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ORIGINAL INVESTIGATION

Diagnostic value of HCMV pp65 antigen detection by FCA for symptomatic and asymptomatic infection: compared to quantification of HCMV DNA and detection of IgM antibody in infants

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Abstract Human cytomegalovirus (HCMV) can cause symptomatic or asymptomatic infection in infants. One hundred and twenty-six infants were assessed clinically for disease in infantile period. Eighty of them were classified as symptomatic infection on the basis of physical, instrumental, and laboratory findings, 5 were demonstrated by following up to have later developed HCMV disease, and the other 41 infants were classified as asymptomatic infection. HCMV DNA was positive in all urine samples of the symptomatic infants detected by quantitative polymerase chain reaction. HCMV-IgM antibody detected by chemiluminescent immunoassay (CLIA) was positive in 62 of the 85 symptomatic infants, but was negative in all of the samples of asymptomatic infants. HCMV pp65 antigen detected by flow cytometry assay (FCA) was positive in 77 of the 85 symptomatic infants and in none of the asymptomatic infants. The coincidence to symptom of HCMV pp65 antigen detection was higher than those of HCMV DNA and HCMV-IgM antibody detection. The sensitivity, specificity, positive prognostic value and the negative prognostic value of HCMV pp65 antigen detection for diagnosis of HCMV infection was 90.6, 100, 100 and 83.7%, respectively. We concluded that detection of pp65 antigen by FCA is more sensitive for diagnosis of HCMV infection than detection of HCMV-IgM antibody and is better than HCMV DNA quantification for distinguishing the symptomatic and asymptomatic HCMV infection in infants.

Keywords HCMV · Flow cytometry · PP65Ag · Real-time PCR · IgM antibody

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Introduction

Human cytomegalovirus (HCMV) infection remains the most common infection worldwide [1]. Infants who acquired HCMV in uterus during maternal primary or reactivated infection may develop severe disease that result in long-term disability [2]. Diagnostic techniques for HCMV detection have greatly improved during recent years with the advent of sophisticated serological and virological methods. However, the significance and usefulness of different assays depend on patient categories. Conventional methods for laboratory diagnosis of HCMV infection include virus culture, serology assay. Virus culture is the "gold standard" method, but is relatively insensitive [3]. Serological test is also used to confirm exposure to HCMV, but it is not reliable since neonates may not have a change in antibody titer or produce detectable HCMV-IgM in case of an active HCMV infection [4]. Quantitative polymerase chain reaction (Q-PCR) for HCMV DNA quantification provides sensitive and specific data for detecting HCMV infection, but results of the method cannot distinguish active or inactive infection. Among the techniques used to confirm an active HCMV infection, detection of the HCMV-specific early pp65 antigen is widely used. The antigenemia assay (AA) has the advantage of quantifying the viral load according to the number of antigen-containing

cells, which correlates well with manifestations of HCMV disease [5, 6]. However, the quantification of antigen-positive cells by the traditional AA under microscopic examination is subjective and time-consuming [5].

With the advent of effective antiviral therapy for human HCMV infection, more rapid and sensitive techniques are required to identify HCMV infection and to monitor the effects of antiviral therapy during the acute stage of the illness [7]. Flow cytometry is an advanced technique, which can be used to analyze a large number of specifically labeled cells in a quantitative manner. The use of flow cytometry in the detection of viral infections has grown to significant proportions [8–11]. In this study, we evaluated the possible use of flow cytometry assay (FCA) for rapid and quantitative detection of HCMV-specific pp65 antigens in peripheral blood samples and for distinguishing symptomatic or asymptomatic infection in HCMV-infected infants.

Materials and methods

Study subjects and specimens

From November 2006 to August 2007, 126 infants in the Affiliated Shengjing Hospital of China Medical University were selected for the study. All of the infants aged less than 1 month when their samples were collected. Based on the physical, instrumental, and laboratory findings, the infants were classified as symptomatic or asymptomatic infection. Symptomatic infection was considered when the infants showed more than one of the following findings: signs and symptoms of systemic involvement such as intrauterine growth retardation, hepatosplenomegaly, skin petechiae/purpura, thrombocytopenia (platelet count <100,000/mm^3), jaundice with direct bilirubin (>3 mg/dL), alanine aminotransferase (ALT) elevation (>80 U/L), pneumonia, neurologic involvement (microcephaly, lethargy/hypotonia, poor sucking, and seizures), and sensorineural defects (chorioretinitis and deafness), and HCMV-associated patterns at neuroimaging including abnormal periventricular hyperechogenicity, intracranial calcifications, ventriculomegaly, hyperechogenicity of lenticolo-striatal vessels, etc. [12].

