Detailed polymorphism study on cytomegalovirus DNA polymerase gene to reveal the most suitable genomic targets for quantitative Real-time PCR

Onur Bilenoğlu[1], Mustafa Altındiş[2], Ersoy Öz[3], Yeliz Yücel-Öz[1]*, Öykü İrigül-Sönmez[1], Can Bora Ünal[1]

[1]Department of Research and Development, Iontek A.Ş., Istanbul, Turkey, [2]Department of Clinical Virology and Microbiology, Sakarya Training and Research Hospital, Sakarya, Turkey, [3]Department of Computer Technologies, Yildiz Technical University, Istanbul, Turkey

ABSTRACT

The human cytomegalovirus (HCMV) is an important human pathogen primarily affecting immunocompromised patients, like transplant recipients or HIV-infected individuals. Early diagnosis of cytomegalovirus (CMV) infection in high-risk patients is essential in order to start preemptive treatments. ***pol*** (UL54) gene encoding for HCMV viral DNA polymerase is a well-defined target for HCMV detection in clinical samples and identifying most highly conserved regions for primer design remains crucial. Though real-time polymerase chain reaction (qPCR) is a rapid and sensitive method for HCMV detection, failure to detect some HCMV strains due to primer and target mismatches have led the researchers to explore more sensitive and reliable methods. Hence, to understand the broader diversity of the ***pol*** mutations in HCMV and to specify the most suitable region for primer-probe design to be used in qPCR assay, we studied both nucleotide and amino acid heterogeneities in 60 HCMV positive samples that were collected to represent national mutational prevalence of ***pol*** gene of HCMV in Turkey. The test was designed with a new set of primers-probe for HCMV detection and quantification based on the sequencing data which revealed the most conserved region on the ***pol*** gene. Statistical probit analysis was applied on qPCR studies which revealed a 95% detection limit of 100 copies/mL. In addition, linearity, reproducibility, and precision of the new test were assessed for diagnostic purposes.

KEY WORDS: Cytomegalovirus; nucleotide variations; real-time polymerase chain reaction; ***pol*** gene
DOI: http://dx.doi.org/10.17305/bjbms.2015.494 *Bosn J Basic Med Sci. 2015;15(3):28-34. © 2015 ABMSFBIH*

INTRODUCTION

The human cytomegalovirus (HCMV) is a double-stranded DNA virus that belongs to the Herpesviridae family. The virus leads to clinical manifestations depending on the patient's age and immune status [1]. Along with immunocompromised patients, transplant recipients, patients with leukemia or lymphoma, and individuals suffering from AIDS are the most susceptible individuals to HCMV infections since the virus can be transmitted prenatally, via transplanted organs or blood transfusion, and through sexual contact [2-4].

Antiviral drugs like ganciclovir (GCV), cidofovir (CDV), foscarnet (PFA) and more recent oral prodrug valganciclovir are anti-HCMV drugs approved for the treatment of HCMV-infected individuals [5-7]. Although, these drugs are proven to reduce the severity of the disease especially in immunocompromised patients and in the pediatric population, it is further shown that a prolonged periods of antiviral therapy with those drugs leads to the gain of resistance mutations on genes like DNA polymerase (***pol***) gene of HCMV and these mutations are the major cause of treatment failure [8,9].

In case of HCMV infection, early and accurate diagnosis remains crucial for susceptible hosts since the development of resistance could be avoided with early preventive antiviral therapy [10]. Molecular diagnosis techniques like polymerase chain reaction (PCR) were commonly used for the early diagnosis, but accuracy of detecting HCMV was reported to be prone to failures due to primer and target mismatches since sequence variation among HCMV strains is very common even on highly conserved regions [11].

