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Feasibility of the Routine Clinical Use of a Multiplex Virus Polymerase Chain Reaction Assay Based on Blood Virus Detection in Hematopoietic Stem Cell-Transplanted Patients

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Abstract

Background: Multiplex virus assays are useful in immunocompromised hosts but still challenging in routine clinical settings in terms of their sensitivity, specificity, reproducibility, and time and cost performances. In recent years, we developed a qualitative multiplex virus PCR assay capable of the simultaneous detection of 13 virus species within 3 h. However, because of the multiple and concomitant nature of this virus assay, it should be validated for qualitative reliability. **Materials and Methods:** As a preclinical examination, this multiplex PCR was able to detect 1.25×10^3 copies/mL of 13 synthesized virus genomes and preserved same virus DNAs by the serial dilution method. Blood samples from 40 patients who underwent hematopoietic stem cell transplantation were then examined by multiplex PCR for 13 virus species, followed by quantitative real-time PCR for all 13 virus species as reference PCR when these patients developed symptoms suggestive of viral infection. **Results:** In 421 cumulative qualitative-quantitative tests, the multiplex PCR certainly detected 1.0×10^3 copies/mL of 5 viruses (CMV, JCV, BKV, HHV-6, ADV) that were frequently detected and thus reasonably analyzed. The positive and negative predictive values of multiplex PCR were 84.2% - 93.3% and 90.7% - 99.0%, respectively, and sensitivity and specificity were 59.0% - 83.3% and 97.2% - 99.2%, respectively, for these 5 viruses. **Conclusion:** From these performances, the multiplex PCR assay may be acceptable in a routine clinical laboratory setting.

Keywords

Multiplex Virus PCR Assay, Routine Laboratory Use, Positive Predictive Value, Negative Predictive Value, Sensitivity, Specificity

1. Introduction

Virus assays are indispensable in immunocompromised hosts but still challenging to perform in routine clinical settings in terms of their sensitivity, specificity, reproducibility, and time and cost performances. In the early era of laboratory medicine, a serological virus assay was typically employed, capable of detecting just a single virus type with a poor time performance [1] [2], although the serological assay is still useful to determine a viral infection as the first, past infections, or re-activation. About three decades ago, virus polymerase chain reaction (PCR) assays were introduced [3] [4] [5] [6] [7], although they were still single-virus assays. However, majority of these studies are remaining as the reference in terms of sensitivity and specificity for respective viruses. In recent years, a number of multiplex virus PCR assays have been developed [8]-[13]; however, these assays have mainly been used for research, not as routine clinical tests, and the number and kind of assayable viruses differ from those of our own assay (13 virus species). In addition, Hwang *et al.* developed a refined multiplex real-time PCR assay in recent years, although assayable viruses were 3 kinds [14].

About 10 years ago, we developed a multiplex qualitative virus PCR assay targeting 12 representative virus species which affect immunocompromised patients, and used it in several clinical studies, including for assessing liver dysfunction [15], hematopoietic stem cell transplantation (HSCT) and miscellaneous immunodeficiency [16], infectious mononucleosis [17], and gastrointestinal virus infection [18]. Through these studies, we observed that this multiplex PCR assay was useful in terms of its sensitivity, specificity, ability for multiple and simultaneous virus detection, and rapid performance. In particular, we considered this virus assay to be indispensable for making an early diagnosis of virus infection or viremia in patients with a history of HSCT, regardless of the setting of a clinical study. For example, we could save a few patients' lives with our multiplex PCR making early diagnoses of life-threatening viral infection such as disseminated visceral varicella-zoster virus infection, meningitis by herpes simplex type 1/2, encephalitis by human herpes virus type 6, colitis by cytomegalovirus, and reactivation of human hepatitis B virus [16]. In addition, a group of Japanese investigators reported viral reactivation after HSCT in a large patient cohort as a clinical study using a multiplex PCR assay system that was basically the same as ours [19].

We therefore proposed the possibility of the routine clinical use of this multiplex virus PCR assay. In the present study, we validated our virus PCR assay for routine clinical use based on virus detection in patients who underwent HSCT in

our institution.

