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M1 enrichment facilitates virus detection in patients with allogeneic hematopoietic stemcell transplantation

Hao Zheng, MM[a], Sai Li, MD[b], Ze-Yin Liang, MD[c], Ru-Li Feng, MD[d], Jin-Xing Lu, MM[a], Yu-Jun Dong, MD[c], Xiao-Ping Chen, MD[a,*]

Abstract

Due to the severe consequences of viral infection in allogeneic hematopoietic stem cell transplantation (allo-HSCT) patients, the routine use of polymerase chain reaction (PCR) screening of viruses is common in clinic, while the sensitivities of molecular methods are not always sufficient for blood samples. We aimed to increase the detection efficiency of viremia in allo-HSCT patients with M1 bead enrichment. Blood samples of allo-HSCT patients with fever were collected. Simultaneously with analyses of real-time PCR without enrichment, M1 bead enrichment followed by real-time PCR was applied to detect possible viruses in these samples, and metagenomic next-generation sequencing analyses were also applied in 10 samples negative with real-time PCR without enrichment. Various species of viruses were detected with M1 enrichment method. Significantly, some viruses that had not been found by real-time PCR without M1 enrichment were also detected by those with M1 enrichment and verified by metagenomic next-generation sequencing analyses. Furthermore, blood samples enriched with M1 beads had lower Ct values of real-time PCR assay than those that had not been treated. In conclusion, M1 bead enrichment increased the detection efficiency of most viruses in hematological malignancy patients.

Abbreviations: ADV = adeno virus, allo-HSCT = allogeneic hematopoietic stem cell transplantation, BKV = BK polyomavirus, CMV = cytomegalovirus, EBV = Epstein–Barr virus, HHV-6 = human herpesvirus-6, HSV = herpes simplex virus, MBL = mannan-binding lectin, mNGS = metagenomic next-generation sequencing, VZV = varicella zoster virus.

Keywords: allo-HSCT, enrichment, M1 beads, virus detection

1. Introduction

Viral infections are the most common and significant cause of mortality and morbidity after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Many species responsible for viral infection are endogenously reactivated.[1] For example, reactivation of cytomegalovirus (CMV) would readily occur in allo-HSCT because approximately 40% to 80% of the world's population has latent infection throughout life.[2] The most severe infection related to CMV is pneumonia, followed by esophagitis, gastritis, and enterocolitis. CMV viremia in the late phase tends to be persistent and life threatening. CMV infection is probably the main reason for unsuccessful transplantation.[3] Another common endogenous reactivation in allo-HSCT is the Epstein–Barr virus (EBV), and the most significant clinical syndrome is posttransplant lymphoproliferative disease.[4] Similarly, upon reactivation, herpes simplex virus (HSV) and varicella zoster virus (VZV) seropositive patients develop life-threatening serious mucocutaneous disease or dissemination, fulminant visceral involvement, and postherpetic neuralgia, unless given antiviral treatment.[5] Endogenous reactivation of human herpesvirus-6 (HHV-6) may result in engraftment delays or graft failure after allo-HSCT. Unusually but significantly, HHV-6 may cause a prominent disease with a facial rash and may be confused with encephalitis or acute graft-versus-host disease.[6]

HZ and SL contributed to this article equally.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval was granted by the Committee on Biomedical Ethics Peking University First Hospital (Research Ethics Committee approval, reference 2021-KY-310). All the methods were performed in accordance with the relevant guidelines and regulations.

[a] *Department of Noscomial Infection, National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention, Beijing, China,* [b] *Department of Laboratory Medicine, HuNan Children's Hospital, Xiangya Medical School, Central South University, Changsha, Hunan, China,* [c] *Department of Haematology, Peking University First Hospital, Beijing, China,* [d] *Clinical Laboratory of Peking University First Hospital, Beijing, China.*

** Correspondence: Xiao-Ping Chen, Department of Noscomial Infection, National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention, No.155 ChangBai Road, ChangPing District, Beijing, China (e-mail: chenxiaoping@icdc.cn).*