This study not only gives insights about mutational prevalence of ***pol*** gene of HCMV in Turkey, it also reveals all the polymorphic regions on ***pol*** gene in order to gain access to the most conserved regions on this specific gene. Furthermore, determining sequence variations on this gene has led us design new target-specific primers and probes for

*Corresponding author: Yeliz Yücel Öz, Iontek A.Ş., Istanbul Technical University Arı 1 Teknokent, Istanbul, Turkey. Tel: +902124815516, E-mail: yyucel@iontek.com.tr

Submitted: 20 April 2015 / Accepted: 11 June 2015

the detection and quantification of HCMV infection under the aim of reducing the progression, morbidity and mortality from HCMV sourced diseases. Construction of this real-time polymerase chain reaction (qPCR) assay has resulted in this very high sensitive, reproducible, precise and linear diagnostic tool that can be used in clinical samples from all different patient populations.

MATERIALS AND METHODS

Clinical specimens

HCMV positive serum samples from 60 patients were used for DNA sequencing studies in order to detect the ***pol*** gene variability.

Sample preparation for PCR

The DNA isolation from HCMV samples was done using QIAamp MinElute Virus Spin Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA isolation was done with extreme caution in order to avoid sample-to-sample cross-contamination.

PCR amplification and DNA sequencing

Overlapping PCR primers were designed to sequence whole ***pol*** gene (Table 1) using Jprimer3 (Iontek®, Turkey). PCR was performed using pfU DNA polymerase (Promega, USA). 50 ng/µl of DNA was added to the reaction mixture containing 0.7 and 0.8 µM of forward and reverse primers respectively. Sequencing of amplified PCR samples was done by (Macrogen Inc., South Korea).

Sample preparation for qPCR

QIAamp MinElute Virus Spin Kit was used to extract DNA from samples. About 5 µl of internal control was added into the lysis buffer. Internal control was incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. Target HCMV DNA and an internal control are co-amplified in a single reaction using sequence-specific primers. The fluorescent signal generated by the target DNA amplification is detected by a probe labeled at the 5' end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification is detected by a second probe labeled at the 5' end with a different reporter molecule, Cy5 through the Cy5 channel.

TABLE 1. Primers used for the amplification of *pol* gene

Primer	Oligonucleotide sequences
CMV_1F	TGCTTCGTAAGCTGTCAGCC
CMV_1R	ACCTCGTACACGGGAAAACC
CMV_2F	AAATCGGCGAGTATCTGCTG
CMV_2R	GTACTGCCGCTTAAAACCCA
CMV_3F	CTGTATAAGGTGGACTCGCAG
CMV_3R	ACATCTGAAACATAGCCGCC
CMV_4F	TAGCGTTGCTGTGTCACCTA
CMV_4R	AAAAACACGGCTCTGAAAAG
CMV_5F	GCAACGCTTTCTACGGTTTT
CMV_5R	ACGGTGCAGGTACAGATCGT
CMV_6F	CGGTTTCTGGCGTATCTTAC
CMV_6R	CCACCAGTCTCAGTCTCAGC

Plasmid construction

For the construction of quantitation standards a 784 bp long region of the HCMV DNA polymerase gene fragment was amplified by PCR with the following forward and reverse primers: 5'-ACATCTGAAACATAGCCGCC-3' and 5'-TACGAGTTCCCTTCCGAATA-3.' The PCR fragment was cloned into a pGEM®-T Easy vector (Promega, USA) according to manufacturer instructions. Dilutions were calibrated with WHO standard dilutions for HCMV (1st WHO International Standard for HCMV for Nucleic Acid Amplification Techniques NIBSC code 09/162). Four plasmid standards were diluted as 5×10^5, 5×10^4, 5×10^3, 5×10^2 copies/mL respectively. Furthermore, pGEM®-T Easy cloning vector (Promega, USA) was also used for the construction of internal control. A 508-bp-long Factor V gene fragment was amplified by PCR with the following homologous primers: 5'-CCTTCGGCAGTGATGGTACT-3' and 5'-TAATTGGTTCCAGCGAAAGC-3.' Ligation of PCR products to pGEM®-T Easy Vector was performed as instructed by the manufacturer. After, ligation was completed, the mixture was used to transform electrocompetent ***Escherichia coli*** Top 10 F' cells.

