Detection of specific lytic and latent transcripts can help to predict the status of Epstein–Barr virus infection in transplant recipients with high virus load*

Barbara Zawilinska1, Anna Kosinska2, Marzena Lenart2, Jolanta Kopec2, Beata Piatkowska-Jakubas3, Aleksander Skotnicki3 and Magdalena Kosz-Vnenchak1,2

1Department of Virology, Chair of Microbiology, Jagiellonian University Medical Collage, 2Laboratory of Molecular Genetics and Virology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 3Chair and Department of Haematology, Jagiellonian University Medical Collage, Kraków, Poland

Received: 27 April, 2008; revised: 16 September, 2008; accepted: 03 November, 2008
available on-line: 17 November, 2008

Epstein–Barr virus (EBV), a member of the family Herpesviridae, is widely spread in the human population and has the ability to establish lifelong latent infection. In immunocompetent individuals the virus reactivation is usually harmless and unnoticeable. In immunocompromised patients productive infection or type III latency may lead to EBV-associated post-transplant lymphoproliferative disorder (PTLD). The aim of our research was to investigate the utility of PCR-based methods in the diagnosis and monitoring of EBV infections in bone marrow transplant recipients. Thirty-eight peripheral blood leukocyte samples obtained from 16 patients were analysed, in which EBV DNA was confirmed by PCR. We used semi-quantitative PCR to estimate the viral load and reverse-transcription PCR (RT-PCR) to differentiate between latent and productive EBV infection. In 14 patients we confirmed productive viral infection. We observed a correlation between higher number of EBV genome copies and the presence of transcripts specific for type III latency as well as clinical symptoms.

Keywords: Epstein-Barr virus, latency, EBV-lymphoproliferative disorder, productive infection, bone marrow transplantation

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous herpesvirus widely distributed in the human population. Primary infection is asymptomatic during early childhood. However, in adolescents or in young adults, nearly half of the cases of primary infections manifest as mononucleosis. EBV infects mainly B lymphocytes and certain epithelial (lymphoepithelial) cells of oropharynx. Like other herpesviruses, EBV has the ability to establish lifelong latent infection in circulating B lymphocytes, so that the virus reactivation can occur, but in immunocompetent individuals it is usually harmless and symptomless (Rickinson & Kieff, 2007). Nevertheless, EBV infection may be life-threatening in immunocompromised patients such as bone marrow or solid organ transplant recipients (Wagner et al., 2002; Tsurumi et al., 2005; Rickinson & Kieff, 2007). In these patients EBV reactivation may lead to persistent lytic infection with high viral load in peripheral blood lymphocytes. EBV-driven B cells can proliferate and progress to immunoblastic lymphoma which is associated with type III EBV latency. In such cases the mortality rate is very high.

*Corresponding author: Barbara Zawilinska, Jagiellonian University Medical School, Chair of Microbiology, Department of Virology, Czysta 18, 31-121 Kraków, Poland; tel.: (48) 12 634 5400; e-mail: mbzawili@cm-uj.krakow.pl

*Presented at the XXXV Winter School “The Structure and Function of Protein and Nucleic Acids” organized by Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 23–27 February, 2008, Zakopane, Poland.

Abbreviations: BALF-2, early gene of Epstein–Barr virus; PBL, peripheral blood leukocytes; EBNA, Epstein–Barr virus nuclear antigen; BZLF-1, immediate-early gene of Epstein–Barr virus; EBV, Epstein–Barr virus; GvHD, graft versus host disease; HHV7, Human Herpesvirus type 7; HSCT, haematopoietic stem cell transplantation; LMP, latent membrane protein; M-MLV, Moloney murine leukemia virus; PTLD, post-transplant lymphoproliferative disorder.
and reaches 50 to 80% (Wagner et al., 2002). Early identification of patients at risk for developing EBV-associated post-transplant lymphoproliferative disorder (PTLD) could reduce morbidity and mortality, thereby improving overall patient management (Rezonable & Paya, 2003).

