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Can the HCMV-pp65-antigenemia assay completely be replaced by HCMV-PCR in monitoring patients after bone marrow transplantation?

Paul R. Lübeck[1], Annemarie Berger[1], Hans Wilhelm Doerr[1], Holger F. Rabenau[1*]

[1]Institute of Medical Virology, Hospital of the Johann Wolfgang Goethe University of Frankfurt, Paul-Ehrlich-Str. 40, 60596 Frankfurt, Germany

***Corresponding author: Rabenau@em.uni-frankfurt.de**

Abstract

Background: Human cytomegalovirus (HCMV) is the most frequent viral pathogen in bone marrow transplant (BMT) patients, where it causes significant morbidity and mortality. The results of the two most frequently used laboratory assays for the detection of active HCMV infection, the pp65-antigenemia assay and the polymerase chain reaction (PCR), often differ in BMT patients. Within the context of limited financial resources it is necessary to economize routine laboratory diagnostic procedures. To evaluate if the pp65-antigenemia assay can be replaced by HCMV-PCR we retrospectively assessed 988 samples from 253 BMT patients without additional information on the patient's clinical presentation or therapy.

Material and Methods: The results of both assay (a commercially available pp65-antigen assay and an in-house quantitative real time HCMV-PCR) were classified into four different quantitative groups: PCR group (PCR-g) 0 = PCR neg. (<200 copies/ml); PCR-g 1 = 200-999 copies/ml; PCR-g 2 = 1,000- 9,999 copies/ml; PCR-g 3 = >9,999 copies/ml; pp65-antigen group (pp65-g) 0 = 0 positive cells/400,000 leucocytes; pp65-g 1 = 1-3 pos. cells; pp65-g 2 = 4-99 pos. cells and pp65-g 3 = >99 pos. cells.

Results: The mean value of PCR copies rises with the number of pp65-antigen (pp65-Ag) positive cells in the antigenemia assay. However, in the pp65-g 0 the mean value and the range of the PCR copies are higher than in the pp65-g 1 (pp65-g 0: 1,188 copies, range: 0-200,000 copies; pp65-g 1: 554 copies range: 0-12,900). There is a statistically significant correlation between the results of the pp65-Ag-assay and quantitative PCR (spearman-correlation: cumulative: 0.49, $p = 0.01$; pp65-g 2: 0.38, $p= 0.01$; pp65-g 3: 0.44, $p = 0.01$). Regarding PCR groups, there is no correlation with the results of the quantitative antigenemia assay. Using any positive PCR result as the reference, the pp65-Ag-assay showed 59% / 77% / 60% / 76% for sensitivity / specificity/ positive predictive value / negative predictive value, respectively and a positive likelihood ratio of 2.6. These values rose with the number of PCR copies and pp65-positive cells. A highly positive result (pp65-g 3 (>99 cells) or PCR-g 3 (> 9,999 copies/ml)) of either test almost certainly excludes the possibility of a negative result of the other test.

Conclusion: When the pp65-Ag result is greater than 3 positive cells per 400,000 leucocytes, the pp65-Ag-assay and the plasma PCR parallel each other. However, highly positive PCR results can be obtained in pp65-Ag-assay negative samples. Our results suggest that both assays complement each other and should be used concomitant.

Keywords: Cytomegalovirus, HCMV, pp65, antigenemia, PCR

Introduction

Human cytomegalovirus (HCMV) is ubiquitous and the seroprevalence rate is 40-100% worldwide [1]. By using a variety of immune escape mechanisms it establishes latent infection, from which it is able to reactivate [2, 3, 4, 5, 6]. Bone marrow transplant (BMT) patients are at particular risk of HCMV infection since their immune system is completely suppressed iatrogenically. HCMV is the most frequent viral pathogen in BMT patients and causes significant morbidity and mortality [7, 8]. Primary infections are both more severe and more often symptomatic than secondary infections. However, BMT patients mostly suffer from HCMV reactivation rather than infection via donor material [9]. Practically all organs can be affected by HCMV infection. Additionally, HCMV infection causes a direct immunosuppression [10, 11] favoring the outbreak of opportunistic infections as caused by bacteria, fungi and protozoa [7]. As a result of the great susceptibility to HCMV infection and the severity of the disease, BMT patients require strict diagnostic monitoring in close intervals especially because the disease proceeds very quickly, occasionally within a week [12]. Therefore the sensitivity of the laboratory method used is of particular importance. The formerly used culture-based assays have been replaced by direct antigen (e.g. pp65-antigen (pp65-Ag)) and DNA detection methods, which are less time consu-

