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ORIGINAL ARTICLE

Evaluation of a standardised real-time PCR based DNA-detection method (Realstar®) in whole blood for the diagnosis of primary human cytomegalovirus (CMV) infections in immunocompetent patients

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Abstract Cytomegalovirus (CMV) DNA detection in blood could, as a supplementary test to serology, improve the accuracy and speed of diagnosis of an acute CMV infection. In this study we evaluated the performance of a commercially available and standardised CMV PCR assay in whole blood for the diagnosis of a primary infection in immunocompetent adults. Moreover, the kinetics of viral DNA was evaluated in order to provide a time frame in which viral DNA could be detected during an acute primary infection. Whole blood samples were collected from 66 patients with an acute CMV infection, 65 patients with an acute Epstein-Barr virus infection, 27 patients with various other acute infections (parvovirus B19, HIV, Toxoplasma gondii), 20 patients with past CMV infections (>1 year) and 20 apparently healthy persons. For CMV DNA detection and quantification a commercially available real-time PCR was applied (RealStar®, altona Diagnostics). The clinical sensitivity of CMV PCR in whole blood for the diagnosis of a recent primary CMV infection was 93.9 % and the diagnostic specificity 99.2 %. In the majority of the patients CMV DNA was not detectable anymore approximately within 4 weeks after the first blood sample was taken. From these data we concluded that, together with a suggestive serological profile, a positive CMV PCR result in whole blood can be regarded as a diagnostic confirmation of a recent CMV infection on a single blood sample in an immunocompetent patient. However, a negative CMV PCR result does not exclude a recent CMV infection.

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Introduction

The diagnosis of a recent (<3 months) primary cytomegalovirus (CMV) infection is mainly based on serology. Despite the high performance of current serological assays, false positive CMV IgM test results can cause diagnostic confusion [1–3]. Supplementary CMV IgG avidity testing is also sometimes hampered by clinical inaccuracy, and in case of negative CMV IgG results, not possible to perform [4–6]. A clinically validated CMV PCR assay for diagnosis of a primary CMV infection could improve the accuracy and speed of diagnosis and be very useful as a supplementary method.

In this study we evaluated the performance of a commercially available and standardised CMV PCR assay for the diagnosis of primary infections in immunocompetent adults. Moreover, the kinetics of virus DNA was evaluated in order to provide a time frame in which viral DNA could be detected during an acute primary infection.

Materials and methods

Patient samples

Group 1 (CMV group) consisted of EDTA whole blood samples from 66 patients (31 males and 35 females, 16–72 years old) with a proven primary CMV infection. The recent CMV infection was attested by the presence of serum CMV IgM combined with either a CMV IgG seroconversion (n=19) or by a significant CMV IgG titre change (>3 fold, n=47) between the first and a follow-up serum sample within 90 days (mean: 22 days). CMV IgM was positive on the first and follow-up sample. The interval between these two diagnostic serum samples was maximally 1 month for 52 patients; for 14 patients the interval was longer than 1 month. The serum

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CMV IgM and IgG measurements were performed on Architect i2000 systems (Abbott Laboratories, Abbott Park, IL) [7].

In the 47 patients with a CMV IgG titre change, CMV IgG avidity was performed using the VIDAS CMV IgG avidity II assay on VIDAS 30 (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions [8, 9]. In 29 patients the CMV IgG avidity could be performed on the first serum sample, and was low (index<0.40) for all of them. In 18 patients the CMV IgG level was too low (<6 U/ml) on VIDAS to perform an IgG avidity on the first sample, and therefore the CMV IgG avidity was performed on the follow-up sample. In these follow-up samples all CMV IgG avidity results were low.

Group 2 (EBV group) consisted of 65 EDTA whole blood samples from 65 patients (26 males and 39 females, 10–53 years old) with a proven primary Epstein-Barr virus (EBV) infection. This relatively large control group was chosen to evaluate the clinical specificity since both CMV and EBV are herpesviruses and primary infections with these two viruses can produce very similar clinical manifestations in immunocompetent patients. The recent EBV infection was attested by the presence of serum EBV viral capsid antigen (VCA) IgM combined with either an EBV VCA IgG seroconversion (n=60) or by a significant EBV VCA IgG titre change (>3 fold, n=5) between the first and a follow-up serum sample within 90 days (mean, 21 days). The EBV nuclear antigen-1 (EBNA-1) IgG was negative in the first two sequential serum samples from each patient. The interval between two diagnostic serum samples was maximally 1 month for 55 patients; for ten patients the interval was longer than 1 month. The serum EBV IgM and IgG measurements were performed on Liaison (DiaSorin, Saluggia, Italy) [10]. The CMV serostatus was not known in these patients.