Numerous studies have confirmed the correlation between high EBV viral load in peripheral blood and the risk of developing EBV-associated post-transplant lymphoproliferative disorder (PTLD) could reduce morbidity and mortality, thereby improving overall patient management (Rezonable & Paya, 2003). However, quantitative methods give no information about the stage of EBV infection. Lytic or productive infection is characterized by expression of > 60 early and capsid structural genes, whereas in the immortalized or continuously proliferating state, typical for type III latency, transcripts for the EBNAs and LMPs genes are present. Therefore, owing to a possibility of developing a severe viral disease, rapid, reliable and highly sensitive diagnostic methods for monitoring EBV infection are needed.

The aim of our research was to investigate the usefulness of polymerase chain reaction (PCR) - based methods: RT-PCR and semi-quantitative PCR, in the diagnosis and monitoring of bone marrow transplant recipients. The main purpose of the applied methods was the detection of EBV transcripts characteristic for lytic as well as type III latency, estimation of viral genome copy number and correlation of the results with patients’ clinical status after transplantation.

**MATERIALS AND METHODS**

**Samples.** We studied 38 samples of peripheral blood leukocytes (PBL) from 16 EBV-positive patients after allogenic haematopoietic stem cell transplantation (HSCT) in different stages of engraftment. Prospective monitoring of our patients has shown that in some recipients EBV loads have fluctuated while in others persisted for more than 3 months. All analysed patients have undergone pre-emptive antiviral therapy with acyclovir or gancyclovir. In our research we also used Namalwa cells (Burkitt lymphoma cell line) as a positive control. Namalwa cells contain two copies of EBV DNA incorporated into the genome (Vernard et al., 2000). As a negative clinical control we used leukocytes obtained from eight EBV-negative patients (confirmed in serological as well as in PCR tests) who had undergone allo-HSCT.

**RNA and DNA extraction.** Eukocytes obtained from recipients were isolated by sedimentation of 3–5 ml EDTA-treated blood samples with 6% dextran solution according to the method described by The et al. (1995). Aliquots of 1×10^6 PBL or Namalwa cells were used for RNA extraction by Trizol Reagent (Invitrogen), per 100 µl of cell suspension) and DNA extraction using Genomic DNA Prep Plus (A&A Biotechnology).

**Viral DNA assay by nested PCR.** To confirm the presence of EBV-1 or EBV-2, two-step PCR for EBNA2 was applied according to Venard et al. (2000) (Table 1). In EBV-positive samples the amount of EBV DNA was confirmed by a semi-quantitative PCR. Ten-fold dilutions of a DNA standard isolated from 1×10^6 Namalwa cells were amplified by PCR and separated by gel electrophoresis. DNA samples from patients were also diluted 10-fold, amplified, and the bands separated on a gel were compared to the standard run in parallel. The calculated EBV load for all samples was expressed as the number of EBV genome copies per 10^6 PBL (Fig. 1A).

**Viral mRNA assay by RT-PCR.** Reverse transcription of RNA samples was performed using oligo-dT primers (Sigma) and Mo-MLV reverse transcriptase (Sigma). The efficiency of RT reaction was verified by PCR for β-actin (Table 1). cDNA samples were used to detect specific EBV transcripts by PCR. All PCR products were separated by electrophoresis and the intensity of the bands was compared and marked as “+”, “++”, or “+++” (Fig. 1B).

Primers were chosen to distinguish between EBV productive and latent infection. For that pur-

