Short Communication

Rapid and Sensitive Identification of Glycoprotein H Genotypes in Clinical Human Cytomegalovirus Samples

Wei Li[†], Ran Tao[†], Xiao Zhang, Qiang Shu, Hui-Hui Gao, Shi-Qiang Shang^{*}, Zhao-Yang Peng, and Hua-Mei Li

Department of Laboratory, Children's Hospital of Zhejiang University School of Medicine, Hangzhou, PR China

SUMMARY: Human cytomegalovirus (HCMV) is a common pathogen that causes persistent infections in immune deficient patients and results in significant morbidity and mortality, particularly among transplant recipients and children. Different HCMV glycoprotein H (gH) genotypes may cause different diseases and affect the severity of these diseases. To develop a sensitive quantitative real-time PCR assay that could rapidly distinguish between two HCMV gH genotypes, primers were designed to target the conserved region of the gH gene. gH1 and gH2 probes were designed to target the two variable regions. Standard HCMV strains (AD169 and TOWNE) and 203 clinical urine samples from HCMV infected children were used for the present study. Based on the primer-probe set used to detect the target gH gene segment of HCMV, our quantitative real-time PCR assay specifically discriminated between HCMV gH1 and gH2 with a detection limit of approximately 10^2 viral copies/ml. Among the 203 clinical urine samples tested, 145 were gH1 positive, 56 were gH2 positive, and 2 were positive for both. Thus, we developed a gH gene-based real time-PCR method that could rapidly, stably, and specifically distinguish between two HCMV gH genotypes. We found HCMV gH1 to be common among children examined in Zhejiang, China.

Human cytomegalovirus (HCMV) is a member of the herpesvirus family that infects most populations worldwide (1). In healthy adults, HCMV infection causes few, if any, symptoms, but infections are often much more severe in immunocompromised patients and congenitally infected infants (2–4). Many reports have focused on the effects of HCMV strain on the biologic properties of the virus, and each clinical HCMV subtype affects the outcomes of HCMV infections (4–6). Glycoprotein H (gH) is an important target for neutralizing antibodies, but is also believed to be involved in viral entry into cells, which suggests that gH genetic variants can affect viral pathogenicity and the clinical outcomes of immunocompromised patients (7–9).

There are two major genotypes for the gH gene, which can be distinguished based on variability at the cleavage site at the gH N-terminus (10). Different HCMV gH genotypes may be associated with disease severity (11–14). Using multiplex PCR plus restriction fragment length polymorphism to discriminate between HCMV gH genotypes is complicated when using clinical samples. Thus, a simple and effective method for HCMV gH genotyping is required.

Recently, real-time PCR assays have been used for pathogen detection, such as identifying bacteria and viruses (4,15,16). The purpose of the present research is to develop a real-time PCR-based method to accurately identify HCMV gH genotypes using clinical samples. In

order to develop a sensitive real-time PCR assay to rapidly distinguish between the two gH genotypes in clinical samples of HCMV infection, gH gene sequences from HCMV strains were downloaded from GenBank.

The Primer Express software package (Applied Biosystems, Foster City, CA, USA) was used for primer and probe design. The target segment of the gH gene used for HCMV identification was also used for conservation analysis using a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Mega 4.0 software (BioDesign Institute, Tempe, AZ, USA). We used the forward primer, TGTTTTCACGCAGGAAGCGG; reverse primer, TGGATCACGCCGCTGAACC; gH1 probe, FAM-CATCCGAAGCGCTGGACCCCTCACG; and gH2 probe, HEX-TATCCGAACCGCTGGACAAAGCG-BHQ1. All the primers and probes used in this study were synthesized by Takara (Otsu, Japan).

The present study was approved by the ethics committee of the Children's Hospital of Zhejiang University School of Medicine, China. With the permission of their parents, a total of 203 urine samples were collected from children with HCMV infections. Standard HCMV strains (AD169 and TOWNE), hepatitis B virus (HBV), Epstein-Barr virus (EBV), and adenovirus strains used in this study were provided by the Children's Hospital of Zhejiang University School of Medicine.

