Original Article

Detection of Human Herpes Viruses CMV, EBV, and HHV6 and their co-infection in renal and allogeneic hematopoietic stem cell transplant recipients by multiplex real-time PCR

*Nazmul Hasan[1], Rummana Rahim[2], Abu Hasan[3], Abu Jafar Mohammad Saleh[4], Mizanur Rahman[5]**

1. Scientific Officer, Molecular Diagnostics, Evercare Hospital Dhaka.
2. Associate Consultant, Molecular Diagnostics, Evercare Hospital Dhaka.
3. Chief Scientific Officer, Molecular Diagnostics, Evercare Hospital Dhaka.
4. Sr. Consultant & Coordinator, Hematology & Stem Cell Transplant, Evercare Hospital Dhaka.
5. Sr. Consultant, Molecular Diagnostics, Evercare Hospital Dhaka.

Address for Correspondence :
Dr. Mizanur Rahman
Sr. Consultant,
Molecular Diagnostics,
Evercare Hospital Dhaka.
mizanur.rahman@evercarebd.com

Submitted: *12 – Dec - 2023*
Accepted: *02 – Feb - 2024*

ABSTRACT

Background: Herpesviruses infection especially Cytomegalovirus (CMV) in transplant recipients cause life-threatening diseases. Furthermore, their management may be complicated by co-infection with other human herpes viruses like Epstein-Barr Virus (EBV) and human herpes virus 6 (HHV6). Besides CMV, no data is available for EBV and HHV6 in transplant recipients in the country.

Study Population: The study included 48 renal transplant recipients, and 36 allogenic hematopoietic stem cell transplant (allo-HSCT) recipients treated at Evercare Hospital in Dhaka, Bangladesh, between February 2015 and November 2023.

Materials and method: The transplant recipients were advised to undergo CMV, EBV, and HHV6 PCR tests based on recommendations from nephrologists and hematologists. The diagnostic method used was multiplex real-time PCR, which allows for the simultaneous detection of multiple pathogens in a single test.

Results: In renal transplant recipients, 43.75% tested positive for herpesvirus infections. Among them, 35.42% had CMV as the sole infection, while 8.33% had co-infections involving CMV, EBV, and HHV6. In allo-HSCT recipients, 88.89% tested positive for herpesvirus infections. Of these, 58.33% had CMV as the only infection, 11.11% had HHV6, and 19.45% had co-infections with CMV, EBV, and HHV6. Interestingly, no cases of EBV infection as a single pathogen were found in either group of patients.

Thus, this study suggests that after renal and allo-HSCT transplant procedures, multiplex real-time PCR for CMV, EBV, and HHV6 may be valuable for the further management of these patients and warrants large scale study.

Keywords: *CMV, EBV, HHV6, Co-infection, Renal transplant, Allo -HSCT, Multiplex PCR.*

INTRODUCTION

Human herpes viruses are herpes simplex virus type 1 (HSV1) and type 2 (HSV2), varicella-zoster virus (VZV), epstein-barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 6 (HHV6), human herpesvirus 7 (HHV7), human herpesvirus 8 (HHV8) or kaposi's sarcoma associated herpes virus (KSHV).[1] Herpes viruses are present in human population all over the world and often reactivated in latently infected immunosuppressed patients[2]. After renal and hematopoietic stem cell transplant, herpesviruses reactivation frequently occurs[3-4]. Among human herpesviruses, EBV,CMV and HHV6 may cause severe and occasionally lethal complications in transplant recipients, such as pneumonitis, enteritis, encephalitis, and viral associated lymphoproliferative disorders etc[5-8]. In transplant recipients it was evident that CMV was an active introducer of some members of the herpesvirus family like EBV and HHV6.[9] CMV can enhance the pathogenicity of other viruses through interaction with the transplant recipients body defense systems. As a result, co-infection occurs and co-infection between CMV and other herpesviruses like EBV and HHV6 were

important cofactors of lethal and severe diseases[10]. Therefore, it is important to screen and monitor for these viruses and diagnose any virus-related diseases as early as possible to ensure the success of transplantation. Though CMV was found positive in approximately one third of renal transplant recipients in early post-transplant period in Bangladesh there is no data of co-infection rate in the country[11]. Hematopoietic stem cell transplants have been started in the country in the recent past and there was no study yet regarding existence of herpes viruses like CMV, EBV and HHV6 alone or co-infection among allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