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BK polyomavirus (BKV) is involved in the etiology of viral hemorrhagic cystitis, as is the case with other polyomaviruses and adeno virus (ADV).[7] Other primary viral infections, such as influenza, respiratory syncytial virus, and ADV, usually cause severe respiratory system infections in all phases after allo-HSCT.[8]

Due to the severe consequences of viral infection in allo-HSCT, the pre-emptive treatment approach has recently replaced prophylaxis, which is ascribed to the development of novel diagnostic methods.[1] The routine use of polymerase chain reaction (PCR) in the clinic, which allows for the quantification of viral DNA, makes it possible to detect viremia earlier.[9] For example, regular monitoring of CMV DNA should be routinely performed in seropositive recipients. Surveillance with EBV-DNA once weekly has been suggested.[10] However, the sensitivity of molecular methods is not always sufficient, especially for blood samples that contain many PCR inhibitors such as hemoglobin, lactoferrin, and IgG.[11]

In a previous study, we developed a type of magnetic bead, that is, M1 beads, which were coated with a recombinant human mannan-binding lectin (MBL) that could increase EBV detection efficiency from patient whole blood.[12] Since MBL was reported to be able to bind many viruses,[13] M1 beads were used to enrich a variety of viruses in the blood of allo-HSCT patients and were analyzed with real-time PCR to investigate virus infection conditions in allo-HSCT patients in this study.

2. Methods

2.1. Patient enrollment and sample collection

Hematological malignancy patients hospitalized at Peking University First Hospital with body temperatures >38.5°C were recruited in the study from August 2021 to August 2022. In this study, an episode was defined as a separate case of clinically suspected infection. Blood samples were drawn by venipuncture using sterile BD Vacutainer lithium heparin tubes (BD Diagnostics, Shanghai, China). Before the assays, all samples were stored at 4°C for no more than 4 days.

2.2. Diagram of the M1 bead enrichment followed by real-time PCR (M1-qPCR) method compared with the standard qPCR (S-qPCR) method

All samples were analyzed simultaneously using both the M1-quantitative real-time polymerase chain reaction (qPCR) method and the standard qPCR (S-qPCR) method. In brief, using the M1-qPCR method, 2 mL of whole blood was enriched with 100 μL of M1 beads, followed by nucleic acid extraction and real-time PCR analyses, as described in following sections. With the S-qPCR method, 0.2 mL of whole blood was applied directly to nucleic acid extraction and real-time PCR analyses, as described in the following sections.

2.2.1. S-qPCR method. The standard method of virus detection was performed with 200 μL of blood, which was also used for viral RNA/DNA extraction using a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (Takara Bio, Dalian, China). The real-time PCR primers and probes used for the viruses are listed in Table 1. Positive results were considered for samples for which the Ct values were lower than 40, whereas those of the negative controls were higher than 40.

2.2.2. M1-qPCR method.

2.2.2.1. M1 bead enrichment. M1 bead enrichment was conducted as previously described.[12] The following procedures were performed in a biosafety cabinet: All materials, including the sample tubes, were sterilized with ultraviolet light in a cabinet for 30 minutes at the beginning of the experiment. Approximately 2 mL of blood was collected from each patient. Briefly, phosphate-buffered saline (PBS) was added to a final volume of 10 mL with 6 mM $CaCl_2$. Next, 100 μL of M1 beads was added to the sample. Negative controls consisting of 10 mL of PBS without the sample were treated simultaneously. The samples were then mixed by inversion in a rotor for 50 to 60 minutes at 37°C. The sample tubes were magnetized for 5 minutes on a magnetic rack. The microbe-bound beads were collected and resuspended in 200 μL of PBS.

2.2.2.2. Viral nucleic acid extraction and detection. Viral nucleic acids were extracted using a TaKaRa MiniBEST Viral RNA/DNA Extraction Kit, according to the manufacturer's protocol. The real-time PCR primers and probes for the viruses (including CMV, EBV, HSV-1/2, VZV, HHV-6, BKV, and ADV) are listed in Table 1.[14–20] Positive results were considered for samples for which the Ct values were lower than 40, whereas those of the negative samples were higher than 40.

2.3. mNGS and analysis

Ten whole-blood samples that were positive by M1-qPCR but negative by S-qPCR were sent to WILLINGMED Tech (Beijing, China) for metagenomic next-generation sequencing (mNGS)

Table 1

Primers and probes for viruses detected.