A urine sample (1 mL) was aseptically placed in a 1.5-mL microcentrifuge tube and centrifuged for 5 min. The supernatant was decanted and the precipitate suspended in 50 μ L of lysis buffer, which contained 10 mmol/L Tris-HCL (pH 7.6), 5 mmol/L EDTA, and 0.5% sodium dodecyl sulphate. The mixture was boiled for 10 min, after which it was centrifuged at 4°C. A supernatant sample (5 μ L) was then used as the template for real-time PCR amplification.

Real-time PCR amplification was performed in a total volume of 50 μ L using an ABI7500 Real time PCR

Received January 15, 2014. Accepted July 10, 2014.
J-STAGE Advance Publication November 25, 2014.

DOI: 10.7883/yoken.JJID.2014.017

*Corresponding author: Mailing address: Children's Hospital of Zhejiang University School of Medicine, 57 Zhugan Xiang, Hangzhou 310003, China. Fax: +86-571-87033296, E-mail: shangshiqiang33@163.com

[†]These authors contributed equally to this work.

System (Applied Biosystems). The reaction mixture included 400 nM of each forward and reverse primer, 100 nM of each gH1 and gH2 fluorescently-labeled specific probe, 1 U of Taq DNA polymerase (Takara), and 5 μ L of template DNA, with water added to give a final volume of 50 μ L per sample. Positive and negative controls were also included. Controls with water instead of template DNA were included in each run using the following conditions: 94°C for 2 min, and 40 cycles of 94°C for 15 s and 60°C for 45 s. The HCMV load was quantified by determining the cycle threshold (CT) (i.e., the number of PCR cycles required for fluorescence to exceed a value significantly higher than background fluorescence). Samples with CT values of 37.0–40.0 were considered to have 0 copies/ μ L. Samples with CT values of <35.0 were quantified by the actual CT value.

However, if the CT value was between 35.0 and 37.0, a second fluorescent quantitative-PCR was requested; if the CT value was not within the range of 37.0–40.0 with the second test, then the actual CT value was used for quantification. Real-time PCR products were sent to Invitrogen for sequencing.

The feasibility of this real-time PCR technique for detecting HCMV DNA was determined using standard HCMV AD169 (gH1) and TOWNE (gH2) strains. Our results showed that the gH probes could specifically discriminate between the gH gene genotypes of AD169 and TOWNE (AD169 CT value was 19.23 with the gH1 probe and TOWNE CT value was 21.45 with the gH2 probe). With this test, no fluorescent signals were detected and there were no cross-reactions with DNA extracted from the human genome, HBV, adenovirus, or

Fig. 1. Sequences of clinical PCR sample products.

EBV.

To estimate the sensitivity of real-time PCR, the targets used were AD169, as representative of HCMV gH1; and TOWNE, as representative of HCMV gH2. To determine the detection range, we prepared a series of 10-fold dilutions from 10^7 copies/mL to 10^2 copies/mL. The detection range of this real-time PCR method was at least 10^2 to 10^7 copies/mL. Data were then subjected to log-linear analysis to generate a standard curve in order to determine unknown copy numbers. These standard curves typically had high R^2 values of >0.99 .

A total of 203 urine samples from HCMV infected children were analyzed by real-time PCR. All of them were tested positive with the gH probes. Identification results showed that 145 samples (71.43%) were gH1 positive, 56 samples (27.59%) were gH2 positive, and 2 samples (0.99%) were positive for both gH1 and gH2. The HCMV gH1 genotype was the most common HCMV genotype detected in Zhejiang Province. Fourteen PCR sample products were randomly selected and sequenced; 10 for HCMV gH1 and 4 for HCMV gH2. After comparing with the reference sequences for the standard HCMV strains, AD169 and TOWNE, the similarity between AD169 and HCMV gH1 was found to be 95%–96% and the similarity between TOWNE and HCMV gH2 was 98%–99% (Fig. 1). Several site mutations have been found in the sequence of the target gH gene segment, however these had little effect on primer conjugation.

In our previous studies, we have shown that real-time PCR is generally acceptable as an accurate, cost-effective method for pathogen identification (15,16). In the present study, we sought to design a quantitative real-time PCR assay to rapidly and specifically identify HCMV gH genotypes in clinical samples. A forward primer and a reverse primer were designed for the conserved region of the gH gene, and two probes were designed for the variable region of the gH gene. Our results showed that this real-time PCR system was specific for HCMV testing. It could simultaneously detect and discriminate between HCMV gH1 and HCMV gH2 through the use of fluorescent hybridization probes in one PCR tube, and with a maximum sensitivity of 10^2 copies/mL. No fluorescent signals were detected and no cross-reactions were found using DNA extracted from the human genome, HBV, EBV, or adenovirus.

