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Regular paper

Multiplex real-time PCR to identify a possible reinfection with different strains of human cytomegalovirus in allogeneic hematopoietic stem cell transplant recipients*

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Human cytomegalovirus (HCMV) infection remains the leading cause of serious contagious complications after allogeneic hematopoietic stem cell transplantation. These infections in HCMV-seropositive recipients can be due to reactivation or reinfection. Different HCMV strains were identified by determining the genotypes isolated from repeatedly tested patients. The UL55 sequences encoding viral glycoprotein B (gB) have been chosen as the target gene. The region, in which the gB precursor protein is cleaved into two fragments by a cellular endoprotease, is characterized by genetic variability, and based on that HCMV is classified into four major genotypes: gB1, gB2, gB3 and gB4. Multiplex real-time PCR assay enabled both, HCMV gB genotyping, as well as simultaneous quantitative assessment of the detected genotypes. This study was carried out in 30 transplant recipients, from whom 105 isolates of HCMV DNA were genotyped. In 40% of recipients, a mixed infection with two or three genotypes was detected. Genotype gB1 dominated in general, and characteristically for mixed infections, the genotype gB3 or gB4 was always present. Although there were no significant differences in the load for each genotype, in case of multiple infections, the number of copies of gB1 genotype was significantly higher when compared to a single gB1 infection. In patients with mixed genotypes, chronic HCMV infections and graft versus host disease were observed more often, as well as antiviral treatment was less effective. It was assumed that these adverse effects can be related to the presence of gB3 and gB4 genotypes.

Key words: HCMV gB genotypes; multiple HCMV infections; reactivation and reinfection; allo-HSCT

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INTRODUCTION

Human cytomegalovirus (HCMV) is a major cause of morbidity and mortality among immunocompromised patients, especially those who undergo transplantation or suffer from either malignant hematologic disease or AIDS. In the absence of any form of preventive intervention, HCMV infection develops in 30% to 80% of transplant recipients, resulting in symptomatic disease in 7% to 33% of them (Ljungman *et al.*, 2010; Linares *et al.*, 2011). HCMV does not only directly cause morbidity and occasional mortality, but also induces many short-term and long-term indirect effects that collectively contribute to allograft rejection and diminished patient survival. Prevention of HCMV infection and disease is, therefore, essential to ensure a successful outcome of transplantation (Kotton *et al.*, 2010; Razonable *et al.,* 2013).

Transplant recipients develop HCMV disease either as a primary infection (when HCMV is transmitted through the transplanted allograft to a HCMV-naive recipient), a reactivation infection (when an endogenous latent virus reactivates after transplantation) or as reinfection (when donor-transmitted virus is superimposed on an endogenously reactivated virus). While primary HCMV infection is considered to be the most serious in solid organ transplant recipients, secondary infections, resulting from virus reactivation or superinfection with different strains, are the highest risk for developing HCMV disease in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients (Boeckh & Nichols, 2004).

The aim of this study was to apply multiplex real-time PCR to determine the genotype of glycoprotein B (gB) of HCMV strains isolated from blood of seropositive patients subjected to allo-HSCT. This glycoprotein, as an integral component of the viral envelope encoded by the UL55 gene, is the main target for both, humoral and cellular immune responses. It also fulfills a key role in the adsorption and penetration of HCMV into the target cell, as well as in the spread of infection from cell to cell. In these processes, the gB molecule serves as the fusion protein and, in cooperation with other membrane glycoproteins of the envelope, triggers an entry fusion and allows entry of the virus into cells (Vanarsdall *et al.*, 2012; Wille *et al.*, 2013).

The region included between codons 448–481 of UL55 gene, in which the gB precursor protein is cleaved into two fragments by a cellular endoprotease, is characterized by genetic variability, enabling the division of HCMV into four major genotypes: gB1, gB2, gB3 and gB4 (Pignatelli *et al.*, 2004; Isaacson & Compton, 2009).

We hypothesized that distinction of superinfection from the episodes of reactivation of virus with the same genotype would be possible by gB genotyping and comparing HCMV isolates obtained from the same recipient, repeatedly tested at different time points after transplantation. Additionally, the consequences of such HCMV

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Abbreviations: HCMV, human cytomegalovirus; gB, glycoprotein B; allo-HSCT, allogeneic hematopoietic stem cell transplantation; PBL, peripheral blood leukocytes; GvHD, graft *versus* host disease; MGB probe, minor-groove DNA-binding probe

infections with multiple or single gB genotype after allo-HSCT were analyzed.