Mutational analysis of HCMV gene fragments

Sequenced ***pol*** gene consists of 3729 bp and it encodes for a protein of 1242 amino acids. The sequence of each sample was determined and compared against the rest of the 60 isolates and the laboratory strain, Ad169.

qPCR assay

A qPCR assay was designed to quantify the HCMV DNA isolated from the serum of HCMV infected patients. Positions of PCR primers and probe were selected following the sequencing of the whole gene, on the most conserved UL54 regions encoding ***pol*** gene of HCMV. The forward and reverse primers were 5'-GCCCAGGTAGGCCGTTAC-3' and 5'-ATCTGCTGTCCGTCAAAGAT-3,' respectively (Eurofins, Germany). The selected probe was labeled with 6-carboxy-fluorescein at the 5' end as the reporter dye and BHQ1 at the 3' end as the quencher; FAM- CTCGTAGTGA AAATTAATGGTGTTGAAC - BHQ1 (Eurofins, Germany). PCR was performed using QuantiTect Probe PCR Kit

(Qiagen, Germany). 50 ng/µl of DNA was added to the reaction mixture containing 0.12 µM probe and 0.7 µM of forward and reverse primers. Forward and reverse primers for the internal control were 5'-CCCCATTATTTAGCCAGGAG-3' and 5'-ATGAGAGACATCGCCTCTGG-3,' respectively. The probe for the internal control was labeled with Cy5 at the 5' end as the reporter dye and BHQ2 at the 3' end as the quencher; Cy5-5'-CTCGTAGTGAAAATTAATGGTGTTGAAC-3'-BHQ2 (Eurofins, Germany). In order to check the detection specificity of ***pol*** primer-probe set, a glycoprotein B (***gB***) region of HCMV was selected. The forward and reverse primers for the ***gB*** region were 5'-CATCATGGTGGTCTACAAGCG-3' and 5'-CGAAAACCGTGCCTCCTAT-3,' respectively (Eurofins, Germany). This time the selected probe was labeled with 6-carboxy-hexachlorofluorescein at the 5' end as the reporter dye and BHQ2 at the 3' end as the quencher; HEX-CTGTAGGAACTGTAGCATTGAGCAAAC-BHQ2 (Eurofins, Germany). PCR conditions for the multiplex reaction were as follow: 15 minutes at 95°C followed by 50 cycles of 30 seconds at 95°C and 1:30 minutes at 54°C. The data acquisition was performed using the IONTEK® Fluorion Detection System (Iontek®, Turkey). Positive controls, consisting of DNA isolated from AD169 virions (ATCC VR-538) was used throughout the study.

RESULTS

Sequencing of ***pol*** gene of 60 clinical specimens and one laboratory strain (CMV Ad169) were completed in this study using six sets of overlapping primers listed in Table 1. Analyzes on different isolates resulted in the finding that specimens carry many variations that represent the mutational prevalence of ***pol*** gene of HCMV in Turkey compared to the AD169 strain (Figure 1).

Although, there are numerous nucleotide changes on the ***pol*** gene, amino acid substitutions are found only in a limited number of positions (Figure 2). Along with the ***pol*** gene variations demonstrated newly in this study, ***pol*** gene also harbors a number of known mutations located in the conserved domains of the polymerase, which are associated with the resistance of HCMV to anti-HCMV drugs like PFA, GCV, and CDV. Clinical data have shown that mutations on the ***pol*** gene that confer resistance to anti-HCMV drugs include T700A, M715V, E756Q, V781I, V787L, L802M, A809V, V812L, T821I, K805Q, D413A [12-15].

The aim of this study was to reveal both known and novel mutations on the ***pol*** gene in order to find conserved HCMV DNA regions suitable for use as real-time PCR target sequences. The overlapping PCR primers were designed to sequence whole ***pol*** gene (Table 1) using Jprimer3 platform (Iontek®, Turkey) which was developed within the framework of the TUBITAK granted project "TUBITAK 3080096 – High Performance Bioinformatic Platform." Designed target-specific PCR primers and the probe listed at Table 2 were analyzed in order to minimize the formation of primer dimers, and other types of secondary structures.

