

Research Article

Comparison of HCMV Loads Using In-house Quantitative CMV PCR, Artus CMV TM PCR and COBAS Amplicor CMV Monitor Test

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Received November 23, 2009; Accepted June 10, 2010

Abstract

A real-time PCR assay based on highly conserved cytomegalovirus glycoprotein B (CMV-gB) gene was developed to quantify the CMV genome loads in human plasma obtained from immunocompromised patients. The sequence comparison from several strains resulted in the best possible set of primers and a probe that directed against the most highly conserved region. The detection limit of In-house TaqMan polymerase chain reaction assay for CMV-DNA was 2.3 log₁₀ copies/ml and the linear measure interval was 2.3 to 8.3 log₁₀ copies/ml (R > 0.99). The In-house TaqMan assay was compared to commercial quantitative PCR test, COBAS Amplicor CMV monitor test and Artus CMV TM PCR test, in the determination of CMV DNA load in plasma samples. The correlation between In-house TaqMan and COBAS assays was statistically significant, R = 0.915 (P < 0.05; n = 27). Also correlation between In-house TaqMan and Artus CMV assays was statistically significant and higher (R = 0.998; P < 0.05; n = 8). When the In-house TaqMan system was evaluated with the Nucleospin[®] RNA virus kit and QIAamp blood minikit, no significant difference was found at the 98.2% confidence interval. Preliminary results indicate that the In-house TaqMan real-time PCR with the designed primer and probe set can be an alternative tool for quantification of CMV loads in clinical routine laboratories.

Key Words: CMV; Real-time PCR; Quantitative

Introduction

Human cytomegalovirus (CMV) infection is frequently found in immunocompromised patients, such as recipients of solid organ transplant and of bone marrow transplant and patients infected with HIV. The CMV DNAemia plays a critical role in the pathogenesis of CMV disease and is considered a major risk factor for the development of CMV disease (Aitken et al.,

1999; Boeckh and Boivin, 1998). Early detection and monitoring of CMV infections are important for preventing the reactivation of endogenous CMV and for the surveillance of recipients of seropo-sitive donor transplants. Over the last decade, diagnosis of CMV infections often requires the detection of virus by cell culture, pp65 antigen detection, or nucleic acid amplification techniques. Both DNA and pp65 antigen detection

methods have largely replaced viral tube culture and shell vial culture techniques in CMV surveillance due to their improved sensitivity, predictive value and rapidity. However, the pp65 antigenemia assay is subjective in reading and requires rapid transportation of specimen to the laboratory (Boeckh et al., 1997). Qualitative PCR is not an appropriate tool in predicting the CMV disease versus latent viral infection because of its low predictive value (Delgado et al., 1992; Fernandez et al., 2002; Humar et al., 2004; Ikewaki et al., 2003). Quantification of CMV loads in the format of a plasma-based PCR has the following advantages: the assay is more sensitive and leukocyte-count independent, and materials can be stored before being processed. Viral loads appear to be important for diagnosis and prediction of CMV disease. Optimal detection of CMV DNA requires primer sequences capable of detecting all the CMV isolates. The glycoprotein B gene (gB) is one of the less variable regions of the CMV genome (Wirgart et al., 1998; Murphu et al., 2003), except for two variable subregions (Shepp et al., 1998; Chou and Dennison, 1991). At the nucleotide sequence level, while major immediate early and DNA polymerase gene sequences from 46 clinical isolates have 87.9 and 92.8% homology to those of AD169, respectively, the gB gene sequences demonstrated a higher homology, i.e. 93% (Wirgart et al., 1998).