One hundred and twenty-six urine specimens, sera and corresponding peripheral blood cells were obtained from the selected infants with the permission of their parents.

Quantification of HCMV DNA by Q-PCR

Q-PCR was performed with a commercially available TaqMan system (Q-CMV Real-Time System, Da An company, China). HCMV AD169 UL123 genomic region (Immediately Early 1) was used for targeting amplification region. DNA was extracted from urine specimens with DNA extract buffer (10 mM EDTA, 10 mM Tris–Cl, 1% (w/v) SDS and 100 μg/ml proteinase K) according to the manufacturer's instructions. Thermal cycling conditions were as follows: 93°C for 2 min, 10 cycles of 93°C for 45 s and 55°C for 1 min, 30 cycles of 93°C for 30 s and 55°C for 45 s. Fluorescence signals were recorded using the optimal excitation/emission wavelengths. An internal quantification standard curve was constructed with dilutions, ranging from 10^3 to 10^7 copies/reaction, of a plasmid carrying the HCMV UL123 gene.

All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. PCR analyses were conducted in triplicate for each sample.

Detection of HCMV-IgM antibody by chemiluminescent immunoassay

Indirect chemiluminescence immunoassays (Liaison IgM assays; DiaSorin, VC, Italy) were used for the quantitative determination of HCMV-specific IgM antibodies. HCMV-IgM antibody was detected on a fully automated random-access analyzer. The principle of the chemiluminescent immunoassay (CLIA) test is based on magnetic microparticle technology with flash light kinetics, and an isoluminol derivative as a label. The CLIA signal is generated by the addition of two trigger solutions, and light is quantified as relative light units. Data reduction is based on a master curve with a two-point recalibration method. Calibration allows recalculation of the working standard curve from the stored master standard curve.

A monoclonal antibody to human IgM is used to coat the paramagnetic particles. During the first step, IgM antibody in serum or plasma is captured onto the magnetic particles after 10 min incubation. Then, HCMV-specific IgM antibody was detected by soluble HCMV antigen together with a monoclonal antibody labeled with an isoluminol derivative. After washing, the chemiluminescence signal was generated. The measurement range is between 8 and 160 arbitrary units (AU)/ml. Positive results of HCMV-IgM are defined as values above 30 AU/ml, borderline results range from 15 to 30 AU/ml, and negative results are defined as values below 15 AU/ml.

Detection of HCMV pp65 antigen by FCA

Red blood cells in 100 μl of whole blood were hemolysed for 8 min at room temperature in 1 ml lyses solution (FACS Lyses Solution, BD Biosciences, CA) and the remaining cells were permeabilized for 10 min in FACS Permeabilizing Solution (BD Biosciences, CA). The cell pellet was

washed with phosphate-buffered saline (PBS buffer) for several times.

Fixed permeabilized cells were stained as previously described [10, 11] using HCMV pp65 monoclonal antibody from mouse (Chemicon Company, CA). Briefly, after removing the fixture and permeabilizing solution by centrifugation at 1,000 *g* for 5 min, cells were washed in PBS containing 4% fetal calf serum (PBS:FCS) and were suspended in 50 µl of the anti-pp65 antibody. For each sample, isotypically matched monoclonal antibody (BD Biosciences, CA) was used as a control. Both tubes were incubated at 4°C for 60 min. After washing and centrifugation twice in PBS, 4 µl of the second antibody, which is FITC-conjugated goat anti-mouse immunoglobulin (BD Biosciences, CA) was added and incubated at 4°C for 30 min. After rinsing two times with PBS:FCS solution, the cells were suspended in 400 µl of PBS and analyzed by a FACS Calibur (BD Biosciences, CA) flow cytometer.

Controls used

Peripheral blood leukocytes (PBL) from patients without HCMV infections were used as negative controls.

At least 100,000 PBLs gated by light scatter were assessed for accuracy. Debris and dead cells were excluded from the analysis by the conventional scatter gating method. All data were expressed in percentage fluorescence scatter form using a region defined according to isotype control analysis. FCA fluorescence results were expressed as the percentage of PBLs carrying pp65 antigen. The percent of positive cells was estimated by setting the level for positive cells not to include the background stained with a non-specific isotype control antibody. According to the Receiver Operating Characteristic (ROC) curve, the threshold for positivity was determined in our study as being 0.05% of positive PBLs.