After the invention of real time PCR technology, PCR assays are becoming widespread tools for monitoring and alternative diagnosis[12] . At the very beginning of PCR technology, pathogen was identified by conventional PCR and later with real time PCR through which single pathogen was detected by each run. However, conventional PCR is laborious and monoplex PCR is expensive due to the use of separate kit for each pathogen. Moreover, there may be more than one virus simultaneously reactivate after renal and hematopoietic stem cell transplant[13-14]. Real time multiplex PCR can resolve this issue by detecting multiple pathogens simultaneously by a single run which may save time and money[15-16].

Here we report the existence of CMV, EBV and HHV6 virus and their co-infection rate detected by multiplex real time PCR in renal and allo-HSCT recipients attended at Evercare Hospital Dhaka, Bangladesh from February 2015 to November 2023.

MATERIAL AND METHODS

Method of data collection: The data of the patients were taken from hospital information system of Evercare Hospital Dhaka (Ex. Apollo Hospitals Dhaka), Bangladesh. This study was carried out between February 2015 and November 2023 among renal and allo-HSCT recipients. Age, sex, type of specimen clinical history etc. were used for data analysis. To protect patients' private information except for the age and sex, all samples were de-identified and patient consent were not needed as data is extracted from the routine test result available in the hospital information system.

Clinical Samples: The samples were sent by the Nephrology and Hematology department of Evercare Hospital Dhaka. A total of 84 samples were collected from the age group of 02-66 years old. Peripheral blood with EDTA was collected from post-transplant recipients who were advised for CMV, EBV and HHV6 by Hematologist and Nephrologist. Then centrifuge the tube and plasma were separated in 2 ml micro-centrifuge tube and plasma were used for DNA extraction. All samples were stored at -20°C until DNA extraction and then extracted DNA was stored at -80°C. All the laboratory works were performed in the molecular laboratory of Evercare Hospitals Dhaka.

DNA extraction & Multiplex Real time PCR: DNA was extracted by using QIAamp DNA Mini (Qiagen, Germany) spin column-based extraction kit. 200 µl of sample was used for DNA extraction. The elution volume was 50 µl. We used CE-IVD approved Sacace CMV/EBV/HHV6 Real-TM commercial multiplex real time PCR kit from Sacace Biotechnologies Srl, Italy. The total PCR volume was 25 µl where 15 µl master mix prepared for each sample, Negative control (NC) and Positive control (PC) and then 10 µl of extracted DNA, NC & PC was added respectively in 0.2 ml PCR strip tube. PCR amplification was done by Rotor-Gene Q (Qiagen, Germany) and QuantStudio-5 Dx (Applied Biosystems) thermocycler according to kit manufacturer's instruction which was programmed as follows: Hold-95˚C for 15 min, 5 cycles of 95˚C for 5s , 60˚C for 20s, 72˚C for 15 s , then 40 cycles of 95˚C for 5s, 60˚C for 20s (30s for QuantStudio-5 Dx) and 72˚C for 15s. Signal was acquired at 60˚C, and analysis was performed on the linear scale. Thresholds were set manually in each run. The fluorescence was detected in ROX/Orange channel for Cytomegalovirus, JOE/Yellow/HEX channel for Epstein Barr Virus, CY5/Red channel for Human Herpes Virus 6 and FAM/Green channel for

amplification of internal control. The recommendations of the manufacturer were strictly followed for DNA extraction and Real time PCR.

RESULTS

Our study population consisted of 84 transplant recipients of which 48 (57.14%) were renal transplant recipients and 36 (42.86%) were allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients.

CMV/EBV/HHV6 in Renal transplant recipients

Out of 48 patients 62.5% were male and 37.5% were female in this group. 46 patients were among 19-65 years old. Among all the patients 21 (43.75%) had at least one pathogen positive cases and of them, 17(35.42%) were only CMV positive cases and 4 (8.33%) were co-infection with CMV, EBV and HHV6 cases (table 1). There are no EBV and HHV6 infections found as a single pathogen in renal transplant recipients. Viral infection rate was higher in male than in female. Among 21 positive cases 66.67% of patients were male and 33.33% were female. Among positive cases, generalized weakness (57.14%), cough (47.61%), fever (38.09%), abdominal discomfort (23.80%), loose motion (23.80%) were the most common symptoms (table 3). Nausea, vomiting, breathing difficulties were also observed.

