genicity. Since the amount of recovered epithelial lining fluid in BALF can vary considerably, the quantitative methodology in this study used data normalization to GAPDH, a housekeeping gene, to correct for this variation [24]. In agreement with previous studies [11–13], we found that quantification of the viral load in BALF may be useful in diagnosing CMV pneumonia. We noted that 3 patients with proven or probable CMV pneumonia had negative or clinically insignificant pp65 antigenemia, highlighting the importance of obtaining samples from the site of infection. Meanwhile, the pathogenicity of HSV-1 was unclear in this study because antiviral therapy did not lead to clinical improvement in 3 of 4 patients diagnosed with possible HSV-1 pneumonia. Previous studies also showed that HSV-1 in BALF may be a marker for underlying clinical conditions rather than a cause of mortality [25, 26]. In clinical practice, however, when there is a high viral load in BALF samples from ALI/ARDS of otherwise unexplained etiology, administration of antiviral agents against HHVs with possible lung pathogenicity (e.g. CMV, HSV-1, and HHV-6) would be an option for treatment.

We must acknowledge that it is difficult to extrapolate our results to all ALI/ARDS patients or to elucidate the etiologic role of HHVs in ALI/ARDS due to the selected group of patients and the retrospective nature of our study without a standardized diagnostic procedure. In addition, we must note that the pathogenic significance of HHVs is often difficult to determine via quantification of the viral DNA in BALF because of the substantial overlap between viral loads in symptomatic and asymptomatic patients [6, 11, 13]. Accordingly, although we have demonstrated that our diagnostic approach was technically feasible and potentially useful in ALI/ARDS patients to detect HHVs in the lung, further studies prospectively collecting all de novo ALI/ARDS patients in order to assess the exact prevalence of HHVs in ALI/ARDS or to investigate the possible impact of common bacterial infections on the reactivation of HHVs are needed. Also, a controlled antiviral treatment trial is warranted to draw conclusions regarding the etiologic role of the detected viruses in the development or worsening of ALI/ARDS.

Our study has some more limitations. First, there was a large heterogeneity in the studied population that precluded assessment of the impact of HHVs on the prognosis. Second, we may have underestimated the prevalence of HHVs because an HHV type with a low viral load in BALF could have been missed in the multiplex PCR assays if the BALF also contained a high viral load of another HHV type. Third, the impact of virus-bacteria coinfections could not be assessed because two thirds of our patients had received antimicrobial agents at the time of the BAL. Forth, oral contamination cannot be excluded because BALF samples obtained without a tracheal tube were possibly contaminated with oropharyngeal secretions by virtue of the technical procedure.

In conclusion, the implementation of multiplex and real-time PCR for HHVs allowed efficient detection and quantification of viral genomic DNA in BALF in selected ALI/ARDS patients of unknown etiology, especially in patients with immunosuppression or endotracheal intubation. In this setting, where clinicians must consider a wide differential diagnosis, the combination of multiplex and real-time PCR for HHVs may represent a useful diagnostic tool for the management of ALI/ARDS.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Analysis of Viral Infection by Multiplex Polymerase Chain Reaction Assays in Patients with Liver Dysfunction

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Abstract

Objective While unexplained liver dysfunction is common, it is sometimes difficult to identify its exact cause. One cause is viral infections. The identification of viruses other than hepatitis B and C that cause liver dysfunction is difficult because no methods to simultaneously identify these viruses have been established. The aim of this study was to quickly and simultaneously identify multiple virus species.

Methods A total of 49 patients with unexplained liver dysfunction and undetermined inflammation were examined. The majority of patients had hematologic malignancies, and some had undergone bone marrow transplantation. Qualitative polymerase chain reactions (PCR) were performed to detect 12 species of DNA virus in whole blood. Quantitative real-time PCR was performed when a specific virus was amplified. In addition, 6 RNA hepatitis viruses were directly assayed by real-time PCR. These 2 PCR steps were completed within 1 hour.

Results The most frequently detected virus in 37 patients with liver dysfunction, was transfusion transmitted virus (38%), which was followed by human herpes virus (HHV) type 6 (35%), Epstein-Barr virus (14%), cytomegalovirus (8%), and rarely hepatitis G virus and HHV-7 (3%). Similar viremia was observed in 12 patients with mild liver dysfunction. The results of the PCR assay were mostly consistent with those of routine virus serological tests.

Conclusion A multiplex viral PCR assay was a useful tool for quickly identifying viruses that possibly cause liver dysfunction. It was also important that liver dysfunction acted as a proband that led to the discovery of serious viremia.

Key words: liver dysfunction, multiplex PCR, real-time PCR, human herpes viruses, hepatitis virus

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Introduction

A clinician often encounters unexplained liver dysfunction; however, it is sometimes difficult to identify the exact cause of the dysfunction because of the many causes of liver dysfunction. One common cause of liver dysfunction is viral infection. Although it is easy to detect hepatitis B virus (HBV) and hepatitis C virus (HCV) because of the established laboratory tests for these viruses, the detection of other viruses that cause liver dysfunction is difficult because the current laboratory methodologies in a hospital have some limitations in terms of quick performance and the limited number of identifiable viral species. Therefore, the prompt and proper diagnosis of viral infections is important when a patient exhibits liver dysfunction. An assay was developed to simultaneously detect 12 kinds of viral DNA genomes in the blood. The assay uses a multiplex polymerase chain reaction (PCR) to identify the viruses, and real-time PCR to determine the viral load. In addition, 6 RNA

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Table 1. Primer and Probe Sequences Employed in Multiplex Qualitative Polymerase Chain Reaction (PCR) Analyses

Virus	Region of amplification	Primer sequence	Probe sequence	Reference
HSV1/HSV-2	polymerase	F: GCTCGAGTGCGAAAAAACGTTC R: TGCGGTTGATAAACGCGCAGT	3'FITC: GCGCACCAGATCCACGCCCTTGATGAGC LcRed604-5': CTTGCCCCCGCAGATGACGCC	3
VZV	gene29	F: TGTCCTAGAGGAGGTTTTATCTG R: CATCGTCTGTAAAGACTTAACCAG	3'FITC: GGGAAATCGAGAAACCACCCTATCCGAC LcRed640-5': AAGTTCGCGGTATAATTGTCAGT	4
EBV	BamH1	F: CGCATAATGGCGGACCTAG R: CAAACAAGCCCACTCCCC	3'FITC: AAAGATAGCAGCAGCGCAGC LcRed640-5': AACCATAGACCCGCTTCCTG	GeneBank V01555
CMV	Glycoprotein	F: TACCCCTATCGCGTGTGTTC R: ATAGGAGGCGCCACGTATTC	3'FITC: TCGTCGTAGCTACGCTTACAT LcRed705-5': ACACCACTTATCTGCTGGGCAGC	5
HHV6	101k gene region	F: ACCCGAGAGATGATTTTGCG R: GCAGAAGACAGCAGCGAGAT	3'FITC: TAAGTAACCGTTTTCGTCCCA LcRed705-5': GGGTCATTTATGTTATAGA	6
HHV7	U57	F: GAAAAATCCGCCATAATAGC R: ATGGAACACCTATTAACGGC	3'FITC: GCCATAAGAAACAGGTACAGACATTGTCA LcRed705-5': TTGTGAAATGTGTTGCG	GeneBank NC001716
HHV8	EB BDLF1ORF21	F: AGCCGAAAGGATTCCACCAT R: TCCGTGTTGTCTACGTCCAG	3'FITC: CCGGATGATGTAAATATGGCGGAAC LcRed705-5': TGATCTATATACCACCAATGTGTCATTTATG	7
BKV/JCV	VP2	F: CACTTTTGGGGGACCTAGT R: CTCTACAGTAGCAAGGGATGC	3'FITC: TCTGAGGCTGCTGCTGCCACAGGATTTT LcRed705-5': AGTAGCTGAAATTGCTGCTGGAGAGGCTGCT	8
Parvo B19	NS1	F: CCGCCAAGTACAGGAAAAAC R: CAGCTACACTTCCACGCA	3'FITC: GCAAAAGCCATTTTAGGCGGGCA LcRed640-5': CACCAGGGTAGATCAAAAAATGCGTGGA	9

BKV/JCV: BK virus/JC virus, CMV: cytomegalovirus, F: Forward, FITC: Fluorescein isothiocyanate, EBV: Epstein-Barr virus, HHV: human herpes virus, HSV: herpes simplex virus, Parvo B19: Parvovirus B19, R: Reverse, VZV: varicella-zoster virus

hepatitis viruses were directly quantified with real-time PCR. Our multiplex PCR combined with real-time PCR was highly useful in the quick diagnosis of viral hepatitis.

Materials and Methods

Patients

Patients with unexplained liver dysfunction, that received medical care in Shinko Hospital, Kobe, Japan, from February to December, 2011 were enrolled in this study. Liver dysfunction in this study was defined as patients that exhibited more than 2 times the normal upper limits of aspartate transaminase (AST) (80 IU/L), alanine transaminase (ALT) (80 IU/L), or alkaline phosphatase (ALP) (720 IU/L) levels or more than 1.5 mg/dL of total bilirubin, with negative serological tests for HBsAg and HCV. Patients with normal liver function or mild dysfunction underwent this viral PCR examination for possible viral infection because of fever or inflammatory signs. These patients were enrolled in this study. Patients who showed positive results for HBV or HCV by serological or molecular examinations were also included in this study in order to examine the possibilility of multiple viral loads. In addition, the serological tests for both HBV and HCV were performed before the multiplex PCR analysis in all patients included in this study.