MATERIALS AND METHODS

Study population. We enrolled thirty HCMV-seropositive patients (12 female and 18 male, a median overall age of 36 years) with active HCMV infection confirmed according to the standard diagnostic procedure, who received allo-HSCT from matched sibling donor between 2004 and 2009, at the Chair of Hematology, Jagiellonian University Medical College in Kraków.

Indications for allo-HSCT were: acute lymphoblastic leukemia (N=7), acute myeloid leukemia (N=11), chronic myeloid leukemia (N=6) and other hematologic diseases (N=6). Serological status of donor-recipient pairs was determined by ELISA using Enzygnost anti-CMV IgG/IgM kits (Simens Healthcare, Germany). Only 4 recipients received cells from seronegative donors.

The patients were monitored at least once a week until 100th day after transplantation, thereafter every 2 weeks for 6 months, or when there were clinical indications.

Active HCMV infection was defined as the detection of viral DNA in two consecutive blood samples. HCMV disease or syndrome was defined according to the published recommendations (Ljungman *et al*., 2002).

All the recipients during the neutropenic phase received standard prophylactic therapy against infections, i.e. oral ciprofloxacin, fluconazole, and acyclovir. Cotrimoxazole was administered as prophylaxis against *Pneumocystis jiroveci* during conditioning until 2 days before transplantation, restarted after engraftment and continued for at least 6 months. For preemptive anti-HCMV therapy in recipients with asymptomatic active HCMV infections, two therapeutic schemes were used, i.e. ganciclovir (5 mg/kg × 2) intravenously for either 14 days or until two consecutive results were negative, or ganciclovir for 7 days followed by 5 days a week until the disappearance of viral DNA in the PCR test. The dose of ganciclovir was modified according to creatinine clearance. In the case of suspected myelosuppression induced by ganciclovir, foscarnet was administered at an inducing dose of 60 mg/kg every 12 hours, and then a supporting dose of 60 mg/kg/day. In the treatment of HCMV disease, either ganciclovir (10 mg/kg/day) or foscarnet (120 mg/kg/day) was used with intravenous immunoglobulin preparations. In individual cases, cidofovir was administered at an inducing dose of 5 mg/kg once a week, followed by a maintenance therapy every other week.

The study on monitoring infections with herpesviruses in allo-HSCT recipients was approved by the Ethical Committee of Jagiellonian University.

Virus isolates and clinical specimens. A hundred and five HCMV DNA samples of clinical isolates obtained during the prospective monitoring of recipients were included in the gB genotyping. The DNA samples were extracted from aliquots of $1x10^6$ peripheral blood leukocytes (PBL) using Genomic DNA Prep Plus (A&A Biotechnology, Poland). Separation of PBL was performed in 6% dextran (MW 60, 000) according to the method described by The and coworkers (1995).

The load of HCMV DNA was quantified by using the Q-CMV Real Time Complete kit (Nanogen Advanced Diagnostics, Italy). The samples were stored at –70°C until tested.

The prototype strains of HCMV: gB1 — Towne (ATCC — VR-977) and gB2 — AD-169 (ATCC — VR-538) were used to prepare calibration curves.

HCMV gB genotyping. Multiplex real-time PCR was used for HCMV gB genotyping, in which the mixture of primers and minor-groove DNA-binding (MGB) probes labeled with VIC and FAM fluorophores allowed the simultaneous detection of not only the genotypes, but also their quantitative assessment. The sequence of primers and probes, and PCR conditions were established according to Pang and coworkers (2008). The amplification products were similar in length, 72–79 bp depending on the genotype. All four genotypes were detected in one reaction, in two separate tubes (separately for gB1 and gB3 and adequately for gB2 and gB4). The reaction mixture with a final volume of 20 µl contained: 10 µl of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 400 nM primers and 200 nM of the corresponding MGB-probes, and 4 µl of DNA isolated from 1×10^6 PBL. The reaction was performed with the Applied Biosystems 7500 Fast Real-Time PCR System. Serially diluted DNA derived from the prototype gB1 and gB2 strains was amplified in parallel with each reaction to determine the calibration curves. Water and DNA extracted from PBL of an HCMV-seronegative donor were used as negative controls of amplification.