Table 1: Distribution of CMV/EBV/HHV6 among renal transplant recipients

Age (Years)	Sex(M/F)		Total Case	Positive (%)	CMV (%)	EBV (%)	HHV6 (%)	Co-infection CMV/EBV/HHV6 (%)
	Total	Positive						
0-5	M-0 F-0	M-0 F-0	0	0				
6-18	M-1 F-0	M-1 F-0	1	1				1
19-45	M-12 F-8	M-6 F-3	20	9	5 3			1
46-65	M-16 F-10	M-7 F-4	26	11	5 4			2
>65	M-1 F-0	M-0 F-0	1	0				
Total (%)	M-30(62.5) F-18(37.5)	M-14(66.67) F-7(33.33)	48 (100)	21 (43.75)	17 (35.42)	0 (0)	0 (0)	04 (8.33)

CMV/EBV/HHV6 in allo-HSCT transplant recipients

Out of 36 patients 72.23 % were male and 27.77% were female in this group. Among them 32 (88.89%) were positive cases with at least one pathogen and of them 21 (58.33%) were positive for CMV, 4 (11.11 %) were HHV6 and 7 (19.45%) were co-infection cases (table 2). There was no single EBV infection found in this group. Viral infection rate was higher in male than in female. Among 32 positive cases 68.75% patients weremale and 31.25% were female. In this group, a higher positivity rate was found in the age group of 19-45 years old. Among 32 positive cases the most prevalent symptoms were generalized weakness (40.62%), nausea (34.37%), vomiting (34.37%) and abdominal discomfort (31.25%) with loose motion, fever, cough and breathing difficulties (table 3).

Table 2: Distribution of CMV/EBV/HHV6 among allo -HSCT recipients

Age (Years)	Sex(M/F)		Total Case	Positive (%)	CMV (%)	EBV (%)	HHV6 (%)	Co-infection CMV/EBV/HHV6 (%)
	Total	Positive						
0-5	M-1 F-0	M-1 F-0	1	1	1			
6-18	M-6 F-0	M-6 F-0	6	6	2		2	2
19-45	M-12 F-7	M-10 F-7	19	17	8 6		1	1 1
46-65	M-7 F-3	M-5 F-3	10	8	2 2		1	3
>65	M-0 F-0	M-0 F-0	0	0				
Total (%)	M-26 (72.23) F-10 (27.77)	M-22 (68.75) F-10 (31.25)	36 (100)	32 (88.89)	21 (58.33)	0 (0)	4 (11.11)	7 (19.45)

Table 3: Clinical symptoms of herpes viruses (CMV/EBV/HHV6) in both group of positive recipients:

Symptoms	Renal transplant recipients no. (%)	allo-HSCT recipients no. (%)
Generalized weakness	12 (57.14)	13 (40.62)
Nausea	2 (9.52)	11 (34.37)
Vomiting	2 (9.52)	11 (34.37)
Loose motion	5 (23.80)	3 (9.37)
Abdominal discomfort	5 (23.80)	10 (31.25)
Fever	8 (38.09)	4 (12.5)
Cough	10 (47.61)	3 (9.37)
Breathing difficulty	4 (19.04)	3 (9.37)

CMV/EBV/HHV6 Co-infections

Co-infection was found in both renal and HSCT groups. In renal transplant recipients total 4 (8.33%) infection found and all were double infection. Among them 3 (6.25%) were CMV and EBV and 1 (2.08%) was CMV and HHV6. There was no triple infection found in this group. In allo-HSCT recipients, a total of 7 (19.45%) co-infections were found. Among them 5 (13.89%) were CMV and HHV6 and 1 (2.78%) was CMV and EBV and 1 (2.78%) was CMV, EBV and HHV6 co-infection (Figure: 1).

