Detection of bacteria, fungi, and viruses by a real-time PCR assay using universal primers and probes from blood in patients with febrile neutropenia.

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ABSTRACT Febrile neutropenia is the main treatment-related cause of mortality in cancer patients. During June 2012 to April 2013, 76 blood culture samples from patients receiving chemotherapy for hematological malignancy and cancer with febrile neutropenia episodes (FNEs) were examined for the presence of bacteria and fungi based on 16S rRNA gene and 18S rRNA combined with real-time PCR amplification and sequencing. Furthermore, we used a loop-mediated isothermal amplification (LAMP) assay for the detection of herpes simplex virus type 1 and 2 (HSV-1,2), varicella zoster virus (VZV), epstein-barr virus (EBV), cytomegalovirus (CMV) and human herpes virus type6 and 7 (HHV-6,7), followed by a real-time PCR amplification assay.

Of these samples, bacteria were identified in 19 of 76 FNEs (25.0%) by a real-time PCR assay and in 9 of 76 (11.8%) by blood culture. In 6 blood culture-positive samples, real-time PCR assay detected the same type of bacteria. No fungus was detected both real-time PCR assay and blood culture. Viruses were identified in 6 of 76 FNE (7.9%).

During antibiotic therapy, all samples were negative in blood culture, but a real time PCR assay yielded a positive result in 2 cases of 2 (100%). The number of bacteria DNA copy and serum CRP titer of patients with FNE did not correlated well.

We conclude a real-time PCR assay could be given higher microbe's detection rate, and need shorter turnaround time, and smaller blood sample than blood culture. Using a real-time PCR assay combined with blood culture improves microbiological documentation in febrile neuropenia episodes. doi:10.11482/KMJ-E40(1)1 *(Accepted on September 5, 2013)*

Key words: **Febrile neutropenia, Real-time PCR assay, Loop-mediated isothermal amplification**

INTRODUCTION

In patients receiving chemotherapy for treatment of cancer, febrile neutropenia episodes (FNEs) are one of the most common adverse event by chemotherapy. When fever occurs in neutropenia patients, they are more likely to develop a serious infectious disease with various microbes[1-4].

The definitions of febrile neutropenia (FN) in Infectious Disease Society of America (IDSA) guidelines are general criteria that should be used

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to identify patients in whom empirical antibiotic therapy must be initiated[5]. Fever is defined as a single oral temperature measurement of ≧ 38.3℃ or a temperature of ≧ 38.0℃ sustained over a one-hour period. Neutropenia is defined as an absolutely neutrophilic count (ANC) less than 500 cells/mm^3 or an ANC that is expected to decrease less than 500 cells/mm^3 during the next 48 hours.

Management of FN is very important, because infections of various microbes are major causes of morbidity and mortality in patients receiving chemotherapy[1-4].

Seventy five percent of FN patients resulted in death, before the era of an empirical antibiotic therapy introduced[2]. Recently, the empirical antibiotic therapy has been used in all patients with FN. We administer antibiotic therapy in consideration of causative microorganism predicted for FN patients as soon as possible, after we undertook blood culture. However, the sensitivity of blood culture remains low, and also it takes time to identify causative microorganism by blood culture, so we cannot give appropriate and specific antibiotic therapy quickly for causative microorganisms.

We set up the microbes detecting system by the real-time PCR method that we can detect bacteria, fungi, and viruses from a blood sample in a short time. Primers targeting the 16S rRNA gene for detecting bacteria[3,4] and 18S rRNA gene for detecting of fungi[6-9] were used for the real-time PCR. Furthermore, we used a loop-mediated isothermal amplification (LAMP) assay for detecting herpes viruses, such as herpes simplex virus type1 and 2 (HSV-1,2), varicella zoster virus (VZV), cytomegalovirus (CMV), epstein-barr virus (EBV) and human herpes virus type6 and 7 (HHV-6,7). If the LAMP assay is positive, we performed a real-time PCR assay.