Statistical analysis. All viral load results were transformed to log10 values. Descriptive statistics were used to calculate the incidence of gB genotypes. The results were expressed as a mean or median ± S.D. Continuous variables were analyzed by the U Mann–Whitney or ANOVA tests, with values of $P < 0.05$ considered significant. Dichotomous variables were analyzed using the chi-square test or Fisher's exact test. The statistical analysis was done using the STATISTICA PL 10.0 software.

RESULTS

The study was performed in 30 allo-HSCT recipients (all HCMV-seropositive), from whom 105 clinical isolates of HCMV were selected for gB genotyping

Table 1. Distribution and prevalence of gB genotypes in 30 allo-HSCT recipients.
Initially, infection with one genotype was found in 26 patients, and co-infection with two genotypes in 4 patients. In subsequent samples, superinfection with one or two new genotypes was demonstrated in 9 recipients (8 from initially single-genotype, and one from the group of co-infection).

Genotypes	Initial single-genotype infections[1] (%)	Mixed infections		Total No. (%) of genotypes[1]
		Co-infection	Superinfection	
gB1	**13** (43.3)	3	4	**20** (66.7)
gB2	**3** (10.0)	1	3	**7** (23.3)
gB3	**5** (16.7)	2	2	**9** (33.3)
gB4	**5** (16.7)	2	1	**8** (26.7)
No. of patients	26	4	9	30

[1]The difference between the gB1 prevalence and other gB genotypes was statistically significant ($P < 0.02$, Fisher's exact test).

in follow-up observation ranging from 2–46 months (mean 15.8 months). Each patient was repeatedly examined for genotyping, an average of 3.5 ± 2.9 times. The mean time between allo-HSCT and the first positive PCR result confirming the presence of HCMV was 53.5 days.

The initial genotyping of HCMV clinical isolates revealed infection with one genotype in 26 (87%) recipients and co-infection with two genotypes in 4 (13%) patients. In analysis of the subsequent samples, superinfection with other genotypes was identified in 9 (30%) recipients (Fig. 1).

The time from the occurrence of the initial genotype to detection of superinfection ranged from 8 to more than 300 days, on average over 3 months. In the remaining patients with only one genotype, secondary infection was also observed, probably as a result of reactivation or reinfection, but due to virus with the same gB genotype. The distribution of the different genotypes, including co-infections and superinfections is summarized in Table 1. Twelve (40%) out of the 30 analyzed recipients were infected with more than one gB genotype of HCMV (2 and 3 genotypes were confirmed in 10 and 2 patients, respectively). Genotype gB1 dominated generally, and for mixed infections it was characteristic that the gB3 or gB4 genotype was always present. Genotype gB3 or gB4 was detected in only 4 out of 18 recipients with a single gB-genotype infection.

The number of viral copies (load) was significantly higher (4.3 times on average) in samples with mixed genotypes than in samples with a single genotype (Fig. 2). Although there were no significant differences in the values of load for each genotype (Fig. 3), in samples with multiple gB-genotypes the copy number of gB1-genotype increased significantly ($P = 0.02$; U Mann–Whitney test). No such relationship was observed for the other genotypes. The results are shown in Table 2.

In the clinical evaluation, because of a small group of patients studied, it was difficult to analyze the impact of the individual genotype on the course of the infection. Therefore, the clinical course of HCMV infection was compared in two groups of allo-HSCT recipients: patients infected with a single gB-genotype (N=18) and with multiple gB-genotypes as a result of co-infection and/or superinfection (N=12).

Asymptomatic infection occurred more frequently in persons infected with one genotype when compared to multiple genotypes (in 33% and 8% recipi-

