Comparison of real-time, and qualitative polymerase chain reaction assays in detection of cytomegalovirus DNA in clinical specimens

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ABSTRACT

Objectives: To compare the real-time (RT), and qualitative (Q) polymerase chain reaction (PCR) assays for detection of Cytomegalovirus (CMV) DNA.

Methods: The study took place in the Department of Microbiology, Erciyes University, Kayseri, and in Iontek Laboratory, Istanbul, Turkey, from August to December 2006. One hundred and seven clinical specimens from 67 patients were included in the study. Cytomegalovirus DNA was investigated using RT-PCR kit (Fluorion Iontek, Turkey) and Q-PCR kit (Fluorion Iontek, Turkey). Deoxyribonucleic acid sequencing was applied to the samples that yielded discrepant results in both assays. MacNemar's Chi Square test was used for statistical analysis.

Results: Of the specimens, 27 were found positive with both assays; 9 with only RT-PCR, and 11 with only Q-PCR assay. Both assays were found negative in 60 of the specimens. There was a good agreement between the 2 assays in 87 (81.3%) of the specimens. There was no statistical significant difference between the assays ($p>0.05$). Two of the 11 samples that RT-PCR negative Q-PCR positive, and 3 of the 9 samples that RT-PCR positive Q-PCR negative were found to be CMV DNA positive by DNA sequencing.

Conclusion: A good level of concordance between RT-PCR and Q-PCR assays for CMV DNA detection has been found.

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Cytomegalovirus (CMV) infections are common, and usually asymptomatic in healthy children and adults; however, the incidence and spectrum of the disease in newborns and in immunocompromized hosts establish this virus as an important human pathogen.[1,2] Serological tests, culture methods, CMV antigenemia test, and molecular methods are applied in the diagnosis of CMV infection. In the immunocompromized patients, diagnosis of CMV infection depends on the CMV viremia due to inadequate serological response, and lengthy culturing methods in which the virus is shown. The CMV antigenemia test and detection of CMV genome in blood indicate viremia.[3,4] The detection of CMV pp65 lower matrix protein in blood leukocytes allows a sensitive and specific detection of CMV. However, pp65 antigenemia has some important pitfalls since the test is completely done manually, requiring the immediate processing of specimens, technical skills, and training for the final reading of the assay.[5] The polymerase chain reaction (PCR) is more sensitive than pp65 antigenemia, however, the clinical value of qualitative (Q)-PCR data is less, as it does not discriminate latent CMV infection from replicating infection.[6-9] The level of CMV DNAemia plays a critical role in the pathogenesis of CMV disease. It is considered a major risk factor for the development of CMV disease,[10] and has been shown to predict CMV disease in AIDS[11] and renal transplant[12] patients. The real-time (RT)-PCR has greatly improved precision in DNA quantitation due to the fact that threshold cycle (C_T) values observed when PCR is still in phase is a more reliable measure than an endpoint measurement of the amplified PCR product.[13-15]

In this study, our aim was to compare RT-PCR and Q-PCR assays for detection of CMV DNA.

Methods. The clinical specimens were received by the Department of Clinical Microbiology, Laboratory of Virology, Erciyes University Medical Faculty, Kayseri, Turkey, for routine diagnosis of CMV infection. The total number of clinical specimens was 107. Ninety-seven sera, 4 stools, 3 cerebrospinal fluids, one bronchoalveolar lavage, one sputum, and one lung

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tissue. The clinical specimens were collected from 67 patients. Forty-three of the patients had malignancy, 12 of them were bone marrow transplant recipient, 8 of them were from kidney transplant recipient, 4 of them were newborn. The CMV DNA was examined with RT-PCR and Q-PCR assays. Viral DNA was extracted from serum using the QIAamp DNA minElute kit (Qiagen, Germany) and the DNA was extracted from other clinical specimens using QIAamp Mini Kit (Qiagen, Germany) according to manufacturer's instructions. The same DNA isolates were used for 2 PCR reactions.