Blood and plasma samples

EDTA-2Na-chelated whole blood (200 μL) was obtained from individual patients who provided their written informed consent. The present study was part of a retrospective analysis of a single institutional clinical study designated the "Multiple Virus-Analytic Study by Multiplex PCR", which had been approved by the institutional review board. The plasma was separated from whole blood by centrifugation at 400-g when 1 or more virus-specific PCR products/signals were detected, and subjected to real-time PCR in order to quantify the number copies of the viral genome. Blood obtained from 12 healthy volunteers with informed consent was subjected to the following virus analyses as negative controls.

Multiplex PCR (1, 2)

DNA was extracted from the whole blood using QuickGene DNA whole blood kit S (FUJIFILM Corporation, Tokyo, Japan) that was installed on an apparatus for the automated purification of nucleic acids (QuickGene-800; FUJIFILM Corporation). The multiplex PCR was designed to qualitatively measure the genomic DNA of 12 viruses; cytomegalovirus (CMV), human herpes virus type 6 (HHV-6), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), human herpes virus type 7 (HHV-7), human herpes virus type 8 (HHV-8), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), and HBV.

The PCR was performed using a LightCycler (Roche, Basel, Switzerland). The primers and probe sequences for these 12 viruses are described in Table 1 (3-9).

Two sets were paired-off with the following capillaries of these 12 viruses; Capillary A: HSV-1, HSV-2, VZV, HHV-6, CMV, Parvo B19, BKV, and JCV, and Capillary B: EBV, HHV-7, and HHV-8. Specific primers for these viruses were used with 0.25 μL AccuPrime Taq polymerase and 1× AccuPrime Buffer I (Invitrogen Corporation, Carlsbad, CA, USA) and 5 ng non-acetylated bovine serum albumin (BSA; Sigma-Aldrich Co., St. Louis, MO, USA), resulting in a final volume of 10 μL for each primer. First, 3 μL of mineral oil (Sigma-Aldrich Co.) was placed in the capillary. Next, 10 μL of reaction mixture was added and centrifuged for 3,000 rpm for 3 s. Finally, 5 μL of probe mix was added to the capillary, which was then capped. The DNA was amplified with 40 PCR cycles at 95℃ (2 s), 58℃ (15 s), and 72℃ (15 s), which was followed by denaturation at 95℃ (1 min).

Fluorescein isothiocyanate-conjugated probe hybridization and LCRed640- or LCRed-conjugated hybridization probes were then mixed with the products by 3,000 rpm centrifugation for 3 s. Specific hybridization was confirmed by a melting curve analysis (10) in which the dissociation of hybridized probes from individual PCR products was seen as the

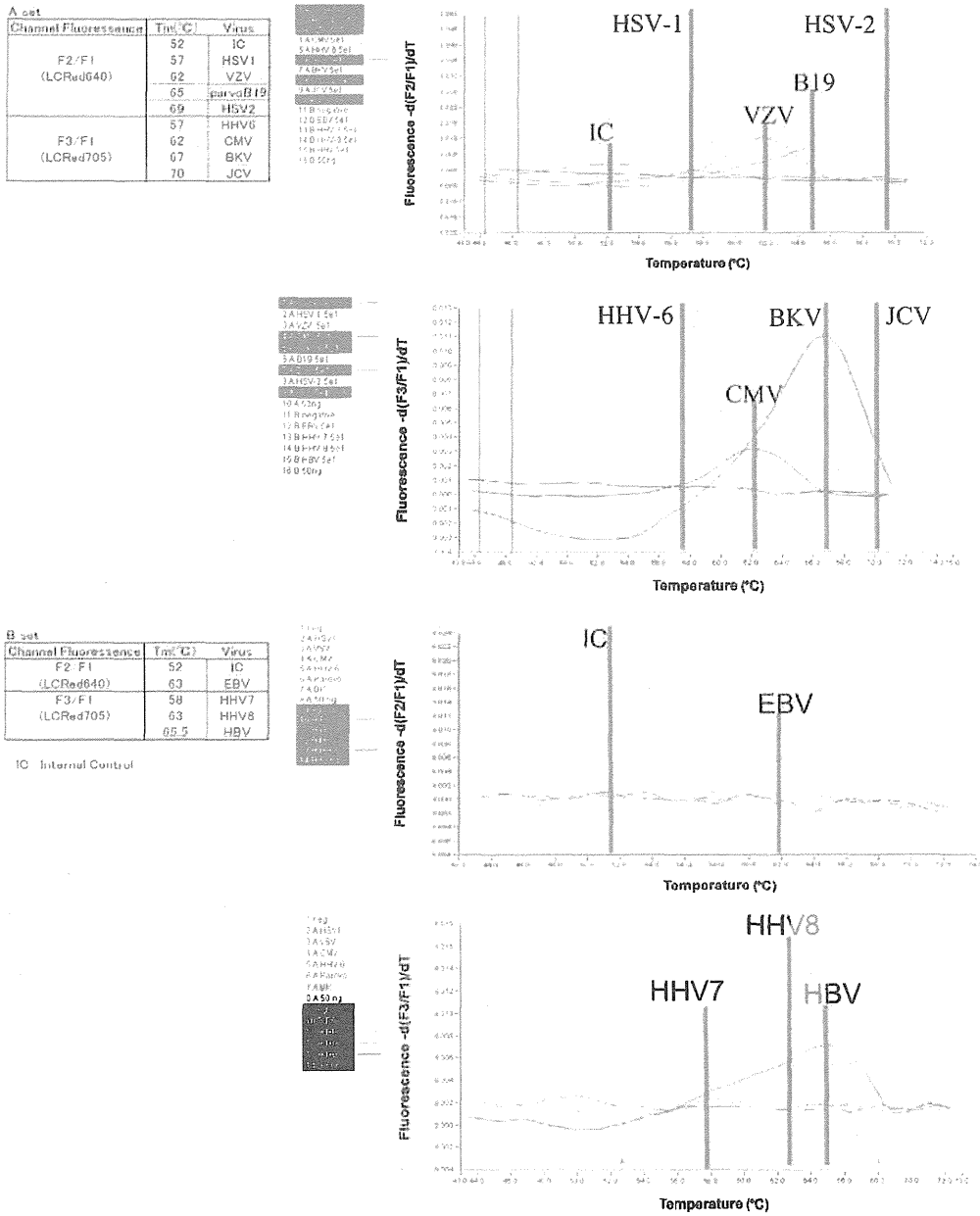

Figure. **Melting curve analysis by virus-specific melting temperature (Tm). A melting curve analysis is used to measure the temperature at which the DNA is split into single chain from double stranded DNA. The PCR products amplified by the primer for each viral sequence hybridize the specific probes labeled by LcRED705 and LcRED640 at 40°C. This hybridized double strand DNA melted/dissociated when it was heated gradually from 40℃ to 80℃. Each viral melting temperature was measured by use of the fluorescence resonance energy transfer (FRET). Releasing the excitation energy causes the fluorescent substance that reached the excitation state to return to the basal state, and that neighboring fluorescent substance obtains the energy and enters into the excitation state. The melting temperature of individual DNA strands is determined based on each viral sequence, the length, and the GC content. BKV/JCV: BK virus/JC virus, CMV: cytomegalovirus, HHV: human herpes virus, HSV: herpes simplex virus, IC: immune complexed, B19: Parvovirus B19, VZV: varicella-zoster virus**

disappearance of fluorescence at the specific dissociation temperature of each virus. The specific temperature for each virus is shown in Figure. The melting curve analysis was performed by denaturing DNA at 95℃ for 1 minute, which was followed by hybridization at 40℃ for 10 s and melting at 40℃ to 80℃ (Ramp rate, 0.2℃/s).

In addition, a sensitivity test of this PCR was performed using known plasmid DNA representatives for the 12 individual DNA viruses. The individual DNA sequences were determined based on the database for DNA viruses, and the

Table 2. Sensitivity of Qulitative Multiplex PCR

	Copies / Tube				
	100	50	25	10	Sensitivity
HSV1	10/10	10/10	10/10	7/10	>25 copies
HSV2	10/10	10/10	10/10	10/10	>10 copies
VZV	10/10	10/10	10/10	10/10	>10 copies
CMV	10/10	10/10	10/10	10/10	>10 copies
EBV	10/10	10/10	10/10	9/10	>25 copies
HHV6	10/10	10/10	10/10	8/10	>25 copies
HHV7	10/10	10/10	5/10	4/10	>50 copies
HHV8	10/10	10/10	6/10	5/10	>50 copies
BKV	10/10	10/10	10/10	10/10	>10 copies
JCV	10/10	10/10	9/10	7/10	>50 copies
HBV	10/10	10/10	6/10	1/10	>50 copies
ParvoB19	10/10	10/10	10/10	10/10	>10 copiee

HSV: herpes simplex virus, VZV: varicella-zoster virus, CMV: cytomegalovirus, EBV: Epstein-Barr virus,
HHV: human herpes virus, BKV: BK virus, JCV: JC virus, HBV: hepatitis B virus, ParvoB19: Parvovirus B19

plasmid DNAs were synthesized by Nihon Techno Service Company, Ibaragi, Japan. The qualitative PCR assay was performed 10 times for each virus using various concentrations of the plasmid DNA and determined the concentration of the plasmid DNA (copy number/tube) as the detection limit when 100% positivity (10/10 assays) was obtained. Table 2 shows that the sensitivity of the PCR varied from more than 10 to 50 copies.