To evaluate the sensitivity of the ***pol*** primers and the probe, a preliminary test was done in order to obtain starting value for the positive cut-off point (i.e., the highest dilution giving a reproducible positive signal). The concentration, at which the sensitivity would be tested, was then chosen around the pre-determined value. Dilution series (consisting of four members; 167, 56, 19, and 6 copies/mL) were prepared and tested in replicates on different days. The results of this experiment (Table 3) were evaluated using statistical probit analysis which yielded in a 95% detection limit of 100 copies/mL. Probit analysis was performed with the software XLSTAT-Pro 7.5.3 + XLSTAT-Dose (www.xlstat.com, NY) and the results are shown in Figure 3.

The linear range of qPCR assay was determined by analyzing a dilution series covering the range 1×10^9-1×10^2 copies/mL. The test was performed with two replicates, and the results are shown in Figure 4. The R^2 value of 0.9998 of the standard curve shown in Figure 5 clearly reveals that the test was highly linear over that range.

Reproducibility and precision data were collected using the quantitation standard of the lowest concentration (5×10^2 copies/mL) calibrated against the WHO Standard for CMV (1st WHO International Standard for HCMV for nucleic acid amplification techniques NIBSC code 09/162). Testing was performed with four replicates, three different lots, on multiple days and by three operators (Table 4).

Additionally, the kit was tested with the pathogens listed in Table 5 for cross-reactivity. No cross-reactivity with the

TABLE 2. *pol* gene target-specific PCR primers and the probe

Primer/ probe	Sequence	Nucleotide position
pol forward	5'- GCCCAGGTAGGCCGTTAC-3,'	1601-1620
pol reverse	5'-ATCTGCTGTCCGTCAAAGAT-3'	1720-1739
pol FAM probe	FAM-CTCGTAGTGAAAATTAAT GGTGTTGAAC- BHQ1	1647-1674

PCR: Polymerase chain reaction

TABLE 3. Determination of highest dilution that gives a reproducible positive signal

Copies/ mL	Numbers of positive sample/ numbers of tested sample	% Positive
167	24/24	100
56	23/24	95.8
19	12/25	48
6	6/25	24