Virus	Upper primer (5'-3') Down primer (5'-3')	Probe (5'-3')
CMV	5'AGCGCCGCATTGAGGA3' 5'CAGACTCTCAGAGGATCGGCC3'	FAM-ATCTGCATGAAGGTCTTTGCCCAGTACATT-BHQ1
EBV	5'CCGGTGTGTTCGTATATGGAG3' 5'GGGAGACGACTCAATGGTGTA3'	FAM-TGCCCTTGCTATTCCACAATGTCGTCTT-BHQ1
HSV-1/2	5'CATCACCGACCCGGAGAGGGAC3' 5'GGGCCAGGCGCTTGTTGGTGTA3'	FAM-CCGCCGAACTGAGCAGACACCCGCGC-BHQ1
VZV	5'CGGCATGGCCCGTCTAT3' 5'TCGCGTGCTGCGGC3'	ROX-ATTCAGCAATGGAAACACACGACGCC-BHQ1
HHV-6	5'TAAATATCGATGCCGCTCTG3' 5'TACGTTCTAGCCATCTTCTTTG3'	FAM-CGCAAACGACAAAGCCA-BHQ1
BKV	5'AGCAGGCAAGRGTTCTATTACTA3' 5'GARGCAACAGCAGATTCYCAACA3'	FAM-AAGACCCTAAAGACTTTCCTTCTGATCTACACCAGTTT-MGB
ADV	5'GCCCACCCTGCTTTATCTTCTC3' 5'CAGGTAGACTGCCTCGATGATG3'	JOE-TGCACTCTGACCACGTCGAAAACTTC-BHQ1

ADV = adeno virus, BKV = BK polyomavirus, CMV = cytomegalovirus, EBV = Epstein–Barr virus, HHV-6 = human herpesvirus-6, HSV = herpes simplex virus, VZV = varicella zoster virus.

analyses. Briefly, whole-blood DNA was quantified using a Qubit dsDNA HS Assay Kit (Life Technologies, Waltham, MA). DNA libraries were constructed by tagmentation. Fragmented DNA and partial adapters were added using a Nextera XT Library Preparation Kit (Illumina) following the manufacturer's protocol. The quality of the libraries was assayed using a High-Sensitivity DNA Kit (Agilent, CA), and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara). For each sample, sequencing was performed on a NextSeq sequencer (Illumina), resulting in 15 to 20 million 75-bp single-end reads on average.

NGS data analysis was performed using the IDseqTM commercial bioinformatics pipeline (Vision Medical, Shanghai, China). Reads aligned to the human reference genome (GRCh38) using Burrows-Wheeler Alignment were excluded from further analyses. The remaining reads for taxonomic assignment were aligned against curated microbial databases consisting of viruses. The results are presented as the number of reads per 10 million.

2.4. Comparison of the detection efficiencies of M1 bead enrichment with anti-EBV antibody enrichment

Using the S-qPCR method described earlier, 11 EBV-positive blood samples were collected and stored at 4°C for no more than 4 days. PBS-simulated samples were prepared by serial dilution of freshly collected EBV particles with PBS. Patient EBV-positive blood samples and PBS-simulated EBV samples were detected with S-qPCR and enriched simultaneously with M1 beads or ant-EBV beads, respectively. The enriched beads were subjected to nucleic acid extraction, followed by real-time PCR. The procedure for anti-EBV bead enrichment was the same as that for M1 bead enrichment. Anti-EBV antibody beads were constructed in a manner similar to the construction of M1 beads. In brief, 400 μg of anti-EBV antibody (anti-EBV gp340/220 envelope protein antibody, Abcam ab6525, Cambridge, UK) was added to 100 μL of protein A beads. Nucleic acid extraction was performed as previously described. EBV was quantified using a commercial qPCR (DaAn Gene, Guangzhou, China).

2.5. Data interpretation and statistical analysis

The detection sensitivity of the M1 and standard methods was calculated for all recruited samples. Discrepancies between these methods regarding their ability to detect microorganisms were analyzed using Cochran Q test (Gretl software ver. 1.9.4., Geeknet Inc, VA). Statistical significance was set at $P < .05$.

