Construction and Evaluation of Cytomegalovirus DNA Quantification System with Real-Time Detection Polymerase Chain Reaction

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ABSTRACT

Background For patients with reactivation of human cytomegalovirus (CMV), a highly sensitive and accurate CMV quantification system is essential to monitor viral load.

Methods We constructed a real-time detection PCR (RTD-PCR) system for CMV DNA and evaluated its linearity, lower detection limit, dynamic range and accuracy using two CMV standards. We used 219 clinical samples derived from 101 patients to compare the system with the pp65 antigen test.

Results The 95% detection limit was determined to be 556 IU/mL (95% CI, 440–797 IU/mL), and the quantification range was between 10^2 and 10^6 copies or IU/mL ($r = 0.996$, 0.999, respectively). The coefficients of variation of inter-assay reproducibility assessed in each three different runs were 2.5% at 1,000 IU/mL and 1.6% at 10,000 IU/mL. The coefficients of variation of intra-assay variability by testing the same samples three times in a single run were 1.8–3.6% and 0.4–1.9%, respectively. The concordance between antigenemia and plasma or serum CMV DNA levels was a good correlation ($r = 0.695$, $P < 0.01$).

Conclusion We constructed the RTD-PCR system which enables accurate evaluation of CMV reactivation by monitoring of viral load in immunosuppressed or immunocompromised patients.

Key words CMV antigenemia, cytomegalovirus, quantification, RTD-PCR

Cytomegalovirus (CMV) is a DNA virus that belongs to the herpes virus family, and often causes severe disease in immunosuppressed or immunocompromised patients. It is one of the most frequently occurring complications after hematopoietic stem cell transplantation and solid organ transplantation.[1, 2]

CMV establishes latency in cells of the myeloid lineage following primary infection. The CMV antigenemia test is the most widely used quantitative assay for the diagnosis and follow-up of CMV infection, which becomes positive prior to the onset of CMV disease.[3–5]

The CMV antigenemia test made it possible to start the administration of antiviral agents as the preemptive therapy when CMV antigen-positive cells were detected in a certain amount. However, the CMV antigenemia test has disadvantages such as long inspection time and complicated procedures. Various reports have been made about the usefulness of real-time PCR to quantify CMV DNA in recent years. One study comparing the usefulness of quantitative PCR system and CMV antigenemia tests for monitoring CMV reactivation showed that quantitative PCR have sensitivity equal to or more than that of CMV antigenemia.[6–9] A quantitative PCR system for CMV DNA is commercially available and so expensive that it is not covered by health insurance in Japan. The aim of this study is to construct an original quantitative PCR system for CMV DNA, and to evaluate the analytical performance.

MATERIALS AND METHODS

Standard and clinical samples

Plasmid DNA, incorporating CMV glycoprotein B (gB) region (UL55), was made as a standard, the copy number of which was calculated based on mass and base length. The CMV DNA reference panels, which are derived from AD169 strain World Health Organization (WHO) International HCMV standard, were purchased from the National Institute for Biological Standards and Control (NIBSC, Ridge, Hertfordshire, UK). This material has been assigned a concentration of 5×10^6 International Units (IU) when reconstituted in 1 mL of nuclease-free water.[10] Archived 219 serum or plasma samples remaining after CMV antigenemia test were available at Tottori University Hospital, and stored at –20 °C until the study. Viral DNA was extracted from 200 μL of serum or plasma using commercial DNA isolation reagents (Qiagen, Tokyo, Japan). This study was

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Abbreviations: CBT, cord blood transplantation; CMV, cytomegalovirus; Ct, cycle of threshold; DNA, deoxyribonucleic acid; gB, glycoprotein B; IU, international unit; pp65 antigen, polypeptide 65 antigen; RTD-PCR, real-time detection polymerase chain reaction; WHO, World Health Organization

approved by the Ethics Committee at Tottori University Faculty of Medicine (approval number 2326).

Primer sets

Primers for the CMV gB region sequence were designed (Takara Bio, Kusatsu, Japan) as follows : forward primer 5'-CCCTTGAGGTAGGGCGGTAG-3', reverse primer 5'-TGCGCGAATTCAACTCGTACA -3', and TaqMan probe (FAM) 5'-ACCTTGTCCTCCACGTACTTTAC-CCGCT-3' (TAMRA).

