Quantitative assessment of viral load in the blood and urine of patients with congenital cytomegalovirus infection using droplet digital PCR

Makoto Yamaguchi M.D., Jun-ichi Kawada, M.D., Ph.D.*, Yuka Torii M.D., Ph.D., Kazunori Haruta M.D., Takako Suzuki M.D., Ph.D., Kazuhiro Horiba M.D., Ph.D., Yoshiyuki Takahashi M.D., Ph.D., and Yoshinori Ito M.D., Ph.D.

Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

***Corresponding Author:** Jun-ichi Kawada, M.D., Ph.D.
Department of Pediatrics, Nagoya University Graduate School of Medicine
65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan
Tel: +81-52-744-2294; Fax: +81-52-744-2974
E-mail: kawadaj@med.nagoya-u.ac.jp

Abstract

Congenital cytomegalovirus infection (cCMV) is a common cause of congenital infections, leading to neurodevelopmental sequelae. Real-time quantitative polymerase chain reaction (qPCR) has been widely used for the diagnosis and assessment of cCMV; however, the correlation between CMV DNA load and the severity of cCMV symptoms has been inconclusive. Droplet digital PCR (ddPCR) offers an improvement over the current qPCR methods through the absolute quantification of viral loads. We compared ddPCR and qPCR results for the quantification of CMV DNA in blood and urine specimens from 39 neonates with cCMV (21 symptomatic and 18 asymptomatic). There was no significant difference in blood CMV DNA loads measured by ddPCR and qPCR, with or without any clinical findings. However, developmental delays at 36 months were significantly more frequently observed in patients with high CMV DNA loads (≥2,950 copies/mL), as measured by ddPCR at diagnosis, than in those with lower CMV DNA loads. The association of urine CMV DNA load with symptoms and developmental delay was not observed. CMV DNA loads in the blood might be used as a predictor of developmental outcomes in cCMV patients, and absolute quantitation of viral loads by ddPCR assay could contribute to the standardization of CMV load measurement.

Keywords: Congenital cytomegalovirus infection, Droplet digital PCR, real-time quantitative PCR, neurological sequelae, developmental delay

Background

Cytomegalovirus (CMV) is one of the most common causes of congenital viral infection affecting infants, with a prevalence of 0.7% worldwide.[1] Congenital CMV infection (cCMV) is the most frequent viral cause of neurodevelopmental sequelae.[2] Most children with cCMV (85%–90%) do not have any clinical findings at birth (asymptomatic infection), while the remaining 10%–15% have clinical findings such as sensorineural hearing loss (SNHL), microcephaly, hepatosplenomegaly, and chorioretinitis (symptomatic infection).[3,4] Antiviral treatment with ganciclovir or valganciclovir has been recommended in symptomatic cCMV.[5]

Diagnosis of cCMV has been performed by viral culture or polymerase chain reaction (PCR) of CMV from saliva or urine samples collected within 3 weeks of birth.[1] Both saliva and urine samples of cCMV contain a high viral load, and postnatal diagnosis of cCMV is preferably performed via real-time quantitative PCR (qPCR). Some neonates with cCMV show high CMV DNA loads in the blood and/or urine; however, the correlation between CMV DNA load and the severity or presence of cCMV symptoms has been inconclusive.[2,6-10]

qPCR has been widely used for CMV diagnosis. However, CMV qPCR has some intrinsic limitations in terms of accuracy, standardization, and precision.[11] The use of the

international standard material provided by the World Health Organization (WHO) has addressed the interlaboratory standardization issue; however, the precision issue remained. Droplet digital PCR (ddPCR) is a high-confidence method for measuring the original target concentration. Therefore, ddPCR demonstrated higher precision than conventional qPCR with higher or equivalent sensitivity.[12] In addition, ddPCR can provide absolute quantitation of viral loads, and recent reports have shown the utility of ddPCR in the diagnosis or disease monitoring of infectious diseases.[12-14] In previous studies, ddPCR for CMV exhibited increased precision compared to qPCR and equivalent sensitivity using clinical samples.[11,15] However, the utility of ddPCR in cCMV patients has not been investigated. In this study, we compared the clinical utility of ddPCR and qPCR in patients with cCMV.