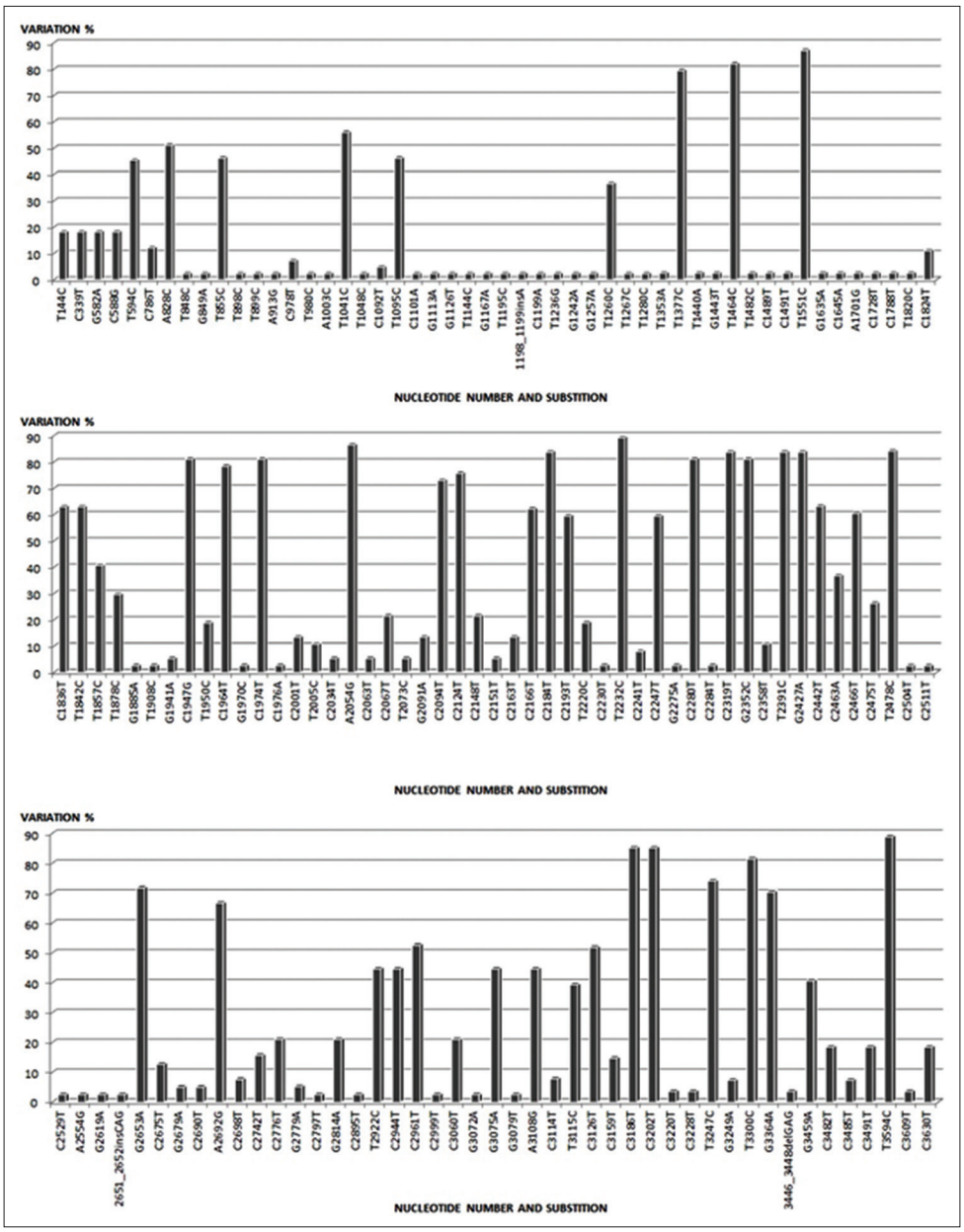

FIGURE 1. Nucleotide variations on ***pol*** gene. Y-axis shows the percentage of variation and X-axis shows the polymorphisms on ***pol*** gene with nucleotide positions.

TABLE 4. Precision and reproducibility data (on C_T and quantitation basis) were obtained by the analysis of the lowest standard (5×10^2 copies/mL)

CMV (500 copies/mL)	Measured quantity (copies/mL)	Standard deviation (IU/mL)	Coefficient of variation (%)	Threshold cycle (Ct)	Standard deviation (Ct)
Intra-assay variability, N=8	504.21	61.59	12.21	35.58	0.68
Inter-lot variability, N=3	500.67	23.56	4.70	34.79	0.30
Inter-operator variability, N=3	500.50	20.54	4.10	34.67	0.26
Total inter-assay variability, N=6	500.59	21.62	4.32	34.75	0.29

CMV: Cytomegalovirus

listed pathogens was observed which has further proven the specificity of the kit. In order to further evaluate the specificity of the test, specifically designed *gB* primers and probe were used. Infectivity of HCMV was previously reported to be enhanced by *gB* gene which was claimed to largely contain conserved nucleotide sequence and hence many studies have used this specific target region for the PCR-depending detection tests [16-18]. On the other hand, recent studies further demonstrated that *gB* region was more prone to polymorphisms than that were previously thought to be [19]. Hence, the design on the *gB* region was carefully done in this study on the most conserved part of this gene. Prior to the specificity test, a startup test was performed in order to obtain an initial value for the positive cut-off point which eventually