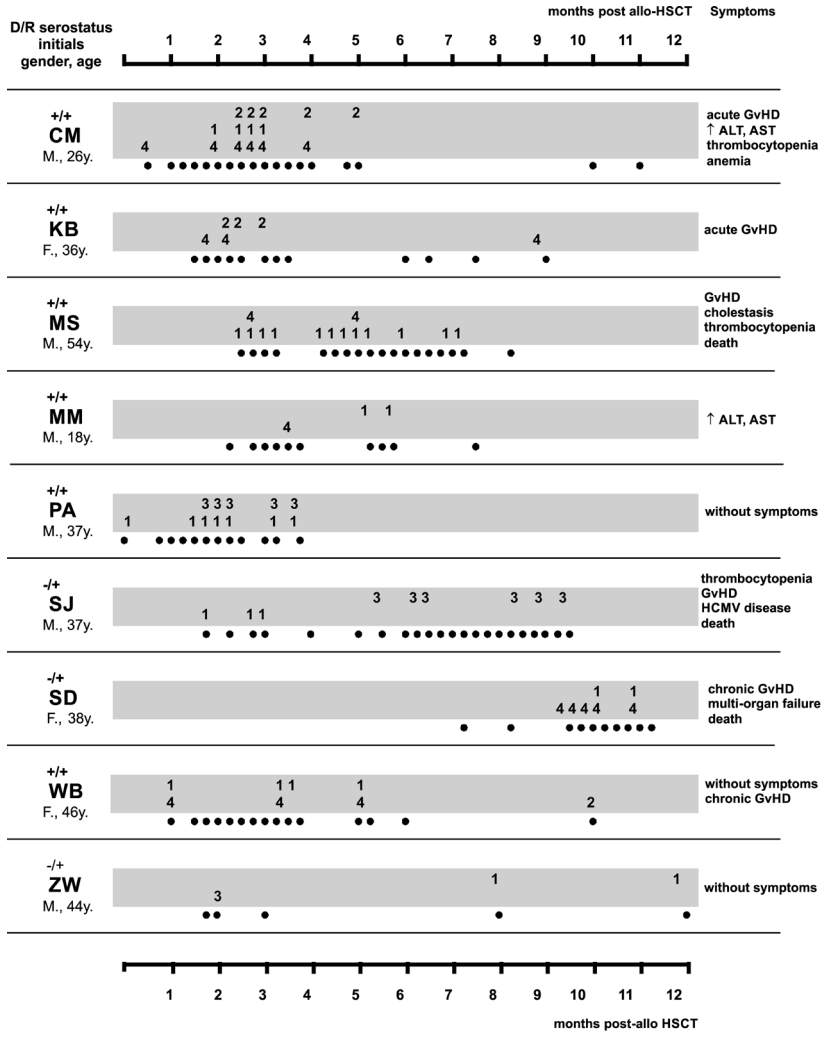

Figure 1. Superinfection with different gB genotypes of HCMV, which was observed in 9 allo-HSCT recipients in the months following transplantation.
Initials of patients, sex and age, and anti-HCMV serologic status between donor–recipient (D/R) at the time of transplantation are presented. Dots indicate a blood sample, in which the presence of HCMV is confirmed, gB genotype type is marked with a digit.

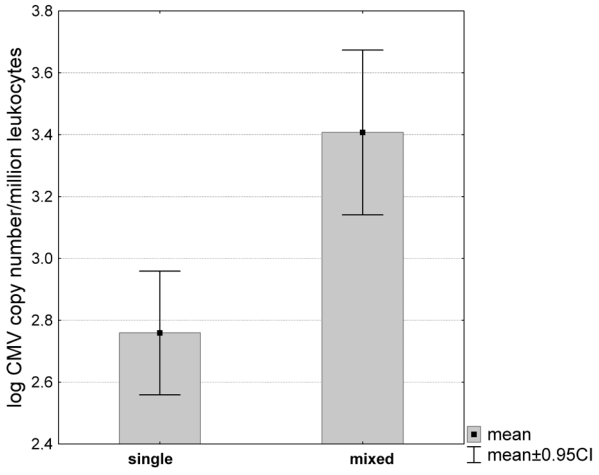

Figure 2. The HCMV load calculated per million PBL in samples with single and mixed gB genotypes estimated by commercial Q-CMV Real Time Complete kit.
The differences were statistically significant ($P=0.0004$, U Mann–Whitney test). CI, confidence interval for mean

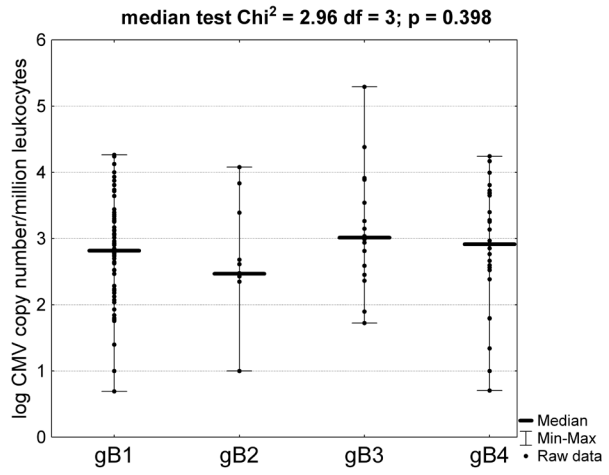

Figure 3. Comparison of the HCMV load for each gB genotype in 105 samples of 30 allo-HSCT recipients.