Materials and Methods

This study retrospectively enrolled 39 consecutive patients with cCMV diagnosed at the Nagoya University Hospital between January 2012 and December 2019. All patients were diagnosed with cCMV by qPCR of their blood or urine within 21 days of age. Patients with cCMV were identified by targeted CMV screening for newborns who had clinical or laboratory signs consistent with cCMV or serological screening in pregnant women.[16] Clinical findings, laboratory findings, hearing by auditory brainstem response, and neurological abnormalities by ultrasonography and/or magnetic resonance imaging were investigated in all patients. Symptomatic infection was defined as the presence of one or more of the following: thrombocytopenia, petechiae, hepatomegaly, splenomegaly, intrauterine growth restriction, hepatitis (as denoted by elevated transaminase and/or bilirubin levels), central nervous system involvement (as denoted by one or more of microcephaly, neuroimaging abnormalities indicative of cCMV), chorioretinitis, and SNHL. Neurological assessment and evaluation with developmental quotient (DQ) were performed at 18 and 36 months by skilled pediatricians, and DQ < 80 was defined as a neurodevelopmental delay.[17]

Whole blood (200 μL) and urine (140 μL) samples were obtained from each patient within 3 weeks of birth. DNA was extracted from whole blood and urine samples

using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) and QIAamp® Viral RNA Mini Kit (Qiagen), respectively. The specimens were eluted in 50 μL of nuclease-free water and stored at -80 ℃.

The WHO international standard material for human CMV containing a total of 5×10^6 international units (IU) was purchased from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, United Kingdom). The WHO standard was diluted with whole blood obtained from a healthy adult volunteer, and serial 10-fold dilutions were made to obtain a concentration from 5×10^2 IU/mL to 5×10^5 IU/mL. DNA was extracted from each concentration and stored at -80 ℃.

qPCR targeting the CMV UL123 gene was performed using the QuantStudio™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), as described previously.[18] A five-point standard curve and a positive and negative control were included in all runs. CMV DNA loads were standardized using the WHO standard material for CMV and are represented as IU/mL.

ddPCR for CMV detection was performed using the QX200 droplet digital PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The primer-probe set was the same as that used for the qPCR assay described above. The ddPCR reaction mixture consisted of 11 μL ddPCR Supermix for Probes (Bio-Rad), 5 U Alu I (Takara Bio Inc.,

Japan), 50 μM CMV primer, 10 μM CMV probe (VIC), and 2.5 μL purified nucleic acid in a final volume of 22.5 μL. The restriction enzyme (Alu I) was used to improve the accuracy of ddPCR by separating tandem gene copies, reducing sample viscosity, and template accessibility. The reaction mixture was loaded into an automated droplet generator (Bio-Rad). PCR amplification was performed using a C100 Touch Thermal Cycler (Bio-Rad) with a thermal profile beginning at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 55 °C for 60 s, 1 cycle at 98 °C for 10 min, and ending at 12 °C. After amplification, the plate was loaded onto a QX200 Droplet Reader (Bio-Rad), and the droplets from each well of the plate were read automatically at 32 wells/h. ddPCR data were analyzed using QuantaSoft analysis software (Bio-Rad), and quantification of the target molecule was presented as the number of copies per PCR reaction.

The association between CMV DNA load and symptoms or developmental delay was statistically assessed using the Mann-Whitney *U* test. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was used to analyze the cutoff value for the CMV DNA load to predict developmental delay at 18 months and 36 months. The cutoff values were considered adequate at an AUC $\geq$ 0.70. Then, Youden's index was used to identify the most appropriate cutoff value for the best relationship between the sensitivity and specificity. All statistical analyses were performed using SPSS version 27

(IBM Corp., Armonk, NY, USA). Statistical significance was set at $p < 0.05$.