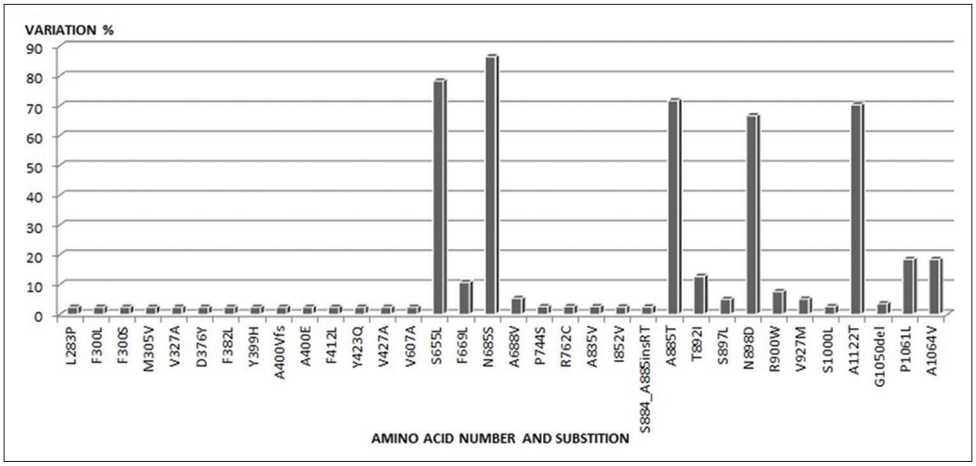

FIGURE 2. Amino acid variations on *pol* gene. Y-axis shows the percentage of variation and X-axis shows the polymorphisms on pol protein with amino acid positions.

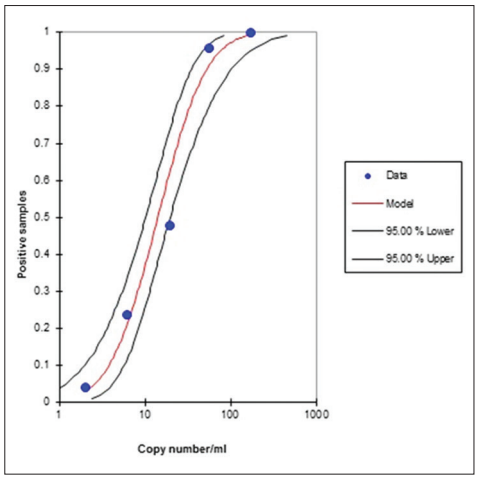

FIGURE 3. Result of probit analysis.

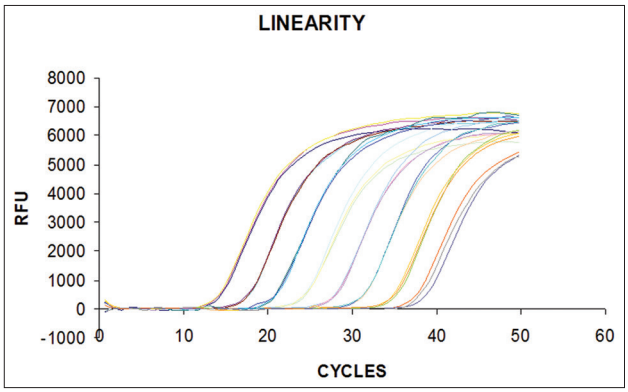

FIGURE 4. Determination of the linear range of real-time polymerase chain reaction. In order to assess the linear range, a dilution series of the highest DNA standard was analyzed by testing each dilution in duplicates.

highlighted the highest dilution giving a reproducible positive signal. Using this pre-determined value, dilution series (1200, 800, 120, and 40 copies/mL) were prepared and tested. The results of this experiment (Table 6) were subjected to statistical probit analysis using XLSTAT-Pro 7.5.3 + XLSTAT-Dose (www.xlstat.com, NY) which yielded in a 95% detection limit

TABLE 5. Result of cross-reactivity tests

Pathogen	CMV/FAM	Internal control Cy5
Parvovirus B19	-	+
TTV	-	+
HPV	-	+
EBV	-	+
HBV	-	+
HSV1	-	+
HSV2	-	+

TTV: Transfusion transmitted virus; HPV: Human papillomavirus; EBV: Epstein-Barr virus; HBV: Hepatitis B virus; HSV: Herpes simplex virus

TABLE 6. Determination of highest dilution that gives a reproducible positive signal