MATERIALS AND METHODS

Patients and blood samples:

Patients with malignancy presenting fever (axillary temperature equal or higher than 38.0℃) and severe neutropenia (ANC less than 0.5×10^9/l) induced by chemotherapy were eligible for this study. Multiple FNEs per patient were allowed to include this study. We took a written informed consent from patients or their parents when they were equal or less than 15 years old. The study was approved by the ethics committee of Kawasaki Medical School. We undertook two sets of blood cultures from adult patients with FNEs and inoculated 10ml of blood each into aerobic bottle and an anaerobic bottle. On the other hand, we collected only 3ml of blood

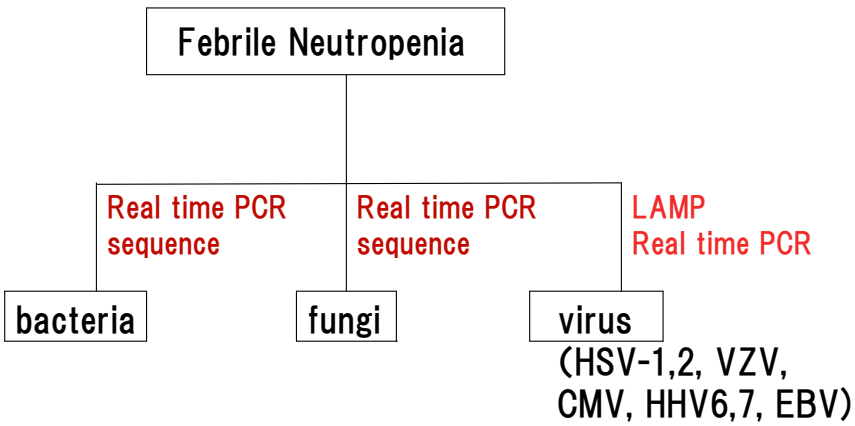

Fig. 1. Nucleic acid amplification tests used in this study for each microbes

into a pediatric aerobic bottle for child patients. Blood culture samples were set in BACTEC™FX (Becton, Dickinson and Company, Tokyo, Japan). β-D Gulcan was measured in each sample. Blood culture samples were also assayed for nucleic acid amplification tests as follows (Fig.1).

Bacteria and Fungus protocol

DNA extraction protocol:

A 400 μl of EDTA-anticoagulated peripheral blood was used for the real-time PCR assay. Sample was stored at -80℃ until DNA extraction. For DNA extraction, QIAamp UCP Pathogen Mini kit (QIAGEN, Basel, Switzerland) was used under the instruction of the manufacture.

A Real-time PCR assay:

The bacterial primer pairs specific for conserved DNA sequences encoding the 16S rRNA gene region were used[3,4]. The real-time PCR reaction mixtures were prepared using 10 μl of enzyme mixture (SsoFast Probes supermix BIO-RAD), 1 μl of 10 μM forward primer (AGTTTGATC (A/C) TGGCTCAG; SIGMA-ALDRICH, Tokyo, Japan), 1 μl of 10 μM reverse primer (GGACTAC (C/T/A) AGGGTATCTAAT; SIGMA-ALDRICH, Tokyo, Japan), 0.5 μl of 10 μM probe (CGTATTACCGCGGCTGCTGGCAC; SIGMA-ALDRICH, Tokyo, Japan) and 5 μl of DNA extract of the sample. The reaction mixture was made up to 20 μl with water.

PCR reactions were performed in the BIO-RAD CFX96 Real-Time System (BIOLAD, Tokyo, Japan) with preliminary denaturation at 95℃ for 1 minute, followed by 40 amplification cycles (with a temperature transition rate of 3.3℃/seconds) of deneturation at 95℃ for 5 seconds, annealing at 53℃ for 10 seconds, and primer extension at 72℃ for 20 seconds.

The fungal primer pairs specific for conserved DNA sequences encoding the 18S rRNA gene region were used[6-9]. Primers bind to conserved regions of the fungal 18S rRNA gene and panfungal oligonucleotide probes were used to bind to a common sequence present in all fungal species. 10 μl of enzyme mixture (SsoFast Probes supermix BIO-RAD, Tokyo, Japan), 0.9 μl of 10 μM forward primer (ATT GGA GGG CAA GTC TGG TG; SIGMA-ALDRICH, Tokyo, Japan), and 0.9ul of 10 μM (reverse primer CCG ATC CCT AGT CGG CAT AG; SIGMA-ALDRICH, Tokyo, Japan), 0.5 μl of 10 μM probes (TTC AAC TAC GAG CTT TTT AAC TG; SIGMA-ALDRICH, Tokyo, Japan) were made up to 20 μl with water, as the reaction mixture.