Results

1. Evaluation of ddPCR and qPCR using the WHO standard.

First, the sensitivity, quantity, and reproducibility of CMV DNA loads were compared between ddPCR and qPCR by measuring the WHO standard material diluted in whole blood. The sensitivity of ddPCR and qPCR was 50 IU/reaction (Supplemental Table 1). Reproducibility was evaluated using the coefficient of variance. The coefficient of variance in ddPCR was smaller than that in the qPCR assay both in the intra-run (intra-assay) and between-run (inter-assay) assays, indicating the high reproducibility of ddPCR. Furthermore, the correlation coefficient of ddPCR was slightly higher than that of qPCR (r^2=0.998 vs. 0.969) (Supplemental Fig. 1).

2. Comparison of ddPCR to qPCR for quantitative detection of CMV DNA in cCMV patients.

CMV DNA loads in whole blood and urine of cCMV patients were evaluated using ddPCR and qPCR. The patient characteristics are shown in Table 1. Among 21 symptomatic patients, SNHL was observed in 13, and abnormal brain imaging was identified in 14. The gestational age and birth weight were also significantly lower in symptomatic patients than in asymptomatic patients. In addition, the DQ was evaluated in 30 and 27 cCMV patients at 18 and 36 months, respectively. Among them,

developmental delay (DQ < 80) at 18 months was observed in 6 and 3 patients with symptomatic and asymptomatic cCMV, respectively. Moreover, developmental delay at 36 months was observed in 7 symptomatic patients and 1 asymptomatic patient with cCMV.

A comparison of CMV DNA loads in whole blood is shown in Figure 1. There was no significant difference in CMV DNA loads measured by ddPCR and qPCR, with or without any symptoms at birth or in abnormal brain imaging. CMV DNA loads in patients with and without developmental delay at 18 months of age were not significantly different. In contrast, CMV DNA loads measured by ddPCR tend to be higher in cCMV patients with developmental delay than in patients with normal development at 36 months of age; however, the statistical significance was marginal (median: 3000 copies/mL vs. 620 copies/mL, $p = 0.059$). No association was observed between urine CMV DNA loads and symptoms, abnormal brain imaging, or developmental delay (Fig. 2).

We examined the cutoff value of CMV DNA loads for predicting developmental delay in whole blood, as measured by ddPCR. The cut-off value of CMV DNA load (2,950 copies/mL) was calculated using the Youden's index of the ROC curve (Fig. 3B). In addition, cCMV patients with CMV DNA loads ≥2,950 copies/mL at diagnosis were more likely to show developmental delay at 36 months than those with lower CMV DNA

loads (4/7 vs. 3/19, p = 0.034) (Fig. 3C). Because most CMV DNA in whole blood are considered cell-associated, each CMV DNA load was converted to CMV DNA load per 10^6 leukocytes, and the cut-off value was recalculated. Similarly, cCMV patients with CMV DNA loads ≥ 270 copies/10^6 leukocytes were more likely to show developmental delays at 36 months (5/8 vs. 2/18, p = 0.014) (Supplemental Fig. 2).

Discussion

We developed ddPCR for CMV DNA measurement and compared the clinical utility of ddPCR and qPCR in patients with cCMV. ddPCR showed comparable sensitivity, better quantifiability, and reproducibility than qPCR. Generally, ddPCR shows better reproducibility and quantifiability than qPCR, especially in measuring low viral loads.[19] Therefore, ddPCR can be applied as a reliable tool for diagnosis, viral load monitoring, and defining therapeutic endpoints. In immunocompromised hosts, viral loads of latently infected viruses, such as CMV, Epstein-Barr virus, and adenovirus, have been used for pre-emptive diagnosis and treatment.[20] However, qPCR for these viruses has not been standardized. The absolute quantitation of viral loads by ddPCR could be applied to standardize viral load measurements and determine cutoff values for diagnosis and treatment initiation.