In conclusion, the real-time PCR method established in this study was applied to identify HCMV gH genotypes in clinical samples. Our primary results show that the HCMV gH1 genotype is prevalent in Zhejiang Province. In further studies, we will focus on the relationship between these HCMV gH genotypes and disease type and severity, based on a larger number of clinical samples from HCMV infected children.

Acknowledgments This work was supported by the National Natural Science Foundation of China (81200486, 81370774), Foundation of Zhejiang Educational Committee (Y201017974), and Key Projects in the National Science & Technology Pillar Program (2012BA104B05).

Conflict of interest None to declare.

REFERENCES

1. Grangeot-Keros L, Cointe D. Diagnosis and prognostic markers of HCMV infection. *J Clin Virol.* 2001;21:213-21.
2. Rasmussen L, Geissler A, Winters M. Inter- and intra-genic variations complicate the molecular epidemiology of human cytomegalovirus. *J Infect Dis.* 2003;187:809-19.
3. Görzer I, Kerschner H, Redlberger-Fritz M, et al. Human cytomegalovirus (HCMV) genotype populations in immunocompetent individuals during primary HCMV infection. *J Clin Virol.* 2010;48:100-3.
4. Paradowska E, Jabłońska A, Studzińska M, et al. Distribution of cytomegalovirus gN variants and associated clinical sequelae in infants. *J Clin Virol.* 2013;58:271-5.
5. Nogalski MT, Chan GC, Stevenson EV. The HCMV gH/gL/UL128-131 complex triggers the specific cellular activation required for efficient viral internalization into target monocytes. *PLoS Pathog.* 2013;9:e1003463.
6. Zhou M, Yu Q, Wechsler A, et al. Comparative analysis of gO isoforms reveals that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and gH/gL/UL128-131 in the virion envelope. *J Virol.* 2013;87:9680-90.
7. Ryckman BJ, Chase MC, Johnson DC. Human cytomegalovirus TR strain glycoprotein O acts as a chaperone promoting gH/gL incorporation into virions but is not present in virions. *J Virol.* 2010;84:2597-609.
8. Vanarsdall AL, Chase MC, Johnson DC. Human cytomegalovirus glycoprotein gO complexes with gH/gL, promoting interference with viral entry into human fibroblasts but not entry into epithelial cells. *J Virol.* 2011;85:11638-45.
9. Vanarsdall AL, Ryckman BJ, Chase MC, et al. Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in *cis* or in *trans*. *J Virol.* 2008;82:11837-50.
10. Chou S. Molecular epidemiology of envelope glycoprotein H of human cytomegalovirus. *J Infect Dis.* 1992;166:604.
11. Madi N, Al-Nakib W, Pacsa A, et al. Cytomegalovirus genotypes gB1 and gH1 are the most predominant genotypes among renal transplant recipients in Kuwait. *Transplant Proc.* 2011;43:1634-7.
12. Guo S, Yu MM, Li G, et al. Studies on genotype of human cytomegalovirus glycoprotein H from infantile clinical isolates. *Zhonghua Er Ke Za Zhi.* 2013;51:260-4. Chinese.
13. Zhou L, Fan J, Zheng SS, et al. Genetic variation within the glycoprotein B and H genes of human cytomegalovirus in solid organ transplant recipients. *Transpl Infect Dis.* 2007;9:73-7.
14. Qian HL, Cai T, Jin HM. Human cytomegalovirus glycoprotein genotypes in the genital tract tissue of tubal pregnancy patients. *J Int Med Res.* 2009;37:385-91.
15. Wu YD, Chen LH, Wu XJ, et al. Gram stain-specific-probe-based real-time PCR for diagnosis and discrimination of bacterial neonatal sepsis. *J Clin Microbiol.* 2008;46:2613-9.
16. Duan QJ, Shang SQ, Wu YD. Rapid diagnosis of bacterial meningitis in children with fluorescence quantitative polymerase chain reaction amplification in the bacterial 16S rRNA gene. *Eur J Pediatr.* 2009;168:211-6.