Table 1. Comparison of quantitative pp65-antigenemia assay and quantitative HCMV-PCR in BMT patients. The results of both tests are classified into the previously described groups.

pp65-group	PCR-copies/ml range	Mean: PCR copies	PCR group 0 0 copies/ml	PCR group 1 200-999 copies/ml	PCR group 2 1000- 9,999 copies/ml	PCR group 3 >9,999 copies/ml
0 (0 pos. cells)	0-200,000 (n=627)	1,188	76% (n=479)	14% (n=85)	9% (n=55)	1% (n=8)
1 (1-3 pos. cells)	0-12,900 (n=134)	554	74% (n=99)	15% (n=20)	10% (n=13)	2% (n=2)
2 (4-99 pos. cells)	0-200,000 (n=168)	7,498	27% (n=45)	27% (n=45)	33% (n=56)	13% (n=22)
3 (>99 pos. cells)	300-200,000 (n=59)	25,885	0% (n=0)	5% (n=3)	58% (n=34)	37% (n=22)

ming and more sensitive [13, 14, 15, 16]. Polymorphonuclear leukocytes (PMN), plasma and whole blood can be used as a sample for the HCMV-PCR. While whole blood testing might detect latent HCMV-DNA, the HCMV structural tegument protein pp65 is a sign of active infection and can be used as a marker of pathogenicity [17]. Quantification of HCMV-antigen or DNA has several indications. It is used to identify patients at high risk for disease and decide whether to start antiviral treatment or not. The result of a quantitative test has to be interpreted in consideration of the patient's underlying disease, e.g. the average viral load and the average number of pp65-Ag positive cells in patients after solid organ transplantation are significantly higher than the corresponding results in BMT patients [18]. Furthermore a higher level of HCMV-antigenemia has a higher positive predictive value for disease [19]. However, the significant threshold for predicting disease differs among patient groups. Since pp65-antigenemia quantitatively decreases when antiviral treatment is administered it can also be used for therapy monitoring [20].

The objective of this study was answer the question if one of both tests (quantitative detection of pp65-antigen or HCMV-PCR) can be replaced or substituted. Within the context of limited financial resources this is of major importance. In the daily routine of laboratory HCMV diagnostic tests there is no demographic or clinical data of patients and their antiviral therapy available in the majority of cases. Therefore we studied 988 samples from 253 patients after bone marrow transplantation retrospectively without additional information on patient's clinical presentation or therapy and assessed the performance of both tests.

Material and methods

Patients and Samples

Samples used in this study were collected for routine diagnostic performed at the Institute for Medical Virology, Goethe University Hospital Frankfurt am Main, Germany. A total of 988 samples from 253 patients after BMT were obtained between 01.02.2003 and 01.08.2008. There was no information available on the timepoint of BMT or the use of anti-HCMV therapy. The tests were mostly performed from one sample. If both assays were not performed at the same day, we compared the results from samples obtained within a maximum interval of 4 days. To ascertain reliable results, samples with low leucocytes counts (< 200,000 cells) were excluded from the pp65-Ag assay and therefore from the retrospective analysis.

Detection of HCMV using pp65-antigenemia assay

Two ml of EDTA or citrate whole blood were processed according to the instructions of the manufacturer (CINAkit; VIVA Diagnostik, Hurth, Germany). One glass slide with two wells per patient was prepared by adding 100 µl of suspended leukocytes, corresponding to 200,000 cells to each well. After drying, one drop of anti-HCMV-pp65-specific 1C3+AYM-1 monoclonal antibodies was added, followed by an incubation period (30 min, 37°C), washing steps and the addition of a secondary fluorescence-marked-antibody. In a separate slide a negative control (not infected neuroblastoma cell culture) as well as a positive control (HCMV strain AD169 infected neuroblastoma cells) was prepared analogous. Using a fluorescence microscope at 400 times magnification two wells per patient were examined. Positive pp65-Ag results for HCMV infection showed apple green fluorescence. Unspecific results were confirmed with the control-slide. The detection of a single colored cell was considered a positive pp65-Ag-assay result. Results were specified as the number of pp65-Ag-positive leucocytes per 400,000 cells.