2. Materials and Methods

2.1. Patients

All patients who received HSCT from May 2015 to July 2021 in Shinko Hospital were included in the present study. A total 40 patients received HSCT, including 24 and 16 allogeneic and autologous transplantations, respectively. The median age of 40 patients was 58 years old (range: 48 - 69 years old), of which 21 and 19 patients were male and female, respectively. The conditioning regimen for allogeneic transplantation was standard fludarabine + 4-day busulfan (Ful-Bu4), cyclophosphamide + total body irradiation, or Ful-Bu4 + melphalan. The conditioning regimens for autologous transplantation for malignant lymphoma and multiple myeloma were ranimustine + etoposide + cytarabine + melphalan (MEAM) and high-dose melphalan, respectively.

2.2. Ethical Statement

The present prospective analysis was a single-institutional clinical study, designated, "The early diagnosis of viral infection by multiplex virus PCR assay in hematopoietic stem cell-transplanted patients", which had been approved by the institutional review board in June 2015 (IRB No.: 1505).

2.3. Blood Samples

EDTA-2Na-chelated whole blood (2 mL) was obtained before and after HSCT after obtaining written informed consent from each patient. After HSCT, the blood sample was collected when individual patients developed symptoms or clinical findings suggestive of viral infection, such as a fever, dyspnea, skin rash, headache, abdominal pain, diarrhea, or a high C-reactive protein (CRP) level. In the present study, individual patients developed multiple such episodes after HSCT. Accordingly, a median of 9.0 (range 4 - 27) multiplex virus PCR assays combined with real-time virus PCR assays were performed per patient.

2.4. Multiplex Qualitative Virus PCR and Quantitative Real-Time PCR Assay

After obtaining the blood sample, we collected the plasma and then extracted DNA using the QIamp MinElute Virus Spin kit (Qiagen, Tokyo, Japan). In this procedure, we obtained 100 μL of DNA solution (BufferAVE) (Qiagen) from 400 μL plasma. Although the methods of the multiplex virus PCR and real-time PCR assays were previously described in detail [15] [16], including the primer and probe sequences for 13 virus species, we modified a few points in the present study.

First, we performed a multiplex virus PCR assay using the GVP-9600 Gene Detection System (Shimadzu Corporation, Kyoto, Japan) and a solid-phase plate consisting of seven-well plates (Opportunistic DNA Viruses Detection Kit) (Shimadzu), in which primers, probes, Taq, and buffer constituents were fixed in

advance as a solid phase on well plates. Each well is for 2 or 3 kinds of virus species and for TBP and GADPH. The probes in the corresponding wells were labeled with 3 kinds of fluorochromes, 6FAM TM, HEX TM, and ROX TM [16]. We purchased the Viruses Detection Kit from Shimadzu Corporation. Second, we added 10 μL DNA solution instead of the previously mentioned 2 μL to the reaction mixture for the multiplex qualitative PCR assay in the present study. Therefore, the reaction mixture at a final volume was 20 μL consisted of 10 μL DNA solution and 10 μL dH_2O. Third, we performed quantitative real-time PCR for all 13 virus species after a qualitative multiplex PCR assay, regardless of the virus detection by multiplex PCR, to validate the accuracy of the multiplex PCR assay using the GVP-9600 Gene Detection System (Shimadzu). Namely, we designated this multiplex PCR followed by real-time PCR for all 13 virus species as one test per blood sample. The lower detection limit of our real-time PCR for 13 virus species was 2.5×10^2 copies/mL.

The 13 virus species examined in the present study were as follows: cytomegalovirus (CMV), JC virus (JCV), BK virus (BKV), human herpes virus type 6 (HHV-6), adenovirus (ADV), Epstein-Barr virus (EBV), parvovirus B19 (ParvoB19), varicella-zoster virus (VZV), human hepatitis B virus (HBV), herpes simplex virus type 1 (HSV-1), HSV-2, HHV-7, and HHV-8. Namely, ADV was an additional target in the multiplex PCR employed in the present study, differing from previous assay system targeting 12 virus species [15] [16] [17] [18].

Real-time PCR for CMV targeted the UL 83 gene domain [20]. Regarding CMV WHO International Standard [7] [21], the value obtained with our real-time PCR can be converted to the Standard value by multiplying by 0.3. This conversion was based on the comparison of the quantification cycle (Cq) value between the Standard system and our real-time PCR, that is, Cq values for 1000 IU of International standard (NIBSC code: 09/162) and our standard of 1000 copy/mL were 30.24 (n = 3) and 28.50 (n = 3), respectively (unpublished data). Regarding HHV-6, our multiplex-real time PCR system detected both the HHV-6A and HHV-6B genomes.