The introduction of a commercial quantitative PCR assay, the COBAS[®] Amplicor[®] CMV monitor test (Roche Diagnostics), has provided a system that is easily used for routine clinical diagnosis. The assay is based on four major processes: specimen preparation; PCR amplification of target DNA using CMV specific complementary primers; hybridization of the amplified products to oligonucleotide probes specific for the target(s); and detection of the probe-bound amplified products by colorimetric determination. Following PCR amplification,

the COBAS Amplicor Analyzer automatically adds denaturation solution to denature the CMV amplicon to form single-stranded DNA. The biotin-labeled amplicon are hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. The amplicon hybridized to the target-specific oligonucleotide probes bound to the magnetic particles is detected by avidin-horseradish peroxidase-tetramethylbenzidine (TMB)-hydrogen peroxide colorimetric reaction. The intensity of the absorbance of the blue complex formed is measured by COBAS Amplicor Analyzer at a wavelength of 660 nm. The results were expressed as DNA copies per milliliter of plasma. The test can quantitate CMV DNA over the range from 400 to 100,000 CMV DNA copies/ml.

Recently, several analyte-specific reagents (ASRs) for CMV DNA have been produced. The Artus[®] CMV TM PCR Kit (QIAGEN, Valencia, CA) produces real-time detection by fluorescence resonance energy transfer (FRET). Its principle is based on fluorogenic probe which consists of a single oligonucleotide with a labeled reporter fluor on the 5' end and a labeled quencher on the 3' end. Signal production in this system relies on 5' exonuclease activity of Taq polymerase causing a diminution of signal quenching in parallel with amplicon production. The fluorescence intensity is monitored for real-time detection.

The In-house TaqMan assay is based on TaqMan-MGB probe. It is TaqMan probe conjugated with minor groove binder (MGB) at 3' end along with the quencher dye. MGB can improve melting temperature of probe, thus increasing probe specificity. It is possible to shorten the length of probes, which can help probe design especially in AT rich region and improve the sensitivity and specificity. This new TaqMan probe also incorporates a non-fluorescent quencher (NFQ) dye that acts as the energy transfer

acceptor from the receptor molecule that does not emit a detectable fluorescent signal of its own. The advantage is that a less sensitive signal with lower fluorescent background is emitted from the reaction. This improves spectral discrimination and makes data interpretation easier.

The usefulness of the COBAS Amplicor CMV has been shown by many groups (Caliendo et al., 2000; Piiparinen et al., 2002). However, the disadvantage of the assay is that CMV loads greater than 10^5 copies/ml are measured inaccurately and that the assay requires a specialized device that seems to be expensive for developing countries. The accurate, rapid, and cost-effective assay is required for the frequent determination of CMV loads in patients at high risk of CMV disease.

Thus, we have developed an In-house quantitative real-time PCR based on highly conserved CMV glycoprotein B (CMV-gB) gene with a TaqMan-MGB probe. We simplified the method by using plasma rather than leukocyte. We determined the agreement in viral loads among a TaqMan system and commercial assays, including COBAS CMV monitor test and Artus CMV TM PCR Kit. In addition, the efficiency of DNA extraction methods was compared between QIAamp[®] DNA Blood mini kit (QIAGEN, Valencia, CA) and Nucleospin[®] RNA virus kit (MACHEREY-NAGEL).

Materials & Methods

Clinical specimens

A total of 42 plasma specimens were kindly provided by the Molecular unit of Siriraj Hospital. All specimens from patients with suspected CMV disease are tested by the COBAS CMV monitor test. Based on the results of COBAS assay, 24 consecutive positive and 18 consecutive negative plasma samples were chosen for the comparison of the two test systems, COBAS CMV monitor test and In-house TaqMan assay.

Of these 42 plasma samples, 5 positive and 5 negative samples were determined by three methods including TaqMan assay, COBAS assay and Artus CMV assay. Additionally, six spiked specimens and four CMV-infected supernatant were included in the comparison of above three methods. The herpes simplex virus type 1 or 2 (n = 5), varicella-zoster virus (n = 5), Epstein-Barr virus (n = 5), hepatitis B virus (n = 5) were analyzed as specificity controls by using an In-house TaqMan assay.