Copies/mL	Number of positive/ number of tested	% Positive
40	12	1
120	12	3
800	16	10
1200	14	14
1600 (standard 4)	23	23

of 1000 copies/mL (Figure 6). This data has further proven the upper hand sensitivity of *pol* region since the sensitivity of the test with *gB* primer-probe set was 1000 copies/mL while sensitivity of the test with *pol* primer-probe set was 100 copies/mL, hence *gB* region was only held for the determination of the specificity of *pol* region. In order to follow-up this aim, a multiplex qPCR was performed on randomly chosen HCMV positive samples which were used throughout the study. HCMV *pol* and *gB* real-time PCR amplifications were resulted in accordance which further supported the specificity of *pol* region (data not shown).

DISCUSSION

Real-time PCR technology has proven to be one of the major methods in the detection and quantification of pathogens. Compared to other methods qPCR introduces many

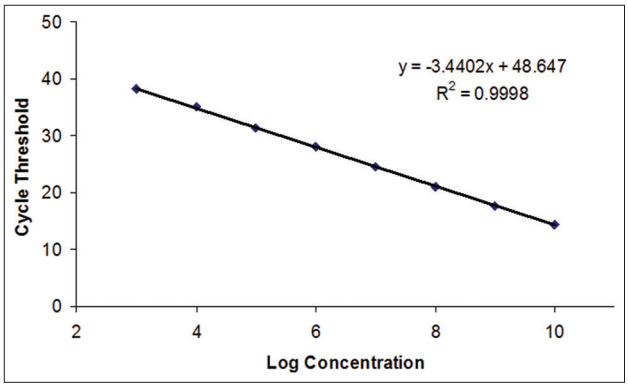

FIGURE 5. Correlation coefficient and regression line.

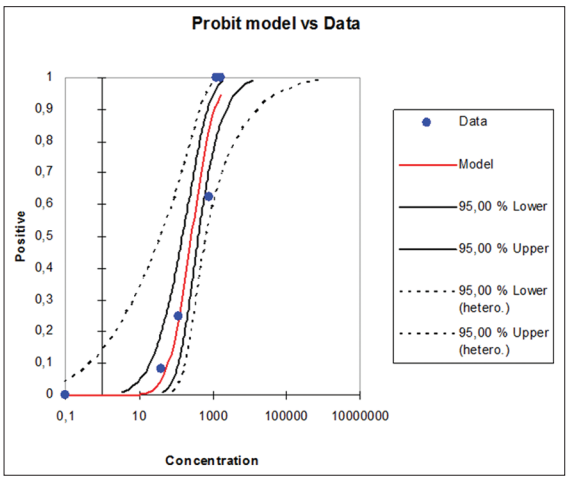

FIGURE 6. Result of probit analysis.

advantages like speed, sensitivity, cost efficiency and practicality. However, the test performance highly depends on the selection of the target region.

In this study we aimed to identify the most suitable target region for qPCR to quantify HCMV. Sequencing of the whole ***pol*** gene of HCMV using overlapping fragments revealed the candidate regions for primer-probe design. Sequencing results also reflected nucleotide changes which lead to amino acid heterogeneities in the ***pol*** gene of HCMV in Turkish patients. A set of primers and probes were designed on the most conserved region of the ***pol*** gene using the Jprimer3 (Iontek®, Turkey) Oligo Design Software. The set was verified using FDS real-time PCR system. Statistical probit analysis revealed a 95% detection limit of 100 copies/mL.

CONCLUSION

Comparison of the nucleotide and amino acid changes in 60 different HCMV isolates, along with the already known substitutions have led us to the most optimum target-specific primer-probe design that can be used to detect HCMV DNA at clinical samples using qPCR. The high reproducibility, precision, sensitivity, specificity and linearity of the assay has proved that it can be a good candidate as a molecular diagnostic tool for HCMV detection.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

REFERENCES

[1] Cha TA, Tom E, Kemble GW, Duke GM, Mocarski ES, Spaete RR. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol 1996;70:78-83.

