Preliminary Report for the Application of Nucleic Acid Sequence-Based Amplification in Detection of Human Cytomegalovirus (HCMV) Late pp67 mRNA for Diagnosis of HCMV Infection

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Abstract

Human cytomegalovirus (HCMV) late pp67 mRNA expression by nucleic acid sequence-based amplification (NASBA) in patients, clinically diagnosed as possible HCMV, probable HCMV disease, and no disease, was evaluated. The RNAs were isolated from 11 whole-blood samples of 11 patients for the specific amplification of the pp67 mRNA. NASBA results were compared to results from PCR assay and serological assay. The HCMV pp67 mRNA could be found in 3 of 11 patients, whereas, HCMV-DNA PCR was positive in 6 of 11 patients. PCR assay for HCMV-DNA in plasma has proved to correlate with clinical diagnosis of HCMV infection. Only 2 patient samples of NASBA positive results coincided with HCMV-DNA PCR. However, the diagnosis of clinically relevant HCMV infection by NASBA was seen. Anti-CMV IgG titers of 1:1,600 or over 1:1,600 were found in 2 of 3 NASBA positve cases and 5 of 6 HCMV-DNA positive cases, whereas, anti-CMV IgM were all negative. These results showed the correlation of HCMV infection detected by NASBA, PCR assay and anti-CMV IgG of the titers up to 1:1,600. Additionally, a low antibody titer of the HIV patient could be diagnosed by NASBA or PCR. In conclusion, pp67 mRNA NASBA appears to be a promising diagnostic tool in analysis of HCMV infection and/or disease. Its diagnostic value should be defined in the specific group for the follow-up of immunocompromised patients, such as organ transplant recipients in future prospective studies.

Key words : pp67 mRNA, NASBA, HCMV-DNA, PCR

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Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus producing infection of individuals in all age groups. Although HCMV infection causes only mild or asymptomatic infection in most healthy individuals, significant morbidity and mortality can occur in two groups of patients, infants exposed to congenital infection and patients with acquired or induced immunosuppression (such as those infected by human immunodeficiency virus (HIV) and transplant recipients)[1,2]. The diagnosis of active HCMV infection at an early stage are very important for a guide of preemptive therapy[3-5]. The current methodologies for diagnosis of HCMV infection in Thailand, rely on the detection of serologic responses to HCMV, viral DNA[6,7] and CMV pp65 antigenemia assay[8].

Serological assay gives only indirect evidence of the presence of the virus and can be problematic because of the immunological disorders occurring in most patients at risk of developing HCMV infection[9,10]. Serologic studies of HCMV in Thai populations indicated a seropositive rate of 93.3-97.3 per cent[11-13]. The usefulness of HCMV serologies for the diagnosis of clinical HCMV infection is limited. However, detection of HCMV-specific IgM antibodies can provide supportive evidence for the diagnosis of HCMV infection.

Although amplification of the viral DNA by PCR proved to be analytical sensitive in the detection of HCMV, it does not necessarily correlate with active infection, due to the possible amplification of latently present viral DNA and/or incomplete viral genomes[14]. The CMV pp65 antigenemia assay detects the lower matrix protein pp65 (UL83) in blood leukocytes by immunocytochemical technique[8]. The number of pp65-positive cells has been shown to correlate with HCMV disease. The test result can be obtained within 1 day, but it requires direct processing of fresh clinical specimens[15,16].

The direct approach in detection of active HCMV infection is the NASBA RNA technology (Nucleic acid sequence-based amplification). This assay detects mRNAs coding for the matrix tegument protein pp67 of HCMV which is only expressed during viral replication. Recently, it has been reported that the monitoring of HCMV infection in renal allograft recipients by detection of pp67 mRNA in blood by NASBA is highly specific and sensitive and that NASBA can be used to monitor the natural history of HCMV infection and the effect of antiviral treatment[14]. Therefore, we preliminarily investigated the prevalence of late pp67 mRNA of HCMV in patients with suspected HCMV infection and/or disease and those of other diseases without HCMV infection. The results of the NASBA assay were compared with the results of HCMV DNA in plasma by PCR and serology.