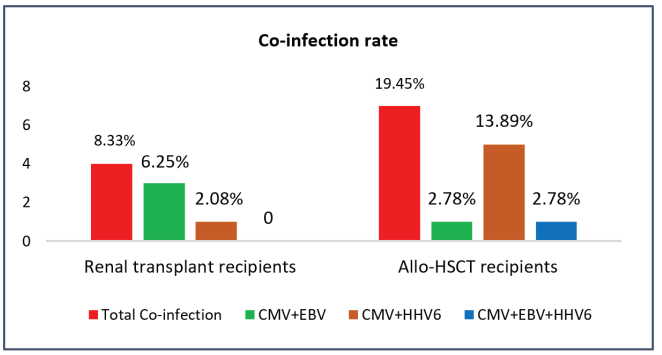

Figure 1: Distribution of CMV/EBV/HHV6 co-infection in renal transplant and allo-HSCT recipients.

DISCUSSION

Human herpes viruses particularly CMV, EBV and HHV6 have a great impact on the health of transplant recipients. Post-transplant infection is a common cause of graft deterioration, morbidity, and mortality[17]. To our knowledge, this is the first study in Bangladesh using the multiplex real time PCR assay for detection of CMV, EBV and HHV6 DNA in renal and allo-HSCT recipients. It was already proved that the multiplex assay is sensitive and specific as the single real time PCR assay which facilitates cost effective diagnosis and may contribute to decrease in the use of antiviral agents, viral complications, and hospitalization of patients[18]. The results of our study demonstrated a

high level of CMV infection in both groups of transplant recipients. CMV alone was positive 35.42 % in renal and 58.33 % in allo-HSCT recipients. Our data was similar with one PCR based study in Bangladesh, where showed that 34.4% renal transplant patients were CMV positive[11]. A study from Brazil has also shown 39.4 % of CMV positive among renal transplant recipients[19]. In renal transplant recipients, we found CMV-EBV and CMV-HHV6 co-infection 6.25% and 2.08%, respectively. The existence of these co-infection reported in other studies were showed CMV-EBV co-infection was 2.6% to 32.7% and CMV-HHV6 co-infection was 9.1 to 29.3 % in solid organ transplant recipients including renal transplant recipients[20-21]. In our study there are no triple infections found in renal transplant recipients. In allo-HSCT recipients, our data was also matched with other study in Pakistan, where CMV detection rate was higher and the positivity rate was 66.1% [22] In this group, CMV-EBV, CMV-HHV6 and CMV-EBV-HHV6 co-infection were found 2.78%, 13.89% and 2.78% respectively. We observed a higher co-infection rate between CMV and HHV6 in our study. Higher co-infection rate was also reported in two previously published studies where co-infection of CMV and HHV6 among positive cases were 52.3% [23] and 52.8% [24] in allo-HSCT recipients.

In this study, we found HHV6 infection as a single pathogen only in allo-HSCT recipients. Interestingly, no cases of EBV infection as a single pathogen were found in either allo-HSCT recipients or renal transplant recipients.

Another important issue that needs to be mentioned here is, the herpes viruses like CMV, EBV and HHV6 can affect the transplant recipient patients through blood and blood products transfusion as they can be transmitted through blood[25]. In the context of Bangladesh, the transfusion medicine department does not screen the blood donor for CMV, EBV and HHV6,[26] although healthy blood donor may be infected by these pathogens. Several studies from many countries exhibited the existence of herpes viruses, especially CMV, EBV and HHV6 infection and their co-infection among blood donors[27-30]. Blood, or blood product transfusion may be needed during treatment or after transplantation. So, it is better to screen herpes viruses CMV, EBV and HHV6 in all blood donors to reduce pathogen transmission in renal and allo-HSCT recipients and multiplex PCR can help to identify all herpes viruses by a single test. Screening of CMV alone may miss cases where infection may happen with other member alone such as we found few cases of HHV6 alone in allo-HSCT recipients.

CONCLUSION

Human herpesviruses remain an important challenge in immunocompromised hosts, like transplant recipients. In our study, we found evidence of herpes virus infection and their co-infection among both types of transplant recipients. Till now, for latent infection, serology remains a useful marker, but molecular based assay needs for current diagnosis of herpes virus infection. Multi-center based large scale research may improve understanding of the burden of CMV, EBV, HHV6 infection and co-infections. Multiplex real time PCR can be used due to its high sensitivity and accurate diagnosis of herpesvirus viruses and their co- infection for better management of transplant recipients.