The assay could directly measure the load with a linear relationship up to 10^9 copies/tube; however, dilution was required when the viral load exceeded 10^{10} copies.

Real-time PCR (1, 2)

Real-time PCR was performed when a positive and specific result was obtained by multiplex PCR for the human herpes viruses. Quantitative reverse transcription (RT)-PCR was performed first for the human RNA hepatitis viruses. Hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), hepatitis G virus (HGV), and transfusion transmitted virus (TTV) we assayed as the infecting organisms of a human hepatitis virus. Real-time PCR was performed using 0.5 Units of Taq DNA polymerase (Thermo Fished Scientific Inc., Waltham, MA, USA), 1 mM dNTPs (Bioline USA Inc., Taunton, MA, USA), 3 mM $MgCl_2$ (NIPPON GENE Co. Ltd., Tokyo, Japan), 0.1 μg Anti-taq high (Toyobo Co. Ltd., Osaka, Japan), 10 ng non-acetylated BSA (Sigma-Aldrich Co.), and One Step PrimeScript Real-time RT-PCR kits (Takara Bio Inc., Shiga, Japan). Each reaction final volume was 20 μL, and the reaction was performed on the Light Cycler DX400 (Roche).

The sequence for the primers and the probes of each virus are shown in Table 3A, B (11-21). The DNA of human herpes virus and HBV were amplified by PCR with the following conditions: denaturation at 95℃ (10 s) and then 50 cycles of PCR (denaturation at 95℃ [1 s], annealing at 60℃ [20 s], and cooling at 40℃ [20 s]). The RNA of the remaining hepatitis viruses was amplified by RT-PCR with following conditions: RT reaction at 42℃ (5 min), denaturation at 95℃ (10 s), 50 cycles of PCR (denaturation at 95℃ [1 s], annealing at 60℃ [20 s], and cooling at 40℃ [20 s]. The value of the viral-genome copy number in the sample was considered to be significant when more than 10 copies/tube were obtained.

The real-time PCR was repeated 8 times on a VZV-positive specimen that contained 10 copies/tube of VZV plasmid. The mean value of cycle numbers required to obtain 10 copies/tube were 28. 28 cycles with a standard deviation (SD) of 0.317 cycles. The distribution coefficient was 0.393, indicating excellent reproducibility of this quantitative PCR. Each cycle number for the reproducibility was tested with the Kolmogov-Smirnov test assuming as normal distribution.

These multiplex PCR and real-time PCR procedures of the present study were performed in the Laboratory of Cell Therapy in Shinko Hospital.

Results

Viruses identified in patients with unexplained liver dysfunction

A total of 49 patients were included in this study. The characteristics and the laboratory data of these patients with unexplained liver dysfunction are described in Tables 4, 6. The majority of the patients had hematologic malignancies and were immune-compromised, especially, those patients who were seen soon after allogeneic bone marrow transplantations. One or more viral species was detected in 28 of the 37 patients with liver dysfunction, and their copy number was determined (Table 4). The most frequently detected virus was TTV (14/37), which was followed by HHV-6 (13/37), EBV (5/37), CMV (3/37), and rarely HGV (1/37) and HHV-7(1/37). Three virus species were detected in 2 patients (Nos. 32 and 36); 2 species were detected in 8 patients; and a single virus was detected in 19 remaining patients. However, no virus genomes were detected in 9 of the 37 patients, indicating that there were other causes of liver dysfunction in these patients at the time of the analysis. One patient that was diagnosed with chronic hepatitis B (No. 36) was infected with both EBV and TTV in addition to HBV, suggesting that co-viral infections may exists in patients with hepatitis B. The incidence of fever or skin rash in this cohort of patients was low (Table 4) presumably due to the immunosuppressive procedures or chemotherapies in the majority of these patients.

Twelve healthy volunteers were examined in the same way as a negative control. Table 4 shows that none of the 12 DNA viruses were detected by the multiplex PCR in these volunteers. As for hepatitis viruses, all 7 hepatitis viruses except TTV were below the detection limit. TTV was detected in 2 of 12 volunteers with a viral load ranging from 5×10^1 to 7.7×10^2 copies/mL (Table 4).

Table 3A. Primer and Probe Sequences in the Assay of Human Herpes Virus (HHV) by Reverse Transcriptase-polymerase Chain Reaction

Herpes virus	Primers and probe sequences	Amplification	Referance
HSV1 and 2	HSV-F: CGCATCAAGACCACCTCCTC	gB	11
	HSV-R: GCTCGCACCACGCGA		
	HSV1-P: JOE-TGGCAACGCGGCCCAAC-TAMRA		
	HSV2-P: FAM-CGGCGATGCGCCCCAG-TAMRA		
VZV	VZV-F: AACTTTTACATCCAGCCTGGCG	ORF29	GeneBankX04370,AJ871403,DQ457052
	VZV-R: GAAAACCCAAACCGTTCTCGAG		
	VZV-P: FAM-TGTCTTTCACGGAGGCAAACACGT-TAMRA		
EBV	EBV-F: CGGAAGCCCTCTGGACTTC	BALF-5	12
	EBV-R: CCCTGTTTATCCGATGGAATG		
	EBV-P: FAM-TGTACACGCACGAGAAATGCGCC-TAMRA		
CMV	CMV-F: CATGAAGGTCTTTGCCCAGTAC	U65-U66	13
	CMV-R: GGCCAAAGTGTAGGCTACAATAG		
	CMC-P: FAM-TGGCCCGTAGGTCATCCACACTAGG-TAMRA		
HHV6	HHV6-F: GACAATCACATGCCTGGATAATG	U37	14
	HHV6-R: TGTAAGCGTGTGGTAATGTACTAA		
	HHV6-P: FAM-AGCAGCTGGCGAAAAGTGCTGTGC-TAMRA		
HHV7	HHV7-F: CGGAAGTCACTGGAGTAATGACAA	U57	15
	HHV7-R: CCAATCCTTCCGAAACCGAT		
	HHV7-P: FAM-CTCGCAGATTGCTTGTTGGCCATG-TAMRA		
HHV8	HHV8-F: CCTCTGGTCCCCATTCATTG	ORF65	16
	HHV8-R: CGTTTCCGTCGTGGATGAG		
	HHV8-P: FAM-CCGGCGTCAGACATTCTCACAACC-TAMRA		
BKV/JCV	BKV & JCVF: GGAAAGTCTTTAGGGTCTTCTACCTTT	ORF	17
	BKV-R: GATGAAGATTTATTYTGCCATGARG		
	JCV-R: GAAGACCTGTTTTGCCATGAAGA		
	BKV & JCV-P: 6FAM-ATCACTGGCAAACAT-MGB		
Parvob19	B19-F: GGGTTTCAAGCACAAGYAGTAAAAGA	NS1	GeneBank M13178,AJ717293,AX003421
	B19-R: CGGYAAACTTCCTTGAAAATG		
	B19-P: FAM-CAGCTGCCCCTGTGG-MGB		

The real-time herpes simplex virus (HSV) PCR is a multiplex PCR that can detect both HSV1 and HSV2 DNA in the same reaction. The optimized gB primer pairs amplify both HSV1 and 2 with equal efficiency, with the 2 type-specific probes labeled with different fluorescent dyes. The HSV1 probe is labeled with JOE at the 5'-end and with TAMRA at the 3'-end. The HSV2 probe is labeled with FAM at the 5'-end and with TAMRA at the 3'-end.
CMV: cytomegalovirus, EBV: Epstein-Barr virus, VZV: Varicella-zoster virus

Table 3B. Primer and Probe Sequences in the Assay of Human Hepatitis Virus by Reverse Transcriptase-polymerase Chain Reaction

	Primer and Probe Sequences	Amplification	Reference
HAV	HAV-F:GGTAGGCTACGGGTGAAACC	5'NC	GeneBank :AB020564、AB020565,
	HAV-R:GCCGCTGTTACCCTATCCAA		AB020568,AB020568,M14707,K02990,
	HAV-P:FAM-TACTTCTATGAAGAGATGC-MGB		X75216,X75215
HBV	HBV-F:GTGGTGGACTTCTCTCAATTTTCTAG	S-gene	
	HBV-R:GGACAMACGGGCAACATACCT		AF090842,AF100309,,X04615
	HBV-P:FAM-TGTCTGCGGCGTTTT		
HCV	GTCTAGCCATGGCGTTAGTA	57NC	GeneBank:
	CTCGCAAGCACCCTATCAGGCAGT		AF009606,AF356827,D14853,AF169004,
	HCV-P:FAM-CTGCGGAACCGGTGAGTACAC-BHQ		AB030907,D50409,D17763
HDV	HDV-F:GCATGGTCCCAGCCTCC	ribozyme I	
	HDV-R:TCTTCGGGTCGGCATGG		18
	HDV-P:FAM- ATGCCCLaGGLtCGGAC-TAMRA		
HEV	HEV-F:GGTGGTTTCTGGGGTGAC	ORF3	
	HEV-R:AGGGGTTGGTTGGATGAA		19
	HEV-P:FAM- TGATTCTCAGCCCTTCGC -TAMRA		
HGV	HGV-F:CGGCCAAAAGGTGGTGGATG	5'NC	
	HGV-R:CGG TAGGGCCAACACCTGTGGA		20
	HGV-P:FAM- CAGGGTTGGTAGGTCGTAAATCCCGGTCA-TAMRA		
TTV	TTV-F:TCCGAATGGCTGAGTTT	ORF2	
	TTV-R:CGAATTGCCCCTTGACT		21
	TTV-P:FAM- ACTCACCTHCGGCACCCGC-iowaBK		