MATERIAL AND METHOD

Patients

From October 1998 to January 1999, a total of 11 patients (aged between 5 months and 66 years) attended or were admitted to the Department of Medicine (transplantation clinic or ward of infectious disease), Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. Of the patients enrolled this study, some were clinically diagnosed as possible HCMV infection, probable HCMV disease, and no HCMV disease.

Samples collection and preparation

5 ml of EDTA blood and plasma samples were collected. Plasma samples were aliquoted and kept at -70°C for detection of anti-CMV IgG, IgM and HCMV DNA. In addition, 0.1 ml of EDTA blood was added to 0.9 ml of NASBA lysis buffer (4.7 M guanidinium thiocyanate; 46 mM Tris, pH 6.4; 20 mM EDTA, 1.2 per cent w/v Triton X-100) and stored at -70°C.

Serology

All plasma were determined for the presence of HCMV IgM and IgG using Vironostika® anti-CMV IgM II and anti-CMV IgG II Microelisa System (Organon Teknika BV, Boxtel, Holland). Both tests were based on sandwich enzyme immunoassay. The positive and negative results were analysed as recommended by the company.

HCMV DNA by PCR

50 µl of DNA were isolated from 200 µl of plasma by High Pure viral nucleic acid kit (Boehringer Mannheim, Germany)[17], kept at -70°C before being used in PCR.

The primers for PCR were from the 4th exon of the HCMV immediate early gene (Eco RI J-fragment of the AD169 strain). Primer A (5'-AGC TGC ATG ATG TGA GCA AG-3') was complementary to the antisense DNA strand and consisted of nucleotides 1767-1786. Primer B (5'-GAA GGC TGA GTT CTT GGT AA-3') was

complementary to the sense DNA strand and consisted of nucleotides 1894-1913[18]. These primers were custom synthesized (Life Technologies, USA), allowing the amplification of a 147 bp fragment.

The PCR of HCMV DNA in plasma extracts were performed in a total volume of 50 µl. The reaction mixture consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.5 µg of primers A and B, 1.2 Units of AmpliTaq DNA polymerase (Perkin Elmer) and 5 µl of DNA extracts. The reaction mixtures were first denatured at 94°C, 4 min, then amplified in 42 cycles of 94°C, 1 min and 65°C, 3 min and extended 4 min at 65°C after the last cycle in the Gene Amp PCR System 2,400 (Perkin-Elmer Corporation, USA). The amplified products were detected by gel analysis. 10 µl of the amplicon was analysed on 2 per cent NuSieve® gel (FMC Bio Product, Rockland, Maine USA) in 1xTBE buffer and visualized by UV transilluminator after staining with ethidium bromide. HCMV strain AD169 was the positive control. Multiple negative controls, aerosol-resistant tips, and separate processing areas were used to minimize the risk of amplicon contamination.

Detection of HCMV late pp67 mRNA

HCMV pp67 mRNA assay was carried out according to the manufacturer's instructions (Organon Teknika, B.V., Boxtel, The Netherlands). Briefly, 1 ml of the lysed-whole-blood suspension, which equaled 100 µl of whole blood input sample, was used. 20 µl of SC RNA containing 3,000 copies of pp67 SC RNA in 1.0 mM Tris buffer, pH 8.5, was added prior to nucleic acid isolation[14], which was performed essentially as described by Boom et al[19]. Briefly, lysates were incubated with 50 µl of activated silica particles carrying adsorbed nucleic acids, were washed twice with 1 ml of wash buffer (5.25 M guanidium thiocyanate, 50 mM Tris, pH 6.4), twice with 1 ml of 70 per cent ethanol, and once with 1 ml of acetone. Finally, after the silica was dried at 56°C for 10 min, the nucleic acids were eluted in 50 µl of 1.0 mM Tris buffer, pH 8.5, and stored at -70°C. The NASBA reaction was performed with two primers which were designed to amplify part of the mRNA encoding HCMV pp67 (the UL 65 gene product). NASBA reactions were carried out as described by Kievits et al[20] in a 20-µl reaction mixture containing 40 mM Tris, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 15 per cent vol/vol dimethyl sulfoxide, 5 mM dithiothreitol, each deoxynucleoside triphosphate at a concentration of 1 mM, ATP, CTP, and UTP each at a concentration of 2 mM, 1.5 mM GTP, 0.5 mM ITP, 2 µg of bovine serum albumin, 0.08 U of Rnase H, 32 U of T7 RNA polymerase, 6.4 U of avian myeloblastosis reverse transcriptase, each primer at a concentration of 0.2 µM, and 5 µl of isolated nucleic acids. Prior to the addition of the enzymes, the NASBA reaction mixtures were incubated for 5 min at 65°C to destabilize the secondary RNA structures and were then cooled for 5 min to 41°C to allow primer annealing. Following the addition of the enzymes, the reaction mixtures were incubated at 41°C for 90 min and were then stored at -70°C.