Protocol

The RTD-PCR reaction was carried out in a 50 μL reaction mixture containing 900 nM forward and reverse primer, 250 nM TaqMan Probe, 2 X TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA) and 5 μL template. The mixture was incubated at 50 °C for 2 minutes for initial activation of uracil-N-glycosylase, followed by inactivation of uracil-N-glycosylase at 95 °C for 5 minutes. Subsequent PCR amplification consisted of 60 cycles of denaturation at 95 °C at 15 seconds and annealing and extension at 60 °C for 1 minute in an ABI7300 Real-time PCR System (Applied Biosystems).

Evaluation of the RTD-PCR assay

Conversion of CMV DNA international units (IU) to copies/mL was evaluated by serial dilution of NIBSC standards from 10^6 to 10^2 IU/mL, with triplicates at every dilution. Linearity of quantification by RTD-PCR system was evaluated as the log recovery for each member of quality control panel. The lower detection limit was determined by 10-fold serial dilutions from 10^3 to 10^1 IU/mL in 16 replicates.

CMV antigenemia test

The CMV antigenemia test is used for detecting viral matrix phosphoprotein (pp65) in peripheral blood leukocytes (PBLs) by indirect immunostain assay. Briefly, EDTA-treated whole blood samples were fractionated by dextran sedimentation and lysis of erythrocytes. The granulocytes were then centrifuged to prepare a cytospin slide (15,000 granulocytes per slide). The cells were then fixed with formaldehyde, sequentially immunostained by monoclonal antibodies C10/C11 (LSI CMV; LSI Medience, Tokyo, Japan), and reacted with goat alkaline phosphatase-labeled anti-mouse immunoglobulin as the second reaction. The examination was performed in duplicate. Measurement of the CMV antigenemia test was performed by LSI Medience.

Statistical analysis

For correlation analysis of obtained data, antigenemia results were reported as $\log_{10}$ pp65 positive cells/30,000 examined and CMV DNA titers were also reported as $\log_{10}$ IU/mL. The differences between titers obtained by both quantitative tests were considered statistically significant for P-value < 0.05, according to Mann-Whitney U test (SPSS statics 21; IBM, Armonk, NY).

RESULTS

Standards calibration and linearity

For the calibration of standards, two standard panels (pCMV gB, NIBSC) were cross-validated in the same reaction. Standard curves for CMV quantification were established for each panel by serially diluting the standards from 10^6 to 10^2 copies/mL (IU/mL for NIBSC standard) and by amplification in triplicates. We generated a log-linear regression plot by plotting the cycle of threshold (Ct) value. The correlation coefficients of the two different standards were 0.996 and 0.999, respectively (Fig. 1). The two standards exactly corresponded to each other; the conversion factor was 1.02–1.07 copies/IU for each concentration (3–7 log copies or IU/mL).

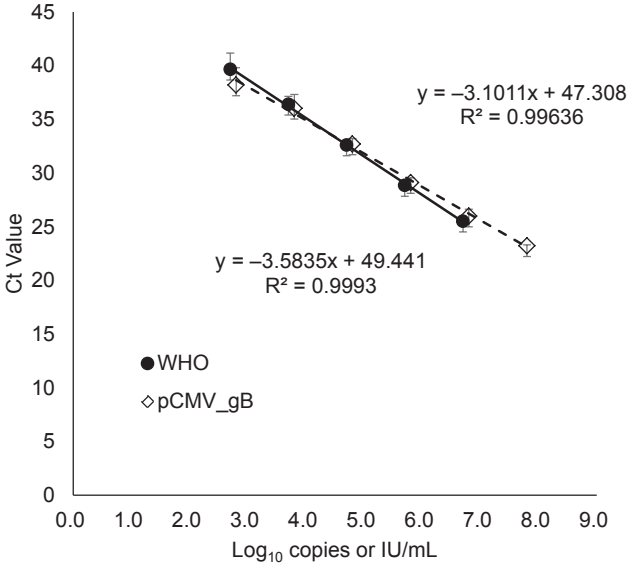

Fig. 1. Standard curves generated by linear regression analysis of the Ct value measured for each amplification versus the $\log_{10}$ virus copy number for each standard dilution. ●, WHO standard panel ranging from 10^6 IU/mL ; ◇, plasmid pCMV_gB with original 10^6 copies/mL. Ct, cycle of threshold; CMV, cytomegalovirus; gB, glycoprotein B; IU, international unit; WHO, World Health Organization; y, Ct value; R^2, coefficient of determination.

Low detection limit

Further validation of the lower limit of detection of RTD-PCR system was obtained by testing multiple replicates of dilutions of the standard. Dilutions (equal to

1000, 500, 250, 125, 63, 31 IU/mL) were tested for 16 replicates at each dilution. Using Probit analysis (IBM SPSS 21), the number of positive results at each dilution was used to calculate the CMV DNA concentration having a 95% probability of detection (Table 1). The 95% detection limit was determined to be 556 IU/mL (95% CI, 440–797 IU/mL).