Group 3 (various infections+healthy controls) consisted of 67 EDTA whole blood samples. In this group 27 EDTA whole blood samples came from patients with various proven acute infections, all possibly producing very similar clinical manifestations in immunocompetent patients: recent parvovirus B19 infections (n=9), recently diagnosed HIV infections (n=9), recent Toxoplasma gondii infections (n=9). The diagnosis of these infections was based on serum IgG seroconversion/significant IgG titre changes (parvovirus B19 and Toxoplasma) and on the combination of clinical information, serology and viral load for HIV. Twenty samples in this group came from patients with a past CMV infection: samples were collected from patients known to have a previous CMV IgG positive result more than 1 year before. Also in this group 20 EDTA whole blood samples from apparently healthy persons (irrespective of the CMV IgG status) were included.

The serological assays used for the diagnosis of the parvovirus B19 infections were performed on Liaison, and the serological assays used for the diagnosis of the HIV and Toxoplasma infections were performed on Architect i2000 systems.

Whole blood DNA extraction

Blood specimens were collected in K3 EDTA tubes (Vacuette, Greiner Bio-One, Kremsmuenster, Austria) and stored at −20 °C up to 3 years until nucleic acids were isolated using the NucliSENS® easyMAG™ system (bioMérieux, Boxtel, The Netherlands) [11, 12]. DNA was extracted from 200 µl of EDTA whole blood and extracts were eluted in 50 µl of elution buffer.

PCR method

The RealStar® CMV PCR Kit 1.0 (altona Diagnostics, Hamburg, Germany, Product No. 021013) is a real-time PCR test for the detection and quantification of human CMV specific DNA. The assay includes quantification standards which are calibrated against the first WHO international standard for human cytomegalovirus (NIBSC code: 09/162). The assay was performed according to the manufacturer's instructions using the LightCycler® 480 II/96 (Roche Diagnostics, Mannheim, Germany). All results were expressed as IU/ml in whole blood.

Analytical evaluation

To verify that the standard curve was correctly obtained with the PCR assay, we compared the standard curve to a dilution series of the original WHO preparation obtained from NIBSC (NIBSC code 09/162, National Institute for Biological Standards and Control, Hertfordshire, UK). The WHO standard for CMV consists of 5 x 10^6 international units (IU) of whole virus human CMV Merlin strain. The lyophilized standard was reconstituted in 1 ml of nuclease-free water as recommended by the manufacturer. This reconstituted standard was diluted in CMV negative whole blood. A dilution series of this whole blood sample was made by using CMV negative whole blood, and every individual diluted sample was extracted and analysed.

The limit of detection (LOD) was defined as the lowest concentration at which≥95 % of test runs on a whole blood sample gave a positive result [13]. The LOD was estimated by extracting the WHO standard, diluted in CMV negative whole blood, 20 times and performing the PCR analysis in a single analysis run.

The lower limit of quantification (LOQ) was estimated from a dilution series of the WHO standard, diluted in CMV negative whole blood. The lowest concentration on this dilution series which was reproducible with an imprecision of maximally 20 % and an accuracy in the range 80–120 %, was considered as the LOQ.

The within-run coefficient of variation (CV) was evaluated by extracting two whole blood samples ten times and performing the PCR analysis in a single analysis run. For the evaluation of the between run CV, two whole blood samples were aliquoted into ten fractions and stored at −20 °C. For every different analysis run, an aliquot was thawed, extracted and analysed.

Results

Analytical evaluation

The LOD and LOQ of the altona CMV PCR in whole blood were estimated to be 500 IU/ml and 1250 IU/ml, respectively. The Pearson correlation coefficient of the standard curve compared to a dilution series of the original WHO preparation at CMV concentrations higher than 1250 IU/ml was 0.9988 ($p = 0.001$).