The amplification products (wild-type and SC RNA) were then diluted in detection diluent and were incubated for 30 min at 41°C with biotinylated pp67-specific capture probe bound to 5 µg of streptavidin-coated magnetic beads and 3 x 10^{11} molecules of a ruthenium-lebeled oligonucleotide detection probe specific for either pp67 mRNA or SC RNA[14]. As a negative control, the detection diluent was also incubated with the wild type RNA probe and the olignucleotide bound to magnetic beads. Following incubation, an assay buffer solution was added and the tubes were placed in an electrochemiluminescence instrument (NASBA QR System; Organon Teknika B.V.) for final reading of the results[21].

RESULTS

Fig. 1 shows the results of patient plasma HCMV DNA obtained by PCR assay. In 11 samples (from 11 patients), 6 were HCMV DNA positive (Table 1).

Table 1 demonstrates the correlation of pp67 mRNA, plasma DNA, serologic diagnostic pattern and clinical findings in patients with suspected HCMV infection or disease and patients of other diseases without HCMV infection. In two patients (patients 1 and 7), HCMV infection was detected by pp67 mRNA NASBA and PCR. Another patient (patient 4), demonstrated pp67 mRNA positive result, but HCMV DNA was negative. Of 8 negative pp67 mRNA results, 4 (patients 5, 8, 10 and 11) had positive results of HCMV DNA and 4 had negative results. In the last case, the blood for detection of pp67 mRNA was taken 3 days after the ganciclovir treatment.

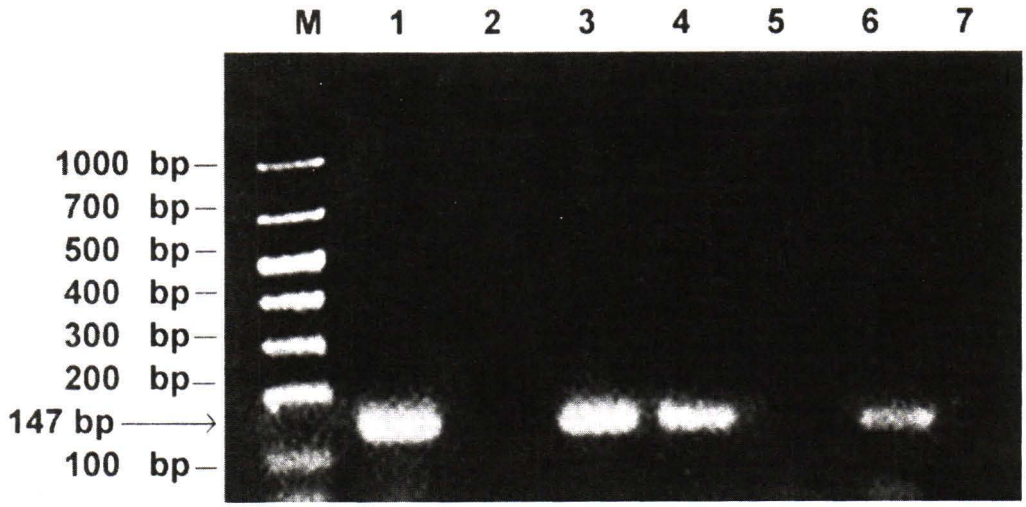

Fig. 1. Detection of HCMV DNA in patient plasma samples by PCR assay. *Lane M*, molecular weight markers, *lane 1*, HCMV AD169, *lane 2-4*, HCMV DNA detected in plasma samples, *lane 5*, negative control, *lane 6*, positive HCMV PCR products.