Table 1. RTD-PCR assay data used for probit analysis

CMV concentration (IU/mL)	Number positive/tested	Number expected positive	Probability
1000	16/16	15.999	1.000
500	14/16	14.583	0.911
250	9/16	8.248	0.516
125	5/16	4.301	0.269
62.5	4/16	2.761	0.173
31.3	2/16	2.143	0.134
0.0	0/16	1.628	0.102

CMV, cytomegalovirus; IU, international unit; RTD-PCR, real-time detection polymerase chain reaction.

Specificity

Twelve normal human sera were tested by both assays and all gave negative results.

Reproducibility study

Inter-assay reproducibility was assessed by repeating 2 samples of different concentrations, 1,000 IU/mL and 10,000 IU/mL, in each of three different runs of RTD-PCR. The coefficients of variations were 2.5% at 1,000 IU/mL and 1.6% at 10,000 IU/mL. Intra-assay variability was evaluated by testing the same samples three times in a single run. The coefficients of variation were 1.8–3.6% and 0.4–1.9% for the low and high concentrations, respectively.

Comparison with CMV antigenemia test

All 219 clinical samples derived from 101 patients were analyzed both by the pp65 antigenemia test and the RTD-PCR system. Results of 90 were negative and 75 were positive with both tests. On the contrary, 42 samples (19.1%) that tested negative by pp65 antigenemia were positive by PCR and showed moderate levels of CMV DNA (mean viral load 2.9 Log copies/mL) (Table 2). Twelve samples were positive by the antigenemia test and negative by RTD-PCR.

In the case of PCR-positive samples more than or equal to 556 (2.7 $\log_{10}$) IU/mL, the CMV DNA levels of 62 pp65-positive samples and 32 pp65-negative samples were compared. The CMV DNA were significantly higher in pp65 positive samples than in negative samples (Fig. 2A). In cases of samples with concordant positive results, the correlation between the number of CMV DNA log copies/mL of plasma or serum and the number of pp65 positive cells per 30,000 PBLs was statistically significant (r = 0.695, P < 0.01) (Fig. 2B). None of the patients developed CMV-related symptoms.

Table 2. Comparison between CMV DNA and pp65 antigen results in the examined samples

	pp65 –	pp65 +	Total
CMV DNA –	90	12	102
CMV DNA + (< 2.7 Log_{10} IU/mL)	10	13	23
CMV DNA + (≥ 2.7 Log_{10} IU/mL)	32	62	94

CMV, cytomegalovirus; IU, international unit ; pp65 antigen, polypeptide 65 antigen ; RTD-PCR, real-time detection polymerase chain reaction.

