Comparison of hybrid capture and reverse transcriptase polymerase chain reaction methods in terms of diagnosing human cytomegalovirus infection in patients following hematopoietic stem cell transplantation

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ABSTRACT

Objectives: Human cytomegalovirus (CMV) is a life threatening cause of infection among hematopoietic stem cell recipients. Developing reliable methods in detecting the CMV infection is important to identify the patients at risk of CMV infection and disease. The aim of this study was to compare the 2 tests- hybrid capture test, which is routinely used in the diagnosis of CMV infection among hematopoietic stem cell recipients, and reverse transcriptase polymerase chain reaction (RT-PCR) detecting UL21.5 mRNA transcripts of the active virus.

Methods: In this prospective study, a total of 178 blood samples obtained from 35 patients following allogeneic hematopoietic stem cell transplantation at the Bone Marrow Transplantation Unit of the Hematology Department, Ibn-i Sina Hospital of Ankara University School of Medicine, Turkey between January 2003 and September 2003 were analyzed. Hybrid capture and RT-PCR using UL21.5 gene transcript method to investigate HCMV in blood samples were performed at the Department of Microbiology and Clinic Microbiology Ankara University School of Medicine, Turkey.

Results: When hybrid capture test was accepted as the golden standard, the sensitivity of RT-PCR was 33%, specificity 100%, false negativity 67%, false positivity 0%, positive predictive value 100%, negative predictive value 74%, and accuracy was 77%.

Conclusion: Improving this test by quantification, and application of additional gene transcripts, primarily the late gene transcripts can help increase the sensitivity and feasibility.

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The hematopoietic stem cell recipients become immunocompromised for a period that may last months. During this time the patients become vulnerable to different kinds of infections. Human cytomegalovirus (CMV) is one of the life threatening causes of infection in this group of patient.[1-3] The infection occurs usually by reactivation of the latent virus within 100 days following bone marrow transplant (BMT).[2-4] The infection rate is approximately 80% among the CMV-seropositive recipients, and 28%

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among the seronegative recipients with a seropositive donor.[4] The CMV pneumonia develops in 30-40% of the infected patients with a mortality rate of 50-92%.[5] Thus, developing more sensitive methods to detect the CMV infection before the onset of clinical findings is important in order to commence the appropriate 'pre-emptive' antiviral therapy at the right time.[6-8]

Monitoring the CMV infection is usually based on, viremia detection by various techniques namely; viral culture, immunocytochemistry or polymerase chain reaction (PCR). As cell culture methods are slow and bothersome, antigenemia test detecting the viral antigens on the cell surface or PCR and hybridization methods detecting the viral DNA are preferred for monitoring the CMV infection. One of the most widely used CMV DNA detection technique is the hybrid capture method. This quantitative approach aims to diagnose CMV viremia, quantifying the virus with PCR was also found to be superior to other methods. These viral DNA detection methods is not informative regarding the replicative status of the virus and the latent viral DNA being interpreted as positive can lead to problems.[8,9] The requirement of more reliable methods has led the researchers to develop techniques that directly detect the viral mRNA. Thus, differentiating the active virus that is capable of mRNA transcription from the latent virus is attempted.[10]

The aim of this study was to compare the results obtained with the hybrid capture and the reverse transcriptase polymerase chain reaction (RT-PCR) method and to evaluate their effect on the detection of CMV infection/disease.

Methods. ***Patients and blood sampling.*** A total of 178 blood samples obtained sequentially with weekly intervals from 35 patients following allogeneic hematopoietic stem cell transplantation (HSCT) at the Bone Marrow Transplantation Unit of the Hematology Department, Ibn-i Sina Hospital of Ankara University School of Medicine, Turkey between January 2003 and September 2003 were analyzed. The Ethical Committee of Ankara University School of Medicine approved the study and informed consent was taken from all patients included in the study. There were 18 female and 17 male patients; aged 19-47 years (mean: 35 years). The origin of stem cells was peripheral blood in 23 (65.7%) patients and bone marrow in 12 (34.3%) patients.

Diagnosis of patients were as follows: acute myeloblastic leukemia (n=17, 48.6%), chronic myelogenous leukemia (n=9, 25.7%), myelodysplastic syndrome (n=2), Hodgkin's disease (n=3), non-Hodgkin's lymphoma (n=1), renal cell carcinoma (n=1), multiple myeloma (n=1), and breast cancer (n=1) (**Table 1**).