2.5. Detection Performance of the Multiplex PCR Assay by Serial Dilution of Virus DNA

The detection performance of multiplex PCR was examined by serial dilution of plasmid DNA for 13 virus species, which were synthesized by Nihon Techno Service Company (Ibaraki, Japan), and preserved DNA for 13 virus species from patients in whom the respective virus genomes had been detected and quantified with real-time PCR in the present study or the previous clinical study ("Multiple Virus-Analytic Study by Multiplex PCR"), which had been approved by the institutional review board in December 2010 (IRB No.: 0939). Our previous studies [15] [16] [17] [18] were performed under this approval. The detection performance test using preserved DNA from blood samples was also approved by the institutional review board (December 2018; IRB No.: 1834), and we provided patients with an opportunity to opt out from this analysis.

2.6. Statistical Analyses

The positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity of multiplex qualitative PCR were calculated according to conventional formulas using a calculator. To compare the mean values, student's *t*-test was used.

3. Results

3.1. Detection Performance of the Qualitative Multiplex PCR Assay Examined with Virus Genomes and Preserved Virus DNA

As a preclinical study, we first examined the detection performance of qualitative multiplex PCR with a known copy number of viruses by serial dilution. The detection performance was examined 2 ways: using synthesized plasmid DNA for 13 respective virus genomes and using preserved DNA from patients previously examined for viremia. With synthesized plasmid DNA, we performed 12-time repeated experiments with an almost 100% yield of positive detection of all 13 virus species at the lowest DNA concentration of 1.25×10^3 copies/mL as well as at higher concentrations (Table 1). With preserved DNA, we performed

Table 1. Detection performance of multiplex virus PCR assays for 13 virus species by serial dilution of synthesized plasmid DNA for respective virus genomes and preserved DNA from patients previously examined.

Viruses	1.25×10^4 and 6.25×10^3 copies/mL	1.25×10^3 copies/mL
CMV	12/12 (5/5)	12/12 (5/5)
JCV	12/12 (5/5)	12/12 (5/5)
BKV	12/12 (5/5)	11/12 (4/5)
HHV-6	12/12 (5/5)	12/12 (5/5)
ADV	12/12 (5/5)	11/12 (5/5)
EBV	12/12 (5/5)	12/12 (5/5)
ParvoB19	12/12 (5/5)	12/12 (5/5)
VZV	12/12 (5/5)	11/12 (4/5)
HBV	12/12 (5/5)	12/12 (4/5)
HSV-1	12/12 (5/5)	11/12 (5/5)
HSV-2	12/12 (5/5)	11/12 (5/5)
HHV-7	12/12 (5/5)	11/12 (3/5)
HHV-8	12/12 (5/5)	12/12 (ND: DNA not available)

Each data point indicates number of positive virus detection per 12 and 5 repeated experiments. The data in the parenthesis indicate the results of experiments using preserved DNA. ND: not done. CMV: cytomegalovirus, JCV: JC virus, BKV: BK virus, HHV-6/7/8: human herpes virus type-6/7/8, ADV: adenovirus, EBV: Epstein-Barr virus, ParvoB19: parvovirus B19, VZV: varicella-zoster virus, HBV: human hepatitis B virus, HSV-1/2: herpes simplex virus type-1/2.

5-time repeated experiments with similar results at DNA concentrations of 1.25 × 10^3 copies/mL as with synthesized plasmid DNA (Table 1). We performed 5-time repeated experiments but not more repeating with preserved DNA because of limited amounts of the DNA.

3.2. Number of Virus PCR Tests and Incidence of Virus Detection in Patients Who Received HSCT

Synthesized plasmid DNA samples have few contaminants, such as protein, which may interfere with PCR. In the detection performance test using preserved DNA, the concentration of contaminants in the sample may have been reduced by serial dilution. Therefore, we examined the performance of our multiplex PCR assay using clinical specimens.

