. 1. The change of viral copy numbers of the three groups at five stages (upon admission, discharge and third, sixth, and ninth month after discharge from hospital). There was a significant difference between the viral loads of the two groups in the five stages based on a MANOVA test ($p < 0.001$).

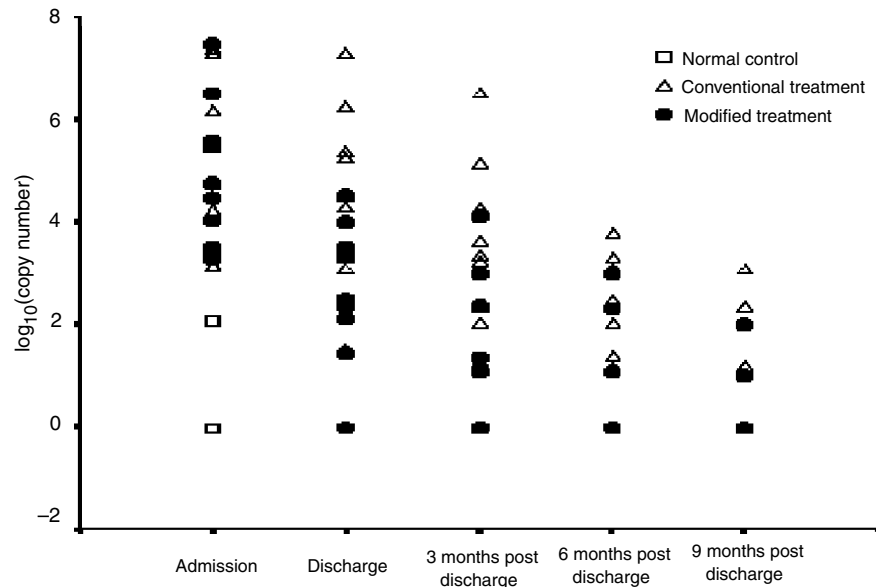
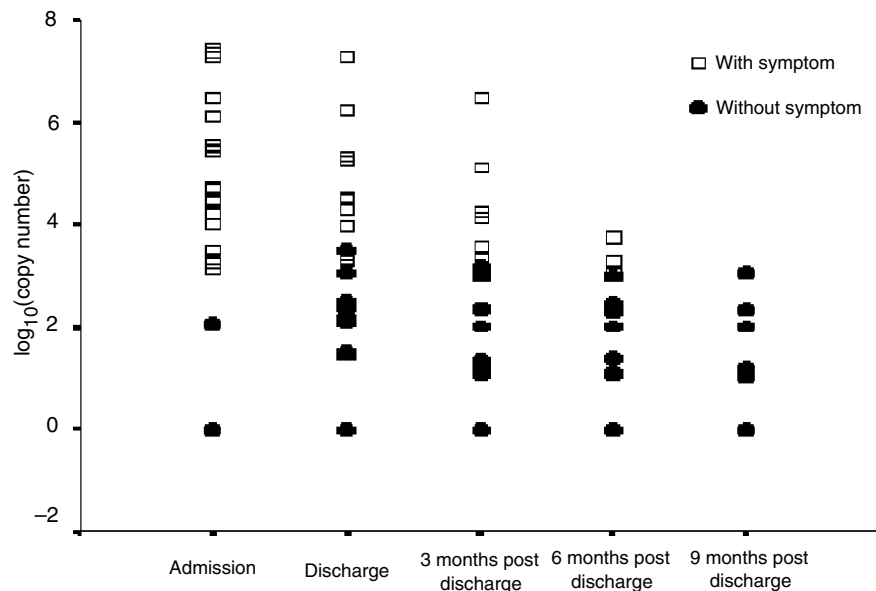


Fig. 2. Comparison of human cytomegalovirus (HCMV) DNA copy number between those with symptoms and asymptomatic cases using *t*-test ($t = 18.28$ and $p < 0.001$).



treatment) namely: mental retardation, microcephaly and sensorineural hearing loss.