HAV: hepatitis A virus, HBV: hepatitis B virus, HCV: hepatitis C virus, HDV: hepatitis D virus, HEV: hepatitis E virus, HGV: hepatitis G virus, TTV: transfusion transmitted virus

Follow-up examination of liver dysfunction and viremia

The study then examined the relationship between the liver dysfunction and viremia. The detected viruses were re-examined after appropriate intervals in 15 of the 37 patients included in the present study. Table 5 shows that the liver dysfunction was improved or normalized in almost all patients reexamined. The load of HHV-6 virus decreased with the improvement of the liver dysfunction in all 6 patients re-examined for this virus. The correlation between the copy number of HHV-6 and the levels of AST, ALT, and ALP was analyzed at 2 periods of the examination using Restricted Maximum Likelihood. The improvement of the

Table 4. Characteristics of Patients with Unexplained Liver Dysfunction and Their Laboratory Data

Patient No.	Age	Sex	Underlying disease	AST	ALT	ALP	T.Bil	LDH	CRP	% of Atyp. lym	Fever	Skin rash	LN swelling	History of transfusion	Virus detected and its copy number
1	82	M	Pulmonary Tbc	67	156	375	0.7	218	1.45	0	±	−	−	None	None
2	65	M	IP	32	95	319	0.8	288	1.40	0	±	−	−	None	None
3	36	F	Septic shock	96	50	802	10.0	489	11.80	1.6	+	−	−	None	HHV-6: 3.9×10^3
4	69	F	ATL post BMT	168	190	241	0.8	356	0.24	0	−	−	−	None	HHV-6: 8.3×10^2
5	36	M	post HPS	56	138	306	0.5	324	0.07	8.0	−	−	−	None	None
6	49	M	Malignant lymphoma	49	110	333	1.0	254	0.71	0.5	−	−	−	RCC	HHV-6: 4.7×10^2
7	70	M	FUO	43	72	1,678	0.7	121	16.93	0	+	−	−	None	None
8	72	F	MDS/AML	119	64	465	0.7	259	0.39	1.6	−	−	−	RCC, PC	HGV: 1.8×10^6
9	53	M	AML, post BMT	39	99	711	0.4	114	0.28	0.4	−	+	−	RCC, PC	HHV-6: 6.1×10^3
10	78	F	Myeloma, Pneumonia	54	117	183	1.7	689	1.97	1.0	±	−	−	RCC, PC	CMV: 3.6×10^4, EBV: 8.0×10^2
11	85	M	MDS	470	215	968	1.9	647	0.11	0	−	−	−	None	TTV: 5.1×10^3
12	66	F	Multiple myeloma	45	80	1,059	1.0	335	18.70	32.4	+	−	−	RCC, PC	None
13	56	M	MDS post BMT	93	93	228	0.8	493	3.45	0.8	+	−	−	RCC, PC	None
14	59	M	Malignant lymphoma	158	326	644	1.3	274	2.12	1.5	±	−	−	None	HHV-6: 1.7×10^2, TTV: 1.2×10^4
15	34	F	ALL	448	481	1,420	2.0	719	12.54	0	+	−	−	RCC, PC	TTV: 1.2×10^5
16	62	M	Malignant lymphoma	137	197	973	2.1	407	7.91	0.5	+	−	−	RCC, PC	None
17	75	F	Drug-induced hepatitis	124	361	526	1.2	185	0.19	0	−	−	−	None	HHV-6: 1.6×10^3
18	36	F	Infectious mononucleosis	511	469	411	0.6	675	0.65	33.5	±	−	+	None	CMV: 1.7×10^4
19	44	F	Infectious mononucleosis	169	352	1,956	1.4	393	0.65	38.9	±	−	+	None	EBV: 1.1×10^5
20	66	F	Autoimmune hepatitis	418	645	602	0.8	329	0.27	0	−	−	−	None	TTV: 5.8×10^3
21	25	F	Infectious mononucleosis	87	114	354	0.9	450	0.91	38.3	+	+	+	None	CMV: 5.7×10^4, HHV-6: 3.6×10^2
22	67	F	ATL	1,189	1,186	2,900	0.5	796	7.66	21.8	+	+	−	RCC, PC	HHV-6: 1.0×10^5
23	65	F	IP	66	76	721	0.5	712	18.98	0	+	−	−	None	None
24	86	M	Malignant lymphoma	481	433	1,182	0.6	576	0.03	0	−	−	−	None	HHV-6: 7.0×10^3, EBV: 6.4×10^2
25	54	M	AIDS	84	43	1,130	0.3	262	1.15	0	±	−	+	None	HHV-6: 1.5×10^3, EBV: 7.1×10^2
26	73	F	Myeloma	241	486	222	2.1	567	3.61	0.5	±	−	−	RCC, PC	HHV-6: 1.7×10^3, TTV: 4.1×10^2
27	70	M	Knee joint MRSA infection	21	29	683	0.9	141	1.89	1.0	±	−	−	None	TTV: 3.5×10^4
28	57	F	Sepsis	158	37	387	0.4	625	33.80	0	+	−	−	None	None
29	50	M	PH	42	84	497	0.2	264	1.85	0	−	−	−	None	TTV: 4.5×10^4
30	50	F	AML	20	86	179	0.3	148	0.06	0	−	−	−	RCC, PC	TTV: 9.0×10^3
31	32	F	AML, post BMT	173	230	275	0.5	193	0.19	2.4	−	−	−	RCC, PC	TTV: 4.4×10^6
32	52	M	ALL, post BMT	26	58	663	0.7	214	15.31	1.2	+	±	−	RCC, PC	HHV-6: 1.2×10^4, HHV-7: 7.7×10^3, TTV: 4.9×10^3
33	44	F	MDS, post BMT	36	84	209	0.9	239	0.17	0.8	−	−	−	RCC, PC	HHV-6: 1.9×10^4, TTV: 2.7×10^5
34	19	M	Malignant lymphoma	48	125	181	0.7	161	0.26	0	−	−	±	None	TTV: 5.5×10^2
35	76	F	Drug-induced hepatitis	578	384	920	2.1	562	0.39	0	−	−	−	None	TTV: 7.3×10^3
36	80	M	Hepatitis B (chronic)	1,502	868	459	3.8	427	1.32	0.4	±	−	−	None	HBV: 1.6×10^8, EBV: 1.5×10^3, TTV: 7.8×10^4
37	57	M	Hepatitis B (acute)	938	1,707	917	3.3	459	1.15	0	±	−	−	None	HBV: 1.5×10^8
12 Healthy volunteers				HHV-1,2, VZV, EBV, CMV, HHV-6,7,8, BKV, JCV, PalvoB19: N.D.											HAV, HBV, HCV, HDV, HEV, HGV: <10 copies. TTV: positive in 2 of 12; 5×10^1–7.7×10^2 copies.

Normal upper limits of AST, ALT, ALP, T-Bil, LDH, and CRP are 40 IU/L, 40 IU/L, 360 IU/L, 1.3 mg/dL, 230 IU/L, and 0.3 mg/dL, respectively. The copy number of each virus listed is expressed in its copy number /mL.

Atyp. Lym: atypical lymphocyte, PC: platelet concentrate, RCC: red cell concentrate, AIDS: acquired immunodeficiency syndrome, ALL: acute lymphoblastic leukemia, ALP: alkaline phosphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, ATL: adult T-cell leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, FUO: fever of unknown origin, HPS: hemophagocytic syndrome, IP: interstitial pneumonia, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, PH: pulmonary hypertension, Tbc: tuberculosis, T.Bil: total bilirubin, MRSA: methicillin-resistant Staphylococcus aureus, N.D: not detected

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Table 5. Patients Reexamined for Liver Dysfunction and Previously Documented Viremia