3. Results

3.1. M1 enrichment significantly increased virus detection efficiency

During this period of study, 220 patients with hematological malignancies were enrolled during their chemotherapy or allogeneic HSCT phase. After excluding 3 cases of noninfectious fever, 217 blood samples were analyzed.

In total, 59 samples were identified as viremia using the M1-qPCR method (sensitivity: 59/217, 27.19%). For S-qPCR, 34 samples were identified (sensitivity: 34/217, 15.67%). The results of the M1-qPCR and S-qPCR methods were consistent with the 35 findings. The S-qPCR method missed 25 viremia findings identified by the M1-qPCR method, of which 10 samples were verified by mNGS. Interestingly, 14 samples were detected as multiple viral infections using either the M1-qPCR or the S-qPCR method. M1-qPCR detected 2 to 3 viruses in 4 samples, but the S-qPCR method detected none; in another 6 samples, M1-qPCR found 2 to 3 viruses while the S-qPCR method detected only 1. The detailed diagnostic results for these methods are shown in Table 2 (following the references).

In 59 samples with viremia, EBV and CMV were detected using the M1 method with significantly higher sensitivity and lower Ct values. Although there was no difference in the Ct values of BKV between these methods, M1-qPCR detected more BKV infection cases than S-qPCR. Furthermore, 1 case of VZV and ADV was detected by M1-qPCR but not by S-qPCR. However, statistical analysis was not possible because of the lack of positive samples (Table 3).

3.2. Comparing EBV enrichment efficiency between M1 beads and beads coated with anti-EBV antibody

As shown in Figure 1A, in PBS-simulated samples, M1 beads and anti-EBV antibody beads were all effective in capturing EBV particles, especially at low EBV concentrations, although anti-EBV antibody beads showed a little improvement at some points of concentration. However, in all 11 patient authentic samples, M1 beads significantly enriched EBV, while beads with anti-EBV antibodies hardly worked (Fig. 1B).

4. Discussion

In this study, we investigated the application of M1 bead enrichment for the detection of possible viruses in patients with hematological malignancies. Our study indicated that the results of M1-qPCR corresponded well with those of S-qPCR. More significantly, the M1-qPCR method had much higher sensitivity than the latter method. Furthermore, mNGS analyses also verified the positive results of M1-qPCR, which were not found by S-qPCR. These results demonstrate that M1-qPCR can indeed increase the sensitivity of viremia detection. Many studies have assessed the performance of new methods by classifying all findings judged as clinically relevant as "true positive."[21] In this study, the sensitivity of the M1-qPCR or S-qPCR method was also calculated for all episodes of cases with body temperatures >38.5°C. We realized that many of these patients might only have bacteremia. Therefore, the sensitivity may have been underestimated. The high sensitivity of M1-qPCR most likely resulted from its ability to decrease the abundance of human nucleic acids in whole blood with M1 beads.[22]

The viruses most often encountered in this study were EBV and CMV, followed by BKV and HSV-1/2, whereas only one case of VZV or ADV was detected solely by M1-qPCR. As reported in other studies, EBV, BKV, and HSV were among the most common viruses found in allo-HSCT patients.[5,23] However, HHV-6, which is also recognized as a commonly encountered virus in allo-HSCT patients,[24] was not found in our patients. Since this study was short of information about patients' clinic information, we could only speculate that the discrepancy might be a result of the different patient populations being utilized since HHV-6 infection usually occurs in the early postengraftment phase.[1] Interestingly, CMV reactivation is strongly associated with EBV reactivation.[25] In this study, CMV was also detected alongside EBV in 4 patients (numbers 173, 56, 38, and 131 in Table 1) with M1-qPCR, but not with S-qPCR. Given the severe consequences of CMV infection in all HSCT patients, M1-qPCR might play a significant role in decreasing the mortality due to CMV infection. In summary, M1 beads can be used to identify all the viruses described earlier.