Discussion

Quantitative, fluorescence-based, RT-PCR assay is a HCMV DNA quantitative detection method based on a normal PCR technique. It possesses the high sensitivity of PCR and specificity of DNA hybridization. Likewise, it has been reported to be an accurate quantitative assay (7, 9–11). Based on the results of our study, clinical symptoms plus the use of RT-PCR showed a very high sensitivity, positive and negative predictive values, which indicate the usefulness of this method in the diagnosis of HCMV infection.

The copy number of HCMV DNA can be monitored during viral replication. Generally, the levels of HCMV DNA in peripheral blood leukocyte are lower in individuals with latent infection than those with acute symptomatic infections (4, 14, and 15). Conventional virologic screening is of limited value for prediction of development of HCMV disease (3–5) and there are only a few data from children. Our results showed that RT-PCR can detect HCMV DNA levels as low as 0 copies/ml to as high as 2.87×10^7 copies/ml that is similar to previous reports (4, 15, 16). RT-PCR showed that it could distinguish latent from acute infection by determining the viral DNA copy number. The viral load is higher in patients with acute infection than those with latent infections. Moreover, the risk of developing HCMV infection can be predicted using 10^3 copies/ml of HCMV DNA as the cut-off value. These observations can be applied when monitoring HCMV infection among immunocompromised patients with malignancy. However, further studies should be undertaken using this cut-off value on a larger number of populations.

Some reports suggest that the risk of development of HCMV disease might be correlated with the viral load, i.e., the higher the viral load value, the more serious the clinical manifestation and the poorer the prognosis (5, 16–18). In contrast, other reports propose that there is no correlation between the viral load values and severity of HCMV disease (15, 19). Based on our results of HCMV hepatitis, children with HCMV DNA copy number $> 10^7$ had sequelae, irregardless of the type of treatment. These were mental retardation, microcephaly and sensorineural hearing loss. Likewise, all of them had severe symptoms with long duration of illness, which might indicate that HCMV was acquired congenitally. We can only assume because all of

our patients came for consult after 1 month of age. Although the number of patients is relatively small to make a definite conclusion, a relationship between HCMV viral load and risk of developing the disease, as well as prognosis were demonstrated. Hence, we considered that the quantitative measurement of HCMV DNA can serve as an important tool for predicting HCMV disease and for prognostication.

Real-time PCR demonstrated the decrease in copy number of HCMV DNA during and after treatment, which could be used for monitoring the response to treatment. The present data showed that both treatment methods were able to decrease the copy number of HCMV DNA in the blood. However, this decrease was earlier noted in the modified treatment group, which was expected because ganciclovir is an accepted method of treatment in HCMV infection. Further details with regards to the two different treatment methods were discussed in another report.

The viral load presented a descending tendency at the last two stages. This may be related to an improvement of the immunity of these children. We also found out that in seven cases of HCMV hepatitis children, the viral DNA was still detectable even after ninth month of discharge. Similar to the study of Fang et al., they were also able to detect HCMV DNA even after 15–40 months treatment (20). Strata et al. (21) reported that HCMV disease could have a 30% recurrence rate after the initial antiviral treatment. Hence, there is a possibility that these patients may become carriers of the virus and warrants further investigation. In conclusion, authors recommend the use of RT-PCR for monitoring activity of HCMV.

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References

1. BOPPANA SB, FOWLER KB, VAID Y, et al. Neuroradiographic findings in the newborn period and long-term outcome in children with symptomatic congenital cytomegalovirus infection. *Pediatrics* 1997; 99: 409–14.
2. NELSON CT, DEMMLER GJ. Cytomegalovirus infection in the pregnant mother, fetus and newborn infant. *Clin Perinatol* 1997; 24: 151–60.
3. BLANK BS, MEENHORST PL, MULDER JW, et al. Value of different assays for detection of human cytomegalovirus in predicting the development of HCMV disease in human immunodeficiency virus-infected patients. *J Clin Microbiol* 2000; 38: 563–9.
4. SCHAFER P, LAUFS R. Experience with quantitative PCP for the management of HCMV disease. *Intervirol* 1996; 39: 204–12.