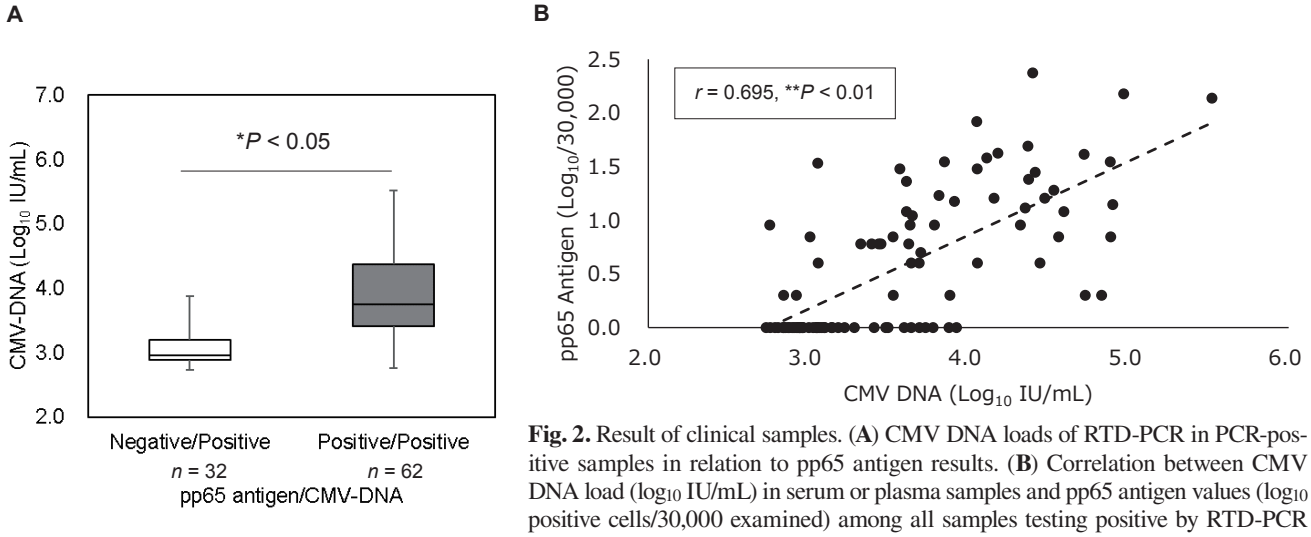

Fig. 2. Result of clinical samples. (**A**) CMV DNA loads of RTD-PCR in PCR-positive samples in relation to pp65 antigen results. (**B**) Correlation between CMV DNA load ($\log_{10}$ IU/mL) in serum or plasma samples and pp65 antigen values ($\log_{10}$ positive cells/30,000 examined) among all samples testing positive by RTD-PCR assay. CMV, cytomegalovirus; IU, international; unit; pp65 antigen, polypeptide 65 antigen; RTD-PCR, real-time detection polymerase chain reaction.