Table 2. Viral load for each gB genotype of HCMV determined in 105 samples of 30 allo-HSCT recipients with regard to single- and multiple-genotype samples.

Genotype	No.	(%)	mean*	S.D.	median*
Single-gB genotype[1] N=79					
gB1	48	(60.76)[3]	2.75[2]	0.65	2.78
gB2	7	(8.86)	2.51	0.24	2.46
gB3	12	(15.19)	3.24	1.08	3.41
gB4	12	(15.19)	2.45	1.15	2.54
Multiple-gB genotype[1] N=26					
gB1	23	(88.5)[4]	3.11[2]	0.81	3.17
gB2	6	(23.1)	3.17	0.69	3.04
gB3	7	(26.9)	3.16	0.58	2.99
gB4	19	(73.1)[4]	3.03	0.87	2.92

*$\log_{10}$ of HCMV copy number /million PBL; S.D., standard deviation; [1]Differences between the median viral loads not significant for individual gB genotypes ($P>0.05$, ANOVA Kruskal–Wallis test); [2]Differences between the gB1 viral loads in samples with single- and multiple-genotype were statistically significant (the Mann-Whitney U test, $P=0.02$); [3]The difference between the gB1 prevalence and other gB genotypes was statistically significant ($P<0.0001$ Fisher's exact test); [4]The difference between the prevalence of gB1 and gB4 genotypes and gB2 and gB3 genotypes was statistically significant ($P<0.001$, Fisher's exact test).

ents, respectively), but this difference was not statistically significant.

HCMV disease developed in individual recipients in both groups and it manifested as gastroenteritis, hepatitis, and retinitis. Increase in transaminases, thrombocytopenia and anemia occurred in both groups of patients with similar frequencies. The differences were not statistically significant. It was characteristic, however, that in patients with mixed genotypes, recurrences and chronic HCMV infections were observed more often, as well as acute or chronic form of graft versus host disease (GvHD). Antiviral treatment results were also less effective in recipients infected with mixed genotypes. The anti-HCMV therapy had to be repeated in 90% of these patients. The results of this analysis are presented in Table 3.

DISCUSSION

The method applied by us, with the use of specific MGB DNA probes of great sensitivity, allowed the simultaneous detection of not only the gB genotypes of HCMV but also their quantitative assessment, which in the case of co-infection gave the opportunity to determine the mutual quantitative proportions between genotypes. The presence of two or three genotypes in the randomly selected HCMV clinical isolates was confirmed in even 25% (26/105) of samples.

Based on the gB genotyping, the preliminary hypothesis about the possible and common reinfections with different strains of HCMV after hematopoietic stem cell transplantation was confirmed. The results showed that the presence of specific antibodies in the transplant recipient fails to protect such person against secondary infections and disease progression. Although differences in the incidence of HCMV disease in the compared groups of patients did not differ significantly, less frequent cases of asymptomatic patients with multiple genotype infections in comparison to infection with only one gB genotype were reported.

In patients with mixed infections, a severe course of infection was observed more frequently, as is evidenced by: significantly higher levels of viral DNA found in peripheral blood, more frequent relapses, the tendency to chronic infection and failure of antiviral therapy.

These adverse effects observed in cases with multiple infections could be related to the presence of gB3 and gB4 genotypes, which give, as already suggested by other authors, greater immuno- and myelosuppression in comparison to gB1 and gB2 genotypes (Fries *et al.*, 1994; Torok-Storb *et al.*, 1997; Randolph-Habecker *et al.*, 2002). Also, Xia *et. al.* (2012) reported statistically significant lower rates of viral clearance in Chinese HSCT-recipients at day 21 of therapy, and more frequent HCMV reactivation/reinfection in patients infected with the gB3 genotype, than in those infected with the gB1 genotype.