The heterogeneity of M1 bead binding was ascribed to the broad binding spectrum of MBL, which can bind to various microbial carbohydrate structures, including viral envelope glycoproteins.[26] As indicated in this study, because BKV has no envelope, its binding efficiency is much lower than that of viruses with envelopes, such as EBV, CMV, and HSV. Interestingly, viruses without envelopes, such as BKV and ADV, could also be captured by M1 beads. Additionally, for some EBV-positive samples (such as numbers 24, 31, 146, and 161 in Table 1), the

Table 2

Viruses and Ct values detected by M1-qPCR and S-qPCR.

Patient number	M1-qPCR (Ct value)	S-qPCR (Ct value)	mNGS (RPTM)
5	BKV (35.98)	-	BKV (300)
76	CMV (37.29)	-	CMV (45)
158	BKV (35.87)	-	BKV (265)
172	BKV (35.62); EBV (35.99)	-	BKV (302); EBV (543)
39	CMV (35.14); BKV (36.20)	-	CMV (521); BKV (132)
43	EBV (33.82); BKV (36.57)	-	EBV (1005); BKV (27)
173	CMV (34.05); EBV (35.66); BKV (32.66)	-	CMV (252); EBV (166)
11	EBV (36.209)	-	EBV (28)
30	HSV-1/2 (35.85)	-	HSV-1 (700)
45	VZV (33.0)	-	VZV (876)
83	EBV (37.36)	-	NA
80	HSV-1/2 (37.78)	-	NA
105	EBV (37.42)	-	NA
112	CMV (36.88)	-	NA
114	EBV (36.98)	-	NA
119	EBV (36.92)	-	NA
135	CMV (35.88)	-	NA
138	CMV (36.8)	-	NA
142	HSV-1/2 (35.95)	-	NA
152	ADV (32.12)	-	NA
167	CMV (38.33)	-	NA
186	CMV (33.74)	-	NA
188	CMV (34.93)	-	NA
206	CMV (38.24)	-	NA
208	CMV (33.98)	-	NA
33	BKV (32.84); EBV (35.95)	BKV (36.79)	NA
37	BKV (32.09); EBV (35.20)	BKV (31.21)	NA
56	CMV (37.12); EBV (32.46)	EBV (38.0)	NA
81	EBV (33.82); HSV-1/2 (36.8)	EBV (36.24)	NA
91	HSV-1/2 (33.1); EBV (38.0)	HSV-1/2 (35.63)	NA
38	CMV (33.35); EBV (36.38); HSV-1/2 (35.82)	CMV (36.28)	NA
40	BKV (32.79); EBV (33.35)	BKV (33.89); EBV (35.56)	NA
41	EBV (32.91); BKV (36.18)	EBV (36.23); BKV (36.23)	NA
42	EBV (26.27); BKV (29.42)	EBV (30.37); BKV (31.05)	NA
131	CMV (32.72);EBV (32.69)	CMV (35.52); EBV (36.72)	NA
9	CMV (33.23)	CMV (36.25)	NA
18	EBV (33.65)	EBV (38.24)	NA
19	EBV (31.37)	EBV (36.43)	NA
24	EBV (26.16)	EBV (33.57)	NA
31	EBV (32.40)	EBV (38.82)	NA
146	EBV (25.05)	EBV (30.23)	NA
161	EBV (26.95)	EBV (31.72)	NA
34	EBV (33.41)	EBV (37.72)	NA
36	EBV (33.29)	EBV (36.62)	NA
68	CMV (34.81)	CMV (36.43)	NA
79	EBV (34.2)	EBV (36.25)	NA
84	EBV (35.0)	EBV (38.05)	NA
86	CMV (34.0)	CMV (36.62)	NA
92	EBV (33.0)	EBV (35.41)	NA
99	EBV (31.85)	EBV (34.42)	NA
102	EBV (33.0)	EBV (36.23)	NA
108	EBV (33.69)	EBV (36.44)	NA
122	CMV (28.92)	CMV (31.78)	NA
123	EBV (32.35)	EBV (35.42)	NA
124	EBV (30.35)	EBV (33.17)	NA
125	EBV (31.18)	EBV (34.68)	NA
133	CMV (34.0)	CMV (37.73)	NA
162	BKV (28.82)	BKV (29.92)	NA
192	BKV (34.05)	BKV (36.62)	NA

Samples marked in the light shadow had Ct values of more than 5 between M1-qPCR and S-qPCR. Samples marked in dark shadows were found to contain ≥2 viruses by M1-qPCR, but none were detected by S-qPCR.