---

Table 1. Targets for primers used in PCR and RT-PCR

| Stage of infection: | Gene/transcript | Product | According to: |
|---------------------|----------------|---------|--------------|
| **EBV**             |                |         |              |
| Confirmation of infection | EBNA2/EBV1 or 2 | 497 bp or 150 bp | Venard et al., 2000 |
| Productive infection | BZLF1          | 608 bp  | Mundle et al., 2001 |
|                     | BALF2          | 238 bp  | Prang et al., 1997 |
|                     |                | 118 bp  |              |
| Type III latency    | EBNA2          | 596 bp  | Venard et al., 2000 |
|                     | LMP1           | 351 bp  | Qu et al., 2000 |
|                     |                | 198 bp  |              |
EBV lytic and latent transcripts in transplant recipients with high virus load

pose, transcripts of productive infection: BZLF1 (immediate-early) and BALF2 (early) and type III latency transcripts for viral oncogenes — EBNA2 and LMP1 — were detected (Table 1). We performed two-step PCRs (nested PCRs) for detection of BALF2, EBNA2 and LMP1 transcripts to increase the sensitivity of the method. PCR for BZLF1 transcript was one-step.

RESULTS

The results obtained for blood samples of EBV-positive patients are presented in Table 2. The presence of EBV-1 was confirmed in all 38 samples of PBL. Basing on the serological tests before transplantation in the donor–recipients pairs, primary EBV-infection could be recognized in two patients (no. 12 and 13), while in the others the presence of EBV-1 was a result of reactivation or possibly reinfection. Samples were collected at different stages of engraftment (from one to 29 months after HSCT).

Isolated RNA was analysed by RT-PCR to differentiate between productive and latent EBV infection and mostly to detect transcripts specific for type III latency, connected with a higher risk for developing PTLD. In all studied samples the transcript for the house-keeping gene β-actin was detected, which confirmed the efficiency of RNA extraction and RT reaction. Moreover, all specific transcripts were present in the Namalwa cell line used as a positive control. In no material obtained from uninfected patients, serving as a negative clinical control (results not shown in Table 2), did we detect any EBV transcripts.

Productive infection was detected in 14 of 16 analysed patients (patients no. 3–16). Almost in all of the samples (83%) a transcript for the BALF2 gene (essential in the lytic phase of the viral replication) was detected, while a transcript for the immediate-early gene BZLF1 was detected only in 19% of materials obtained from four patients (patients no. 6, 9, 12 and 13). It is possible that the difference in the number of positive samples for productive infection of EBV is a consequence of the different sensitivity of one-step and two-step PCR. It is also possible that the detection of further stages of EBV lytic infection (i.e. for late genes) may be much more useful in the diagnostics of bone marrow transplant recipients.

Transcripts for viral oncogenes EBNA2 and LMP1, specific for type III latency of EBV, were detected in 5 of 16 patients (patients no. 4, 9, 12, 13 and 15) although expression of the BZLF1 and/or BALF2 genes, characteristic for lytic infection, was also confirmed. In patients no. 1 and 2 only LMP1 transcript was present, which confirmed the latent stage of EBV infection, but not type III latency.

The aim of the semi-quantitative PCR analysis was to estimate the number of EBV viral genome copies in the DNA samples studied. A number of EBV genome copies (between 2 000 and 20 000 per $1 \times 10^6$ leukocytes) was demonstrated for 8 out of 16 patients, and in six of them (patients no. 3, 4, 12, 13, 14 and 15) the higher viral load correlated with symptoms such as graft versus host disease (GvHD), increased levels of transaminases, thrombocytopenia, and fever. For patient no. 15, between 7 to 9 months after transplantation, we observed an increase of the EBV copy number from 200 to 20 000 per $1 \times 10^6$ PBL without clinical symptoms (data not included in Table 2). Furthermore, samples of patient no. 14 collected after 1.5 and 2 months following HSCT were only positive for the BALF2 transcript specific for productive infection and the estimated EBV genome copy number was low – 200 per $1 \times 10^6$ PBL. Because in these sample the presence of HHV7 was also detected, the high level of transaminases may be a result of a mixed infection. In the sample collected from the same patient 3 months after transplantation, we additionally confirmed the LMP1 transcript that accompanied an increase of the EBV genome number to 2 000 per $1 \times 10^6$ PBL and the appearance of thrombocytopenia.

Figure 1. Examples of gel electrophoresis pattern of PCR products.