PCR reactions were performed in the BIO-RAD CFX96 Real-Time System (BIOLAD, Tokyo, Japan) with preliminary denaturation at 95℃ for 1minute, followed by 40 amplification cycles (with a temperature transition rate of 3.3℃/seconds) of deneturation at 95℃ for 5 seconds, annealing and primer extention at 60℃ for 30 seconds, with a single fluorescence acquisition step at the end of extention for the fungal PCR assay.

Sequencing and phylogenetic identification:

Bacterial sequence and analyze were used PCR products, not real-time PCR products.

PCR was performed in a 50 μl reaction mixture containing 4 μl of 0.2mM dNTP mixture, 1 μl of 10 μM forward primer (AGTTTGATC (A/C) TGGCTCAG; SIGMA-ALDRICH, Tokyo, Japan), 1 μl of 10 μM reverse primer (GGACTAC (C/T/A) AGGGTATCTAAT; SIGMA-ALDRICH, Tokyo, Japan), and 10 μl of DNA extract of the sample.

PCR was carried out in a 2720 Thermal Cycler (Applied Biosystems, Foster cuty, CA) with preliminary denaturation at 95℃ for 5min, followed by 35cycles of amplification consisting of denaturation at 94℃, primer annealing at 50℃, and elongation at 72℃, each lasting for 30 seconds.

Sequencing was performed by ABI 3130xl

Genetic Analyzer by BigDye reaction (Applied Biosystems) with forward primer or reverse primer. For identification, sequences were compared with sequences of known bacteria listed in official databases using the BLAST program available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Viral protocol

We used a LAMP assay for the detection of HSV-1,2, VZV, CMV, EBV and HHV-6,7. If a LAMP assay is positive, we performed a real-time PCR for these viruses.

DNA extraction protocol:

A 400 μl of EDTA-anticoagulated blood was used for the assay. The sample was stored at -80℃ until it was applied for DNA extraction.

For DNA extraction, QIAamp DNA Blood Mini kit (QIAGEN, Basel Switzerland) was used under the instruction of the manufacture. DNA was stored at 4℃ until tested.

LAMP methods:

Primers and reaction steps were referred to previous described methods for detection of HSV-1,2, VZV, CMV, EBV and HHV-6,7[10-16].

Real-time PCR:

Primers, probes and PCR protocol were referred to previous described methods for detection of HSV-1,2, VZV, CMV, EBV and HHV-6,7[17-20].

Statistical analysis:

For statistical analysis, Stat View 5.0® software (SAS Institute, Inc., Cary, NC, U.S.A.) was used. The results were compared between the two groups using the Mann-Whitney U test. To examine the correlation among factors, Spearman's rank correlation coefficient (non-parametric method) was used. The date were considered statistically significant if p-value was less than 0.05.

RESULTS

Population Characteristics

A total of 76 patients with FNEs were admitted to the Departments of Pediatrics, the Department of Hematology, and the Department of Respiratory Medicine in Kawasaki Medical School Hospital during June 2012 to April 2013. Twenty two of 76 FNEs revealed positive results by blood culture and/or nucleic acid amplification tests. Characteristics and Laboratory data of 76 patients are listed in Table 1. Median age of patients was 38 years (range, 1-72 years). Among 76 patients of FNEs, 62 patients