Detection of HCMV using quantitative PCR

DNA was extracted from 200 µl EDTA plasma using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the instructions of the manufacturer. HCMV-DNA was quantified on 10 µl of the extracted DNA using the ABI PRISM 7900 (PE Applied Biosystems, Weiterstadt, Germany) as previously described [21, 22, 23]. The dynamic range for quantification is approximately 3 $\log_{10}$, with 200 copies/ml as the lower and 200,000 copies/ml as the upper limit of detection. As an internal control a defined amount of murine CMV virus was used for the measurement of the efficiency of extraction and amplification [21, 22, 23]. After an initial heating of 95° C for 15 min, 40 cycles consisting of denaturation (15 sec at 94° C) and annealing (1 min at 60° C) were run. The signals detected by the ABI PRISM 7900 were analyzed using SDS-software. Quantification of results was performed using an external standard curve.

Classification of quantitative results

The results of both tests are classified into four quantitative groups. Group 0 represents negative results, group 1 slightly positive results, group 2 moderately positive results and group 3 highly positive results. The results of the quantitative antigenemia (pp65) and quantitative PCR are expressed as the number of HCMV positive cells / 400,000 leucocytes and the number of HCMV copies per milliliter, respectively. In detail: PCR group (PCR-g) 0 = PCR neg. (<200 DNA copies/ml); PCR-g 1 = 200-999 copies/ml; PCR-g 2 = 1,000-9,999 copies/ml; PCR-g 3 = >9,999 copies/ml; pp65-Antigen group (pp65-g) 0 = 0 positive cells; pp65-g 1 = 1-3 pos. cells; pp65-g 2 = 4-99 positive cells and pp65-g 3 = >99 positive cells/400,000 leucocytes.

Statistical analysis

Group-specific mean, standard deviations of the mean and spearman correlations were calculated using the program SPSS Statistics 17.0 (SPSS inc., Chicago, Illinois, USA).

Table 2. Comparison of pp65-antigenemia and quantitative HCMV-PCR. Mean, standard deviation and spearman-correlation for different groups. * significant correlation, p<0.01.

pp65-Ag-pos. cells	Mean	Standard deviation	Spearman-correlation	Standard deviation	Mean	PCR copies/ml	n
0	0	0	-	11,258	1,188		627
1-3	2	1	0.07	1,873	554		134
4-99	25	23	0.38*	24,589	7,498		168
>99	339	245	0.44*	50,460	25,885		59
	1	5	-	0	0	0	623
	9	20	0,05	229	399	200-999	153
	71	143	-0.01	2,218	3,472	1,000-9,999	158
	205	277	0.13	62,854	55,489	>9,999	54
cumulative	25	100	0.49*	19,236	3,650	cumulative	988

95%-confidence intervals [CI] for proportions and sensitivities, specificities, positive and negative predictive values and positive likelihood ratios were calculated using the program BiAS für Windows 9.01 (epsilon Verlag, Hochheim, Darmstadt, Germany 2007).