Venous sampling was performed and 2 tubes containing 5 mL of EDTA blood were collected from every patient. One tube was used for "hybrid capture" test while the other was used for RT-PCR.

Analysis was performed in the same day at the Department of Microbiology and Clinic Microbiology Ankara University School of Medicine, Turkey.

Detection of CMV DNA - Hybrid capture method. This test is a nucleic acid hybridization method relying on detection of the chemiluminescence and it is applied in accordance with its manufacturers' instructions (Digene, Hybrid Capture Assay Version 2.0). Briefly, 3.5 ml of whole blood in a tube containing EDTA was used. After lysing the red cells, the leukocytes were pelleted. Cell pellets were denatured and then transferred to hybridization tubes. A CMV probe containing unlabeled RNA was added to the tubes, and the tubes were incubated for 2 hours at 70^{O}C. Then, they were transferred to capture tubes coated with anti-RNA-DNA antibodies and were incubated for 60 min with shaking at 1,100 rpm. An alkaline phosphatase-conjugated antibody specific for RNA-DNA hybrids was then added, and the tubes were incubated for 30 minutes at room temperature. After washing, the substrate was added, the chemiluminescence were detected by a luminometer and reported as relative light unit (RLU). The CMV DNA, expressed as picograms per milliliter (pg/ml), was quantified by comparing 3 reference positive standards in a calibration standard curve in accordance with the manufacturers' instructions.

During the interpretation of results, samples with "radiation value (RLU) $\geq$ the positive cut off value" are considered as positive. The RLU values lower than the positive cut off value while being higher than or equal to 75% of this value are considered as suspected positive and in this case the test was repeated one week later with another blood sampling belonging to the same patient. Samples with RLU levels lower than 75% of the positive cut off value were accepted as consisting of lower amounts of CMV DNA than the detection limit of the test or no CMV DNA at all and considered as negative.

The RT-PCR method. *Obtaining mononuclear cells*. Histopaque 1119 (Sigma) was used for obtaining the mononuclear cells to be used in RT-PCR method.

The RNA isolation. A kit (QIAMP Blood Mini Kit-Quiagen) able to isolate RNA from blood cells was used in order to isolate RNA from the mononuclear cells obtained from patients. This method depended on RNA binding to a silica gel membrane. During the process, cells were fragmented by lysis, the RNAses

in the medium were neutralized by denaturation and RNA was released along with other molecules. Consequently, ethanol was added to the medium in order to increase the binding affinity of RNA. The formed solution was then loaded to the spin colon and following centrifugation; the binding of RNA with the silica-gel structure in the tube was acquired. Contaminants were washed away from the membrane. The total RNA was dissolved into water (30 μl) not containing RNAse and stored at –80^{O}C.

Transcription of RNA to cDNA. The RNA obtained was transcripted into DNA by using reverse transcriptase enzyme. With this aim a laboratory kit (Revert AidTM First Strand cDNA Synthesis Kit-Fermentas) containing this enzyme and its buffer were used in accordance with the manufacturers' instructions. In this method, cDNA synthesis was commenced at the nonspecific spots over the RNA sequence by random hegzamer primers.

Confirming the existence of cDNA. In the samples obtained after treatment with reverse transcription, the existence of cDNA was confirmed with PCR by using glyceraldehyde-3-phosphate dehydrogenase primers (5'-TTC CCG TTC AGC TCA GGA TGA CCT TGC CC-3' and 5'-AAG GCT GAG AAC GAA GCT TGT CAT CAA T-3').[11] The reaction took place with the presence of 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U Taq polymerase (Fermentas) in the medium. Thermal cycling was adjusted as follows; at 94^{O}C for 5 minutes, then for 26 cycles at 94^{O}C for 30 seconds, then at 55^{O}C for 30 seconds, than at 72^{O}C for 60 seconds and finally at 72^{O}C for 10 minutes. The PCR product obtained was electrophoresed in agarose gel with 0.5 mg/ml ethidium bromide for the presence of 500 bp of amplicon.