Table 1. Characteristics of the patients with febrile neutropenia

Patient charactreristic	Value
Median age (years)	38 (1 ～ 72)
Male (%)	8 (44)
No.(%) of patients with diagnosis	
Acute Lymphoblastic Leukemia	4 (22)
Acute Myeloid leukemia	3 (17)
Non Hodgkin lymphoma	5 (2)
NK/T cell Lymphoma	1 (6)
Hepatoblastoma	1 (6)
Lung cancer	4 (22)
Use of central venous catheter (%)	14 (78)
Median temperature (℃)	38.2 (38.0-38.5)
Median time of fever prior to admission (min)	45 (5 ～ 105)
WBC (/ μl)	439 (10 ～ 4,000)
Neutro (/ μl)	82 (0 ～ 861)
CRP on set (mg/dl)	4.35 (0.35 ～ 29.66)
CRP max (mg/dl)	10.78 (0.88 ～ 31.40)
Fever period (days)	4.5 (1 ～ 11)

Table 2. Bacterial culture results and molecular diagnosis results of patients with febrile neutropenia episodes in this study
NHL : non-Hodgikin's lymphoma, HB : hepatoblastoma, AML : acute myeloid leukemia, ALL : acute lymphoblastic leukemia

Sample			Blood culture	Real-time PCR and PCR, sequence, and identification		
Patients	Age	diagnosis		SQ mean	BLAST	Bit score, identities
7	66y	NHL	Negative	24.24	*Enterococcus faecium*	1,356, 99%
11	1y7m	HB	*Streptococcus mitis*	37.39	*Streptococcus mitis*	1,352, 99%
12	1y7m	HB	Negative	-	*Streptococcus mitis*	346, 91%
14	85y	NHL	Negative	0.71	*Acinetobacter junii*	612, 84%
18	43y	NK/T cell Lymphoma	*Klebsiella pneumoniae*	1.20×10^5	*Klebsiella sp*	1,308, 98%
20	7y6m	AML	*Neisseria sp*	-	Negative	-
21	11y7m	ALL	Negative	2.11	*Acinetobacter sp*	412, 73%
22	16y8m	AML	Negative	-	*Bacillus sp*	503, 84%
23	11y7m	ALL	*Streptococcus mitis*	57.91	*Streptococcus mitis*	1,327, 99%
24	7y6m	AML	*Streptococcus mitis*	48.38	*Streptococcus mitis*	1,328, 99%
25	3y8m	ALL	Negative	-	*Enterobacter sp*	1,040, 96%
26	16y8m	AML	Negative	-	*Enterobacter sp*	1,325, 98%
27	16y8m	AML	Negative	7.48	*Enterobacter sp*	1,188, 97%
35	7y6m	AML	*Streptococcus mitis*	-	Negative	-
36	5y10m	ALL	Negative	0.68	*Streptococcus gordonii*	333, 79%
37	16y8m	AML	*Streptococcus mitis*	5.80	*Streptococcus mitis*	1,061, 95%
38	16y8m	AML	*Streptococcus mitis*	3.21	*Streptococcus mitis*	355, 93%
39	11y7m	ALL	Negative	-	*Acinetobacter sp*	191, 89%
40	11y7m	ALL	Negative	3.91	*Acinetobacter sp*	1,055, 94%
46	7y6m	AML	*Streptococcus mitis*	3.15	Negative	-
48	16y8m	AML	Negative	5.61	*E. Coli*	309, 77%
63	13y1m	NHL	Negative	-	*Staphylococcus epidermidis*	619, 91%

NHL : non-Hodgikin's lymphoma, HB : hepatoblastoma, AML : acute myeloid leukemia, ALL : acute lymphoblastic leukemia

with FNEs were admitted to the Department of Pediatrics, 10 patients with FNEs to the Department of Hematology and 4 patients with FNEs to the Department of Respiratory Medicine. Seventy one out of 76 patients with FNEs (93.4%) had hematologic malignancies.

Mean WBC count and neutrophil count were 434/μl (range, 10-4,000) and 82/μl (range, 0-861). The mean duration from onset of FN to defervescing in normal temperature was 4.5 days (range, 1～11).

Comparison of the rate of detection between blood culture and nucleic acid amplication test:

All patients were performed blood culture once or twice on the first day of the febrile episodes, and a positive result was obtained in 9 FNEs, such as *Streptococcus mitis, Klebsiella pneumoniae, Streptococcus mitis, Neiseria sp*.

On the other hand, the real-time PCR assay was positive in 19 FNEs.