Results

A total of 988 samples from 253 patients after BMT were tested with quantitative HCMV-PCR and quantitative pp65-Ag-assay. The results of both tests were classified into four groups (Tab. 1). 479 (48.5%) samples were negative in the pp65-Ag-assay and below the detection limit in the HCMV-PCR. 148 (15.0%) samples were negative in the pp65-Ag-assay but PCR positive (PCR-g 1: n = 85; PCR-g 2: n = 55; PCR-g 3: n = 8), while 144 (14.6%) samples were positive in the pp65-Ag-assay but HCMV-PCR negative and 217 (22.0%) samples were positive in both tests. When the pp65-Ag-assay result was negative (pp65-g 0), 76.4% (n = 479) of the results of the PCR were negative as well (Tab. 1). Nevertheless, 13.6% (n = 85) of the PCR results were slightly positive (PCR-g 1), 8.8% (n = 55) were moderately positive (PCR-g 2) and 1.3% (n = 8) were highly positive (PCR-g 3). The mean of PCR copies in this group was 1,188 copies/ml and the range was 0-200,000 copies/ml. When the pp65-Ag-assay result was slightly positive (pp65-g 1), 73.9% of the results of the PCR were negative (PCR-g 0; n = 99), 14.9% were slightly positive (PCR-g 1; n = 20), 9.7% were moderately positive (PCR-g 2; n = 13) and 1.5% were highly positive (PCR-g 3; n = 2). The mean of PCR copies was 554/ml and the range was 0-12,900 copies/ml. Table 1 shows, that the mean value of HCMV-PCR copies/ml and the number of the pp65-Ag-group show a parallel rising. However, in the pp65-g 0 the mean value of the PCR copies is higher than in the pp65-g 1 (pp65-g 0: 1,188 copies/ml; pp65-g 1: 554 copies/ml). The standard deviation and the range of PCR copies show similar characteristics (pp65-g 0: PCR range: 0-200,000 copies/ml, standard deviation of mean: 11,258 copies/ml; pp65-g 1: PCR range: 0-12,900 copies/ml, standard deviation of mean: 1,873 copies/ml). Furthermore, a higher grading in the pp65 group correlates with a higher grading in the HCMV-PCR group.

Pp65-antigenemia and quantitative PCR (Tab. 2; p = 0.01) are statistically significant correlated. Samples of the pp65 groups 2 and 3 also positively correlated with the quantitative HCMV-PCR (Tab. 2; p = 0.01). In contrast, regarding PCR groups, there is no significant correlation with the results of the quantitative pp65-Ag-assay. Using results of the plasma HCMV-PCR and pp65-Ag-assay, respectively, as the reference, the relative sensitivities, negative predictive values and the likelihood ratios both rose with the number of PCR copies/ml and pp65-Ag-positive cells (Tab. 3). A highly positive result (pp65-g 3 (>99 positive cells) or PCR–g 3 (> 9,999 copies/ml)) of either test almost certainly excludes the possibility of a negative result of the other test.

Discussion

HCMV is still the most frequent viral pathogen in BMT patients and causes significant morbidity and mortality [7, 8]. The quantification of the viral load is frequently performed for general diagnosis and therapy monitoring [24, 25]. The objective of this study was to compare the two mostly used laboratory methods in quantitative HCMV diagnostic, pp65-Ag-assay and HCMV-PCR, in BMT patients and clarify if one of these tests is obsolete in certain situations of the daily routine of laboratory HCMV diagnostic, that is to say regardless of the availability of clinical data on patients or their antiviral therapy. Preemptive therapy based on pp65-Ag detection is associated with a reduction in the incidence of CMV disease in allogeneic stem cell transplantation (SCT) recipients [26]. This kind of standard procedure for the prevention of HCMV disease minimizes antiviral therapy side effects and the risk of late-onset HCMV disease [27, 28]. Pre-emptive therapy should be administered once more than one or two pp65-Ag positive cells in 200,000 leucocytes are detectable [29]. The major advantages of the pp65-Ag testing are the short processing time (less than 6h) and the lack of requirement of a highly specialized laboratory. Disadvantages are the need of immediate processing (<6 – 8 h) and the, since it cannot be automated, time-consuming slide interpretation, which also adds a subjective component. The use of the flow cytometry assay, however, seems adequate for the detection of pp65- antigen and avoids the manual slide testing [30]. In order to obtain a significant pp65-Ag result, a sufficient number of granulocytes are required. Therefore, the test may be unreliable when the leucocytes count is low, e.g. early after bone marrow or SCT or during a period of severe neutropenia [12]. In previous studies real-time HCMV-PCR appeared to be more sensitive than immunofluorescence [31, 32, 33]. To increase the sensitivity of the pp65 antigenemia test we examined 400,000 leucocytes instead of 200,000 cells, which is more common. Analogous to the pp65-Ag-assay the quantitative PCR is highly predictive for HCMV disease in BMT patients [34]. Both plasma and whole blood samples [35] can be used as a source of viral DNA. Since HCMV can be found latently in monocytes, we used plasma analogous to the procedure for the detection of HIV [36]. The viral load is associated with disease development [35]. Unlike the pp65-Ag-assay it can be used during periods of severe cytopenia as well [37]. Our data showed that the range and the mean of the viral load were both higher in patients with a negative pp65-Ag-assay result than in patients with a