Nested PCR. In this study, the target of nested PCR was a region belonging to one of the immediate early genes of CMV. This region started from the 27080th base of the unique long (UL) part of the virus genome and extended to the 27277th base. After this gene region was transcribed under intracellular conditions, it was removed out of the transcript with intron splicing, with a length of 83 bp. The length of this gene before splicing was 258 bp and the length of the spliced part was 175 bp. Nested PCR was used with external and internal primer sequences that could differentiate the spliced and non-spliced regions. According to this, the primer sequences were as follows:

SLG1: 5'-CTA TGG ATC TTG AGC TTA CT-3'
SLG2: 5'-TCG CTG CCA TCT CCG TCT GT-3'
SLG3: 5'-GTG ACC TTG ACG GTG GCT TT-3',
and
SLG4: 5'-CGT CAT ACT CCC CGG AGT AA-3'[12]

Reaction volume of both PCR phases were kept at 50 ml and the cycles were adjusted as follows; at 94^{O}C for 1 minute, at 55^{O}C for 2 minutes, and after 30 cycles at 72^{O}C for 3 minutes, and the final extension was at 72^{O}C for 7 minutes. Thermal cycles took place with the presence of 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U Taq polymerase (Fermentas) in the medium. At the end of the first phase, one μl of PCR product obtained was added to the reaction mixture of the second phase. Every sample was studied twice.

Electrophoresis. Two percent agarose gel was prepared for electrophoresis. After the gel took its solid form, it was placed in the electrophoresis tank and 15 μl DNA was mixed with loading buffer (6 X agarose gel loading dye, Amresco) and the mixtures were placed in wells. The gel was electrophoresed at 200 V for 10 minutes. Molecular weight marker (Fermentas Gene RulerTM 50 bp DNA labder) was loaded for comparison purposes. Sterile water was used for negative control. Staining of gel was performed according to its manufacturer's instructions (SYBR Green I Nucleic Acid Gel Stain, Roche, 1999). The stained gel was investigated under 254 nm ultraviolet transluminator (Vilber Lourmat). The RNA existence belonging to the target region was observed as a band at the point corresponding to 175 bp of length.

Statistics. Sensitivity was calculated as the proportion of the individuals with CMV infection who are correctly identified by the RT-PCR test. Specificity was calculated as the proportion of CMV infection free individuals that tested negative. Positive predictive value was calculated as the proportion of test positive individuals who actually have CMV infection. Similarly the negative predictive value is the proportion of patients with a negative result who are CMV infection free.[13]

Results. ***Patients.*** Before transplantation CMV immunoglobulin G (IgG) was positive and immunoglobulin M (IgM) was negative in all patients. In donors, all were IgM negative and all but one was IgG positive. Eight (22.9%) patients died during the course of the study. Six of them were CMV negative and 2 were positive. Stem cell resources of all of these 8 patients were the peripheral blood. Only 2 patients died from CMV infection. The causes of mortality of the other patients were graft versus host disease (GVHD), sepsis, thrombotic thrombocytopenic purpura (TTP), and recurrence of primary disease.

Results of Digene hybrid capture test. A total of 178 blood samples from 35 patients were studied. Among these samples, stem cell source was peripheral blood in 113 samples and bone marrow in the remaining

Table 1 - Diagnosis of patients undergoing hematopoietic stem cell transplantation.

Disease	Number of patients N=35 (%)
Acute myeloblastic leukemia	17 (48.6)
Chronic myelogenous leukemia	9 (25.7)
Myelodysplastic syndrome	2 (5.7)
Hodgkin's disease	3 (8.5)
Non-Hodgkin's lymphoma	1 (2.8)
Renal cell carcinoma	1 (2.8)
Multiple myeloma	1 (2.8)
Breast cancer	1 (2.8)

Table 2 - Frequency of cytomegalovirus infection according to the source of stem cell.

HCMV infection	Peripheral blood (%)	Bone marrow (%)	Total
HCMV infection (+)	7 (30.4)	5 (41.7)	**12**
HCMV infection (-)	16 (69.6)	7 (58.3)	**23**
Total	**23**	**12**	**35**

HCMV - human cytomegalovirus

Table 3 - Differences between duration of positive CMV results and peak virus levels in terms of the type of transplanted tissue.

Tissue values	Peripheral blood	Bone marrow
Mean duration of positive results (days)*	121.8	45.6
Mean peak viral load (pg/ml)	305.5	204

*$p<0.05$, CMV - cytomegalovirus

Table 4 - Relation between acute graft versus host disease (GVHD) and cytomegalovirus.