The real-time PCR assay detected *Enterococcus faecium, Streptococcus mitis, Acinetobacter junii, Acinetobacter sp, Klebsiella sp, Bacillus sp, Enterobacter sp, Streptococcus gordoni* and *Staphylococcus epidermidis* (Table 2).

Table 3. The correlation between Blood culture and a real-time PCR assay

	Blood culture positive	Blood culture negative	total
Real-time PCR positive	6	13	19
Real-time PCR negative	3	54	57
total	9	67	76

In 60 of 76 (79%) FNEs, blood culture and the real-time PCR assay gave the same results, regardless of positive and negative results.

Of these samples, bacteria were identified in 19 of 76 FNEs (25.0%) by real-time PCR and in only 9 of 76 (11.8%) by blood culture. In 6 of 9 blood culture-positive samples, the real-time PCR assay detected the same type of bacteria (No.11, 18, 23, 24, 37, 38).

Comparing with blood culture, the real-time PCR assay for bacteria had a sensitivity, specifity, and positive and negative predictive value of 67%, 81%,

32% and 95%, respectively (Table 3).

In this study, fungi were not detected in 76 FNEs by blood culture and the real-time PCR assay. β-D gulcan was negative in all cases.

Comparison of the detection speed between blood culture and real-time PCR:

It took an average of 9 hours 59 minutes for blood culture to become positive in this study. It took another 24 hours more so that causative bacteria were identified.

It took an average of 4 hours for obtaining positive results by the real-time PCR assay and another 12 hours for the sequence results (data not shown).

Molecular Results and Clinical course:

The number of bacteria DNA copy and serum CRP titer of patients with FNE did not correlated well (Fig.2).

As for No.14 patient, *Acinetobacter junii* was detected from blood, but the quantity of bacteria DNA copy was as few as 0.71 copy/μl and very small amount. However, his serum CRP titer was high as 21.44mg/dl. He died because of encephalitis two days after the FN onset. On the contrary, No. 18 patint was detected *Klebsiella sp* from blood, and the quantity of bacteria was as many as 1.20×10^5 copy/μl. However, her serum CRP titer was not so high as 6.17 mg/dl. She was started with biapenem for FN. But she did not respond well, then she was switched from biapenem to doripenem and vancomycin on Day 4. In consideration of a catheter-related infection, her central-vein catheter was removed on Day 5. She defervesced on Day 6.

The antibiotic therapy was not given to 74 of 76 FNEs before the FN onset, so FN developed in only two patients (No.7, No.12) during the preventive antibiotic therapy. No.7 patient was collected blood culture during the tazobactam/piperacillin treatment. The result of blood culture was negative, however *Enterococcus faecium* was detected by the real-time PCR assay. Similarly, No.12 patient was collected blood culture during doripenem and amikacin treatment. The blood culture was negative, however *Streptococcus mitis* was detected by the real-time PCR assay.

Nine patients with FNEs who were detected *Enterococcus faecium, Acinetobacter junii* and *sp,*

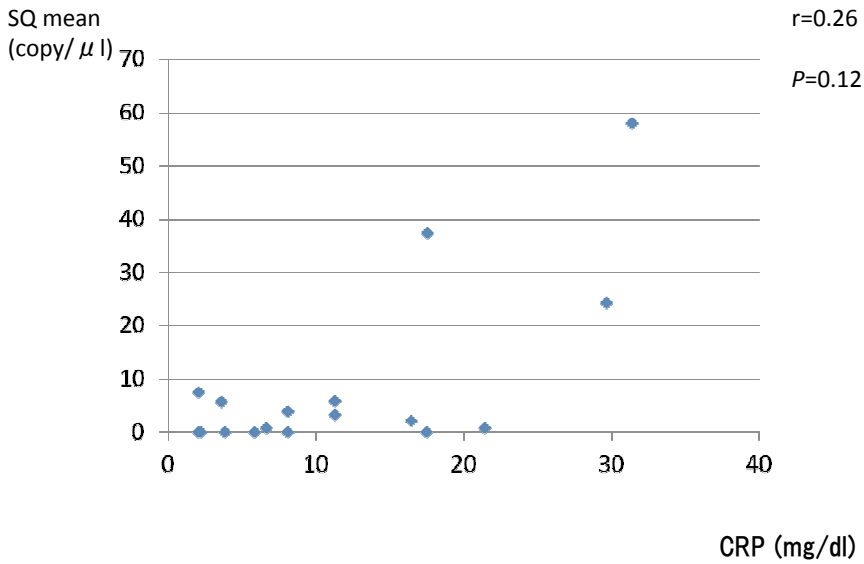

Fig. 2. The correlation between a number of bacterial DNA copy and serum CRP titer.