A. PCR for EBNA2/EBV1. Serial dilution of DNA obtained from $10^6$ Namalwa cells (upper panel) and DNA obtained from two selected patients’ samples (bands no. 1–3 and 4–6). PC, positive control, DNA isolated from Namalwa cell line (band no. 7).

B. RT-PCR for LMP1 showing different intensity of gel bands; bands no. 1 and 12, molecular marker, no. 2–10 selected samples, no. 11, positive control from Namalwa cells.

for developing PTLD. In all studied samples the transcript for the house-keeping gene β-actin was detected, which confirmed the efficiency of RNA extraction and RT reaction. Moreover, all specific transcripts were present in the Namalwa cell line used as a positive control. In no material obtained from uninfected patients, serving as a negative clinical control (results not shown in Table 2), did we detect any EBV transcripts.

Productive infection was detected in 14 of 16 analysed patients (patients no. 3–16). Almost in all of the samples (83%) a transcript for the BALF2 gene (essential in the lytic phase of the viral replication) was detected, while a transcript for the immediate-early gene BZLF1 was detected only in 19% of materials obtained from four patients (patients no. 6, 9, 12 and 13). It is possible that the difference in the number of positive samples for productive infection of EBV is a consequence of the different sensitivity of one-step and two-step PCR. It is also possible that the detection of further stages of EBV lytic infection (i.e. for late genes) may be much more useful in the diagnostics of bone marrow transplant recipients.

Transcripts for viral oncogenes EBNA2 and LMP1, specific for type III latency of EBV, were detected in 5 of 16 patients (patients no. 4, 9, 12, 13 and 15) although expression of the BZLF1 and/or BALF2 genes, characteristic for lytic infection, was also confirmed. In patients no. 1 and 2 only LMP1 transcript was present, which confirmed the latent stage of EBV infection, but not type III latency.

The aim of the semi-quantitative PCR analysis was to estimate the number of EBV viral genome copies in the DNA samples studied. A number of EBV genome copies (between 2 000 and 20 000 per $1 \times 10^6$ leukocytes) was demonstrated for 8 out of 16 patients, and in six of them (patients no. 3, 4, 12, 13, 14 and 15) the higher viral load correlated with symptoms such as graft versus host disease (GvHD), increased levels of transaminases, thrombocytopenia, and fever. For patient no. 15, between 7 to 9 months after transplantation, we observed an increase of the EBV copy number from 200 to 20 000 per $1 \times 10^6$ PBL without clinical symptoms (data not included in Table 2). Furthermore, samples of patient no. 14 collected after 1.5 and 2 months following HSCT were only positive for the BALF2 transcript specific for productive infection and the estimated EBV genome copy number was low – 200 per $1 \times 10^6$ PBL. Because in these sample the presence of HHV7 was also detected, the high level of transaminases may be a result of a mixed infection. In the sample collected from the same patient 3 months after transplantation, we additionally confirmed the LMP1 transcript that accompanied an increase of the EBV genome number to 2 000 per $1 \times 10^6$ PBL and the appearance of thrombocytopenia.
Table 2. Results of RT–PCR and semi–quantitative PCR compared with clinical symptoms