As shown in Table 2, a total of 40 patients received allogeneic (n = 24) or autologous (n = 16) HSCT. The virus test (multiplex PCR followed by real-time PCR for 13 virus species) was performed until episodes suggestive of viral infection disappeared in patients after HSCT. However, in four patients who received allogeneic HSCT, the disappearance of the suggestive episode could not be confirmed because of early death due to engraftment failure (one patient) or recurrence of underlying neoplasm (three patients). As a result, the median number of tests per patient was 9.0, resulting in a total of 421 tests being performed, including those before HSCT, in all 40 patients. The number of tests per patient in allogeneic HSCT (11.0) was significantly higher ($P < 0.01$) than that in autologous HSCT (7.0). However, the incidence of virus detection in cumulative tests by real-time PCR (at least one virus detection) and the number of virus species per

Table 2. Number of virus PCR test and virus detection in patients who received HSCT.

	Total HSCT (n = 40)	Allogeneic HSCT (n = 24)	Autologous HSCT (n = 16)
Cumulative No. of virus PCR test	421	301	120
No. of virus test/patient	Median: 9.0 (range: 4 - 27)	Median: 11.0* (range: 4 - 27)	Median: 7.0 (range: 4 - 14)
Incidence of virus detection	56.5%/421 tests	56.5%/301 tests	56.7%/120 tests
Patients with virus detection	39/40 (97.5%)	24/24 (100.0%)	15/16 (93.8%)
No. of virus species detected	Median: 3.0 (range: 0 - 6)	Median: 3.0 (range: 1 - 5)	Median: 2.0 (range 0 - 6)
No. of patient with positive test before HSCT	11 (CMV, HHV-6, JCV, BKV, EBV)	6 (CMV, HHV-6, JCV, BKV, EBV)	5 (CMV, BKV, JCV)

One virus PCR test means multiplex PCR for 13 virus species followed by real-time PCR for all 13 virus species. HSCT: Hematopoietic stem cell transplantation. *: P< 0.01 when compared with test number in autologous HSCT.

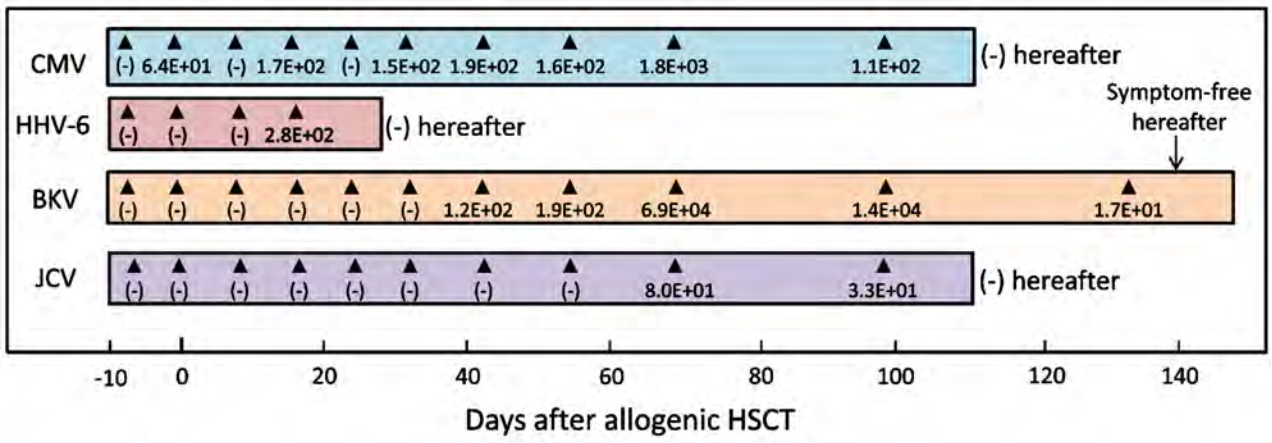
patient were not significantly different between allogeneic and autologous HSCT (Table 2). In 11 patients, one or 2 virus species were detected at low copy numbers before HSCT. We showed a representative time course of virus detection in Figure 1 in a patient in whom 4 virus species were detected.

3.3. Detection of 13 Virus Species by Real-Time PCR in Patients Who Received HSCT

Regarding positive tests on real-time PCR, the most frequently detected virus was CMV (33.3%), followed by BKV (15.7%), JCV (14.5%),, HHV-6 (10.2%), EBV (6.4%), ADV (4.8%), ParvoB19 (1.7%), VZV (0.5%), and HBV (0.5%) among 421 cumulative tests. HSV-1/2 and HHV-7/8 were not detected in this study. Although the pattern of virus detection was similar between allogeneic and autologous HSCT, ADV was detected only in one test in an autologous HSCT patient. Symptomatic virus disease was observed only in one allo-transplanted patient who developed ADV cystitis with a favorable outcome.