ACKNOWLEDGEMENT

We thank the following individuals for their contributions to this study: Abu Sobhan Murad, Mr. Malay Biswas, Abdus Sabur.

REFERENCES

1. Fishman JA. Overview: cytomegalovirus and the herpesviruses in transplantation. Am J Transplant. 2013;13 Suppl 3:1-8. doi:10.1111/ajt.12002
2. Timbury, M. C., and E. Edmond. 1979. Herpesviruses. J. Clin. Pathol. 32: 859–881.
3. Jenkins, F. J., D. T. Rowe, and C. R. Rinaldo, Jr. 2003. Herpesvirus infections in organ transplant recipients. Clin. Diagn. Lab. Immunol. 10:1–7.
4. Yoshikawa, T. 2004. Human herpesvirus 6 infection in hematopoietic stem cell transplant patients. Br. J. Haematol. 124:421–432.

5. Limaye, A. P., M.-L. Huang, W. Leisenring, L. Stensland, L. Corey, and M. Boeckh. 2001. Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem-cell transplant recipients. J. Infect. Dis. 183:377–382.
6. Razonable, R. R., R. A. Brown, A. Humar, E. Covington, E. Alecock, C. V. Paya, and the PV16000 Study Group. 2005. Herpesvirus infections in solid organ transplant patients at high risk of primary cytomegalovirus disease. J. Infect. Dis. 192:1331–1339.
7. Hentrich, M., D. Oruzio, G. Ja¨ger, M. Schlemmer, M. Schleuning, X. Schiel, W. Hiddemann, and H.-J. Kolb. 2005. Impact of human herpesvirus-6 after haematopoietic stem cell transplantation. Br. J. Haematol. 128:66–72.
8. Gruhn B, Meerbach A, Hafer R, Zell R, Wutzler P, Zintl F. Pre-emptive therapy with rituximab for prevention of Epstein-Barr virus-associated lymphoproliferative disease after hematopoietic stem cell transplantation. Bone Marrow Transplant. 2003; 31: 1023–5
9. Sia IG, Patel R. New strategies for prevention and therapy of cytomegalovirus infection and disease in solid-organ transplant recipients. Clin Microbiol Rev. 2000;13(1):83-121
10. Anderson-Smits C, Baker ER, Hirji I. Coinfection rates and clinical outcome data for cytomegalovirus and Epstein-Barr virus in post-transplant patients: A systematic review of the literature. Transpl Infect Dis. 2020;22(6):e13396. doi:10.1111/tid.13396
11. Zannat H, Mamun AA, Salahuddin AZ, et al. Clinical Profiles and Outcomes of Cytomegalovirus Positive Renal Transplant Patients in Early Post-Transplant Period. Mymensingh Med J. 2023;32(2):371-377
12. Mackay, I. M., K. E. Arden, and A. Nitsche. 2002. Real-time PCR in virology. Nucleic Acids Res. 30:1292–1305
13. Niesters, H. G. M. 2004. Molecular and diagnostic clinical virology in real time. Clin. Microbiol. Infect. 10:5–11.
14. Pradeau, K., L. Couty, J.-C. Szelag, P. Turlure, F. Rolle, P. Ferrat, D. Borsessoule, Y. L. Meur, F. Denis, and S. Ranger-Rogez. 2006. Multiplex real-time PCR assay for simultaneous quantitation of human cytomegalovirus and herpesvurus-6 in polymorphonuclear and mononuclear cells of transplant recipients. J. Virol. Methods 132:77–88
15. Templeton, K. E., S. A. Scheltinga, N. F. C. Beersma, A. C. M. Kroes, and E. C. J. Claas. 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol. 42:1564–1569
16. Traverso, M., M. Malnati, C. Minetti, S. Regis, S. Tedeschi, M. Pedemonte, C. Bruno, R. Biassoni, and F. Zara. 2006. Multiplex real-time PCR for detection of deletions and duplications in dystrophin gene. Biochem. Biophyse Res. Commun. 339:145–150.
17. Evans R, Bhagani S, Haque T, Harber M. Infectious Complications of Transplantation. Practical Nephrology. 2014;829-856.Published 2014 Mar 1.doi:10.1007/978-1-4471-5547-8_71