RT-PCR. The RT-PCR based on Taqman chemistry was performed by Fluorion CMV QNP 2.1, real-time PCR Kit (Iontek, Turkey). A 224 bp region within the glycoprotein B (gB) gene is amplified. Ten microliters of extracted DNA were added to the plate containing 15.25 μL of the reaction mixture. A 0.25 μL internal control was added to the reaction mixture. The PCR was performed under the following conditions: after 13.30 minutes at 95°C, the samples were submitted 50 cycles, with each cycle consisting of a step at 95°C for 30 seconds, followed by step at 54°C for one minute and 30 seconds. Amplification and detection were performed by ICycler detection system (Biorad, USA). The quantification range of real time PCR was $5x10^3$-10^6 copy/mL. No amplification results were reported as <500 copy/mL. The results between 500-5000 copy/mL were reported as <5000 copy/ml. The results greater than $5x10^6$ copy/mL were reported as >$5x10^6$ copy/mL.

Q-PCR. The Q-PCR was performed by Fluorion CMV QL 1.0 kit (Iontek, Turkey) that contains the same primers as the Fluorion CMV QNP 2.1, real-time PCR kit from the CMV gB gene. The detection limit of the qualitative PCR is 1000 copy/mL. Ten microliters of extracted DNA were added to the plate containing 15 μL of the reaction mixture. The PCR was performed under the following conditions: after 13.30 minutes at 95°C, the samples were submitted 50 cycles, with each cycle consisting of a step at 95°C for 30 seconds, step at 54°C for one minute, followed by a step at 72°C for one minute. Amplification was performed by GeneAmp® PCR System 9700 (Applied Biosystems, USA). The amplification products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

DNA sequencing. The discrepant results obtained in 2 assays were confirmed by DNA sequencing at Iontek Laboratory in Istanbul, Turkey. A gB fragment was amplified, and concentrated products were sequenced directly with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, USA). The sequencing products were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Abi Prism, USA).

The results of both tests were analyzed using the MacNemar's Chi Square test.

Results. Cytomegalovirus DNA was investigated in 107 clinical specimens by RT-PCR and Q-PCR. Of the specimens, 27 were found positive with both assays; 9 with RT-PCR only, and 11 with Q-PCR assay only. Both assays were found negative in 60 of the specimens. There was a good agreement between both tests in 87 (81.3 %) of the specimens. Statistically, there was no significant difference between the results of the 2 PCR assays (Mc Nemar, $p>0.05$). The results of 2 PCR assays are shown in **Table 1**. Cytomegalovirus DNA was found positive in 36 of the specimens by RT-PCR; DNA level was found as 10^4 copy/mL in 2 of them, and <5000 in 34 of them. Amplification of internal controls was detected in all of the RT-PCR negative samples. Discrepant results were obtained from 20 serum samples. Two of the 11 serum samples that RT-PCR negative Q-PCR positive were found CMV DNA positive by DNA sequencing. Three of the 9 samples that RT-PCR positive qualitative PCR negative had a clearly readable chromatogram with identified CMV sequence. The sequencing results of the remaining the 6 samples were not good as the viral load of the samples were low.

Discussion. Cytomegalovirus causes asymptomatic infection in people with normal immune system, in immunocompromized patients like solid organ and bone marrow transplant recipients, and AIDS, also in infants after intrauterine infection, it causes serious morbidity and mortality.[1,2] Detection of CMV DNA is important in the diagnosis of CMV disease, however, the Q-PCR does not discriminate latent CMV infection from replicating infection.[6-9] During follow up for CMV infection, the Q-PCR provides cut off levels of CMV DNA as clinical markers to predict infection progress and identify high-risk patients requiring pre-emptive anti-CMV therapy.[5] The RT-PCR has improved accuracy and increased the linear range of the quantitation of PCR tests. It has also significantly decreased the turnaround time needed for amplification, detection, and quantification.[13,14]

Table 1 - The results of real-time polymerase chain reaction (PCR) and qualitative PCR assays to detect cytomegalovirus DNA in clinical specimens.