3.4. Relationship between Positivity by Real-Time PCR and Multiplex Qualitative PCR

As shown in Figure 2, we plotted all real-time PCR positive results alongside the results of qualitative multiplex PCR (qual), showing the copy number of viruses and virus species on the vertical and horizontal axes, respectively. The presence of two horizontal plots means a true positive test (*i.e.* a positive test), while only one real-time PCR-positive plot in red color means a false negative multiplex PCR test at the indicated copy number. Because real-time PCR detects viruses even at low copy numbers, we examined the virus copy number at which false negative multiplex PCR test results would be obtained. Many false negative tests were observed below copy numbers of 1×10^2/mL, but small number between 1×10^2/mL and 1×10^3/mL, and none above 1×10^3/mL except for 2 tests in HHV-6

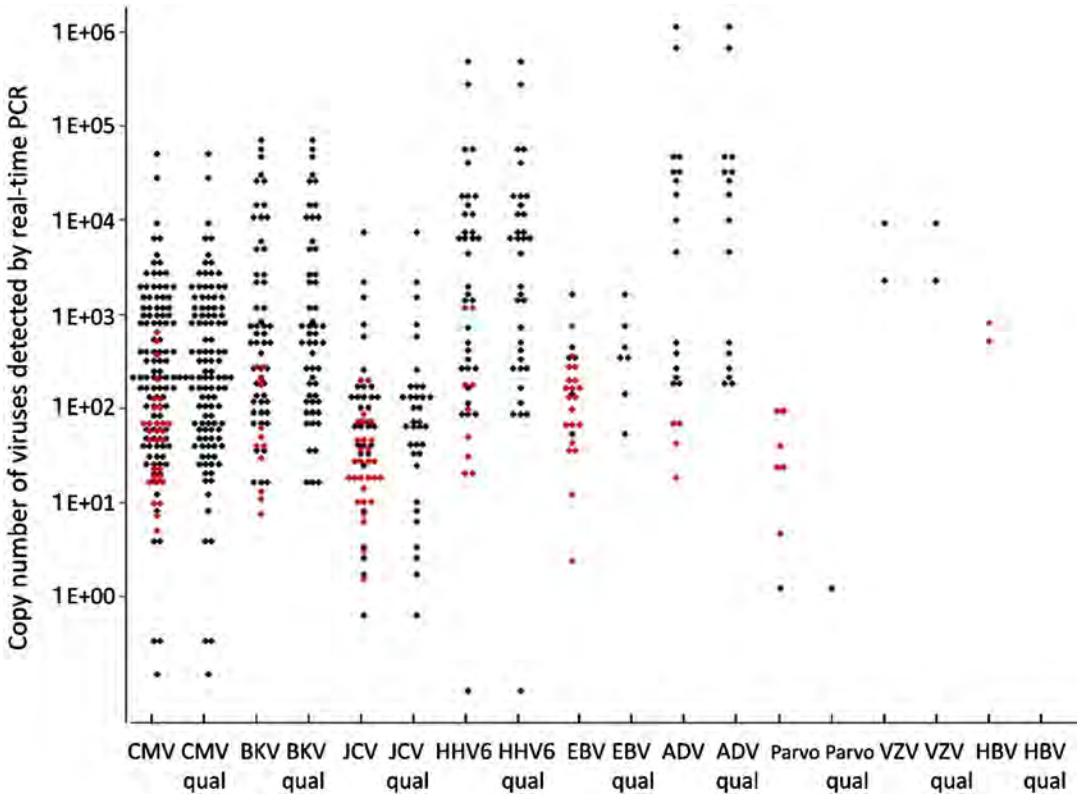


(−): no virus detection, HSCT: hematopoietic stem cell transplantation. CMV: cytomegalovirus, HHV-6: human herpes virus type-6, BKV: BK virus, JCV: JC virus.

Figure 1. A representative time course of virus detection from a 51-year-old male who underwent allogeneic hematopoietic stem cell transplantation.

(1.1 and 1.2 × 10^3 copies/mL) in each virus species, indicating that multiplex PCR certainly detects these viruses above copy numbers of 1 × 10^3/mL even with clinical specimens (Figure 2).