- = negative, ADV = adeno virus, BKV = BK polyomavirus, CMV = cytomegalovirus, EBV = Epstein–Barr virus, HHV-6 = human herpesvirus-6, HSV = herpes simplex virus, NA = not applicable, qPCR = quantitative real-time polymerase chain reaction, RPTM = reads per ten million, S-qPCR = standard quantitative real-time polymerase chain reaction, VZV = varicella zoster virus.

differences in Ct values between M1-qPCR and S-qPCR were >5. According to the speculation that a 1 Ct difference means a 3.33-fold difference in quantity deduced from the log values, M1 beads might have enriched EBV from at least 3 mL of whole blood (0.2 mL × 3.33 × 5 = 3.33 mL). In fact, only 2 mL of whole blood was used for enrichment by M1 beads in this study. Thus, why could M1 beads enrich more viruses than the amount that theoretically exists in 2 mL of whole blood?

To answer this question, we compared the capture efficiency of the M1 beads with that of magnetic beads coated with

Table 3

Diagnostic performances of M1 method compared with standard method.

	M1 method		Standard method		
Pathogen	**Nu**	**Ct (mean ± SD)**	**Nu**	**Ct (mean ± SD)**	***P***
EBV	35	31.67 ± 2.75	23	35.50 ± 2.31	<.01
CMV	19	33.00 ± 1.78	7	35.80 ± 1.75	<.05
BKV	13	32.31 ± 2.36	7	33.67 ± 2.72	>.05
HSV-1/2	6	35.88 ± 1.56	1	35.63	NA
VZV	1	33.00	0	-	NA
ADV	1	32.12	0	-	NA

- = negative, ADV = adeno virus, BKV = BK polyomavirus, CMV = cytomegalovirus, EBV = Epstein–Barr virus, HSV = herpes simplex virus, NA = not applicable, Nu = number, VZV = varicella zoster virus.