Nested PCR

Nested PCR was performed to amplify CMV DNA. The primers used for CMV PCR corresponded to the nucleotide sequence of glycoprotein B (HCMV-gB, accession no. X04606) of strain AD169. The sequences of the primers in the first PCR were 5' AAACATAGCGGACCGTGAG 3' as a forward and 5' ACCTTCCTCAAAAACCCCT 3' as a reverse. The sequences of the primers in the second PCR were 5' GTCGGAGTCTTTCAAGTGTC 3' as a forward and 5' TTGATCTATACTCGACAGCG 3' as a reverse.

First and second PCR primers were mixed in a final volume of 50 μ l, which included 5 μ l of template DNA, 0.5 μ l of Taq DNA polymerase (3.5 U/ μ l, Roche), 1 μ l of each primer (20 μ M), 4 μ l of dNTP (2.5 mM), 5 μ l of reaction buffer and 33.5 μ l of distilled water. Each of the mixtures was subjected to 30 cycles of amplification under 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute using a DNA thermal cycler. The amplified PCR products were electrophoresed on 1.5% agarose gels and visualized with UV light as a single band of 398 bps after staining with ethidium bromide.

Nucleic acid extraction

For COBAS CMV monitor test, nucleic acid was extracted using the MagNA Pure total nucleic acid kit according to the manufacturer's recommendations. For the In-house TaqMan and the Artus CMV assays, nucleic acid was

extracted using a Nucleospin[®] RNA virus kit. Nucleic acid was extracted from 150 µl of plasma and the sample was eluted in 37.5 µl. The QIAamp[®] DNA Blood mini kit was additionally used for observing the impact of extraction methods on viral loads. A 200 µl volume of plasma was extracted and eluted in 50 µl.

In-house TaqMan assay

DNA was extracted from 150 µl of plasma, and after binding to a silica membrane, was eluted in 37.5 µl of elution buffer by use of a Nucleospin[®] RNA virus kit. For the PCR, 5 µl of DNA eluate (corresponding to 20 µl of original plasma sample) was added to a 20 µl reaction mixture.

Part of the gB gene from the CMV isolates was aligned by Clustal W (freeware, www.ebi.ac.uk/clustalw/) to verify the conservation of gene region. The target region in the CMV

AD169 genome (accession no. X17403) was glycoprotein B (gB), at nucleotide 80917 to 80986. TaqMan-MGB probe and primers were designed with Primer Express software (version 2.0, Applied Biosystems, Foster City, CA). TaqMan-MGB probe was designed between primers. The base sequences of the primers used were as follows: upstream, 5' CGCTGCTCTGCGTCCA 3'; downstream, 5' CTCGCCTTACACCAACGA 3'. An oligonucleotide probe: 5' FAM CAGGCTTACCAGATGCT NFQ 3' (Figure 1). The thermal cycling conditions were 1 cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 sec and at 60°C for 1 minute. TaqMan PCR assay was applied on ABI PRISM 7500 (Applied Biosystems, Foster City, CA). The data were collected at the annealing step (60°C) of each cycle, and threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit.

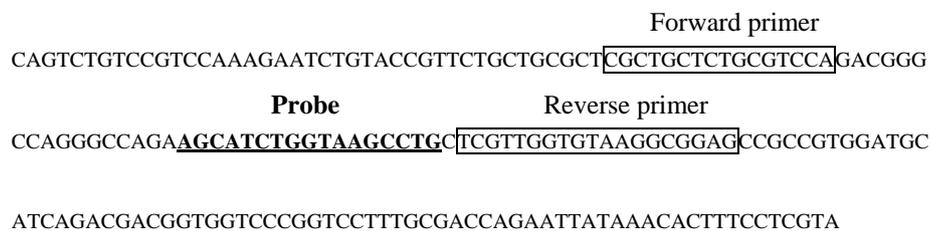


Figure 1 Schematic representation of the primers and probe

In-house TaqMan assay standard

The target DNA used for standard curves was seven 10-fold serial dilutions (10^6 copies to 1 copies) of a plasmid DNA containing the primer-spanning region of the gB gene. The amplicon was generated by nested PCR (as described above) using CMV AD169 genomic DNA and was subsequently cloned into multicloning sites of 3,929 bp pCR2.1 plasmid DNA (Invitrogen, Carlsbad, Calif.) in order to construct the gB-pCR2.1