5. SPECTOR SA, WONG R, HSIA K, PILCHER M, STEMPIEN MJ. Plasma cytomegalovirus (CMV) DNA load predicts CMV disease and survival in AIDS patients. *J Clin Invest* 1998; 101: 497–502.
6. SCHALASTA G, EGGERS M, SCHMID M, ENDERS G. Analysis of human cytomegalovirus DNA in urines of newborns and infants by means of a new ultra rapid real-time PCR system. *J Clin Virol* 2000; 19: 175–85.
7. NAJIOULLAH F, THOUVENOT D, LINA B. Development of a real-time PCR procedure including an internal control for the measurement of HCMV viral load. *J Virol Methods* 2001; 92: 55–64.
8. PEDIATRIC BRANCH OF CHINESE MEDICAL ASSOCIATION. Diagnostic scheme of human cytomegalovirus infection. *Chin J Pediatr* (in Chinese) 1999; 37: 441.
9. LIVAK KJ, FLOOD SJ, MARMARO J, GIUSTI W, DEETZ K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 1995; 4: 357–62.
10. GAULT E, MICHEL Y, DEHEE A, BELABANI C, NICOLAS JC, GARBARG-CHENON A. Quantification of human cytomegalovirus DAN by real-time PCR. *J Clin Microbiol* 2001; 39: 772–5.
11. NITSCHKE A, STEUER N, SCHMIDT CA, LANDT O, SIEGERT W. Different real-time PCR formats compared for the quantitative detection of human cytomegalovirus DNA. *Clin Chem* 1999; 45: 1932–7.
12. YAO MH. Treatment of infants with HCMV hepatitis combining Chinese traditional and Western medicines. *J Zhejiang Tradit West Med* [in Chinese] 1995; 5: 12–13.
13. LI XQ, LI YM, DUAN MX. Combined therapy of Chinese and western medicine in infants with HCMV hepatitis (26 cases). *TCM Res* 1998; 11: 38–39.
14. STAGNO S, REYNOLDS DW, TSANTOS A, FUCCILLO DA, LONG W, ALFORD CA. Comparative serial virologic and serologic studies of symptomatic and subclinical congenitally and natively acquired cytomegalovirus infections. *J Infect Dis* 1975; 132: 568–77.
15. FERREIRA-GONZALEZ A, FISHER RA, WEYMOUTH LA, et al. Clinical utility of a quantitative polymerase chain reaction for diagnosis of cytomegalovirus disease in solid organ transplant patients. *Transplantation* 1999; 68: 991–6.
16. FOX JC, GRITHS PD, EMERY VC. Quantification of human cytomegalovirus DNA using the polymerase chain reaction. *J Gen Virol* 1992; 73: 2405–8.
17. SALTZMAN RL, QUIRK MR, JORDAN MC. High levels of circulating cytomegalovirus DNA reflect visceral organ disease in viremic immunosuppressed patients other than marrow recipients. *J Clin Invest* 1992; 90: 1832–8.
18. GERDES JC, SPEES EK, FITTING K, et al. Prospective study utilizing a quantitative polymerase chain reaction for detection of cytomegalovirus DNA in the blood of renal transplant recipients. *Transplant Proc* 1993; 25: 1411–3.
19. RASMUSSEN L, MORRIS S, ZIPETO D, et al. Quantitation of human cytomegalovirus DNA from peripheral blood cells of human immunodeficiency virus-infected patients could predict cytomegalovirus retinitis. *J Infect Dis* 1995; 171: 177–82.
20. FANG F, DONG YS. Follow-up survey of infantile cytomegalovirus hepatitis: effect on child's development and nervous system. *Chin J Pediatr* (in Chinese) 1990; 28: 124–8.
21. STRATTA RJ, SHAEFER MS, MARKIN RS, et al. Clinical patterns of cytomegalovirus disease after liver transplantation. *Arch Surg* 1989; 124: 1443–50.