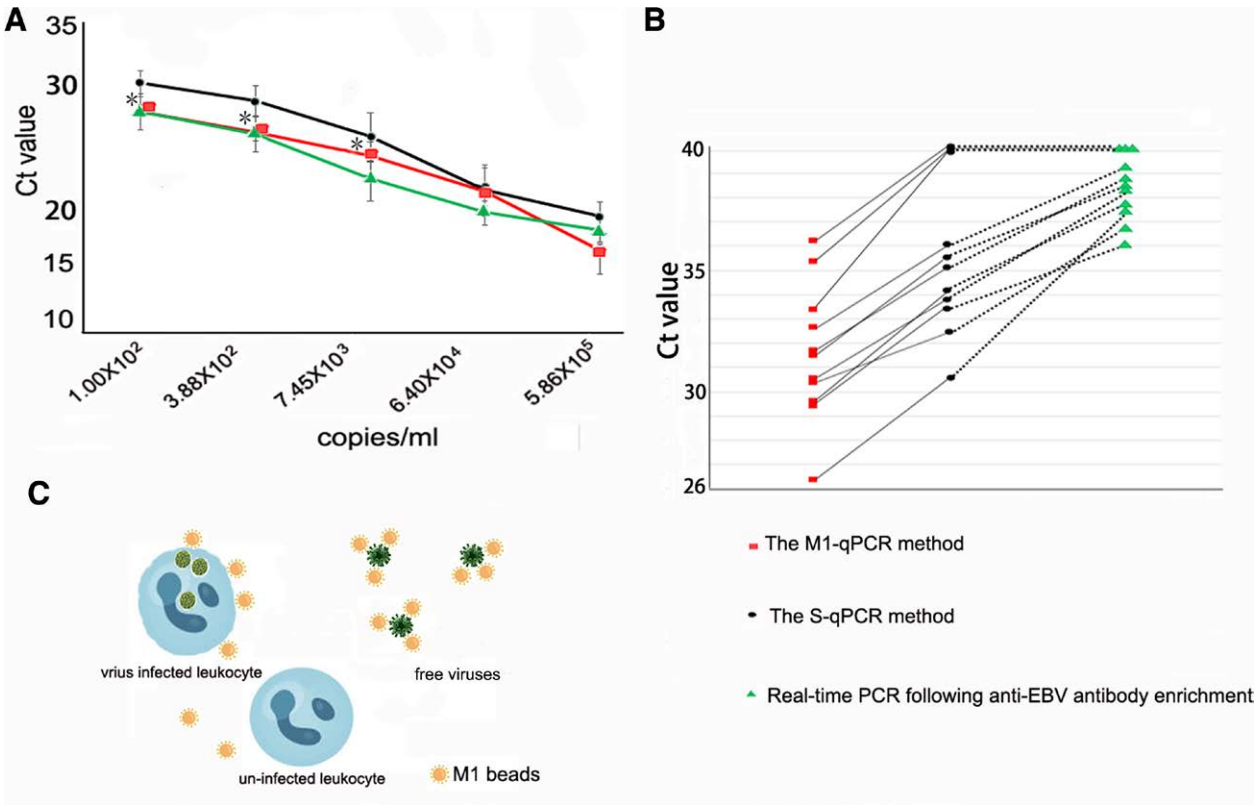

Figure 1. M1 bead enrichment increased virus detection sensitivity in patient blood samples more efficiently than in PBS-simulated samples. (A) Real-time PCR results of PBS-simulated EBV samples analyzed with standard qPCR, M1-qPCR, or qPCR after anti-EBV antibody beads enrichment. Results are the mean standard deviation (SD) values from assays of 5 repetitions. *$P < .05$; (B) Real-time PCR results of 11 EBV-positive patient blood samples assayed with standard qPCR, M1-qPCR, or qPCR after anti-EBV antibody beads enrichment. (C) Diagram of M1 enrichment facilitating pathogen detection by capturing either the pathogen itself or infected leukocytes. EBV = Epstein–Barr virus, PBS = phosphate-buffered saline, PCR = polymerase chain reaction, qPCR = quantitative real-time polymerase chain reaction.

anti-EBV envelope protein antibodies. Using these 2 magnetic beads, PBS-simulated samples containing only free EBV particles and authentic specimens with both free EBV particles and EBV-infected lymphocytes were analyzed. Based on these results, we speculated that EBV viruses could not be efficiently captured by typical anti-EBV antibodies in the blood, because almost all EBV viruses live in host lymphocytes.[27] However, M1 beads may even enrich EBV from EBV-infected blood cells, which provides a suitable explanation for the significant differences between M1 bead enrichment and anti-EBV antibody bead enrichment. The main functional structural domain of M1 is the carbohydrate recognition domain, similar to MBL, while MBL can bind to cells infected with pathogens.[28,29] We speculated that M1 beads could bind to both viruses and infected host cells (Fig. 1C). Thus, M1-qPCR can be applied to detect almost all viral nucleic acids in human blood.

At present, the sensitivity of molecular methods is not always sufficient for whole-blood testing. Thus, pretreatment, especially pathogen enrichment, is necessary even for mNGS.[30] Significantly, blood samples treated with M1 beads had higher sensitivities and lower Ct values than untreated samples. However, we realized that M1-qPCR, similar to other methods applying pretreatment procedures, has the limitations of additional hands-on time, cost, and ease of batching. However, these limitations can be overcome by applying automated instruments.

5. Conclusion

M1 bead enrichment significantly increased the detection sensitivity of various viruses in allo-HSCT patients. With some modifications and improvements, it can also be applied for virus detection in other patients.

Author contributions

Data curation: Hao Zheng, Sai Li.
Investigation: Hao Zheng.
Methodology: Hao Zheng, Sai Li, Ze-Yin Liang.
Software: Hao Zheng, Sai Li.
Formal analysis: Ze-Yin Liang, Ru-Li Feng, Xiao-Ping Chen.
Supervision: Jin-Xing Lu, Yu-Jun Dong.
Conceptualization: Xiao-Ping Chen.
Writing – original draft: Xiao-Ping Chen.
Writing – review & editing: Xiao-Ping Chen.

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