We then analyzed the median copy number on positive tests and false negative tests. As shown in Table 3, the median copy number on positive tests for CMV, JCV, and BKV ranged from 6.9 × 10^1 to 5.2 × 10^2 copies, indicating that multiplex PCR could detect these 3 virus species at low copy numbers. In contrast, the median copy numbers of HHV-6 and ADV were 5.1 × 10^3 and 2.2 × 10^4 copies, respectively. These high median copy numbers were attributed to the presence of patients who had viremia with high copy numbers of HHV-6 or ADV, and there were no false negative tests at copy numbers above 1 × 10^3 except for 2 tests in HHV-6 as mentioned above. The median copy numbers of false negative tests in CMV, JCV, BKV, HHV-6, ADV, and EBV ranged from 4.2 × 10^1 to 1.3 × 10^2 copies, indicating that false negative results only occurred at low copy numbers of these viruses.



All real-time PCR positive results from 9 virus species are plotted alongside the results obtained by multiplex PCR (qual), showing the copy number of viruses and virus species on the vertical and horizontal axes, respectively. The presence of two horizontal plots means a true positive test (*i.e.* a positive test), while only one real-time PCR-positive plot means a false negative multiplex PCR test (in red color) at the indicated copy number. Data of HSV-1/2 and HHV-7/8 are not shown because of no virus detection in the present analysis. CMV: cytomegalovirus, BKV: BK virus, JCV: JC virus, HHV-6: human herpes virus type-6, EBV: Epstein-Barr virus, ADV: adenovirus, Parvo: parvovirus B19, VZV: varicella-zoster virus, HBV: human hepatitis B virus.

Figure 2. Relationship between positivity on real-time PCR and on multiplex qualitative PCR.

3.5. Frequency of False Positive and False Negative Tests with the Multiplex Virus PCR Assay

As shown in Table 4, the frequency of false positive tests for CMV, JCV, BKV,

Table 3. Virus copy number in positive and false negative PCR tests.

	Copy number of positive tests (/mL blood) (median & range)	Copy number of false negative tests (/mL blood) (median & range)
CMV	2.0×10^2 copies (3.5×10^{-1} - 5.0×10^4) N = 112	5.4×10^1 copies (7.2×10^0 - 6.2×10^2) N = 28
JCV	6.9×10^1 copies (1.7×10^0 - 7.2×10^3) N = 36	2.7×10^1 copies (1.7×10^1 - 2.0×10^2) N = 25
BKV	5.2×10^2 copies (1.5×10^1 - 6.9×10^4) N = 55	4.2×10^1 copies (7.6×10^0 - 2.9×10^2) N = 11
HHV-6	5.1×10^3 copies (1.0×10^{-1} - 4.7×10^5) N = 35	7.3×10^1 copies (1.9×10^1 - 1.2×10^3) N = 8
ADV	2.2×10^4 copies (1.7×10^2 - 1.1×10^6) N = 16	4.2×10^1 copies (1.8×10^1 - 7.0×10^1) N = 4
EBV	3.4×10^2 copies (5.2×10^1 - 1.6×10^3) N = 7	1.3×10^2 copies (2.4×10^0 - 2.9×10^2) N = 20

Positive test: Positive result both in multiplex qualitative and quantitative PCR tests; false negative test: negative result in multiplex qualitative PCR but positive in quantitative PCR tests. NaN: not a number; namely, too small data for significant calculation.

Table 4. No, of positive, false positive, negative, and false negative tests and % of false positive and negative tests based of 421 qualitative-quantitative PCR tests.

	Positive tests	False positive tests	% of false positive tests	Negative tests	False negative tests	% of false negative tests
CMV	112	8	6.7	273	28	9.3
JCV	36	3	7.7	357	25	6.5
BKV	55	4	6.8	351	11	3.0
HHV-6	35	5	12.5	373	8	2.1
ADV	16	3	15.8	395	4	1.0
EBV	7	2	(22.2)	392	20	4.9
ParvoB19	1	0	(0)	414	6	1.4
VZV	2	3	(60.0)	416	0	(0)
HBV	0	0	(0)	419	2	(0.5)
HSV-1	0	0	(0)	421	0	(0)
HSV-2	0	0	(0)	421	0	(0)
HHV-7	0	0	(0)	421	0	(0)
HHV-8	0	0	(0)	421	0	(0)

Values in the parenthesis are results from the calculation based on very small data. CMV: cytomegalovirus, JCV: JC virus, BKV: BK virus, HHV-6/7/8: human herpes virus type-6/7/8, ADV: adenovirus, EBV: Epstein-Barr virus, ParvoB19: parvovirus B19, VZV: varicella-zoster virus, HBV: human hepatitis B virus, HSV-1/2: herpes simplex virus type-1/2.