Table 3. Relative sensitivities, specificities, positive and negative predictive values and positive likelihood-ratio for different HCMV-PCR and pp65-Ag reference groups. The numbers in brackets indicate 95% confidence intervals. Values of the positive likelihood-ratio indicate the probability of a positive test result in infected compared to non-infected. Values >10 or <0.1 are considered very well and values >3 and <0.3 are considered good, all other values are considered bad.

	Performance of non-reference test				
Reference	Sensitivity	Specificity	Pos. predictive value	Neg. predictive value	Positive likelihood-ratio
PCR 200-999 copies/ml	44% (36.4- 52.7%)	77% (73.4- 80.1%)	32% (25.9- 38.8%)	85% (81.7- 87.8%)	1.9 (1.5 – 2.4)
PCR 1,000 – 9,999 copies/ml	65% (57.2- 72.6%)	77% (73.4- 80.1%)	42% (35.5- 48.1%)	90% (86.8- 92.2%)	2.8 (2.4 – 3.4)
PCR > 9,999 copies/ml	85% (72.9- 93.4%)	77% (73.4- 80.1%)	24% (18.3- 30.9%)	98% (96.8- 99.3%)	3.7 (3.1 – 4.4)
pp65-Ag 1-3 pos. cells	26% (18.9- 34.4%)	76% (72.9- 79.7%)	19% (13.7- 25.6%)	83% (79.6- 85.9%)	1.1 (0.8 – 1.5)
pp65-Ag 4-99 pos. cells	73% (65.9- 79.7%)	76% (72.9- 79.7%)	45% (39.4- 51.5%)	91% (88.7- 93.7%)	3.1 (2.6 – 3.7)
pp65-Ag > 99 pos. cells	100% (93.9- 100%)	76% (72.9- 79.7%)	29% (22.5- 35.2%)	100% (99.3- 100%)	4.2 (3.7 – 4.9)
PCR total	59% (54.2- 64.5%)	77% (73.4- 80.1%)	60% (54.9- 65.2%)	76% (72.9- 79.7%)	2.6 (2.1 – 3.0)
pp65-Ag total	60% (54.9- 65.2%)	76% (72.9- 79.7%)	59% (54.2- 64.5%)	77% (73.4- 80.1%)	2.6 (2.2 – 3.0)