| Patient [sex/age] | Serostatus^ D/R | Time after HSCT [months] | EBV copy no/10^6PBL | Detected EBV transcripts | Symptoms and additional herpesvirus infection |
|------------------|-----------------|--------------------------|---------------------|-------------------------|-----------------------------------------------|
|                  |                 |                          |                     |                         |                                               |
| 1                | [-/+]           | 8                        | 200                 | – – – +                 | chronic GvHD, liver and kidney failure        |
| [F/20]           |                 |                          |                     |                         |                                               |
| 2                | [-/+]           | 6                        | 200                 | – – – +                 | no symptoms                                   |
| [F/21]           |                 |                          |                     |                         |                                               |
| 3                | [+/-]           | 2.5                      | 2 000               | – + – –                 | thrombocytopenia, CMV infection               |
| [F/36]           |                 | 17                       | 200                 | – – – +                 | no symptoms                                   |
| 4                | [+/-]           | 3                        | 200                 | – + + + +               | no symptoms                                   |
| [M/35]           |                 | 19                       | 2 000               | – + + + +               | GvHD, increased level of transaminases        |
| 5                | [+/-]           | 29                       | 200                 | – + – –                 | increased level of transaminases thrombocytopenia, HHV–7 infection |
| [F/23]           |                 |                          |                     |                         |                                               |
| 6                | [+/-]           | 16                       | 200                 | – + – –                 | no symptoms                                   |
| [M/39]           |                 | 18.5                     | 200                 | – + – –                 | no symptoms                                   |
| 7                | [+/-]           | 1                        | 200                 | – + – –                 | no symptoms                                   |
| [M/22]           |                 | 2.5                      | 200                 | – + – –                 | no symptoms                                   |
| 8                | [-/+]           | 2.5                      | 200                 | – + + – –               | no symptoms                                   |
| [F/27]           |                 | 3                        | 200                 | – + – –                 | no symptoms                                   |
| 9                | [+/-]           | 2                        | 200                 | – + – –                 | no symptoms                                   |
| [M/23]           |                 | 3                        | 200                 | + – +++ +               | no symptoms                                   |
| 10               | [-/+]           | 6                        | 200                 | – + – –                 | relapse of malignancy                         |
| [F/43]           |                 |                          |                     |                         |                                               |
| 11               | [+/-]           | 1.5                      | 2 000               | – + – –                 | no symptoms                                   |
| [M/55]           |                 |                          |                     |                         |                                               |
| 12               | [-/-]           | 4.5                      | 20 000              | + – – – –               | chronic GvHD, fever, increased level of transaminases, thrombocytopenia |
| [M/35]           |                 | 6.5                      | 2 000               | + + + + + +             | increased level of transaminases, thrombocytopenia |
| 13               | [-/-]           | 7                        | 2 000               | + + + + + +             | increased level of transaminases, thrombocytopenia |
| 14               | [-/-]           | 17.5                     | 200                 | – + – –                 | increased level of transaminases, thrombocytopenia |

^ Serostatus: D/R = donor/recipient
A low number of EBV genome copies, 200 per $1 \times 10^6$ PBL, was usually found in samples of “healthy” recipients. The increasing viral load observed in patient no. 4, as well as the decreasing EBV load in patient no. 3 correlated well with their clinical status.

In our study patients were examined for EBV transcripts between 1 and 29 months after HSCT. For most of the patients, samples were collected within a year, on average $4.7 \pm 2.8$ months after HSCT, and also later, except for patients no. 5 and 6, whose samples were examined only late after transplantation. The highest risk of developing PTLD has been reported for the first year after transplantation, when the recipients’ effective cytoxic immune response against EBV-infected B lymphocytes was recovering (Bhatia et al., 1996). In our patients we did not observe such complication, neither have we found typical type III latency, without a productive gene expression. However, in samples with reasonably high EBV copy number (> 200 per $10^6$ PBL) the EBNA2 and/or LMP1 transcripts, characteristic for type III latency, were detected significantly more often, at 67% vs. 30% in low-load samples (Fisher exact test, $P=0.03$).

**DISCUSSION**

Developing reliable and highly sensitive methods for Epstein–Barr virus detection is crucial for the growing number of immunosuppressed patients. In immunocompetent seropositive hosts, EBV is controlled by cell-mediated immunity and the presence of viral genome is usually undetectable in PBL (Hopwood et al., 2002). Immunosuppressive therapy may increase the number of peripheral EBV-driven B cells. In our allo-HSCT patients we observed transient EBV appearance in blood cells. From our studied group three patients (no. 9, 12 and 13) were persistent EBV carriers for more than 3 months. In one carrier, low copy number (200 copies/$10^6$ PBL) was detected, whereas in two others a high viral load was observed as a result of primary infection. Increasing virus load was associated with clinical symptoms. Because for some patients we had confirmed other herpesviruses in the same samples (Zawilinska et al., 2006), it cannot be concluded definitively that these symptoms were caused by EBV only.