HCMV	Acute GVHD (+) (%)	Acute GVHD (-) (%)	Total
HCMV (+)	8 (66.7)	4 (33.3)	**12**
HCMV (-)	10 (43.4)	13 (56.6)	**23**
Total	**18 (52.9)**	**17 (47.1)**	**35**

HCMV - human cytomegalovirus

65 samples. In 12 patients out of 35 (34.3%) hybrid capture test revealed CMV infection in at least one of the blood samples. Number of positive samples ranged between 1 and 5 in every patient infected. When the patients were analyzed depending on the type of transplantation, rate of CMV infection was 30.4% (n=7) in 23 patients undergoing HSCT with the peripheral blood and 41.7% (n=5) in 12 patients undergoing HSCT with the bone marrow. Although the rate of infection was higher among patients in whom the stem cell source was the bone marrow, Fisher's Exact test revealed no statistically significant difference ($p>0.05$) (**Table 2**).

Preemptive ganciclovir treatment was given to these patients with viremia. Suspected positive results were seen in at least one sample in 8 of 23 patients in whom CMV infection was negative. A total of 12 patients were infected with CMV and the first positive results were seen on the 90th day of transplantation as an average (earliest 15 days and latest 220 days).

In HSCT patients, the duration between the transplantation and the first positive CMV result was analyzed with the aim of finding out whether there was any difference in terms of the source of stem cell and it was demonstrated that in 7 patients in whom the source was peripheral blood, the mean duration of positive results was 121.8 days (minimum 32 days and maximum 220 days) while in the remaining 5 patients in whom the source was bone marrow, this duration was 45.6 days (minimum 15 days and maximum 120 days). The CMV viremia occurred earlier in bone marrow recipients compared with peripheral blood ($p<0.05$). In all patients, virus titers ranged between 0 and 830 pg/ml. In patients infected with CMV, the peak virus titers were between 15-830 pg/ml with an average of 263.5 pg/ml.

When the relation between the source of stem cell and peak values of viral load were analyzed, mean peak value was 305.5 pg/ml in 7 patients in whom the source was peripheral blood and 204 pg/ml in 5 patients in whom the source was bone marrow ($p>0.05$) (**Table 3**). Among the 12 patients with positive CMV results, 2 patients were diagnosed with CMV disease (16.6%). Incidence of CMV disease was 5.71% overall. Pneumonia developed in both of these 2 patients. In one of these patients, clinical pneumonia, fever, and symptoms of gastroenteritis were observed and acute GVHD was diagnosed and the patient died at the 4th month. The other patient died at the 3rd post-transplantation month from GVHD and CMV pneumonia. The remaining 10 patients developed no clinical symptom of CMV disease. In the only patient whose donor was CMV seronegative, a total of 9 blood samples were taken; 2 of which showed suspected positive results and this

Table 5 - Characteristics of patients with human cytomegalovirus (HCMV) infection.

Patients	HCMV peak values (pg/ml)	HCMV disease	Death	Acute GVHD	RT-PCR (+)	Hybrid capture
1	42	-	-	-	+	+
2	383	-	-	+	-	+
3	149	-	-	+	-	+
4	18	-	-	-	-	+
5	830	+	+	+	+	+
6	20	-	-	-	-	+
7	697	+	+	+	-	+
8	29	-	-	+	+	+
9	830	-	-	+	+	+
10	121	-	-	-	-	+
11	28	-	-	-	-	+
12	15	-	-	+	-	+

GVHD - graft versus host disease, RT-PCR - reverse transcriptase polymerase chain reaction

Table 6 - Statistical evaluation of reverse transcriptase polymerase chain reaction.

Statistical evaluation	(%)
Sensitivity	(33)
Specificity	(100)
False negative	(67)
False positive	(0)
Positive predictive value	(100)
Negative predictive value	(74)
Accuracy	(77)

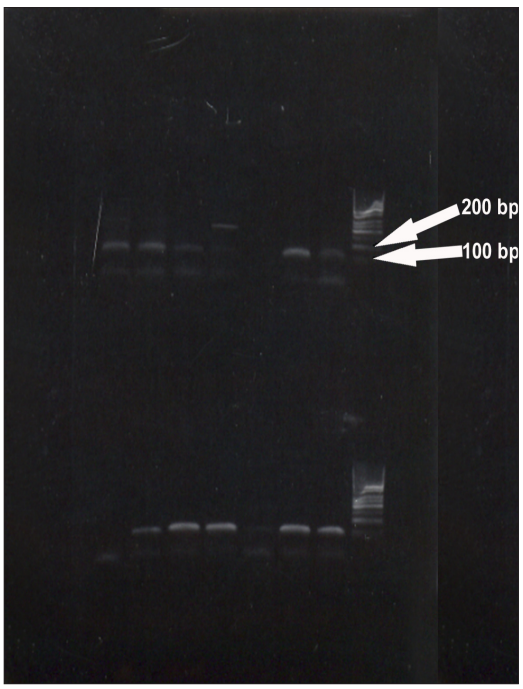

Figure 1 - The positive sample on gel electrophoresis.

patient showed no other pathological laboratory and clinical findings, or both.