plasmid. Plasmid DNA from cultured bacteria was extracted and purified with PurelinkTM Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, Calif.). The plasmid standard DNA concentration was calibrated by spectrophotometry at 260 nm. The original number of CMV copies was determined by the optical density (OD) values against the molecular weights of different dilutions of this plasmid DNA. The standard curve was established by plotting each CMV-gB DNA standard against

the corresponding Ct values. A correlation coefficient of > 0.99 was required for the acceptable slope value of the standard curve. The salmon sperm (10 $\mu\text{g/ml}$) was added to the dilution series of standard to prevent the DNA aggregation and DNA adhering.

PCR Inhibition

Inhibition of amplification due to remaining constituents in the DNA extract, was examined. Five CMV-negative clinical samples were spiked with 50 copies of genomic CMV DNA. All spiked samples were assessed the CMV loads by use of In-house TaqMan assay.

COBAS[®] AmpliCor[®] CMV monitor test

CMV viral DNA was quantitated by coamplifying a 365 bp fragment of the CMV DNA polymerase gene in the presence of a quantitative standard (QS), added into each individual specimen at a known copy number during specimen processing so that specimen preparation, PCR amplification and detection of the target could be monitored. The DNA extraction from plasma samples by isopropanol precipitation and the following PCR amplification and quantitative detection by enzyme immunoassay were performed according to the manufacturer's recommendations.

Artus[®] CMV TM PCR Kit

The Artus CMV assay targets a 105-bp region of the major immediate-early antigen. The assay was performed according to the manufacturer's recommendations. Briefly, CMV DNA was first isolated from 200 μl of plasma by commercial viral DNA extraction kit. Processed specimens were then added to the PCR Master Mix. Amplification and detection were performed using the ABI PRISM 7500 (Applied Biosystems Inc., Foster City, CA) with the following parameters: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 55°C for 1 min. The results were

quantified using CMV DNA at predetermined concentrations provided by the manufacturer.

Statistical Analysis

Student's *t*-test was used for the statistical analysis. CMV viral loads results were expressed as mean (SD) of the \log_{10} transformed data. Differences were considered statistically significant for *P*-value < 0.05 . Pearson correlation and linear regression were used to determine the agreement of methods.

Results

Primer and probe design

The gB gene region of CMV strain AD169 was selected as the target for amplification because it is supposed to be relatively conserved among isolates. Primers and probe were designed to be specific, which was determined by alignment with gB gene of other isolates including TOWNE, Toledo, Merlin, HAN38, HAN20, HAN13, JP, 3157, 3301.

Performance characteristic of In-house TaqMan assay

Based on the constructed standard curve, the lowest limit of detection of the established In-house TaqMan assay was 1 copy/reaction (200 copies/ml plasma). The linear range was 2.3 to 8.3 \log_{10} copies/ml (Figure 2). Of each PCR run, the correlation coefficient was at least 0.99 and the slopes varied from -3.2 to -3.4. No cross-reactivity was observed when the specificity of the assay was tested for other human herpesviruses (herpes simplex virus type 1 and 2, varicella-zoster virus, Epstein-Barr virus) and hepatitis B virus; data not shown. The reproducibility of the In-house TaqMan assay was assessed using standard plasmid DNA as shown in Tables 1 and 2. As expected, the assay has the greater precision at higher CMV levels than the sample with lower CMV level.