Enterobacter sp , E.coli and *Bacillus sp*. by the real-time PCR assay presented acute diarrhea, but blood culture was negative in all cases (No.7, 21, 22, 25, 26, 27, 39, 40).

On the other hand, as to 9 patients with FNEs who were detected *Streptococcus mitis*, *Streptococcus gordonii* and *Neisseria* associated episodes regardless of blood culture and the real-time PCR assay, oral mucositis developed in all these 9 cases (No.11, 12, 20, 23, 24, 35, 36, 37, 38). Two out of 9 patients with FNEs who were detected *Streptococcus mitis* were complicated with pleuritis (No.37, 38). One patient with FNE who was detected *Klebsiella pneumoniae* developed catheter-related infection (No.18).

As for No. 63, *Staphylococcus epidermidis* was detected, but there was no other symptom than fever.

In 11 cases with the real-time PCR positive results, FN was successfully treated by empirical therapy (Table 4). However 11 cases of blood culture or real-time PCR positive results, fever continued after the administration of empirical therapy more than 48 hours, they were quickly switched to other antimicrobial therapy, then they were successfully treated with the following antibiotic treatment.

A LAMP assay and a real-time PCR assay for the detection of human herpes virus type1 to 7

Viruses were detected in 6 of 76 FNEs (7.9%) by LAMP methods (Table 5).

EBV was detected with No.15 patient and No.18

Table 4. Clinical courses of patients with febrile neutropenia episodes

Patients	Blood culture	Real-time PCR and PCR, sequence	CRPmax (mg/dl)	antibiotics	Clinical course
7	Negative	*Enterococcus faecium*	29.66	MEPM+VCM	Improved with ET
11	*Streptococcus mitis*	*Streptococcus mitis*	17.52	CZOP+AMK	Registant to ET
12	Negative	*Streptococcus mitis*	17.52	DRPM+AMK	Improved with ET
14	Negative	*Acinetobacter junii*	21.44	TAZ/PIPC+AMK	Resistant to ET
18	*Klebsiella pneumoniae*	*Klebsiella sp*	6.17	BIPM	Resistant to ET
20	*Neisseria sp*	Negative	3.41	CFPM+AMK	Resistant to ET
21	Negative	*Acinetobacter sp*	16.47	CFPM+AMK	Improved with ET
22	Negative	*Bacillus sp*	5.88	CFPM+AMK	Resistant to ET
23	*Streptococcus mitis*	*Streptococcus mitis*	31.40	CFPM+AMK	Resistant to ET
24	*Streptococcus mitis*	*Streptococcus mitis*	20.85	CFPM+AMK	Resistant to ET
25	Negative	*Enterobacter sp*	3.84	CZOP+AMK	Improved with ET
26	Negative	*Enterobacter sp*	2.06	CFPM+AMK	Improved with ET
27	Negative	*Enterobacter sp*	2.06	CFPM+AMK	Improved with ET
35	*Streptococcus mitis*	Negative	15.54	CZOP+AMK	Resistant to ET
36	Negative	*Streptococcus gordonii*	6.63	CZOP+AMK	Improved with ET
37	*Streptococcus mitis*	*Streptococcus mitis*	11.25	CFPM+AMK	Resistant to ET
38	*Streptococcus mitis*	*Streptococcus mitis*	11.25	CFPM+AMK	Registant to ET
39	Negative	*Acinetobacter sp*	8.12	CFPM+AMK	Improved with ET
40	Negative	*Acinetobacter sp*	8.12	CFPM+AMK	Improved with ET
46	*Streptococcus mitis*	Negative	17.50	CZOP+AMK	Resistant to ET
48	Negative	*E. Coli*	3.62	CFPM+AMK	Improved with ET
63	Negative	*Staphylococcus epidermidis*	3.62	CFPM+AMK	Improved with ET

MEPM : meropenem, VCM : vancomycin, CZOP : cefozopran, AMK : amikacin, DRPM : doripenem, TAZ/PIPC : tazobactam/piperacillin, BIPM : biapenem, CFPM : cefepime, ET : empirical therapy.