Eighteen of 35 patients (52.9%) were diagnosed with acute GVHD. The rate of acute GVHD was 66.7% in 8 out of 12 patients in whom CMV infections were positive, and 43.4% in 10 out of 23 patients in whom CMV infection was negative. Although rate of GVHD was higher in the presence of CMV infection, there was no statistically significant difference ($p>0.05$) (**Table 4**).

Reverse transcriptase PCR results. A total of 178 blood samples belonging to 35 patients were analyzed with RT-PCR method. Ultimately, a total of 5 samples belonging to 4 patients were positive for CMV mRNA (**Figure 1**). All but one of these samples showed concurrent positive results on the hybrid capture test. This negative sample showed a positive hybrid capture test result in the following week (29 pg/ml).

Three patients showed positive results with both RT-PCR and Hybrid Capture methods in the same sample. One of these patients demonstrated clinical findings of CMV disease as well. On the day these samples were recorded as positive, the CMV DNA levels were 228 and 1550 pg/ml. The other 2 patients with positive results showed no pathological clinical findings. With Hybrid Capture method, viral load of the samples taken from the patients were 383 and 830 pg/ml (**Table 5**).

The traditional "gold standard" method for CMV infection detecting is the isolation of virus in cell culture, which may require 4 weeks or more. Therefore, newer, more rapid, and more sensitive assays, such as the antigenemia assay, molecular amplification assays, and DNA hybridization assays,

have been developed and accepted as gold standards in different laboratories.[14-17] In the context of the present work, we used Hybrid Capture as the golden standard method for CMV detection (the routine method that is used for the clinical management), and consequently CMV infection definition. When hybrid capture test was accepted as the golden standard, the sensitivity of RT-PCR was 33%, specificity 100%, false negativity 67%, false positivity 0%, positive predictive value (PPV) 100%, negative predictive value (NPV) 74%, and accuracy was 77% **(Table 6)**.

Discussion. Human cytomegalovirus disease is an important cause of morbidity and mortality in patients undergoing HSCT. Most centers worldwide have reported the rate of CMV infection as 32-70% in seropositive patients.[10,18] Developing CMV disease being is reported in 5% of seronegative patients with seropositive donors, 14% of seropositive patients with seronegative donors, and 12% of seropositive patients with seropositive donors.[19] In diagnosis of CMV infection, the most important finding is demonstration of the virus in blood. The consequent preemptive treatment decreases the rate of CMV disease from 80% to 10-15%.[10,20]

Today, the rapid and sensitive molecular diagnostic methods contribute to the monitorization of CMV infection. However, most of the viremic patients does not develop CMV disease. Thus, methods capable of differentiating active and latent virus are still being investigated. This search has drawn the attention of researchers to detect the mRNA transcripts of the replicating virus.[12] The mRNA transcripts of the virus can be synthesized to cDNA and detected by molecular biology methods. The disadvantage of this method is the possible false positive results, as cDNA is synthesized from the mRNA transcripts of the CMV genome that is in DNA structure, but as the sequence of the mRNA transcript is the same with the original genome, possible DNA contamination of the samples can lead to false positive results.[10,21] In some CMV genes the first mRNA are further spliced and a shorter transcript is formed. This spliced mRNA is shorter from both the first synthesized mRNA and the original DNA. Detecting these transcripts in order to monitor viral replication might help to overcome the possibility of DNA contamination.[12]

To date, several gene transcripts including immediate early (IE), major capsid protein (MCP), UL83, ppUL32, and pp67 have been used to detect CMV infection in patients with immunosuppression.[22] However, studies performed over patients undergoing HSCT are still limited, sometimes with conflicting results. We investigated the presence of UL21.5 gene transcript in the blood samples of such patients. This transcript is chosen as it is a late gene specific for CMV replication and as it was reported to determine the progression of CMV disease.[12] The UL21.5 gene expresses a protein also named as R27080 and although the functions of R27080 are not precisely known, it is believed to be a virokine regulating the immune response.[23] At the late phase of infection, during transcription, mRNA and protein of UL21.5 were reported as being produced in excess amounts in vivo. Thus, this protein is believed to be an appropriate target in the detection of active virus.[24,25]