Statistical analysis

Calculation of the mean, standard deviation (±SD) values and correlation were carried out using an SPSS 11.5 software package. After normalization of the pp65 antigen values of FCA fluorescence by percentage, the correlation between the pp65 antigen values of FCA and quantities of HCMV DNA or HCMV-IgM antibodies were evaluated with Pearson's correlation coefficient. The relationships between the diagnostic values of HCMV pp65 antigen detected by FCA and those of quantification of HCMV DNA, detection of HCMV-IgM antibody were analyzed by linear regression test. ROC curve was used to compare the diagnostic value of certain parameters by FCA.

Results

Clinical outcome

The study population consisted of 126 infants. Among them, 80 infants were classified as symptomatic infection on the basis of physical, instrumental, and laboratory findings, 5 were demonstrated by following up to have later developed HCMV disease, and the other 41 infants were classified as asymptomatic infection. A total of 126 consecutive samples from infants were received by our Laboratory for the simultaneous determination of pp65 antigenemia in PBL, quantitative HCMV DNA in urine and HCMV-IgM antibody in sera.

Correlation between detection of HCMV pp65 antigen and quantification of HCMV DNA for diagnosis

Out of the 126 infants, HCMV DNA was positive in 106 infants by Q-PCR. HCMV pp65 antigen was positive in 72 of the 80 symptomatically infected infants and in all of the 5 infants who were demonstrated as symptomatic infection by following up, but in none of the 41 asymptomatically infected infants. Further more, samples of the eight symptomatically infected infants, who were negative in FCA test, were collected 7–10 days later and five of them were demonstrated as pp65 antigen positive by FCA. Seventy-seven (61.1%) of the infants were positive for both HCMV pp65 antigen and HCMV DNA.

Infants with detectable pp65 antigen had significantly higher HCMV DNA levels (median 59,230 copies/ml; range 1.0×10^4–1.47×10^6) than those of pp65-negative infants (median 2,830 copies/ml; range 1.0×10^3–1.58×10^4) ($p < 0.0001$). The amounts of HCMV pp65 antigen in blood cells were statistically correlated to HCMV DNA levels of infected infants ($p < 0.0001$) (Fig. 1).

Correlation between detection of HCMV pp65 antigen and HCMV-IgM antibody for diagnosis

Sera and whole blood specimens of the 126 infants were collected and used for detection of HCMV-IgM antibody by CLIA and pp65 antigen by FCA, respectively. Among the 126 infants, 62 of the 80 symptomatically infants were positive for HCMV-IgM antibody, but was negative in all of the samples of asymptomatic infants and the infants who were demonstrated as symptomatic infection by following up. HCMV pp65 antigen was positive in 72 of the 80 symptomatically infected infants and in all of the 5 infants who were demonstrated as symptomatic infection by following up. Among the 15 infants, who were positive in FCA and negative in CLIA, the value of IgM antibody

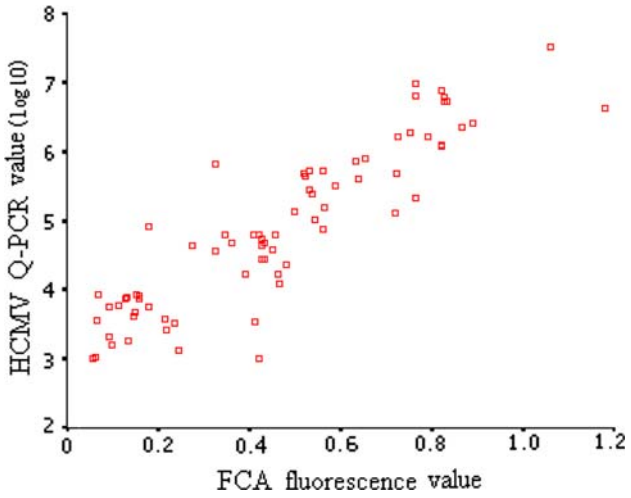

Fig. 1 Correlation between fluorescence value of pp65 antigen in FCA and LOG value of HCMV DNA copies in 77 HCMV-infected infants. Q-PCR: LOG value of HCMV DNA copies. FCA fluorescence: fluorescence value of pp65 antigen

in 13 samples was in borderline range (15–30 AU/ml) and that of 2 infants was below the borderline range in CLIA test.

Infants containing detectable pp65 antigen had significantly higher levels of HCMV-IgM antibody (median of Arbitrary Units/milliliter 46.6 AU/ml; range 8.0–98.5) than those of pp65-negative infants (median 18.3 AU/ml; range 8.0–34.2) ($p < 0.0001$). The overall amounts of pp65-containing cells were statistically correlated to HCMV-IgM levels of infected infants ($p < 0.0001$) (Fig. 2).