Table 1. Correlation of pp67 mRNA, plasma DNA, serologic diagnostic pattern and clinical findings in 11 cases of suspected HCMV infection and those with other diseases without HCMV infection.

Pattern Patients	Details	Laboratory diagnostic				Clinical findings relating to HCMV infection
		pp67 mRNA	HCMV DNA in plasma	HCMV IgM	HCMV IgG	
1	Late symptomatic HIV infection	+	+	-	1:400	probable* HCMV pneumonia
2	Relapse ANLL[a](M1), febrile neutropenia	-	-	-	1:800	no HCMV infection
3	Seizure and polyradiculopathy	-	-	-	1:400	no HCMV infection
4	Post liver transplanttation for 4 months, disseminated aspergillosis	+	-	-	1:1,600	possible** HCMV infection
5	LRKT[b] and chronic allograft dysfunction	-	+	-	> 1:1,600	possible HCMV infection
6	ESRD[c] due to bilateral polycystic kidney disease	-	-	-	1:100	no HCMV infection
7	ESRD	+	+	-	> 1:1,600	possible HCMV infection
8	Lupus nephritis	-	+	-	> 1:1,600	possible HCMV infection
9	Guillian-Barre Syndrome	-	-	-	1:800	no HCMV infection
10	Neonate hepatosplenomeagaly	-	+	-	1:1,600	possible HCMV infection
11	Chronic renal failure with hypertension, CDKT[d] 4 years	-	+	-	> 1:1,600	probable HCMV retinitis

[a]ANLL : acute non-lymphoblastic leukemia
[b]LRKT : living-related kidney transplantation
[c]ESRD : end stage of renal disease, patient 6 and 7, admitted for cadaveric donor kidney transplantation
[d]CDKT : cadaveric donor kidney transplantation
*probable : clinical findings compatible with HCMV infection, evidence of improvement with ganciclovir treatment
**possible : clinical findings suggested HCMV infection, no ganciclovir treatment

2 of 3 NASBA positive cases had high titers of anti-CMV IgG (1:1,600 or over 1:1,600) and 5 of 6 positive cases by PCR assay, anti-CMV IgG titers were also high as shown in Table 1. Six cases of high titer were correlated to the clinical diagnosis, whereas, anti-CMV IgM results were negative in all of the patients in this study.

DISCUSSION

HCMV infection/disease has considerable consequences for the prognosis and management of immunosuppressed individuals. Early diagnosis of an active infection is essential in order to initiate antiviral therapy. The expression of HCMV late pp67 mRNA, in circulating blood leukocytes is considered to directly reflect HCMV replication and dissemination in the infected host and should cease upon effective blockage of viral polymerase by antiviral agents, such as ganciclovir[14]. We, therefore, studied the diagnostic usefulness of pp67 mRNA NASBA, plasma DNA PCR and serology in 11 patients clinically diagnosed as possible HCMV infection, probable HCMV disease, and no HCMV disease.

The results of our study show that 3 of 11 patients were pp67 mRNA NASBA positive. Only two patients of NASBA positve were plasma DNA-positive. One of the NASBA positive patients was plasma DNA-negative, whereas, there were 6 of 11 patients who showed plasma DNA-positive and 4 of 6 patients were negative by NASBA assay. In this study, it would seem that NASBA lacks sensitivity, compared to the PCR assay. HCMV infection was based on clinical diagnosis, and the pp67 mRNA was detected only once in each patient. The time duration of pp67 mRNA during which HCMV infection occurred, is not known. As seen in the last case, the blood sample was taken for detection of HCMV infection 3 days after ganciclovir treatment, or pp67 mRNA may express early. NASBA which failed to detect the pp67 mRNA may due to primer and target mismatches. It should be noted, however, that the results in this study are based on a low number of patients.

However, clinical relevances for the detection of active HCMV infection by NASBA are seen. Similarly, the results of plasma DNA PCR have proved to correlate with the clinical diagnosis.