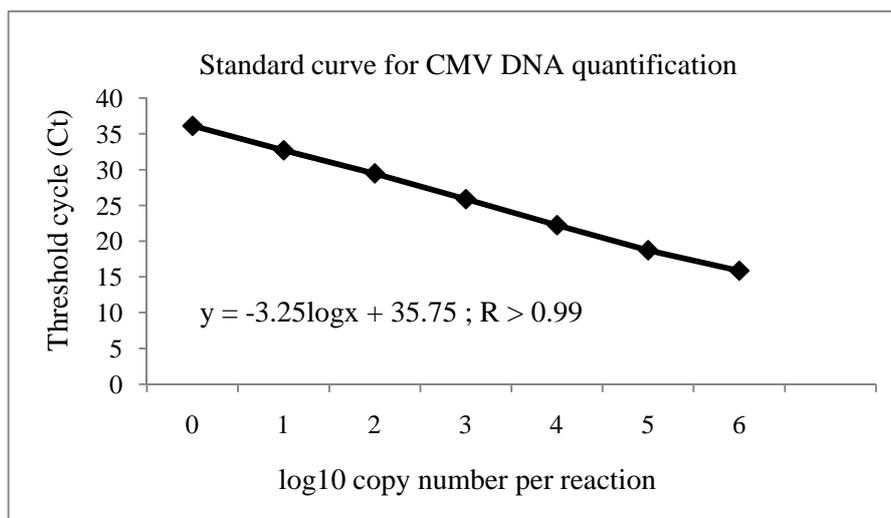


Figure 2 PCR cycle number is plotted against log concentration of the standard dilutions. The linear regression coefficient is close to 1.

Table 1 Inter-assay of the In-house TaqMan assay using standard plasmid DNA

Nominal conc. (log ₁₀ copies)	No. of samples tested	Mean (SD) (log ₁₀ copies)	CV (%)
4.0	9	3.95(0.06)	1.48
3.0	9	2.99(0.08)	2.51
2.0	9	1.91(0.13)	7.01

Table 2 Intra-assay of the In-house TaqMan assay using standard plasmid DNA

Nominal conc. (log ₁₀ copies)	No. of samples tested	Mean (SD) (log ₁₀ copies)	CV (%)
4.0	10	3.89(0.04)	1.02
3.0	10	2.87(0.07)	2.39
2.0	10	1.81(0.17)	9.39

Inhibition of PCR amplification was tested with 5 extracted CMV-negative clinical samples by adding 50 copies of genomic AD169. The viral loads were detected in all samples with the quantity within 0.5 log₁₀ of the expected copy number, indicating no inhibition (Herrmann et al., 2004).

Agreement between the In-house TaqMan assay and COBAS CMV monitor test

A comparison was performed with 42 plasma samples from patients with suspected CMV infection, 6 CMV-spiked samples and 4 super-natants from CMV-infected cells (Table 3). A total of 31 samples that had detectable viral loads by the COBAS CMV monitor test were evaluated by the In-house TaqMan assay.

Four samples that were positive by the COBAS CMV monitor test and negative by the In-house TaqMan assay had a viral loads of 3.0 log₁₀ copies/ml in average by the COBAS CMV monitor test. For the 27 samples that were positive by both assays, the population mean(SD) was 4.37(1.38) log₁₀ copies/ml for the In-house TaqMan assay and 4.22(1.10) log₁₀ copies/ml for the COBAS CMV monitor test. Of the 21 negative samples by the COBAS CMV monitor test, one sample had detectable viral loads of 2.69 log₁₀ copies/ml in the In-house TaqMan assay. The correlation between the In-house TaqMan and COBAS assays was statistically significant (R = 0.915, P < 0.05; n = 27) (Figure 3).

Table 3 Comparison of the qualitative results of In-house TaqMan and COBAS Amplicor assays

No. Of samples	Results obtained by TaqMan	Results obtained by COBAS
27	+	+
20	-	-
4	-	+
1	+	-
Total	28 positive	31 positive