HHV-6, and ADV ranged from 6.7% to 15.8%. The frequency of false negative tests was able to be evaluated in EBV in addition to these 5 viruses, ranging from 1.0% to 9.3%.

3.6. PPV, NPV, Sensitivity, and Specificity of the Multiplex Virus PCR Assay

As shown in Table 5, the PPV and NPV in CMV, JCV, BKV, HHV-6, and ADV ranged from 84.2% to 93.3% and from 90.7% to 99.0%, respectively. The NPV of EBV was 95.1%. The sensitivity and specificity of CMV, JCV, BKV, HHV-6, and ADV ranged from 59.0% to 83.3% and from 97.2% to 99.2%, respectively.

4. Discussion

In the present study, we evaluated whether or not the detection performance of our multiplex virus PCR assay with clinical specimens differed from that using synthesized virus plasmid DNA or preserved virus DNA, and observed equal performances of multiplex PCR assay in both evaluations in CMV, JCV, BKV, HHV-6, and ADV, which were frequently detected and thus reasonably analyzed (Table 1 and Table 3, Figure 2).

These 5 virus species are important to check for after HSCT, and we were able

Table 5. Positive and negative predictive values, sensitivity, and specificity based on 421 qualitative-quantitative PCR test.

	Positive predictive value (%)	Negative predictive value (%)	Sensitivity (%)	Specificity (%)
CMV	93.3	90.7	80.0	97.2
JCV	92.3	93.5	59.0	99.2
BKV	93.2	97.0	83.3	98.9
HHV-6	87.5	97.9	81.4	98.7
ADV	84.2	99.0	80.0	99.2
EBV	(77.8)	95.1	(25.9)	(99.5)
ParvoB19	(100.0)	98.6	(14.3)	(100.0)
VZV	(40.0)	(100.0)	(100.0)	(99.3)
HBV	(0)	99.5	(0)	(100.0)
HSV-1	(0)	(100.0)	(0)	(100.0)
HSV-2	(0)	(100.0)	(0)	(100.0)
HHV-7	(0)	(100.0)	(0)	(100.0)
HHV-8	(0)	(100.0)	(0)	(100.0)

Values in the parenthesis are results from the calculation based on very small data. CMV: cytomegalovirus, JCV: JC virus, BKV: BK virus, HHV-6/7/8: human herpes virus type-6/7/8, ADV: adenovirus, EBV: Epstein-Barr virus, ParvoB19: parvovirus B19, VZV: varicella-zoster virus, HBV: human hepatitis B virus, HSV-1/2: herpes simplex virus type-1/2.

to reasonably calculate the PPV, NPV, sensitivity, and specificity of our multiplex PCR assay based on the results of real-time PCR as the reference. The PPV of these 5 viruses ranged from 84.2% (ADV) to 93.3% (CMV) (**Table 5**), values that appeared satisfactory for routine clinical use. The number of false positive tests affects the PPV value. Although the reason for the false positive results with these five viruses (**Table 4**) is unclear, the results may be due to differences in regents used between multiplex PCR and real-time PCR. In the clinical laboratory, however, confirmation and quantification by real-time PCR is recommended when a positive result is obtained by multiplex virus PCR; therefore, the adverse effects of false positivity, including cross-reaction with unknown microorganisms, would be able to be avoided. In contrast, the NPV in the 5 viruses ranged 90.7% (CMV) to 99.0% (ADV) (**Table 5**), which was acceptable for routine laboratory use.

The sensitivity for these 5 viruses ranged from 59.0% (JCV) to 83.3% (BKV), showing rather low values (**Table 5**). Sensitivity is affected by the number of false negative test results; therefore, these values may have been attributable to the large number of false negative tests below the virus copy number of around 1×10^2/mL. Because the detection of viruses at these low copy numbers are not always needed in clinical practice, the sensitivity for these 5 viruses may be satisfactory after excluding false negative tests at low copy numbers. In contrast, the specificity of these 5 viruses ranged from 97.2% (CMV) to 99.2% (JCV and ADV) (**Table 5**), which was acceptable in routine laboratory setting.