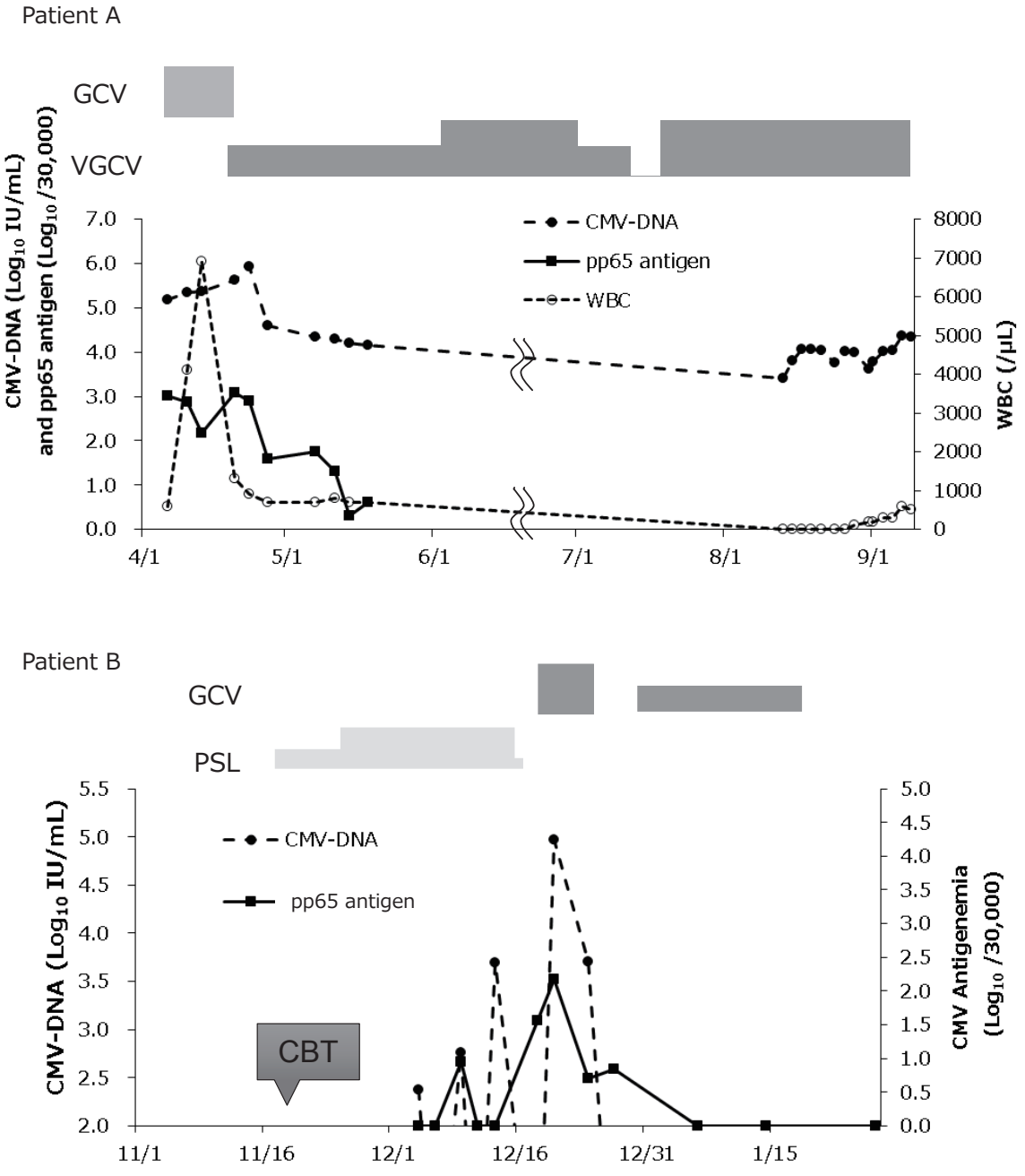

Fig. 3. Patterns of pp65 antigen (■) and CMV DNA (●) in two patients. Patient A with diffuse large B-cell lymphoma developed bone marrow suppression. It became impossible to undergo the pp65 antigenemia test because of WBC (○) decreased in number. Patient B underwent CBT. The period and dose of preemptive and steroid therapies are shown. CBT, cord blood transplantation; CMV, cytomegalovirus; GCV, ganciclovir; IU, international unit; pp65 antigen, polypeptide 65 antigen; PSL, prednisolone; VGCV, valganciclovir; WBC, white blood cell.

Case study

We followed two immunosuppressed patients who had both tests (Fig. 3). Patient A with diffuse large B-cell lymphoma developed bone marrow suppression. By anti-viral therapy, pp65 antigen decreased immediately, while CMV DNA also decreased, however, remaining at high levels. After a while, it became impossible to perform the pp65 antigenemia test because of leukopenia. Patient B underwent cord blood transplantation (CBT). After CBT, both tests showed similar transient positive results.

DISCUSSION

The amount of CMV DNA in blood is a result of the dynamic state between viral replication and clearance. Real-time PCR is a promising diagnostic approach for CMV infection after hematopoietic stem cell transplantation or during chemotherapy since it is more suitable for standardization and easier to handle than the conventional antigenemia assay. Furthermore, PCR system using plasma or serum can be used during leukocytopenia while the antigenemia assay does not produce reliable data without sufficient leukocytes.

Currently, after the introduction of pre-emptive therapy with ganciclovir by monitoring with the CMV antigenemia test, CMV disease including CMV-related pneumonia is nearly preventable.[11, 12] We developed a novel CMV DNA quantification system using an RTD-PCR system targeting the gB gene. The coefficient of correlation between DNA quantity and Ct value was $r > 0.99$, indicating good linearity. And good reproducibility and a wide dynamic range (10^2–10^6 IU/mL) were shown. The lower limit of quantification (556 IU/mL) is inferior to that of the commercial PCR kit (150 copies/mL, Roche Diagnostics, Basel, Switzerland). There are two methods of RTD-PCR that amplify CMV US17 region (US17-PCR) and immediate early (IE) gene (IE-PCR). Different sensitivity and threshold to start ganciclovir were reported for each method.[13] And the thresholds of the viral load to initiate preemptive therapy were also different according to clinical situations. For example, in the study of solid organ transplant recipients evaluating RTD-PCR of plasma and pp65 antigenemia, an antigenemia threshold value of 20 positive cells/20,000, was equivalent to 2500 copies/mL (2275 IU/mL) for antiviral treatment.[14] In case of hematopoietic stem cell transplant recipients from unrelated donors, a much lower threshold of CMV DNA titer was used for preemptive therapy.[15]

The present study shows a good correlation between antigenemia and plasma or serum CMV DNA levels ($r = 0.695$). The novel assay also showed more usefulness in monitoring the CMV reactivation of patients with leukopenia or myelosuppression than antigenemia test (patient A in Fig. 3). Both antigenemia and CMV DNA load deceased after the initiation of therapy. In addition, antigenemia assay and RTD-PCR system showed a similar trend (patient B in Fig. 3). These results indicate that using the RTD-PCR system to monitor quantities of CMV DNA could be useful in ganciclovir treatment.

Twelve samples were pp65 positive and CMV DNA negative while 42 samples were reverse. The targets of the two methods are different; RTD-PCR amplifies free CMV DNA in plasma and the CMV antigenemia test detects pp65 antigens in peripheral blood leukocytes. Which target is appropriate for monitoring CMV infection for clinical use remains to be determined. Their combination may explain virus status more accurately.

In conclusion, we constructed a RTD-PCR system which has sufficient sensitivity, specificity, accuracy, and reproducibility. Quantification can be performed within three hours without false-positive results. Based on our results, the RTD-PCR system appears to be as useful as any commercial CMV quantification system. Furthermore, it may be useful for patients to consider comprehensively the results of RTD-PCR and CMV antigenemia test.

The authors declare no conflict of interest.

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