Patient No.	Age	Sex	Time of reexamination	AST	ALT	ALP	T.Bil	LDH	CRP	Fever	Skin rash	LN swelling	Virus detected and its copy number
3	36	F	after 8 M	29	11	337	0.3	420	3.76	±	−	−	HHV-6: not detectable
4	69	F	after 4 M	34	34	186	0.8	272	0.14	−	−	−	HHV-6: 4.3×10^1
6	49	M	after 4 M	37	70	347	0.4	207	0.06	−	−	−	HHV-6: 1.0×10^1
8	72	F	after 6 M	48	19	289	0.8	227	2.11	−	−	−	HGV: 5.3×10^4
9	53	M	after 4 M	19	28	465	0.5	197	0.03	−	−	−	HHV-6: 1.0×10^3
11	85	M	after 2 W	615	670	1,739	2.0	649	0.24	−	−	−	TTV: 3.5×10^3
18	36	F	after 6 days	152	278	430	0.7	417	0.38	±	−	+	CMV: not reexamined
19	44	F	after 9 days	140	344	1,062	0.8	246	0.05	±	−	±	EBV: not reexamined
20	66	F	after 2 M	17	26	139	0.4	147	3.13	−	−	−	TTV: 9.0×10^3
21	25	F	after 2 M	21	12	221	0.5	161	0.02	−	−	±	CMV:undetectable, HHV-6: not detectable
22	67	F	after 3 M	26	6	278	0.9	328	5.43	−	−	−	HHV-6: 1.4×10^3
27	70	M	after 2 W	78	72	494	2.9	257	2.34	±	−	−	TTV: 1.3×10^6
30	50	F	after 1 M	24	74	158	0.3	217	0.05	−	−	−	TTV: 6.0×10^3
32	52	M	after 2 W	23	33	479	0.9	330	1.38	±	−	−	HHV-6: 1.3×10^3, HHV-7: 5.2×10^3, TTV: 3.1×10^5
33	44	F	after 1 M	38	44	234	0.9	209	0.10	−	−	−	HHV-6: not detectable, TTV: 1.7×10^5
34	19	M	after 2 M	28	56	160	0.9	146	0.55	−	−	±	TTV: not detectable
35	76	F	after 3 M	29	13	776	1.0	244	0.45	−	−	−	TTV: not reexamined
36	80	M	after 3 W	94	172	258	25.1	272	2.07	±	−	−	HBV: 1.6×10^5, EBV: 1.5×10^3, TTV: 1.76×10^6
37	57	M	after 1 M	16	20	223	0.9	132	0.30	−	−	−	HBV: not reexamined

Normal upper limits of AST: ALT: ALP: T-Bil: LDH: and CRP are 40 IU/L: 40 IU/L: 360 IU/L: 1.3 mg/dL: 230 IU/L: and 0.3 mg/dL: respectively.
AIDS: acquired immunodeficiency syndrome, ALL: acute lymphoblastic leukemia, ALP: alkaline phostphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, ATL: adult T-cell leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, FUO: fever of unknown origin, HPS: hemophagocytic syndrome, IP: interstitial pneumonia, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, PH: pulmonary hypertension, Tbc: tuberculosis, T.Bil: total bilirubin

Table 6. Characteristics of Patients with Normal Liver Function or Mild Dysfunction Regardless of Positive Viral Polymerase Chain Reaction

Patient No.	Age	Sex	Underlying disease	AST	ALT	ALP	T.Bil	LDH	CRP	Fever	Skin rash	LN swelling	Virus detected and its copy number
38	60	M	AML, post BMT	30	78	348	0.7	106	0.17	−	−	−	CMV: 7.6×10^2
39	19	F	AML, post BMT	28	28	205	0.5	383	0.82	−	−	−	HHV-6: 3.1×10^3
40	58	F	Ulcerative colitis	43	40	262	0.2	152	15.95	+	−	−	CMV: 3.5×10^2, EBV: 1.9×10^2
41	44	M	AML, post BMT	45	74	232	1.3	200	0.13	−	±	−	HHV-6: 1.1×10^3, TTV: 1.2×10^2
42	63	F	AML	34	40	260	0.4	204	2.05	±	−	−	HHV-6: 6.5×10^3
43	86	M	MDS/AML	28	46	318	1.1	408	17.52	+	+	−	HHV-6: 4.6×10^3
44	65	M	MPD/AML, post BMT	15	18	218	0.8	149	0.26	−	−	−	TTV: 9.2×10^3
45	58	M	Malignant lymphoma	75	71	328	0.9	174	0.11	−	−	−	HHV-6: 1.1×10^4, TTV: 8.9×10^3
46	58	F	Malignant lymphoma	42	79	164	1.2	187	0.65	±	−	−	TTV: 6.3×10^2
47	74	F	Malignant lymphoma	24	14	157	0.4	2,113	3.97	+	−	−	CMV: 5.9×10^3 TTV: 3.8×10^3, HSV-1: 1.5×10^6
48	67	M	MPD/AML, post BMT	14	19	188	1.3	232	0.14	−	−	−	CMV: 1.0×10^6, HHV-6: 1.9×10^2
49	45	M	AML, post BMT	16	53	190	0.7	188	11.24	+	−	−	TTV: 1.4×10^4

ALP: alkaline phosphatase, ALT: alkaline transaminase, AML: acute myeloid leukemia, BMT: bone marrow transplantation, CRP: C-reactive protein, LDH: lactate dehydrogenase, LN: lymph node, MDS: myelodysplastic syndrome, T.Bil: total bilirubin

HHV-6 load was significantly correlated with the levels of AST, ALT, and ALP between the 1st and 2nd examinations, and the correlation coefficients ranged from 0.9435 to 1.0000. Patient 21 had CMV-infectious mononucleosis, and CMV was undetectable after 2 months of treatment with ganciclovir, with normalized liver function. The liver dysfunction was improved after treatment with ganciclovir in another patient with CMV-infectious mononucleosis (patient 18), although the viral load was not reexamined. Patient 19 had EBV-infectious mononucleosis, and the liver dysfunction was also improved with the resolution of fever and lymph node swelling, suggesting the natural course of this disease. On the other hand, the relationship between the changes in liver dysfunction and the TTV load was unclear in 7 patients in that were re-assayed for TTV, and TTV became undetectable in patient 34 with the improvement of the liver dysfunction.

Incidence of liver dysfunction in each viral infection

Twelve patients that did not exhibit overt liver dysfunction regardless of viremia are described in Table 6. The incidence of TTV, HHV-6, CMV, EBV, and HSV-1 infections were 6/12, 6/12, 4/12, 1/12, and 1/12, respectively. The frequency of individual viral infections was similar to that of the patients with liver dysfunction (Table 4). These results suggested that these viral infections do not always cause overt liver dysfunction; therefore, the incidence of liver dysfunction was calculated in each case of viremia listed in Tables 3, 5. The results showed 70% TTV, 68% HHV-6, 43% CMV, and 83% EBV.

Consistency of the results obtained by multiplex PCR in comparison to those obtained by routine viral examination

The results obtained by multiplex PCR combined with real-time PCR and we compared with those obtained with commercially available virus examinations (Mitsubishi Chemical Medience Corporation, Tokyo, Japan), which are routinely employed in this institute. Attending physicians performed routine virus examinations independent of the current assay system in order to investigate the cause of the liver dysfunction or the inflammation. Table 7 shows that the results obtained with the PCR assay system were consistent with those obtained with routine virus tests except for the results of EBV in patients 18, 21, and 40. EBV was not detectable in the PCR assay in the first 2 patients, while the routine EBV serological test was positive for the VCA-IgM antibody. In contrast, the PCR assay system gave a positive result regardless of the negative VCA-IgM antibody determined by routine examination in patient 40. The assay was re-examined in the first 2 patients with a preserved DNA specimen with negative results. In addition, Table 8 shows that the results for HBV and HCV by the PCR were consistent with those by the commercially available method (SRL Inc., Hachioji, Japan).

In addition, the viral load of ParvoB19 was examined in a patient (52-year-old man) that came to the hospital because of anemia and a very low reticulocyte count. A bone marrow aspirate showed pure red cell aplasia (PRCA) with re-

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Table 7. Consistency of the Results Obtained by Multiplex Polymerase Chain Reaction (PCR) with those Obtained by Commercially Available Viral Examination

Patient No.	Viral examination by multiplex PCR	Viral examination by commercial laboratory test*	Results of viral serological tests*	Consistency
5	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA IgG (+)	yes
7	CMV (−)	CMV antigenemia (−)	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA IgG (+)	
11	CMV (−)	CMV serological test	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EA-IgG (−), EBNA IgG (+)	
	HSV (−)	HSV-1 serological test	IgM (−), IgG (+)	
18	CMV (+)	CMV serological test: reactivation/IgM antibody (+)	IgM (+), IgG (+)	yes
	EBV (−)	EBV serological test: primary infection	VCA-IgM (+), VCA-IgG (+), EA-IgG (−), EBNA-IgG (+)	no
19	EBV(+)	EBV serological test: primary infection	VCA-IgM (−), VCA-IgG (+)	no
20	CMV (+)	CMV pp65 antigenemia assay: positive	IgM (−), IgG (+)	yes
21	CMV(+)	CMV pp65 antigenemia assay: positive	IgM (+), IgG (+)	yes
		CMV serological test: reactivation/IgM antibody (+)		
	EBV(-)	EBV serological test: primary infection	VCA-IgM (+), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	no
29	CMV (−)	CMV serological test: previous infection	IgM (−), IgG (+)	yes
	EBV (−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	
37	CMV(−)	CMV serological test: previous infection	IgM (−), IgG (+)	yes
	EBV(−)	EBV serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EA-IgG (+−), EBNA-IgG (+)	
	HSV-1(−)	HSV-1 serological test: previous infection	IgM (−), IgG (+)	
40	CMV(+)	colon biopsy**; immunohistochemistry: positive	IgM (−), IgG (+−)	yes
	EBV(+)	EB serological test: previous infection	VCA-IgM (−), VCA-IgG (+), EBNA-IgG (+)	no

CMV: cytomegalovirus, EBV: Epstein-Barr Virus, HSV: herpes simplex virus

*: Performed by Mitsubishi Chemical Medience Corporation, Tokyo, Japan. Positivity of serological test was determined based on positive IgM antibody.