Results	Real-time PCR		Total
	Positive	Negative	
Qualitative PCR			
Positive	27	11	38
Negative	9	60	69
Total	**36**	**71**	**107**

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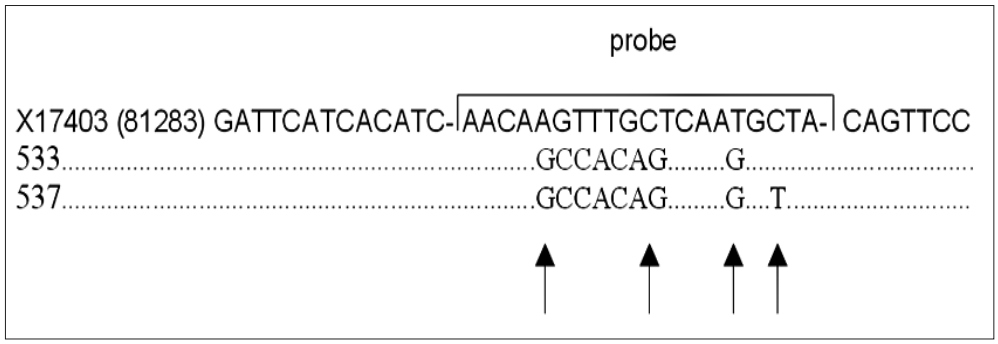

Figure 1 - Sequences of glycoprotein B region from 2 samples from the cytomegalovirus-infected patient compared to sequences in Genbank.

Wirgart et al[16] compared the RT-PCR (ReSSQ) and the Q-PCR assays for detection of CMV-DNA. The association between the Q-PCR and the ReSSQ CMV assay was in agreement for 90% of the samples. In another study, RT-PCR was found the most sensitive test detecting 92% of the CMV positive findings; Cobas Amplicor CMV Monitor detected 80%, pp65 test 88% of the positive findings.[17] Caliendo et al[18] compared the qualitative Amplicor CMV test and quantitative Cobas Amplicor CMV Monitor test, and found that the quantitative Cobas Amplicor CMV Monitor was more sensitive than the qualitative Amplicor assay.

In our study, there was a good agreement between the RT-PCR and the Q-PCR assays in 87 (81.3%) of the specimens. The targets of CMV PCR tests change. The DNA polymerase gene, gB, and major immediate early genes can be the target. The gB gene is overall, one of the less variable regions of the CMV genome and has been used as a target of PCR assays.[19,20] Schaade et al[21] reported a sequence variant (C630T) in the CMV gB gene that, although detectable in their real-time quantitative PCR assay, could not be accurately quantified. The C630T sequence variant in the CMV gB gene was also reported in other studies.[19,22] In addition to C630T sequence variant, Nye et al[22] found multiple mutations within the probe hybridization sites of RT-PCR that targeted CMV gB gene in 2 specimens. In the same study, the CMV DNA was detected in these 2 specimens with RT-PCR that targeted CMV DNA polymerase gene. Furthermore, discrepant results were obtained from 20 serum samples in this study. Two of the 11 serum samples that RT-PCR negative Q-PCR positive were found to be CMV DNA positive by DNA sequencing. In these samples, there were multiple mutations in the probe-binding region, which explains the negativity of the RT-PCR assay (**Figure 1**). We wanted to investigate the CMV DNA in these 2 samples by PCR that specify DNA polymerase gene, however, we could only study one sample as we have already used the other sample for the other tests. The sample was found to be CMV DNA positive with Cobas Amplicor CMV Monitor test.

Herrmann et al[23] designed a duplex quantitative RT-PCR assay to detect both DNA polymerase gene and the gB gene of CMV, and they found 2 obvious cases in which the detected copy number was considerably lower for gB than for polymerase gene. They found multiple mutations in the primer region of PCR that targeted gB gene in these 2 cases. Using more than one target to avoid false negatives due to rare or newly arising variants that escape detection during the initial validation should be considered.

In this study, 9 samples that RT-PCR positive Q-PCR negative indicates a higher sensitivity of RT-PCR assay. Three of them were confirmed as CMV DNA positive by DNA sequencing, however, the remaining 6 gave unsatisfactory results due to their low concentration. All of them had <5000copy/mL CMV DNA.

We conclude that a good level of concordance between the RT-PCR and the Q-PCR assays for CMV DNA detection has been found. However, DNA sequencing should be useful for confirmation of RT-PCR negative and Q-PCR positive results. These results should be verified on further studies with large number of specimens.

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