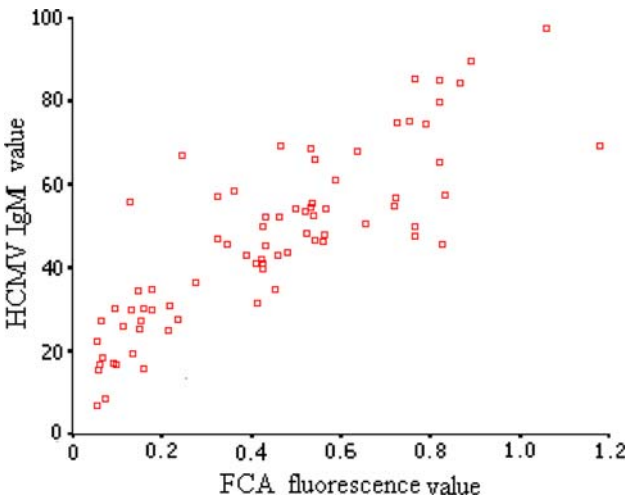

Fig. 2 Correlation between the fluorescence value of PP65 antigen in FCA and the Arbitrary Units of HCMV-IgM in CLIA in 77 HCMV-infected infants. HCMV-IgM value: AU/mL (Arbitrary Units/milliliter). FCA fluorescence: fluorescence value of PP65 antigen. The AU value of IgM negative results were calculated based on the median of 8 AU/ml

Sensitivity, specificity, PPV and NPV of different tests for diagnosis of HCMV symptomatic infection

Evaluation of the value of different tests for diagnosis of HCMV symptomatic infection showed that the sensitivity of HCMV pp65 antigen detection was 90.6%, and that of HCMV DNA quantification and HCMV-IgM detection was 100 and 72.9%, respectively. The specificity of HCMV pp65 detection, HCMV-IgM detection was 100%, and that of HCMV DNA quantification was 48.9% only. The positive prognostic value (PPV) of HCMV pp65 antigen detection, HCMV DNA quantification and HCMV-IgM detection was 100, 80.2 and 100%, respectively. The negative prognostic value (NPV) of HCMV pp65 antigen detection, HCMV DNA quantification and HCMV-IgM detection was 83.7, 100 and 64.1%, respectively (Table 1).

Correlation between different tests and diagnosis of HCMV symptomatic infection

HCMV pp65 antigen was positive in 72 of the 80 symptomatically infected infants and in all of the 5 infants who were demonstrated as symptomatic infection by following up, but in none of the 41 asymptomatically infected infants ($p < 0.001$). Among the eight symptomatically infected infants, who were negative in FCA test, five were demonstrated as pp65 antigen positive by FCA 7–10 days later. The coincidence of HCMV pp65 detection with symptoms of infants was 93.7% (118/126). While, those of HCMV DNA quantification and IgM antibody detection were 83.3% (105/126) and 81.8% (103/126), respectively (Table 1).

Moreover, symptomatic-infected infants had more amounts of pp65-containing cells (median 0.624, range 0.046–1.180) than that of asymptomatic patients (median 0.039, range 0.026–0.048; $p = 0.029$). Similarly, median of HCMV DNA levels did differ significantly between symptomatic (72,000 copies/ml, LOG value is 4.73) and asymptomatic patients (4,370 copies/ml, LOG value is 3.32). Also, median of HCMV-IgM levels between symptomatic (40.6 AU/ml) and asymptomatic patients (14.2 AU/ml) were significantly different (Table 1).

Discussion

HCMV infections remain an important cause of morbidity and mortality in infants. Virological surveillance with highly sensitive techniques is required for the early identification and pre-emptive treatment of the infection before the development of HCMV disease [13, 14]. Therefore, at neonatal period, it is essential to use appropriate and sensitive tests for diagnosis of HCMV infection. HCMV pp65 antigen

Table 1 Diagnostic value of different assays for HCMV symptomatic or asymptomatic infection in 126 infants

Specimen	Assay	Result	No. of infants		Coincidence with symptom (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
			Symptomatic[a]	Asymptomatic					
Blood	Antigenemia	Pos	77	0					
		Neg	8	41	93.7 (118/126)	90.6 (77/85)	100 (41/41)	100 (77/77)	83.7 (41/49)
	Median[b]		0.624	0.039					
	Range		0.046–1.180	0.026–0.048					
Urine	DNA	Pos	85	21					
		Neg	0	20	83.3 (105/126)	100 (85/85)	48.9 (20/41)	80.2 (85/106)	100 (20/20)
	Median[c]		4.73	3.32					
	Range		3.12–7.50	3.00–4.37					
Sera	IgM antibody	Pos	62	0					
		Neg	23	41	81.8 (103/126)	72.9 (62/85)	100 (41/41)	100 (62/62)	64.1 (41/64)
	Median[d]		40.6	14.2					
	Range		8.0–98.50	8.0–25.2					