Generally, in PCR amplification reaction in the presence of multiple virus species, viruses with low amplicon size may be advantageous in the PCR detection. Although we did not perform the competition experiments, multiple virus detection at low copy number was frequently observed in the present study, which was typically shown in **Figure 1** and **Figure 2**. Therefore, the competition between multiple viruses in our PCR amplification might be unlikely.

In the present study, the detection performance of multiplex PCR showed certain virus detection at more than 1.0×10^3 virus copies/mL in analyses with clinical specimens. Regarding preemptive therapy for CMV infection after allogeneic HSCT, a study showed that three positive cells per two slides in a CMV antigenemia test (LSI Medience Corp., Tokyo, Japan) was a reasonable criterion for starting preemptive therapy for CMV disease in high-risk patients [22]. In our previous study using the same multiplex and real-time PCR assay system, 3 positive cells per two slides corresponded to 2.4×10^3 CMV copies/mL plasma [16]. Because our multiplex PCR assay definitely detects CMV genome/DNA at more than 1×10^3 copies/mL, delay in CMV preemptive treatment due to false negative result would be unlikely. Indeed, in the present study and previous study [16], none of patients developed life-threatening CMV disease because of early treatment with gancyclovir based on regular virus check with the multiplex PCR.

HHV-6 is also important and should be frequently monitored in HSCT patients because it rapidly proliferates and often causes encephalitis when its blood

load is high [23]. Ogata *et al.* reported that more than 1×10^4 HHV-6 DNA copies/mL plasma was the threshold value causing encephalitis using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan) [24]. Our multiplex PCR assay can detect HHV-6 DNA at $\geq 1 \times 10^3$/copies mL plasma with certainty; therefore, delay in HHV-6 preemptive treatment due to false negative test would also be unlikely. In fact, none of patients developed critical HHV-6 encephalitis in our institution because of frequent HHV-6 monitoring with the multiplex PCR and early treatment with foscavir. In another study, Inazawa *et al.* described 2 patients who developed HHV-6 encephalitis at a minimum blood viral load of around 3.8×10^3 copies/μg DNA ($=10^4$ copies/1mL blood) using the same virus PCR system as us [25]. Of note, a standardized real-time PCR assay system has not yet been established [26]. In relation to multicenter comparison of real-time PCR assay system [26], our real-time PCR is similar to the PCR assays 1, 2, 4, and 6 shown in the study, and our measuring value is also similar to those of these 4 PCR systems.

Regarding time performance of our multiplex PCR assay, simultaneous screening of 13 virus species within 3 h may be superior to single-virus real-time PCR to detect viruses possibly involved in unknown infection or inflammation. Blood PCR analysis also appears to be enough to predict organ viral infection as previously described [16].

As for the cost of our multiplex PCR assay, one multiplex qualitative PCR assay for 13 viruses costs about $131 US. When one virus is detected, one real-time PCR assay is performed to determine the viral load, coming to a cost of $34 US. Therefore, our multiplex PCR assay shows reasonable cost performance as a routine clinical laboratory test. In addition, this confirmation and viral load determination by real-time PCR takes about 45 min, leading to early intervention of anti-viral treatment if needed.

The limitations associated with the present study include the rather small number of HSC-transplanted patients included; small number of positive tests for JCV, HHV-6, and ADV; and few or no virus detection of EBV, ParvoB19, VZV, HVB, HSV-1/2, and HHV-7/8. Regarding these limitations, we previously examined viremia in another cohort of HSC-transplanted patients using a similar multiplex PCR assay system that detects all the same virus species except for ADV [16], and the virus detection performance was nearly the same as in the present study. With this background, the first two limitations may not be major weak points of this study. However, continued improvement of the ADV detection performance is required. Regarding the third limitation, however, it would be difficult to obtain an appropriate number of positive tests to conduct reasonable analyses, as even in our previous patient cohort, which contained more high-risk patients [16], positivity for these eight viruses was only sporadic.

5. Conclusion

Our multiplex qualitative virus PCR assay for 13 virus species may be suitable

for clinical laboratory use in terms of its sensitivity, specificity, reproducibility, and time and cost performance.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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