18. Wada K, Kubota N, Ito Y, et al. Simultaneous quantification of Epstein-Barr virus, cytomegalovirus, and human herpesvirus 6 DNA in samples from transplant recipients by multiplex real-time PCR assay. J Clin Microbiol.2007;45(5):1426-1432.doi:10.1128/-JCM.01515-06
19. Santos SDRD, Bafi AT, Freitas FGR, Azevedo LCP, Machado FR. Prevalence of cytomegalovirus disease in kidney transplant patients in an intensive care unit. Prevalência de doença por citomegalovírus em transplantados renais em unidade de terapia intensiva. Rev Bras Ter Intesiva.2017;29(4):436-443.-doi:10.5935/0103-507X.20170070
20. Anderson-Smits C, Baker ER, Hirji I. Coinfection rates and clinical outcome data for cytomegalovirus and Epstein-Barr virus in post-transplant patients: A systematic review of the literature. Transpl Infect Dis. 2020;22(6):e13396. doi:10.1111/tid.13396
21. Humar A, Asberg A, Kumar D, et al. An assessment of herpesvirus co-infections in patients with CMV disease: correlation with clinical and virologic outcomes. Am J Transplant. 2009;9(2):374-381. doi:10.1111/j.1600-6143.2008.02501.x
22. Iftikhar R, Farhan M, Khan M, et al. Cytomegalovirus Infection Post-Allogeneic Stem Cell Transplantation: Experience from a Country with High Seropositivity. Transplant Cell Ther. 2023;29(8):521.e1-521.e7. doi:10.1016/j.jtct. 2023.04.023
23. Tormo N, Solano C, de la Cámara R, et al. An assessment of the effect of human herpesvirus-6 replication on active cytomegalovirus infection after allogeneic stem cell transplantation. Biol Blood Marrow Transplant. 2010;16(5):653-661. doi:10.1016/j.bbmt.2009.12.003
24. Wang LR, Dong LJ, Lu DP. [Prevalence of human herpesvirus-6 in allogeneic hematopoietic stem cell transplant recipients in correlation with cytomegalovirus infection]. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2006 Dec;14(6):1204-9. Chinese. PMID: 17204195.
25. Shaz BH. Transfusion Transmitted Diseases. Transfusion Medicine and Hemostasis. 2009:361–71. doi: 10.1016/B978-0-12-374432-6.00066-X. Epub 2009 May 20. PMCID: PMC7150182.
26. Islam MB. Blood transfusion services in Bangladesh. Asian J Transfus Sci. 2009; 3(2):108-110. doi:10.4103/0973-6247.53880
27. Traore L, Tao I, Bisseye C, Diarra B, Compaore TR, Nebie Y, Assih M, Ouedraogo A, Zohoncon T, Djigma F, Ouermi D, Barro N, Sanou M, Ouedraogo RT, Simpore J. Molecular diagnostic of cytomegalovirus, Epstein Barr virus and Herpes virus 6 infections among blood donors by multiplex real-time PCR in Ouagadougou, Burkina

Faso. Pan Afr Med J. 2016 Aug 3;24:298. doi: 10.11604/pamj.2016.24.298.6578. PMID: 28154653; PMCID: PMC5267872.

28. Hudnall SD, Chen T, Allison P, Tyring SK, Heath A. Herpesvirus prevalence and viral load in healthy blood donors by quantitative real-time polymerase chain reaction. Transfusion. 2008 Jun;48(6):1180-7. doi: 10.1111/j.1537-2995. 2008.01685.x. Epub 2008 Apr 15. PMID: 18422852.
29. Kothari A, Ramachandran VG, Gupta P, Singh B, Talwar V. Seroprevalence of cytomegalovirus among voluntary blood donors in Delhi, India. J Health Popul Nutr. 2002 Dec;20(4):348-51.
30. Furui Y, Satake M, Hoshi Y, Uchida S, Suzuki K, Tadokoro K. Cytomegalovirus (CMV) seroprevalence in Japanese blood donors and high detection frequency of CMV DNA in elderly donors. Transfusion. 2013 Oct;53(10):2190- 7