**: Performed in our institution

Table 8. **The Comparison of Hepatitis Virus PCR with Routine Method**

Age	Sex	Type of hepatitis	Qualitative PCR	Quantative PCR	Commercially available PCR*
79	F	HBV	positive	1.88×10^{9}	7.94×10^{7}
69	F	HBV	positive	4.39×10^{7}	2.51×10^{7}
57	M	HBV	positive	1.49×10^{8}	1.25×10^{8}
80	M	HBV	positive	1.59×10^{9}	1.26×10^{9}
79	M	HBV	positive	1.60×10^{8}	7.94×10^{6}
63	F	HBV	positive	2.90×10^{3}	5.01×10^{3}
75	M	HBV	negative	1.00×10^{1}	$<3.91\times10^{3}$
63	M	HCV	N.E.	2.51×10^{6}	2.00×10^{6}
70	F	HCV	N.E.	7.94×10^{4}	1.00×10^{5}
46	M	HCV	N.E.	3.98×10^{5}	2.51×10^{6}
80	M	HCV	N.E.	2.51×10^{6}	1.00×10^{7}
66	M	HCV	N.E.	5.01×10^{3}	1.00×10^{4}
71	F	HCV	N.E.	6.31×10^{5}	1.26×10^{6}
85	F	HCV	N.E.	2.51×10^{6}	6.31×10^{6}

HBV: hepatitis B, HCV: hepatitis C, N.E.: Not examined
*: performed by Special Research Laboratory, Hachioji, Japan

sidual giant proerythroblasts. A diagnosis of PRCA due to ParvoB19 was made, and the assay for ParvoB19 showed 6.9×10^{7} copies/mL in accordance with a positive qualitative PCR result and a positive serological IgM test for ParvoB19 (performed by SRL).

Discussion

The present study found a high incidence of viremia in patients with unexplained liver dysfunction and undetermined inflammation. The high proportion of hematologic malignancies including allogeneic bone marrow transplantation, that were underlying diseases in the patients included in this study may explain the high incidence of viremia. The multiplex PCR procedure appeared to be very useful in the clarification of uncertain liver dysfunction and inflammation. The patients in this cohort turned out to be highly immunodeficient and susceptible to viral infection. The identification of the high incidence of viremia may lead to better management of these patients.

TTV (22) was the most frequently detected virus in the present study; however, the relationship between TTV-positivity and the history of transfusion was unclear as shown in Table 4, thus suggesting a previous TTV infection in these TTV-positive patients. All patients in whom TTV was detected exhibited mild to moderate hepatitis, as observed in previous reports (22), except for patients 11 and 15. The lever dysfunction improved after a short time in these 2 patients, suggesting a transient exacerbation of TTV-related liver dysfunction by immunosuppressive treatment. This possibility, however, should be elucidated in the future in a larger cohort of immune-deficient patients because the relationship between the changes of liver dysfunction and TTV load was unclear in the follow-up examination. Similar incidences of HHV-6 viremia and TTV were observed in the present study. Apart from TTV that is widely distributed in the healthy population (22, 23), HHV-6 viremia is considered to be re-activation of this virus (24); therefore, this viremia indicates a severe immune-compromised condition. The high incidence of HHV-6 viremia in the present study could be explained by the high proportion of patients that underwent allogeneic or autologous hematopoietic stem cell transplantation or patients with hematologic malignancies, or AIDS. HHV-6 viremia occasionally advances to encephalitis or pneumonitis if its load is high (25). Therefore, identification of HHV-6 viremia is very important, and antiviral treatment is required. The present study, found that liver dysfunction was improved as the load of HHV-6 decreased in all 6 patients that were re-assayed for HHV-6 (Tables 4, 5). This suggests that liver dysfunction may be closely related to HHV-6 infection. While 2 of 17 patients in whom HHV-6 was detected showed normal liver function (Table 6), which is consistant with previous reports describing that hepatitis is not a major clinical manifestation of HHV-6 infection (26). In contrast, HHV-6-related hepatitis is reported in patients that underwent heart transplantation (27); therefore, the exact relationship between HHV-6 infection and hepatitis in immune-compromised patients should be clarified in the future. It was also important that the liver dysfunction acted as a proband that led to the discovery of serious viremia in the present study.

EBV and CMV infections can be categorized into 2 groups; primary infections that cause infectious mononucleosis (IM), and re-activation of both viruses. The present study, identified 2 patients with IM due to CMV (28) and 1 with EBV-IM (29). IM can be easily diagnosed because of its characteristic clinical picture. However, the multiplex PCR assay had definite advantages because it provided rapid results. The clinical significance of the re-activation of these 2 viruses is similar to that of HHV-6, and again in this situation, the assay system appeared to be highly useful.

There were some discrepancies regarding EBV detection between the results obtained by multiplex PCR and those obtained by routine serological tests as previously described. Patients 18 and 21, showed re-activation or primary infection patterns for both CMV and EBV when examined by routine serological methods, while EBV was not detected by the assay system. EBV-IM appeared to be atypical in these 2 patients because of the normal white cell count. Furthermore, cross-reactivity of EBV-specific IgM antibodies with CMV-antigens (30) or false positive EBV IgM serological tests (31) occasionally observed. Therefore, their IM may have been CMV-induced. EBV was detected in Patient 40, regardless of the previous infection pattern determined by the serological method. This patient had ulcerative colitis and was in an immune-deficient state. Persistent EBV viremia is likely in such conditions, and normal immune responses that produce the IgM antibody may be suppressed. Although the assay system appears to be reliable, further improvement of our system is necessary.

The authors state that they have no Conflict of Interest (COI).

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10 プライマー/プローブの設計の手順② マルチプレックスPCRの場合

北條浩彦，清水則夫

マルチプレックスPCRは，1つのPCR反応液中で複数のターゲット遺伝子を同時に増幅し検出する方法である．この方法は，1回のPCRで多くの情報（データ）を得ることができることから貴重なサンプルの有効利用と迅速な解析に優れている．しかし，このマルチプレックスPCRを実行するためには，細密なプライマー設計とPCR反応条件の検討が必要である．

マルチプレックスPCRとそのポイント

マルチプレックスとは「多重化」の意味である．マルチプレックスPCRは，1つのPCR反応系（反応チューブ内）で複数の異なるターゲット遺伝子を同時に増幅し（複数ターゲット遺伝子/1反応系），それらを識別して検出（解析）する方法である．通常の方法（1ターゲット遺伝子/1反応系）と比べて，マルチプレックスPCRは同じ量の鋳型DNAからより多くのデータを得ることができる．このため迅速な解析や網羅的な解析，そして貴重なサンプルの有効利用に長けている．このような解析を可能にするのは，①異なるターゲット遺伝子を同時に増幅させる特異性の高いPCRプライマーセットと②増幅したそれぞれのPCR産物を識別する異なる蛍光波長をもった数種の蛍光物質である．よって，特異性が高く相互干渉のないPCRプライマーセットの設計と細密なPCR条件の検討，そして識別可能な異なる蛍光波長をもった蛍光物質の組み合わせがマルチプレックスPCR実行の重要なポイントとなる．

プライマーデザインの簡単な方法

文献から定量用にデザインされたプライマーセットを見つけ出して表のプライマーダイマーチェックソフトで確認して相性のよいものを選択する．例えば，実践編-15のウイルスの迅速検査実験は本方法を使用している．

プライマーを初めからデザインする方法

デザインソフトを使用してプライマーをデザインする（表）．いずれも以下の点を考慮して選択またはデザインする．③のプライマーダイマー形成の可能性については，図1の3種類について考慮する必要がある．

①Primer Tm値を合わせる．各プライマーの差がTm値±5℃以内（できれば2℃以内）になるように設定する．

②デザインされたプライマーの特異性をチェックする．プライマー配列をGenBank BLAST解析で特異性を確認する．GenBank DNA BLAST

表　本項で扱ったウェブアプリケーションとそのURLなど

Webアプリ名	URL	有償/無償	特徴など
プライマーダイマーチェック			
PriDimerCheck	http://biocompute.bmi.ac.cn/MPprimer/primer_dimer.html	フリー	—
IDT OligoAnalyzer	http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/	フリー	—
デザインソフト			
MPprimer	http://biocompute.bmi.ac.cn/MPprimer/	フリー	セット数に制限がないが多数のデザインは困難
PrimerStation	http://ps.cb.k.u-tokyo.ac.jp/mquery.php?language=ja	フリー	ヒトゲノムのみ
PrimerPrex	http://www.premierbiosoft.com/index.html	有料	お勧めするソフト．一度に100セットまで