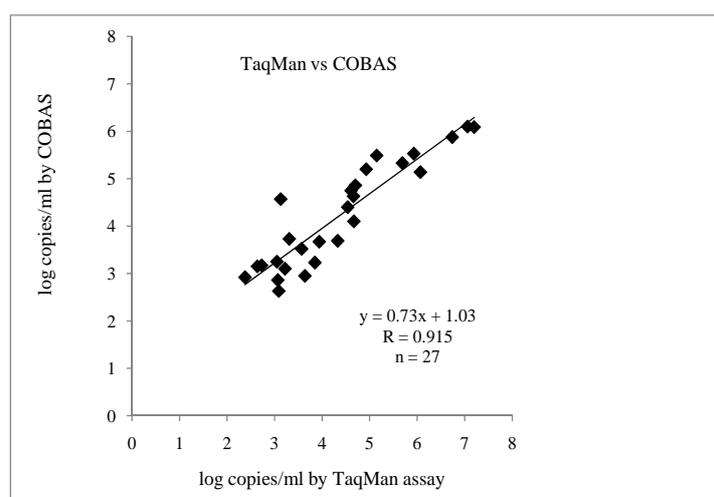


Figure 3 The scatter diagram and the regression line show the relation of CMV loads for each sample measured by both the In-house TaqMan assay and the COBAS CMV monitor (R = 0.915, P<0.05).

Agreement between the In-house TaqMan assay and Artus CMV assay

Sixteen plasma samples and four CMV-infected supernatant samples were examined by the In-house TaqMan and the Artus CMV assays using the Nucleospin[®] RNA virus kit. CMV

DNA was detectable in 8 samples by both tests. As shown in Table 4, the viral loads tested by In-house TaqMan and Artus CMV assay showed a statistically significant and strong correlation ($R = 0.998$, $P < 0.05$; $n = 8$).

Table 4 Comparison of mean viral loads and SD obtained by the In-house TaqMan assay and the Artus CMV assay using same extraction methods

Assay	Mean (SD) (log ₁₀ copies)
TaqMan	5.03(2.03)
Artus CMV	4.98(1.97)

Comparison of In-house TaqMan and extraction methods

Efficiency of DNA extraction methods between the Nucleospin[®] RNA virus kit and QIAamp[®] DNA blood mini kit was compared by

using CMV spiked samples. Of 5 spiked samples and 4 CMV infected supernatants, the mean viral loads and SD for the two methods are shown in Table 5. No significant difference was found at the 98.2% confidence interval.

Table 5 Comparison of mean viral loads and SD obtained by the In-house TaqMan assay using different extraction methods

Extraction Methods	Mean (SD) (log ₁₀ copies)
Nucleospin [®] RNA virus kit	5.96(1.34)
QIAamp [®] DNA blood mini kit	5.65(1.39)

Discussion

Before the introduction of PCR techniques, the antigenemia test was considered as the gold standard to detect CMV disease (Boeckh et al., 1997; Piiparinen et al., 2002; Tiziano et al., 2006). However, quantitative CMV DNA based on real-time PCR was used more frequently in the management of CMV disease in immunocompromised patients (Aitken et al., 1999; Humar et al., 2004; Yun et al., 2003). The real-time PCR assay can

be completed by use of single tube operation. It effectively prevents contamination. Most of procedures are performed by computer processing except for sample preparation. It has improved accuracy and can be monitored in a real time manner.

As mention above, real-time PCR based on TaqMan-MGB probe technology should provide better detection performance than that based on common probes. The assay have been proved and published in quantifying HHV-7 genome

and HBV genome (Fernandez et al., 2002; Zhao et al., 2005). Ikewaki et al. (2003) reported the real-time PCR is more suitable for monitoring CMV reactivation in adult T-cell leukemia (ATL) patients than the antigenemia and nested PCR assays.

In the present study, we have developed a highly sensitive real-time TaqMan quantitative PCR assay for detection of CMV DNA in human plasma. The assay uses two primers and one TaqMan-MGB probe in one reaction. The new primer and probe regions were designed within highly conserved glycoprotein B gene region. Within the gB gene, a clustered distribution of nucleotide substitutions at only a few distinct positions was fewer comparable with major immediate early (MIE) gene. No major difference in nucleotide sequence homology between isolates from the patients and the AD169 and the new reference strains was found (Wirgart et al., 1998).