[2] Chou SW. Acquisition of donor strains of cytomegalovirus by renal-transplant recipients. N Engl J Med 1986;314:1418-23. http://dx.doi.org/10.1056/NEJM198605293142205.

[3] 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;17:355-63. http://dx.doi.org/10.1002/rmv.544.

[4] Torres-Madriz G, Boucher HW. Immunocompromised hosts: Perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients. Clin Infect Dis 2008;47:702-11. http://dx.doi.org/10.1086/590934.

[5] Chrisp P, Clissold SP. Foscarnet. A review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs 1991;41:104-29. http://dx.doi.org/10.2165/00003495-199141010-00009.

[6] Lalezari JP. Cidofovir: A new therapy for cytomegalovirus retinitis. J Acquir Immune Defic Syndr Hum Retrovirol 1997;14:S22-6. http://dx.doi.org/10.1097/00042560-199700001-00005, http://dx.doi.org/10.1097/00042560-199700001-00006.

[7] Pescovitz MD, Rabkin J, Merion RM, Paya CV, Pirsch J, Freeman RB, et al. Valganciclovir results in improved oral absorption of ganciclovir in liver transplant recipients. Antimicrob Agents Chemother 2000;44:2811-5. http://dx.doi.org/10.1128/AAC.44.10.2811-2815.2000.

[8] Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Biron KK. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature 1992;358:162-4. http://dx.doi.org/10.1038/358162a0.

[9] Baldanti F, Underwood MR, Talarico CL, Simoncini L, Sarasini A, Biron KK, et al. The Cys607-->Tyr change in the UL97 phosphotransferase confers ganciclovir resistance to two human cytomegalovirus strains recovered from two immunocompromised patients. Antimicrob Agents Chemother 1998;42:444-6.

[10] Kotton CN. CMV: Prevention, diagnosis and therapy. Am J Transplant 2013;13:24-40. http://dx.doi.org/10.1111/ajt.12006.

[11] Zweygberg Wirgart B, Brytting M, Linde A, Wahren B, Grillner L. Sequence variation within three important cytomegalovirus gene regions in isolates from four different patient populations. J Clin Microbiol 1998;36:3662-9.

[12] Erice A. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev 1999;12:286-97.

[13] Weinberg A, Jabs DA, Chou S, Martin BK, Lurain NS, Forman MS, et al. Mutations conferring foscarnet resistance in a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis. J Infect Dis 2003;187:777-84. http://dx.doi.org/10.1086/368385.

[14] Gilbert C, Boivin G. Human cytomegalovirus resistance to antiviral drugs. Antimicrob Agents Chemother 2005;49:873-83. http://dx.doi.org/10.1128/AAC.49.3.873-883.2005.

[15] Chou S, Lurain NS, Thompson KD, Miner RC, Drew WL. Viral DNA polymerase mutations associated with drug resistance in human cytomegalovirus. J Infect Dis 2003;188:32-9. http://dx.doi.org/10.1086/375743.

[16] Chou SW, Dennison KM. Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding

neutralization-related epitopes. J Infect Dis 1991;163:1229-34. http://dx.doi.org/10.1093/infdis/163.6.1229.

[17] Fox JC, Griffiths PD, Emery VC. Quantification of human cytomegalovirus DNA using the polymerase chain reaction. J Gen Virol 1992;73:2405-8. http://dx.doi.org/10.1099/0022-1317-73-9-2405.

[18] Guiver M, Fox AJ, Mutton K, Mogulkoc N, Egan J. Evaluation of CMV viral load using TaqMan CMV quantitative PCR and comparison with CMV antigenemia in heart and lung transplant recipients. Transplantation 2001;71:1609-15. http://dx.doi.org/10.1097/00007890-200106150-00021.

[19] Novak Z, Chowdhury N, Ross SA, Pati SK, Fowler K, Boppana SB. Diagnostic consequences of cytomegalovirus glycoprotein B polymorphisms. J Clin Microbiol 2011;49:3033-5. http://dx.doi.org/10.1128/JCM.01039-11.