The within-run CV's, calculated on $\log_{10}$ expressed results, were 0.9 % (mean CMV concentration 4.58 $\log_{10}$ IU/ml) and 11.7 % (mean CMV concentration 2.75 $\log_{10}$ IU/ml). The between-run CV's were 1.7 % (mean CMV concentration 4.25 $\log_{10}$ IU/ml) and 5.7 % (mean CMV concentration 3.89 $\log_{10}$ IU/ml).

Clinical evaluation—sensitivity and specificity

For the evaluation of the diagnostic sensitivity and specificity of the CMV PCR test, only the first whole blood sample of each patient from the CMV group (group 1) was used. For the calculation of the CMV PCR specificity, samples from group 2 (EBV group) and group 3 (various infections+healthy controls) were used.

The diagnostic sensitivity of the altona CMV PCR in whole blood for the diagnosis of a recent primary cytomegalovirus infection was 93.9 % (62/66). Serological results from the four CMV PCR negative patients are shown in Table 1. From the 62 positive samples, five samples had a CMV DNA concentration between the LOD and LOQ. The average viral load of the 57 samples with a result above the LOQ was 16 189 IU/ml (range, 1418–144 222 IU/ml).

The diagnostic specificity of the altona CMV PCR in whole blood was 98.5 % (130/132). From the two apparently false positive CMV PCR samples (1708 IU/ml and 1327 IU/ml), one was retrospectively shown to be from a patient with a co-infection of parvovirus B19 and cytomegalovirus: a CMV IgG seroconversion could be shown. After elimination of this sample from the specificity calculation, a final specificity of 99.2 % (130/131) was obtained. The other clinically false positive CMV PCR result was observed in a patient with a recently diagnosed HIV infection (seroconversion<5 months, immunoblot confirmed). There were no serological arguments for a recent primary CMV infection in this sample (negative CMV IgM, high CMV IgG and high CMV IgG avidity). In this patient the positive CMV PCR result could possibly be explained by either a CMV reinfection or a CMV reactivation.

Evaluation of CMV DNA kinetics over time

For the evaluation of the CMV DNA kinetics over time, only whole blood samples from group 1 (CMV group) with no more than 30 days between the first sample (with negative or low positive IgG) and a first sequential sample were used. At least 1 extra follow-up sample had to be available for each patient after the first two sequential samples, so in total at least three samples per patient had to be available. Only patients with a positive CMV PCR result on the first sample were included.

Using these selection criteria, 122 samples from 29 patients were eligible for DNA kinetics evaluation. The results of the viral DNA kinetics are shown in Fig. 1. As can be observed, in the majority of the patients CMV DNA was not detectable anymore approximately within 4 weeks after the first blood sample was taken. In a few cases the CMV DNA could not be detected anymore within a few days after the first blood sample. From 14 of the 29 patients one or more whole blood samples taken between 30 and 90 days after the first blood sample were available and in two of these patients CMV DNA was still detectable (patients no 18 and 27). From 15 of the 29 patients a whole blood sample taken 90 days or later after the first blood sample was available and none of these samples was positive.

Discussion

While CMV DNA detection methods are often used in the context of solid organ or stem cell transplantation, only few studies have evaluated CMV PCR assays in immunocompetent patients. In these studies both in-house based methods as well as commercially available methods were used, and very different clinical performances have been described, with clinical sensitivities of CMV PCR in whole blood or plasma ranging from 20 % to 84 % [14–17]. This broad sensitivity range is not only due to method differences but probably also to the choice of specimen (whole blood vs. plasma), and more importantly, the selection of samples (i.e. stage of the CMV infection) used for the evaluation of these assays. In our population of patients with a very recent and proven CMV infection, we found a high clinical sensitivity (93.9 %) using a commercially available and standardised CMV PCR assay in whole blood. This timing of the sample in function of the CMV infection stage also forms a major drawback for the application of a CMV PCR as a diagnostic tool in this context: as previously described, and as confirmed in this study, CMV

Table 1 Detailed results from the four patients with a recent cytomegalovirus infection but a negative CMV PCR result on the first diagnostic sample. CMV IgM and IgG were determined using Architect i2000 and interpreted according to the cut-offs suggested by the manufacturer, i.e. CMV IgG values≥6 U/ml, and CMV IgM index values≥1.00 were considered positive. The number of days represents the interval between the consecutive samples, with day 0 being the day of the first diagnostic sample. *LOQ* lower limit of quantification