slightly positive pp65-Ag result (Tab. 1). To ascertain reliable results, samples with low leucocytes counts were excluded. A defect in leucocytes avoiding virus replication could be one possible explanation for the observed high viral load in pp65-Ag negative patients [38]. In this case viremia could be caused by organs instead of leucocytes [18]. Furthermore it must be pointed out that two different compartments were examined. The plasma HC CR detects MV released into the blood plasma during active replication, while the pp65-Ag-assay spots an intraleucocytic protein. It has been shown that HCMV replication in vivo is a highly dynamic process with a doubling time of approximately one day [39]. To assess the sensitivity, specificity, positive and negative predictive values and the positive likelihood ratio, we defined any positive result of the reference test as a positive result. This was performed for the pp65-Ag- and PCR-groups 1, 2 and 3 separately and for all groups together. Performing a qualitative comparison, which means using any positive HCMV-PCR result as the reference, our pp65-Ag-assay showed a sensitivity of 59% and specificity of 77%. The positive and negative predictive values were 60% and 76%, respectively. The positive likelihood ratio was 2.57. Yakushiji et al. found a sensitivity of 55.4% and a specificity of 95.5% for the pp65-Ag-assay [40]. Using plasma PCR as reference, Boeckh et al. obtained the following results for the pp65-Ag-assay: 62% / 88% / 45% / 94% for sensitivity / specificity / positive predictive value / negative predictive value [37]. While the sensitivity of the pp65-Ag-assay was about 60% in all three studies, the specificity in our study was much lower. This might be due to the fact that in 14.6% of our samples the pp65-Ag-assay was positive while the PCR was negative. This is an unexpected observation, since the PCR is usually reported to me more sensitive than the pp65-Ag-assay [31, 32]. However, it can be interpreted as a success of our attempt to increase the sensitivity of the pp65-Ag-assay by examining 400,000 leucocytes instead of 200,000. Also, 68.8% of these samples were only slightly positive (pp65-g 1) and none was highly positive in the pp65-Ag-assay. A latent infection could occur without the release of virions into the plasma. In this case no HCMV-DNA copies would be detectable in plasma samples. Boeckh et al. assume that signs of CMV reactivation can be first detected in polymorphonuclear cells due to phagocytosis of virus DNA originating from infected cells other than leucocytes [37]. Due to rapid phagocytosis viral load in plasma is low during this episode of early reactivation however and therefore cannot be detected by plasma PCR [37]. Also, using PCR groups as reference, there was no correlation with the results of the quantitative pp65-Ag-assay (Tab. 2). In contrast to that, using a highly positive pp65-Ag-assay result (>99 pp65-Ag-positive cells) as reference, the sensitivity / specificity / positive predictive value / negative predictive value and the positive likelihood ratio of the HCMV-PCR were 100% / 76% / 29% / 100% and 4.24. The sensitivity, the negative predictive value and the positive likelihood ratio of the pp65-Ag-assay rose with the number of HCMV-DNA copies/ml used as reference (Tab. 3). Although the performance of the PCR was similar to the pp65-Ag-assay, the separate values were lower (Tab. 3). This indicates that if a highly positive pp65-Ag-assay result is obtained, it is almost certainly not associated with a negative HCMV-PCR result, while it is more probable that a highly positive HCMV-PCR result is accompanied by a negative pp65-Ag-assay result. In conclusion, it is more probable to predict a positive HCMV-PCR result based on the pp65-Ag-assay than vice versa. When the pp65-Ag result is at least moderately high, quantitative antigenemia and HCMV-PCR seem to parallel each other (pp65-g 2: correlation-coefficient: 0.38, $p<0.01$) and pp65-g 3: correlation-coefficient: 0.44, $p<0.01$; Tab. 2). The correlation coefficient of HCMV-PCR and pp65-Ag-assay has been reported to be about 0.59 to 0.84 dependent on the study population [41, 42]. In our study, information on the administration of antiviral drugs was not included, so that our correlation-coefficient of 0.49 (Tab. 2) could have been higher if treated and untreated patients could have been distinguished. After administration of antiviral therapy the number of pp65-Ag-positive cells drops faster than the number of HCMV-DNA copies/ml, which might be due to a higher susceptibility to therapy in leucocytes [18]. This could also explain high numbers of HCMV-DNA copies/ml in pp65-Ag negative patients. An intermittent rise of antigenemia after the initiation of antiviral therapy has also been observed [29]. However, it does not appear to indicate antiviral resistance in an asymptomatic patient. The underlying mechanism might be the phagocytosis of pp65 protein from destroyed infected leukocytes [43]. In the daily routine of laboratory diagnostic samples have to be assessed independent from patient's clinical presentation. It is most important that no infection is missed.

Our study has some limitations. Because we do not include any information about the clinical appearance of a patient, the date of transplantation or the administration of antiviral therapy - as it is the common everyday reality of virological laboratory diagnostic - the presented results are a simple correlation of the test results from the pp65-Ag-assay and the HCMV-PCR. Also, the correlation of both assays could be altered since we compared the results from samples obtained within a maximum interval of 4 days if both assays were not performed at the same day.

In summary, in economically difficult times with limited financial resources in the medical field, it is crucial to achieve optimal results while minimizing the financial burden on the health care system. This could be accomplished by omitting diagnostic tests. Our results suggest that this is not the case

for the pp65-Ag-assay and the HCMV-PCR which complement each other and should be used concomitant. However, under certain conditions, when the pp65-Ag-assay result is greater than 3 positive cells per 400,000 leucocytes, the pp65-Ag-assay and the plasma HCMV-PCR parallel each other. If the pp65-Ag-assay is used exclusively, e.g. in smaller laboratories where no HCMV-PCR is available, it has to be noticed that sometimes highly positive PCR results can be obtained even though the pp65-Ag-result is negative.

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