In comparing NASBA and plasma DNA PCR results to anti-CMV IgG, we found that only high antibody titer (up to 1:1,600) could be seen. Moreover, 6 cases of high IgG titer were relevant to the clinical diagnosis of HCMV infection. Although, the four fold rising titer of anti-CMV IgG was not done in this study, anti-CMV IgG could analyze HCMV infection, only if the individuals had high titers, up to 1:1,600. Additionally, a case of low anti-CMV IgG titer of HIV patient could be diagnosed by NASBA or PCR. Anti-CMV IgM was not detected in all of the subjects.

In conclusion, pp67 mRNA NASBA proved to be a diagnostic tool in the analysis of HCMV infection and/or disease. Further investigations are needed to prove the sensitivity and diagnostic value in specific groups of patients, especially in transplant recipients such as kidney and bone marrow transplantation. Such studies are proceeding.

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การตรวจหาสารพันธุกรรม Late pp67 mRNA ของเชื้อ Human Cytomegalovirus โดยการเพิ่มปริมาณของยีนแบบ Nucleic Acid Sequence-Based Amplification ในเลือดผู้ป่วยที่สงสัยว่ามีภาวะติดเชื้อ Human Cytomegalovirus

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ได้ศึกษาการตรวจหา late pp67 mRNA โดยวิธี Nucleic acid sequence-based amplification (NASBA) ในเลือดผู้ป่วยที่น่าจะมีภาวะติดเชื้อจาก HCMV และผู้ป่วยติดเชื้อที่ไม่ได้เกิดจาก HCMV โดยศึกษาเปรียบเทียบกับการตรวจหา HCMV-DNA ใน plasma ด้วยวิธี PCR และการตรวจทาง Serology จากการศึกษาในผู้ป่วยทั้งหมด 11 ราย ตรวจพบ pp67 mRNA 3 ราย และ HCMV-DNA ใน plasma 6 ราย มีเพียง 2 รายที่ได้ผลตรงกัน แต่ผลการตรวจ pp67 mRNA จากเลือดผู้ป่วยทั้ง 3 รายนี้สัมพันธ์กับภาวะติดเชื้อ HCMV HCMV-DNA จาก plasma ผู้ป่วย 6 ราย ก็เข้ากันได้กับอาการของโรคเช่นเดียวกัน ส่วนผลจากการตรวจหา anti-CMV IgG ในรายที่มี titer ตั้งแต่ 1:1,600 ขึ้นไป พบว่าสัมพันธ์กับการตรวจหา pp67 mRNA 2 รายใน 3 ราย และสัมพันธ์การตรวจด้วย PCR ใน plasma จากผู้ป่วย 5 ใน 6 ราย แต่การตรวจหา anti-CMV IgM ได้ผลเป็นลบทุกราย นอกจากนี้ยังพบว่า การตรวจหา pp67 mRNA หรือ HCMV DNA ช่วยการวินิจฉัยภาวะการติดเชื้อและโรคติดเชื้อจาก HCMV ในผู้ป่วยติดเชื้อเอดส์ที่มี anti-CMV titer ต่ำได้ ผลการวิจัยนี้บ่งชี้ว่า สามารถใช้สารพันธุกรรม pp67 mRNA โดยเทคนิค NASBA เป็น marker ในการตรวจวินิจฉัยภาวะการติดเชื้อและโรคติดเชื้อจาก HCMV ได้ โดยน่าที่จะศึกษาวิจัยเพื่อประเมินประสิทธิผลในการบ่งชี้ภาวะการติดเชื้อและโรคติดเชื้อ HCMV ของ pp67 mRNA ในผู้ป่วยที่มีภาวะภูมิคุ้มกันบกพร่องจำเพาะกลุ่ม เช่น ผู้ป่วยมีภาวะติดเชื้อหรือเป็นโรคติดเชื้ออันมีสาเหตุมาจาก HCMV ภายหลังจากได้รับการปลูกถ่ายอวัยวะ เป็นต้น

คำสำคัญ : pp67 mRNA, NASBA, HCMV-DNA, PCR

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