Patient	Day	CMV IgM [index]	CMV IgG [U/ml]	CMV DNA
1 (male, 38 years)	0	4.33	Negative	Negative
	25	3.90	12	Negative
	96	1.83	88	Negative
2 (female, 58 years)	0	5.41	9	Negative
	38	3.39	110	Not performed
3 (male, 33 years)	0	3.79	15	Negative
	7	16.12	44	Positive (< LOQ)
	14	15.24	53	Negative
4 (male, 31 years)	0	1.97	Negative	Negative
	8	9.11	42	Not performed
	151	2.90	125	Not performed

DNA in blood is detectable in the majority of patients only during the first few weeks of the primary CMV infection [16, 18]. Consequently, an estimation of the time frame of the primary CMV infection outside the very acute phase, which is often attempted with CMV IgG avidity testing, is therefore not feasible with CMV viral load measurements.

As a serological approach currently remains the cornerstone of the diagnosis of recent primary CMV infections in immunocompetent patients, follow-up samples are often required to show significant CMV IgG level changes. If however a follow-up sample cannot be taken, PCR testing could be important if CMV IgG is still negative on the first sample and therefore CMV IgG avidity testing cannot be performed, or in cases suggestive of a very acute infection where there is doubt on the correctness of the CMV IgG avidity result [5, 6]. Since in our evaluation CMV PCR was negative in four out of 66 patients (6.1 %) with a recent CMV infection, it should be stressed that a negative altona CMV PCR does not exclude a recent primary CMV infection, even during the very acute phase of a primary infection.

A very high specificity (99.2 %) for the diagnosis of a primary CMV infection was observed in our study. Although such a high specificity was expected in this patient population, in our sample selection the CMV serostatus was not known in 57 % of patients, forming a limitation of our study. Probably a high fraction of these samples with unknown serostatus will have been CMV IgG negative. It is therefore possible that in daily practice such a high clinical specificity for the diagnosis of a primary infection is not obtained in a CMV IgG seropositive population since CMV reinfections or reactivations can also give positive CMV PCR results in blood.

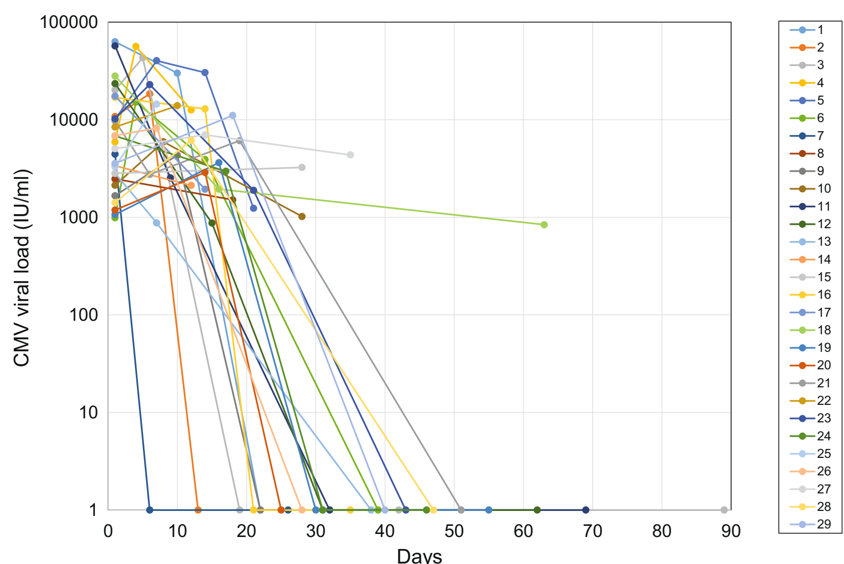

Fig. 1 CMV DNA kinetics in whole blood from 29 patients with an acute CMV infection. On the abscissa, day 0 represents the day the first blood sample was taken from a patient. Only samples taken within 90 days after the first blood sample are shown. On the ordinate the CMV viral loads are shown on a logarithmic scale and undetectable CMV viral loads are expressed as fictitious 1 IU/ml

In conclusion we can say that, together with a suggestive serological profile (positive CMV IgM and negative or weakly positive CMV IgG), a positive CMV PCR result in whole blood can be regarded as a diagnostic confirmation of a recent primary CMV infection on a single blood sample in an immunocompetent patient. However, a negative CMV PCR result does not exclude a recent CMV infection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not required.

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