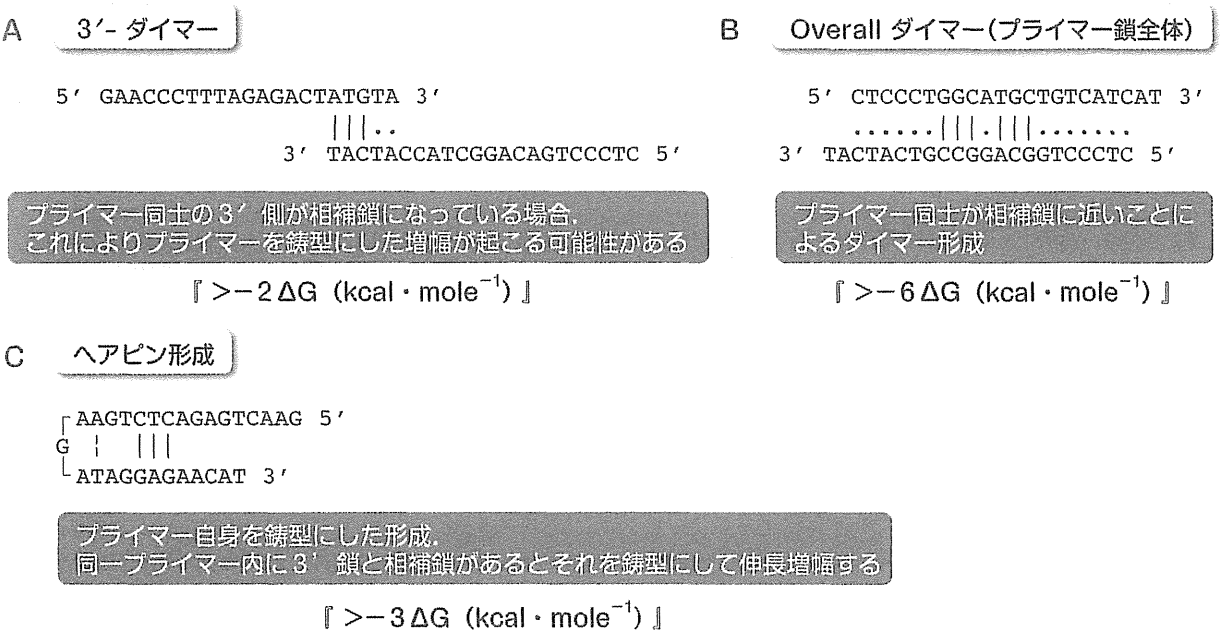

図1　望ましくないプライマーダイマーヘアピン構造の例
『』は，IDT OligoAnalyzerによって出力される望ましい値を示す

(http://blast.ncbi.nlm.nih.gov/Blast.cgi) からnucleotide blastを選択，配列をEnter Query Sequenceに入れてヒトサンプルであったらDatabaseのところにHuman genomic + transcriptにチェックを入れてBLASTボタンを押す．

③プライマーダイマー，ヘアピン形成のチェック．

④増幅産物サイズ．増幅産物の長さは短い方がよいがプローブ検出用にある程度必要なため100～300 bp程度が適当である．

1. MPprimerデザインの実際

Input DNA template欄にFASTA形式のDNA配列を入力する．このソフトはPCR産物を電気泳動でサイズを確認するデザインになっているのでProduct Size Rangesをすべて100～300 bpにしてデザインする．デザインされたプライマーについて，MPprimerにあるPriDimerCheckを使って，プライマーダイマーの確認を行い，最適なプライマーを選択する（図2）．

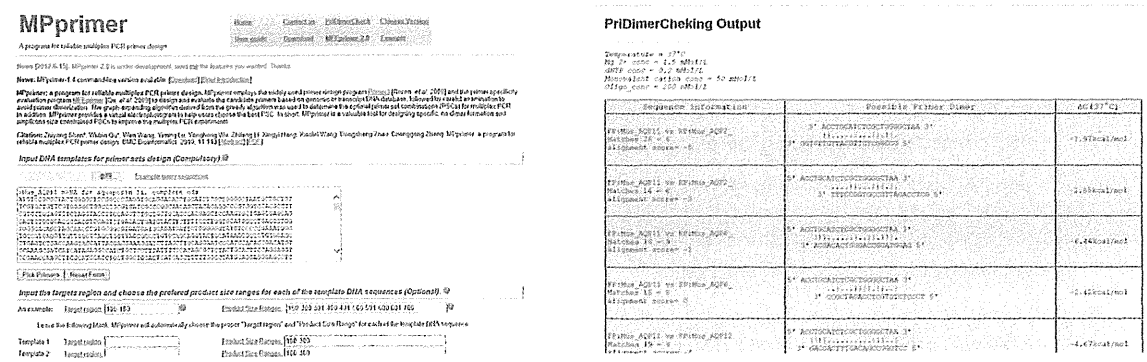

図2　MPprimerの入力画面とPriDimerCheckの結果画面

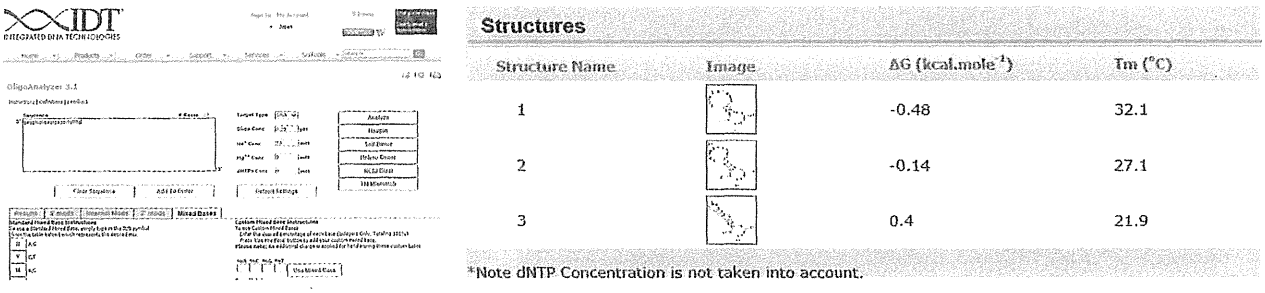

図3　IDT OligoAnalyzerの入力画面とヘアピン構造予測の結果

2. IDT OligoAnalyzerによるヘアピン形成チェック

IDT OligoAnalyzerの sequence欄にプライマー配列を入れてヘアピン構造を確認する（図3）.

ハイブリプローブ設計のポイント

ハイブリプローブ※を設計する際には，PCR反応後に入れるためプライマーやプローブの相性を見る必要はなく，プローブ配列は増幅内であればどこでもよい．ただし，反応内のすべてのプライマー配列や他のターゲット増幅産物との相同性は確認しておくこと．

Tm値を**Current Protocols in Molecular Biology**に準拠し，次の式から算出している．

$$(\text{Tm値}) = 60.8 + 0.41 \times [\text{G,Cの割合}(\%)] - \frac{500}{(\text{総塩基数})}$$

しかし，ここで求めたTm値がそのまま融解曲線分析でのTm値（実測値）にはならないことに注意が必要である．経験的には，求めたTm値より5℃高い温度が融解曲線分析でのTm値となる．LcRedとFITC標識プローブのうち，Tm値の低いプローブの値が反映される．またLcRedを**実践編-15**では使用したが，FRETの原理を用いるため，例えばLcRed705の代わりに安価なCy5.5など同じ波長であれば別の色素でも構わない．

※　ハイブリプローブを用いたPCRの試薬とプライマー濃度：専用試薬が市販されているが，Taq ポリメラーゼの3′エキソヌクレアーゼ活性を不活化しているものと特異性を増強するタンパク質を添加しているものがあり，清水らはタンパク質が添加されている方を使用している（AccuPrime™ Taq DNA Polymerase）．また通常の試薬でも増幅するが，プライマーセットが多いときはTaq ポリメラーゼ濃度を2～3倍にするとよく増幅される．また各プライマー濃度は初めは0.2 μMで実験するとよい．その後は0.1～0.3 μMで調整する．

15 ウイルス感染症を診断する
ウイルスゲノムの定性的検査と定量的検査

清水則夫，渡邊 健，外丸靖浩

リアルタイムPCR活用の目的とヒント

ウイルスは分離培養が難しいため，PCRによりウイルスゲノムを直接検出する手法はウイルス感染症の診断に欠かせない技術となっている．一方，健常人にも多くのウイルスが持続感染していることが知られており，ウイルスゲノムが検出されてもすぐに病気の原因と断定することはできない場合もある．われわれの研究室では，PCR法によるウイルスゲノムの検出法として定性的検査法と半定量的検査法の２種類を開発し，検査対象ウイルスの種類・目的・検査時間・検体量などにより定性的検査系と定量的検査系を使い分けている．

はじめに

ウイルス感染症が疑われる疾患の原因ウイルスを特定する際，一般には臨床症状などから予想されるウイルスを個別に検査する手法がとられている．しかしこのような検査では，予想外のウイルス感染が見逃されたり，複数のウイルスの重複感染や主たる病因ウイルスの感染が見逃される危険性がある．われわれは，多くのウイルスを網羅的・迅速・安価に検出することが可能になればウイルス感染症をより適切に診断できると考え，キャピラリーPCR装置を使用しマルチプレックスPCR法による多種類のウイルスを同時に検出できる検査法（定性検査）を開発した．さらに，多種類のウイルスの同時定量と検査系の自動化を目的にプレートタイプのリアルタイムPCR装置を使用した網羅的ウイルス検出法を開発した．

本項では，迅速性に重点を置いたキャピラリーPCR装置による定性的検査法と，一般に普及しているプレートタイプのリアルタイムPCR装置を用いた半定量的検出法と検査系の自動化に関する取組みを紹介する．