In this study, CMV load in the whole blood measured by ddPCR during the neonatal period was marginally significantly higher in cCMV neonates with developmental delay at 36 months of age. Previous reports have shown that patients with high CMV DNA loads are likely to have SNHL in the neonatal or later period.[6,9] However, an association between CMV DNA loads and neurological sequelae, including developmental delay, has not been fully investigated.[2,7,8] Lanari et al. showed that CMV

DNA load in the neonatal period with >100 copies/10^5 polymorphonuclear leukocytes was associated with neurological sequelae at 12 months.[7] Moreover, Forner et al. have analyzed the relationship between the CMV load in whole blood at birth and the development of late-onset neurological sequelae in asymptomatic cCMV. They found that the risk of neurological sequelae increased dramatically to a range of 3,000–30,000 copies/mL.[8] However, SNHL was included in neurological sequelae in these studies, and most patients did not have neurological sequelae other than SNHL. Although another report evaluated the association between CMV load in the neonatal period and developmental delay, no difference was observed.[2] Therefore, the association between CMV load measured in the neonatal period and developmental delay has been inconclusive.

Antiviral treatment is considered for symptomatic patients with cCMV. However, it is sometimes challenging to differentiate symptomatic cCMV patients from asymptomatic ones during the neonatal period. Moreover, some patients who were asymptomatic at birth develop late-onset neurodevelopmental sequelae. CMV DNA loads in the blood might be useful to evaluate the disease severity of cCMV; however, high CMV DNA loads are sometimes observed in asymptomatic patients. Therefore, the clinical significance of CMV DNA load in cCMV has not yet been established. Our results

suggest that cCMV patients with high CMV DNA loads (≥2,950 copies/mL), as measured by ddPCR, tended to show developmental delay. The recent study including 120 cCMV patients has shown that the median CMV DNA load in patients with neurological involvement was 6600 copies/mL. Therefore, a high CMV load of ≥ 2,950 copies/mL obtained using ddPCR would be a reasonable consideration in this study.[2] Detailed neurological evaluation and careful follow-up may be required in both symptomatic and asymptomatic cCMV patients with high CMV DNA loads. Absolute quantitation of viral loads by ddPCR assay could contribute to the standardization of CMV load measurement in cCMV patients.

This study has some limitations. First, this study was retrospective, and only a small number of patients with cCMV were evaluated. Second, universal cCMV screening was not performed in this study. Most symptomatic patients were identified by hearing screening or neurological abnormalities on ultrasonography, which might have resulted in a high prevalence of developmental delay among symptomatic patients. Moreover, most symptomatic cCMV patients in this study did not have life-threatening manifestations, such as sepsis-like illness and severe end-organ involvement, which might be attributed to the lack of significant differences in CMV DNA loads between symptomatic and asymptomatic patients.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Authors' Contribution Statement

MY, JK, KHo, and YI conceived and designed the experiments. MY performed the experiments and analyzed the data. JK, TY, KHa, TS, and YI collected the clinical samples and data. MY and JK wrote the manuscript. YT and YI supervised the study. All authors have read and approved the final manuscript.

Funding

This research is supported by the "Project for Baby and Infant in Research of Health and Development to Adolescent and Young Adult (BIRTHDAY)" of the Japan Agency for Medical Research and Development (AMED) (JP19gk0110037 to YI) and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (21K07748 to JK).

Conflict of Interest

We declare no conflicts of interest.