PPV positive prognostic value, *NPV* negative prognostic value, *Pos* positive, *Neg* negative

[a] Symptomatic infants include the five infants who were demonstrated as symptomatic infection by following up

[b] FCA values were obtained by percentage gating and calculating on the population of interest

[c] Log value of HCMV DNA copies

[d] AU value: arbitrary units (AU)/ml. The AU value of negative results were calculated based on the median of 8 AU/ml

in blood cells is a marker of active HCMV infection [15]. Flow cytometry is an advanced technique, which can be used to analyze a large number of specifically labeled cells in a quantitative manner. To evaluate the possible use of FCA for rapid and quantitative detection of HCMV-specific pp65 antigens in peripheral blood cells and for distinguishing symptomatic or asymptomatic infection in HCMV-infected infants, we compared the result of FCA with those of Q-PCR and CLIA.

Q-PCR is a sensitive and specific method for detection of virus infections. Several studies have addressed that Q-PCR for HCMV DNA quantification could be used for monitoring HCMV replication conveniently, which is less influenced by the quality of sample and is easy for standardization and automation [16–18]. Detectable HCMV DNA in urine samples indicates HCMV exist in body. HCMV pp65 antigenemia is known to be clinically useful for the diagnosis of HCMV infection in neonate and immunocompromised patients [19, 20]. Traditional method for detection of HCMV pp65 antigenemia is to count the numbers of peripheral blood cells labeled by fluorescence-conjugated pp65 antibody under microscopy. However, the examination is subjective and time-consuming [5]. Flow cytometry is a modern technique that can quantify labeled cells automatically. Our results showed that 77 (61.1%) of the 126 studied infants were positive for HCMV pp65 antigen. Infants with detectable pp65 antigen had significantly higher HCMV DNA levels than those of pp65-negative infants. A significant correlation between pp65 antigenemia and HCMV DNA levels was demonstrated using a non-parametric test, despite the wide dispersion of HCMV DNA levels observed. Our results were in agreement with that of previous studies [20–22].

Serological method has been widely used for diagnosis of virus infection, due to its easy performance and available commercial reagent. HCMV-specific IgM antibody is a reliable marker for indicating recent infection. Presence of HCMV-IgM in serum has been demonstrated in infants with disseminated HCMV infection and/or HCMV disease. In our study, we compared the diagnostic value of pp65 antigen detection by FCA to that of IgM antibody detection by CLIA in infants. The result showed that detection of HCMV pp65 antigen is more sensitive (61.1%, 77/126) than detection of IgM antibody by CLIA (49.2%, 62/126). In addition, infants containing detectable pp65 antigen had significantly higher levels of HCMV-IgM antibody than those of pp65-negative infants. From a diagnostic standpoint, quantitative determination of serum IgM is a less sensitive parameter than pp65 antigenemia. The results indicated that negative result of HCMV-IgM antibody in infants cannot elucidate infection, due to the fact that some infants may show no antibody response following HCMV infection. Distinguishing symptomatic and asymptomatic HCMV infection by laboratory method is more important for clinical treatment. In our study, quantification of HCMV DNA by Q-PCR showed the highest sensitivity and the lowest specificity among the methods studied. The coincidence of HCMV pp65 detection, HCMV DNA quantification and IgM antibody detection with symptoms of infected infants was 93.7, 83.3 and 81.8%, respectively. The data

showed that all of the three methods studied were useful to distinguish HCMV symptomatic and asymptomatic infection. Compared with the symptoms of infected infants, the sensitivity, specificity, positive and negative prognosis value of pp65 antigen detection for diagnosis were 90, 100, 100 and 83.7% respectively. The coincidence of HCMV pp65 detection with symptoms of infected infants was higher than those of HCMV DNA quantification and IgM antibody detection. Although eight patients in symptomatic group were negative in detection of pp65 antigen, five of them were demonstrated as pp65 antigen positive by FCA 7–10 days later. These data showed that detection of HCMV pp65 antigen in peripheral blood cells by FCA was the best method for distinguishing symptomatic and asymptomatic HCMV infection in infants. Detection of pp65 antigen by FCA is more sensitive for diagnosis of HCMV infection than detection of HCMV-IgM antibody and is better than HCMV DNA quantification for distinguishing the symptomatic and asymptomatic HCMV infection in infants. HCMV pp65 antigen detection by FCA showed great advantage in diagnosis of HCMV active infection.

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