In most reports EBV DNA levels in patients with PTDL are significantly higher than in healthy recipients (Hopwood et al., 2002). In our patients the highest level of virus loads was 20000 copies per $10^6$ PBL although in none of them PTDL was observed. So we additionally analysed the expression of specific EBV transcripts which reflected EBV productive infection or latency. Progressive features, ranging
from reactive hyperplasia to monomorphic B cell lymphoma (Nalesnik, 1998; Barrett, 2000), difficulties in PTLD diagnosis and usually a fatal outcome of the disorder (Wagner et al., 2002) are reasons for the application of new diagnostic methods for detection of EBV infection and PTLD.

In our investigation we were able to detect productive infection in the majority of studied patients (even asymptomatic ones) in contrast to Bergallo and co-workers who confirmed such infection in only one of 30 asymptomatic renal recipients (Bergallo et al., 2007). The results of our study indicate also that there is a correlation between the higher number of EBV genome copies and the presence of transcripts specific for type III latent infection. It may be proposed that the appearance of EBV-infected B lymphocytes with type III EBV latency can induce excessive viral replication, or that an increased number of lytic EBV-infected B cells results in the expression of type III latent genes in other infected lymphocytes. The quantitative methods more often applied in the diagnosis of EBV infection, such as real-time PCR (Savoie et al., 1994; Bai et al., 2000; Jebbink et al., 2003; Wagner et al., 2004), can be useful in predicting the risk of developing PTLD, even though the risk is directly related to EBV latent infection in contrast to other herpesviruses, in which case the productive phase of the life cycle causes virus-associated diseases in transplant recipients (Rayes et al., 2005).

To conclude, these preliminary results indicate that PCR-based assays seem to be useful in the monitoring of EBV infection in bone marrow transplant recipients. Semi-quantitative PCR is regarded as a valuable tool for estimating EBV genome copy number and also allows evaluation of the risk for patients, since a high amount of EBV DNA is one of the major risk factors for developing PTLD. However, our results and another study show that an increased viral load in transplant recipients is not always predictive of PTDL (Tysarowski et al., 2007). Using the RT-PCR method in our study additionally allowed us to differentiate between productive and latent infection. Therefore, application of several methods, rather than only one, for monitoring transplant recipients seems to be more reliable and gives much more information about EBV infection. Early identification of patients at risk for developing PTLD could reduce PTLD-related morbidity and mortality by appropriate patient management.

Acknowledgement

This research was partially supported by grants N404 090 32/3224 and N401 082 32/1930 from the Ministry of Science and Higher Education.

REFERENCES

Bai X, Rogers BB, Harkins PC, Sommerauer J, Squires R, Rotondo K, Quan A, Dawson DB, Scheuermann RH (2000) Predictive value of quantitative PCR-based viral burden analysis for eight human herpesviruses in pediatric solid organ transplant patients. J Mol Diagn 2: 191–201.

Barrett Chl (2000) Post-transplantation lymphoproliferative disease. Herpes 7: 4–8.

Bergallo M, Costa C, Baro S, Musso T, Balbo L, Merlino Ch, Cavallo R (2007) Multiplex-nested RT-PCR to evaluate latent and lytic Epstein Barr virus gene expression. J Biotechnol 128: 462–476.

Bhatia S, Ramsay NK, Steinbuch M, Dusenbery KE, Shapiro RS, Weisdorf DJ, Robison LL, Miller JS, Neglia JP (1996) Malignant neoplasms following bone marrow transplantation. Blood 87: 3633–3639.

Cazzik K, Bzowska M, Dobrucki J, Pryjma J (1999) Heat-shocked monocytes are resistant to Staphylococcus aureus-induced apoptotic DNA fragmentation due expression of HSP 72. Infect Immun 67: 4216–4222.

Hopwood PA, Brooks L, Parratt R, Hunt BJ, Bokhari M, Thomas JA, Yacoub M, Crawford DH (2002) Persistent Epstein-Barr virus infection: unrestricted latent and lytic viral gene expression in healthy immunosuppressed transplant recipients. Transplantation 74: 194–202.