As the rate of CMV infection is high in patients undergoing allogeneic HSCT, the study is performed in this patient group. The high CMV seropositivity rate in recipients (100%) and donors (97%) included in the study has been repeatedly demonstrated by studies from Turkey.[26,27] In the surveillance studies of Peggs et al[28] in England and D'Agaro et al[29] in Italy, seropositivity of CMV infection among patients undergoing allogeneic HSCT was found to be 51% and 76%.

In our study, 12 of 35 patients (34.3%) were infected with CMV. Following HSCT, CMV infection rate ranges between 32% and 70%.[5,10,28-30] The relative decrease in our rates and the diversity of rates reported in various studies can be due to, clinic characteristics of patients, different treatment protocols applied, and other risk factors associated with CMV infection.[31-36]

Cytomegalovirus viremia does not always progress to CMV disease in many patients and viremia can remain subtle depending on the immune status of the host or the treatment protocol applied.[18] In accordance with this, in our study only 2 of the 12 (16.6%) patients who were infected with CMV progressed to CMV disease and overall incidence of this disease was 5.71%. In general, rate of CMV disease in HSCT recipients with CMV infection was reported as 8.8-18.7%.[5,10,28,30,37,38] Our rate of CMV disease (16.6%) was in accordance with these studies. There are many studies on a positive or no relationship between CMV infection and GVHD.[29,30,37] We could not determine a significant relation between the presence of CMV infection and development of GVHD in our patient population ($p>0.05$).

Today, peripheral stem cells are more frequently used in allogeneic stem cell transplantation. This type of transplantation carries the advantages of rapid neutrophil and thrombocyte engraftment and higher levels of cytotoxic and helper T cells.[39] However, there are thoughts regarding the peripheral blood being used as the source of stem cells theoretically increasing the risk of CMV infection. Although abundant amounts of leucocytes present in the peripheral blood of the

seropositive donor and given to the patient is said to increase the risk of infection, there are also too many pass-by among the effector T cells of the donor and recipient, which may contribute to the defense system of the host.[40] In our study, the rate of CMV infection in recipients of bone marrow compared with peripheral blood occurred slightly higher (41.7% versus (vs) 30.4%, $p>0.05$) and earlier (45.6 days vs 121.8 days, $p<0.05$). Trenschel et al[41] reported that the mean time of the CMV infection onset in patients following bone marrow and peripheral blood transplantation was 47 days and 39 days. Maeda et al[39] reported no difference between the sources of stem cells (60 days). In our study the mean time in patients in whom bone marrow is the source was 45.6 days and this value is in accordance with the published data. However, the mean time of patients in whom peripheral blood is the source being 121.8 days is definitely a later period than reported in the literature. This can be explained by differences in the diagnosis, characteristics, and treatment protocols.

The reasons for our targeting mRNA in mononuclear cells, are that CMV replication takes place in these cells and the common belief of infected monocytes carrying risks of CMV infection and GVHD.[42,43] In our study a total of 35 patients were analyzed and 12 of them showed positive results in terms of CMV infection when analyzed with the Hybrid Capture method while only 4 with the RT-PCR method. These 4 patients that showed positive results with the RT-PCR method had concurrent positive results on the hybrid capture test. Only 2 of 12 patients with CMV infection progressed into CMV disease and only one of these cases was detected with RT-PCR. Although the sensitivity of RT-PCR in our study (33%) was lower than the one reported by Boriskin et al (100%), our specificity (100%), PPV (100%), and NPV (74%) were in accordance with the published data.[8,44] As a result, CMV continues to be an important viral agent in patients undergoing HSCT. Hybrid capture test is a common method used with diagnostic aim. However, more rapid and reliable methods are still required. We used UL21.5 gene transcript test and believe that this is a possible alternative with high specificity and PPV. However, its low sensitivity limits the widespread use of this test. Improving this test, making it able to quantify results, and experimenting with various gene transcripts, primarily the late gene transcripts can help this method to become more feasible. Thus, further studies are needed.

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