The distribution of CMV gB genotypes seems to be associated with geographic and/or demographic differences among patients. In our study, the frequency of occurrence of each genotype assessed by a single genotyping assay was similar to that described with the use of distinct techniques in solid organ and bone marrow

Table 3. Comparison of clinical symptoms and course of HCMV infection in 30 allo-HSCT recipients infected with single gB-genotype and multiple gB-genotypes (percentages are shown in brackets)

	single gB-genotype N = 18	multiple gB-genotype N = 12	*P**
asymptomatic infection	6 (33)	1 (8)	0.13
HCMV disease	3 (17)	2 (17)	0.69
thrombocytopenia/anemia	6 (33)	6 (50)	0.29
increase in transaminases	4 (22)	4 (33)	0.39
acute GvHD	3 (17)	8 (67)	0.01
chronic GvHD	4 (22)	7 (58)	0.05
chronic HCMV infection [1]	1 (5)	6 (50)	0.01
recurrences of HCMV infection	2 (11)	9 (75)	0.001
No. requiring re-therapy/No. treatment	4/10 (40)	9/10 (90)	0.029
genotype gB3/gB4	4 (22)	12 (100)	0.000

[1]considered as chronic when HCMV in blood was maintained for > 3 months, *Fisher's exact test

cells' transplant recipients in the European countries and North America. A characteristic observation in our research was the dominance of gB1 genotype over the other genotypes, which was also confirmed by others (Fries *et al.*, 1994; Rubalova *et al.*, 2010; Pang *et al.*, 2008; Manuel *et al.*, 2009). In contrast, some data reported in HSCT recipients from Japan and China indicated different distribution of gB genotypes with the dominance of gB2 or gB3 genotypes (Wada *et al.*, 1997; Wu *et al.*, 2010). However, Xia *et. al.* (2012) revealed with a prospective analysis of 102 HSCT recipients that the gB1 genotype was the most prevalent among Chinese patients.

Possibly due to monitoring of HCMV infection for months following transplantation, and repeated genotyping , the incidence of a mixed infection has been affected in our studies, i.e. it has increased from 13% to 40% of recipients. This result differs markedly from the frequency cited by other authors, showing such gB-multiple infections much less often, i.e. in 2.5% (Torok-Storb *et al.*, 1997), 6.3% (Fries *et al.*, 1994) and in 17% of allo-HSCT recipients (Rubalova *et al.*, 2010). Furthermore, these authors used RFLP (Restriction Fragment Length Polymorphism) method for determination of the gB genotype, which could also result in a lower frequency of detection of a mixed infection (Sowmya *et al.*, 2007).

Comparison of viral load for gB1, gB2, gB3 and gB4 genotypes showed no significant differences, but the mean and median values in the samples, in which the gB3 genotype was present, were the highest (Fig. 3). A similar relationship was reported by Manuel and coworkers (2009) in patients subjected to organ transplantation, when the copy number was evaluated in DNA extracted from whole blood. The role of the gB3 genotype in the pathogenesis of HCMV infection appears to be particularly interesting in the context of the study by Torok-Storb and coworkers (1997), analyzing mortality in recipients of bone marrow cells due to myelosuppression, which showed a significant correlation of the increase in this parameter with infections caused by gB3 and gB4 genotypes of HCMV. The risk of death associated with neutropenia in patients infected with these genotypes was more than 12-fold higher than in patients infected with gB1 and gB2 genotypes. In our study we found a significant association between mixed infections and the presence of gB3 and gB4 genotypes, which could be important in the development of chronic HCMV infection and GvHD in enrolled recipients.

In patients with mixed infections, co-infections with two or three genotypes were characterized by a significantly higher load ($P = 0.0001$) than in blood samples, in which only one genotype was present, and these differences for the assessed individual genotypes were preferably revealed for gB1 genotype (Fig. 2 and Table 2). This trend may indicate interactions between co-existing genotypes and stimulation of HCMV replication of one genotype by another one. Lack of significantly higher values of load for the other genotypes in mixed infections can probably be explained by the small number of samples, not representative of a full statistical analysis, in which they were detected. It should be noted that some authors have also pointed out a higher number of HCMV copies in mixed infections when compared to those caused by a single genotype (Pang *et al.*, 2008; Manuel *et al.,* 2009). On the other hand, Humar *et al.* (2003) did not observe such differences, but the adverse effects of a mixed infection were connected with longer time necessary to eradicate the virus. In allo-HSCT recipients with multiple genotype infections enrolled in our study, antiviral therapy results were also significantly worse than in patients with single-genotype infections.

In conclusion, simultaneous identification of HCMV gB genotypes with measurement of their loads in tested samples could provide important information for allo-HSCT recipients with HCMV viremia. Understanding the relationship between different HCMV strains may be very important for comprehension of the pathogenesis of this disease and improvement of the clinical management of immunocompromised patients.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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