Jebbink J, Bai X, Rogers BB, Dawson DB, Scheuermann RH, Domiati-Saad R (2003) Development of real-time PCR assays for the quantitative detection of Epstein-Barr virus and cytomegalovirus, comparison of TaqMan probes and molecular beacons. J Mol Diagn 5: 5–20.

Mundle S, Allampillam K, Attah Rashid K, Dangerfield B, Cartledge J, Zeitzler D, Atenya E, Alvi S, Shetty V, Venugopal P, Raza A (2001) Presence of activation-related mRNA for EBV and CMV in the bone marrow of patients with myelodysplastic syndromes. Cancer Lett 164: 197–205.

Nalesnik MA (1998) Clinical and pathological features of posttransplant lymphoproliferative disorders (PTLD). Springer Semin Immunopathol 29: 325–342.

Prang N, Hornem M, Jäger M, Wagner HJ, Wolf H, Schwarzman FM (1997) Lytic replication of Epstein-Barr virus in the peripheral blood: analysis of viral gene expression in B lymphocytes during infectious mononucleosis and in the normal carrier state. Blood 89: 1665–1677.

Qu L, Green M, Webber S, Reyes J, Ellis D, Rowe D (2000) Epstein-Barr virus gene expression in the peripheral blood of transplant recipients with persistent circulating virus loads. J Infect Dis 182: 1013–1021.

Rayes N, Seehofer D, Lullius SG, Stein A, May G, Kahl A, Frei U, Neuhaus P, Meisel H (2005) Monitoring of human cytomegalovirus, HHV-6 and HHV-7 infection in kidney transplant recipients by molecular methods to predict HCMV disease after transplantation: a prospective study. Ann Transplant 10: 23–28.

Rezonen RR, Paya CV (2003) Herpesvirus infections in transplant recipients: current challenges in the clinical management of cytomegalovirus and Epstein-Barr virus infections. Herpes 10: 60–65.

Rickinson AB, Kieff E (2007) Epstein-Barr virus. In Virology. Fields BN, Knipe DM, Howley PM, eds, pp 2655–2700. Lippincott-Raven Publishers, Philadelphia.

Savoie A, Perpete C, Carpentier L, Joncas J, Alfieri C (1994) Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of
pediatric transplant patients and risk of lymphoproliferative disorder. *Blood* 9: 2715–2722.
The TH, van den Berg AP, Harmsen MC, van der Bij W, van Son WJ (1995) The cytomegalovirus antigenemia assay: a plea for standardization. *Scan J Infect Dis* (Suppl) 99: 25–29.
Tsurumi T, Fujita M, Kudoh A (2005) Latent and lytic Epstein-Barr virus replication strategies. *Rev Med Virol* 15: 3–15.
Tysarowski A, Fabisiewicz A, Paszkiewicz-Kozik E, Kulik J, Walewski J, Siedlecki JA (2007) Usefulness of real-time PCR in long-term follow-up of follicular lymphoma patients. *Acta Biochim Polon* 54: 135–142.
Venard V, Carret AS, Pascal N, Rihn B, Bordigoni P, Le Faou A (2000) A convinient semi-quantitative method for the diagnosis of Epstein-Barr virus reactivation. *Arch Virol* 145: 2211–2216.
Wagner HJ, Rooney C, Heslop H (2002) Diagnosis and treatment of posttransplantation lymphoproliferative disease after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 8: 1–8.
Wagner HJ, Cheng YC, Huls MH, Gee AP, Kuehnle I, Krance RA, Brenner MK, Rooney CM, Heslop HE (2004) Prompt versus preemptive intervention for EBV lymphoproliferative disease. *Blood* 103: 3979–3981.
Zawilinska B, Bulek K, Kopec J, Kosz-Vrenchak M (2006) In situ detection of DNA and mRNA of human cytomegalovirus to distinguish different forms of viral infection in leukocytes. *Acta Biochim Polon* 53: 457–461.