References

1. Suresh B, Mdsarmdmsphmsmd B, April L. Palmer MDAAMDMGMMDPJS, M.D DIBMDRWTJMDZNMDN, et al. Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns. *N Engl J Med* 2011;22:2111-2118.
2. Marsico C, Aban I, Kuo H, et al. Blood viral load in symptomatic congenital cytomegalovirus infection. *J Infect Dis* 2019;219(9):1398-1406.
3. Boppana SB, Ross SA, Fowler KB. Congenital cytomegalovirus infection: clinical outcome. *Clin Infect Dis* 2013;57;Suppl 4 (Suppl:S178-S181):S178-S181.
4. Dollard SC, Grosse SD, Ross DS. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Rev Med Virol* 2007;17(5):355-363.
5. Kimberlin DW, Jester PM, Sánchez PJ, et al. Valganciclovir for symptomatic congenital cytomegalovirus disease. *N Engl J Med* 2015;372(10):933-943.
6. Walter S, Atkinson C, Sharland M, et al. Congenital cytomegalovirus: association between dried blood spot viral load and hearing loss. *Arch Dis Child Fetal Neonatal Ed* 2008;93(4):F280-F285.
7. Lanari M, Lazzarotto T, Venturi V, et al. Neonatal cytomegalovirus blood load and risk of sequelae in symptomatic and asymptomatic congenitally infected newborns. *Pediatrics* 2006;117(1):e76-e83.
8. Forner G, Abate D, Mengoli C, Palù G, Gussetti N. High cytomegalovirus (CMV) DNAemia predicts CMV sequelae in asymptomatic congenitally infected newborns born to women with primary infection during pregnancy. *J Infect Dis* 2015;212(1):67-71.
9. Bradford RD, Cloud G, Lakeman AD, et al. Detection of cytomegalovirus (CMV) DNA by polymerase chain reaction is associated with hearing loss in newborns with symptomatic congenital CMV infection involving the central nervous system. *J Infect Dis* 2005;191(2):227-233.
10. Smiljkovic M, Le Meur JB, Malette B, et al. Blood viral load in the diagnostic workup of congenital cytomegalovirus infection. *J Clin Virol* 2020;122:104231-104231.
11. Sedlak RH, Cook L, Cheng A, Magaret A, Jerome KR. Clinical utility of droplet digital PCR for human cytomegalovirus. *J Clin Microbiol* 2014;52(8):2844-2848.
12. Luo J, Luo M, Li J, et al. Rapid direct drug susceptibility testing of Mycobacterium tuberculosis based on culture droplet digital polymerase chain reaction. *Int J Tuberc Lung Dis* 2019;23(2):219-225.
13. Wouters Y, Dalloyaux D, Christenhusz A, et al. Droplet digital polymerase chain reaction for rapid broad-spectrum detection of bloodstream infections. *Microb Biotechnol*

2020;13(3):657-668.
14. Rutsaert S, Bosman K, Trypsteen W, Nijhuis M, Vandekerckhove L. Digital PCR as a tool to measure HIV persistence. *Retrovirology* 2018;15(1):16-16.
15. Hayden RT, Gu Z, Ingersoll J, et al. Comparison of droplet digital PCR to real-time PCR for quantitative detection of cytomegalovirus. *J Clin Microbiol* 2013;51(2):540-546.
16. Torii Y, Yoshida S, Yanase Y, et al. Serological screening of immunoglobulin M and immunoglobulin G during pregnancy for predicting congenital cytomegalovirus infection. *BMC Preg Childbirth* 2019;19(1):205.
17. Yamada H, Tanimura K, Fukushima S, et al. A cohort study of the universal neonatal urine screening for congenital cytomegalovirus infection. *J Infect Chemother* 2020;26(8):790-794.
18. Kawada J, Torii Y, Kawano Y, et al. Viral load in children with congenital cytomegalovirus infection identified on newborn hearing screening. *J Clin Virol* 2015;65:41-45.
19. Yang D, Hu T, Wu X, Li K, Zhong Q, Liu W. Droplet-digital polymerase chain reaction for detection of clinical hepatitis B virus DNA samples. *J Med Virol* 2018;90(12):1868-1874.
20. Hiwarkar P, Gaspar HB, Gilmour K, et al. Impact of viral reactivations in the era of pre-emptive antiviral drug therapy following allogeneic haematopoietic SCT in paediatric recipients. Bone Marrow Transplant 2013;48(6):803-808.