キャピラリーPCR装置を使用した網羅的・迅速検査系

本検査法は，マルチプレックスPCRにより１本のキャピラリー内で最大32種類の遺伝子が検出可能である．測定はPCR40分，融解曲線解析10分の合計50分程度ときわめて短時間で完了し，単一項目の検出を行う場合と同等の検出感度がある．

図１にマルチプレックスPCR法による，複数ウイルスの同時・迅速・高感度ウイルス検査系

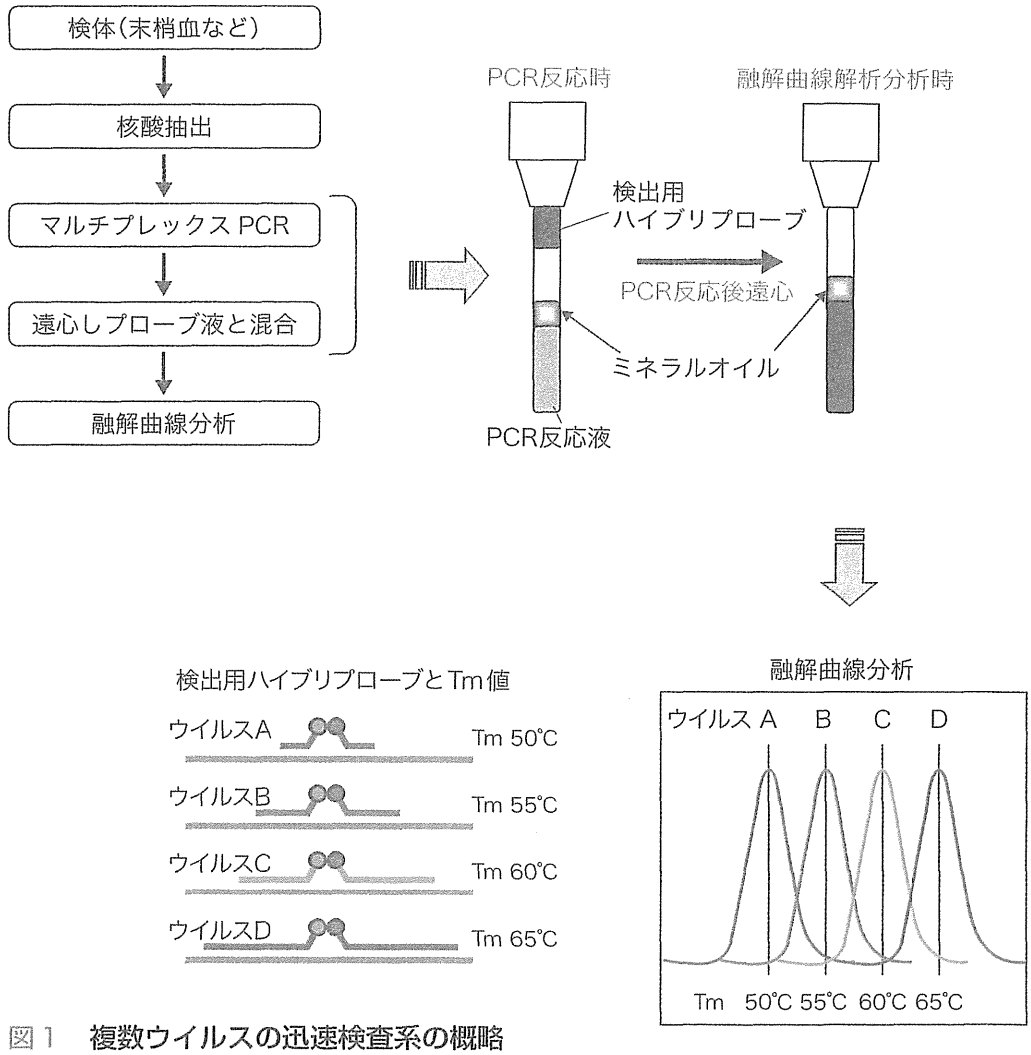

図1 複数ウイルスの迅速検査系の概略

の概略を示す．サンプルから核酸を抽出し，マルチプレックスPCRを行う．その後，検出用ハイブリプローブMixを加えて増幅配列にハイブリダイズさせ，FRETによる蛍光を検出し融解曲線分析（Melting Curve Analysis）を行い，複数のウイルスを同時・定性的に検出・識別するシステムである．図1に示してあるようにハイブリプローブのTm値が違うため，どのウイルス遺伝子が増幅されたか融解曲線分析により得られたピークのTm値から判定される．さらに，内在性コントロール遺伝子（IC）も加え，PCR反応が進まないために生じる偽陰性を防止している．

はじめにPCRを行い終了後に検出用ハイブリプローブを混合するため，お互いの相性を考慮する必要性が低下しプライマー・プローブの設計が容易になる．

検出用ハイブリプローブの設定可能なTm値の範囲は50～75℃であり，プローブ同士のTm値の差が3℃以上あればウイルス種を区別可能なため，合計8種類の異なるピークを区別できる．LightCycler® 2.0では，アクセプター色素としてLcRed640，610，670，710の4種類の蛍光波長が使用可能であるため，理論上は1本のキャピラリーで4×8＝32種類のウイルス種が検出可能なことになる．

準備

例として，ウイルス12種類を同時に検出する系を示す．なお，1度に32本測定できるため同時測定が可能なサンプル数は15検体である．

□ LightCycler® 2.0（ロシュ・ダイアグノスティックス社）

□ DNAウイルス定性用試薬増幅酵素＋Bufferセット（日本テクノサービス株式会社 #D001-1）
詳細については日本テクノサービス株式会社へ直接問合せる．

□ サンプル（検体）DNA　約1 μg
DNAの精製度は結果に影響を及ぼすので非常に重要である．

□ プライマー，ハイブリプローブ*1
株式会社日本遺伝子研究所などで購入可能．

	検出ウイルス名
Aセット	単純ヘルペスウイルス（Herpes simplex virus：HSV-1, HSV-2） 水痘・帯状疱疹ウイルス（Varicella-Zoster virus：VZV） パルボウイルスB19（Parvovirus B19：B19） ヒトヘルペスウイルス6型（Human Herpes Virus type 6：HHV-6） サイトメガロウイルス（Cytomegalovirus：CMV） BKウイルス（BKV），JCウイルス（JCV）
Bセット	EBウイルス（Epstein-Barr virus：EBV） ヒトヘルペスウイルス7型（Human Herpes Virus type 7：HHV-7） ヒトヘルペスウイルス8型（Human Herpes Virus type 6：HHV-8） B型肝炎ウイルス（Hepatitis B virus：HBV）

＊1　ウイルスセットのプライマー，プローブ配列は参考文献1，2を参照．

□ LightCycler® Capillaries（20 μL）

□ LightCycler® Centrifuge Adapters
あらかじめ4℃で冷却しておく．

□ LightCycler® 2.0 Sample Carousel（20 μL）

□ LC Carousel Centrifuge 2.0

□ ミネラルオイル（M8662-SVL，シグマアルドリッチ社）

□ 微量高速遠心機
1.5 mLチューブが遠心できるもの．

プロトコール

以下，Aセットを例にして示す（Bセットも同様）．

1. マスターミックスの作製

❶ プライマーミックスの作製

各プライマーセットを混合し10×濃度に調製しておく．

HSV primer F*2	20 μL
HSV primer R	20 μL
CMV primer F	80 μL

CMV primer R	80 μL
HHV6 primer F	40 μL
HHV6 primer R	40 μL
B19 primer F	60 μL
B19 primer R	60 μL
BKV JCV primer F	40 μL
BKV JCV primer R	40 μL
VZV primer F	20 μL
VZV primer R	20 μL
Total	520 μL

＊2　各プライマーは100 pmol/μLに調製したものを用いる.

❷ 内在性コントロール遺伝子（IC）β-グロビンプライマーミックスの作製

プライマー濃度を4 pmol/μLに調製する.

β-グロビン primer F	4 μL
β-グロビン primer R	4 μL
Nuclease free water	92 μL
Total	100 μL

❸ マルチプレックスPCR用マスターミックスの作製

＜1反応分＞

Primer（❶で調製したもの）	0.60 μL
IC primer（❷で調製したもの）	0.40 μL
Buffer	1.50 μL
定性用増幅酵素	0.25 μL
dH_2O	2.25 μL
Total	5.00 μL

2. 反応液の調製

試薬の調製は，あらかじめ4℃で冷却しておいたLightCycler Centrifuge AdaptersにLightCycler Capillaries（20 μL）を立てて行う.

❶ ミネラルオイルを3 μLずつキャピラリーに入れる

❷ マスターミックスを5 μLずつ入れる

❸ テンプレートを0.2 μg添加してピペッティングで混合し，ヌクレアーゼフリー水で全容量10 μLに調製する＊3

＊3　ここでは全容量10 μLにしているが最大で倍の20 μLまで増やせる.

❹ キャピラリーをアダプターごと高速微量遠心機で1,000 × g（3,000 rpm）で3秒遠心する＊4

＊4　キャピラリーの蓋が開いているため，ミストの発生によるコンタミネーションの危険がある. それを避けるため高速で遠心しないこと.