According to our results, the high efficiency of the amplification was showed by the slope average -3.3 with correlation coefficient of at least 0.99. The detection limit of the assay was 200 copies/ml of plasma and the linear range was between 200 to 2×10^8 copies per ml. The percent coefficient of variation (%CV) values ranged from 1.02% to 9.39% in intraassay analysis and from 1.48% to 7.01% in interassay analysis. The quantitative inhibition was also assessed by adding of 50 copies genomic DNA. No inhibition effect was observed.

A comparison between the In-house TaqMan assay and the COBAS CMV monitor test showed a good concordance with statistical significance ($R = 0.915$, $P < 0.05$). However, the correlation between In-house TaqMan and Artus CMV assays was significantly higher with $R = 0.998$, $P < 0.05$. The analytical sensitivity of both In-house TaqMan and Artus CMV assay reached 200 copies/ml while that of COBAS Amplicor monitor test demonstrated 400 copies/ml. All

defined by positivity rate greater than 95%. In 1 of the 28 COBAS-negative and In-house TaqMan-positive cases, the copy number was less than 500 copies/ml, indicating that the difference in detection capacity was due to the higher sensitivity of the In-house TaqMan assay. In contrast, in 4 of the 31 In-house TaqMan-negative and COBAS-positive cases, the explanation for the discrepancy between the methods would be mismatching nucleotides in the primer and/or probe regions used in the In-house TaqMan assay. A GenBank search showed that no nucleotide variation occurred in the primer and/or probe regions used in the In-house TaqMan assay (data not shown). The undetectable CMV viral load was also demonstrated in the same four samples by the Artus CMV assay. An alternative explanation could be different yields of DNA extraction. For COBAS CMV monitor test, nucleic acid was extracted using the MagNA Pure total nucleic acid kit. The extraction is based on bead technology resulting in high quality nucleic acid, free from cross contamination. While the In-house TaqMan and the Artus CMV assays, nucleic acid was extracted using a Nucleospin[®] RNA virus kit as mentioned above. As expected, when the In-house TaqMan and the Artus CMV assays were compared using the same extraction method, the viral loads were in close agreement (99.8% confidence interval). Although, a smaller correlation in viral loads was observed when the In-house TaqMan assay was evaluated using two different extraction methods, up to 98.2% confidence interval. HCMV loads were demonstrated in good agreements between the different tests using the same extraction method and between different extraction methods using the same test. Our results are in agreement with Vincent et al (Vincent et al., 2009). All clinical samples were processed using same extraction method and tested using three sets of reagents including TaqMan, dual hybridization probes,

and labeled primer system. No significant differences in sensitivity between the three sets of reagents were observed.

The assay performance is variable value depending on various factors, for example chemistry, reagents, target sites, efficiency of amplification, or cycling conditions. In addition, other factors such as types of specimens, steps of sample preparation and design of plasmid standard should be considered and assessed. Furthermore, the differences in assay performance may be present when a large number of samples are tested (Gouarin et al., 2007; Yun et al., 2000; Vincent et al., 2009). It is recommended to further evaluate this In-house TaqMan assay on large panels of viral isolates and a wider range of extraction methods. More studies are needed to determine the threshold of positivity that would be considered indicative of active CMV disease.

Conclusion

The In-house TaqMan assay can accurately, reproducibly and rapidly quantify the CMV viral loads. The assay showed a good correlation with the commercial COBAS Amplicor Monitor test and Artus CMV assay. We conclude that the In-house TaqMan assay for the quantitative detection of CMV using TaqMan-MGB probe is possible to quantitate CMV loads in human plasma. The wider linearity of TaqMan assay from 200 to 2×10^8 copies/ml comparable with COBAS (from 400 to 1×10^5 copies/ml) makes it easier to detect and monitor the patients with very low or high viral loads.

Acknowledgements

The authors wish to thank the Molecular unit of Siriraj Hospital to provide the clinical samples with known CMV quantitative data. Also, we thank the Department of Microbiology, Faculty of Medicine, Chulalongkorn University for a part of CMV quantitative data.

We are grateful to Professor Naoki Inoue at National Institute of Infectious Diseases, Japan for excellent advice.

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