Figure Legends

Figure 1

The CMV load in whole blood was measured using ddPCR (upper row) and qPCR (lower row). Whole blood CMV DNA loads were compared between cCMV patients with and without any symptoms at birth, abnormal brain imaging, and developmental delay at 18 and 36 months of age.

CMV, cytomegalovirus; ddPCR, droplet digital PCR; qPCR, quantitative PCR; cCMV, congenital CMV infection.

Figure 2

CMV load in urine was measured with ddPCR (upper row) and qPCR (lower row). Urine CMV DNA loads were compared between cCMV patients with and without any symptoms at birth, abnormal brain imaging, and developmental delay at 18 and 36 months old.

CMV, cytomegalovirus; ddPCR, droplet digital PCR; qPCR, quantitative PCR; cCMV, congenital CMV infection.

Figure 3

ROC curves derived from CMV load in whole blood as measured with ddPCR and the presence of developmental delay in congenital CMV patients at (A) 18 months and (B) 36 months old. Black dots indicate the best cutoff values. (C) Association between CMV load in whole blood as measured by ddPCR and developmental delay at 36 months old. P-value was calculated by Fisher's exact test.

ROC, receiver operating characteristic; CMV, cytomegalovirus; ddPCR, droplet digital PCR. Developmental delay was defined as a developmental quotient < 80.

Supplemental Figure 1

Regression analysis of measured values of ddPCR and qPCR against assigned CMV copy number. Each dilution was assayed in four replicate reactions.

CMV, cytomegalovirus; ddPCR, droplet digital PCR; qPCR, quantitative PCR.

Supplemental Figure 2

CMV loads in the whole blood were converted to CMV loads per 10^6 leukocytes. These ROC curves were derived from CMV loads and the presence of developmental delay in congenital CMV patients at (A) 18 months and (B) 36 months. Black dots indicate the

best cutoff values. (C) Association between CMV load converted to CMV DNA loads per 10^6 leukocytes and developmental delay at 36 months (C). P-values were calculated using the Fisher's exact test.

CMV, cytomegalovirus; ROC, receiver operating characteristic. Developmental delay was defined as a developmental quotient < 80.

Table 1

Characteristics of cCMV patients

	Symptomatic (n = 21)	Asymptomatic (n = 18)	P value
Gestational age (weeks)	38(36.0-39.0)	39.0 (38.0-40.0)	0.008
Birth weight (grams)	2,580 (2,040-2,831)	2,814 (2,580-3,122)	0.018
Hearing loss	13/21 (62%)	0/18 (0%)	<0.001
Abnormal brain image	14/21 (67%)	0/18 (0%)	<0.001
Developmental delay at aged 18 months (DQ < 80)	6/17 (35%)	3/13 (23%)	0.691
Developmental delay at aged 36 months (DQ < 80)	7/13 (54%)	1/14 (7%)	0.013

Data of gestational age and birth weight are median (IQR, interquartile range)

cCMV, congenital Cytomegalovirus infection; DQ, developmental quotient

Supplemental Table 1

Comparison of within-run (intra-assay) and between-run (inter-assay) variability for ddPCR and qPCR using the WHO standard

		ddPCR				qPCR			
	assigned copy number (IU)	**5**	**50**	**500**	**5,000**	**5**	**50**	**500**	**5,000**
intra-run (n = 4)	**Mean**	n.d	15	140	1,875	n.d	16	631	4,320
	SD	-	4	10	78	-	7	18	260
	CV	-	0.267	0.071	0.042	-	0.438	0.029	0.060
inter-run (n = 3)	**Mean**	n.d	12	156	1,821	n.d	32	601	3,783
	SD	-	3	20	39	-	20	90	533
	CV	-	0.250	0.128	0.021	-	0.625	0.149	0.141

ddPCR, Droplet DigitalPCR; qPCR, quantitative PCR; SD, standard deviation; CV, coefficient of variance (=SD/Mean); n.d, not detected