Table 5. Results of viral molecular diagnosis results in patients with FNEs

sample			LAMP	Real-time PCR
Number	Age	diagnosis		
15	43y	NK/T cell Lymphoma	EBV	EBV 20.2
18	43y	NK/T cell Lymphoma	EBV	EBV 161.0
31	11y7m	ALL	HHV6	HHV6 859.0
32	11y7m	ALL	HHV6	HHV6 530.0
61	12y1m	ALL	HHV6	HHV6 2.6×10^4
62	12y1m	ALL	HHV6	HHV6 2.7×10^4

patient. The quantity of EBV of the samples was 20.2 copy/μl in No.15 patient, and 161copy/μl in No.18 patient by the real-time PCR assay, respectively. The samples were collected in No.15 patient and No.18 patient from the same patient whose clinical diagnosis was NK/T cell Lymphoma in different time, and No.18 patient accompanied a catheter-related infection caused by *Klebsiella pneumoniae*.

HHV6 was detected with No.31, 32, 61 and 62. These 4 cases were collected from the same patients. No.31 and No.32 were collected in the same time. Similarly, No.61 and No.62 were sampled in same time. The patient was a 11-years-old 7 months girl with acute lymphoblastic leukemia (ALL), at the nadir period of consolidation chemotherapy (No.31, 32) and maintenance chemotherapy (No.61, 62). She was extra high risk ALL, but she did not undergo hematopoietic stem cell transplantation and was treated with only chemotherapy.

DISCUSSION

Blood culture currently remains the gold standard in microbiological diagnosis of FN, although it becomes positive 8-36 hours after sampling[21]. It took an average of 9 hours 59 minutes in our study. Furthermore, it took another 24 hours more so that causative bacteria and fungi were identified. We used empirical antibiotics in consideration of causative bacteria or fungi predicted for FNEs. However it takes much time for the identification of causative bacteria by blood culture, so we cannot give appropriate and specific antimicrobial therapy quickly. In our study, it took an average of 1 hour 30 minutes for a real-time PCR assay and another average of 6 hours 30 minutes for the sequence. The real-time PCR was able to identify causative bacteria and fungi more quickly than blood culture.

The real-time PCR assay for detection of causative microbes has been proving to be sensitive and specific with the additional advantage of rapid results. However general use of a real-time PCR assay in clinical practice remains limited due to the lack of standardization and it's high cost[22]. Real-time PCR techniques have a general limitation in being too sensitive. A broad-range PCR assay is more vulnerable due to contamination than a species-specific PCR assay[3]. Contamination from environment, labwares or enzymes sometimes may happen to occur. There was no cut-off threshold (Ct) -value to distinguish real infections from baseline level of contamination DNA in negative sample[2].

We postulated that a copy number helped the determination of real clinical infection from contamination. However we could not find a good correlation between bacterial copy number and serum CRP titer (Fig.2). Rastogi R *et al*. reported many organisms have a single rRNA operon and the actual number is known to vary between 1 and 15[22,23]. Because various species of bacteria were detected in this study, it might be hard to conclude a correlation between the number of copy and disease severity. In an adult population, Schabereiter-Gurtner CS *et al*.[2] reported an increase in positivity from 16.9% (23/136) with blood culture to 24.3% (33/136) with a broad-range real-time PCR assay. In children, Nakamura A *et al*.[1] detected bacteria in 3 cases (13%) by blood culture and in 11 cases (48%) by PCR in 23 FNEs, Santolaya ME *et al*.[22] increased positivity from 16.3% (29/177) by blood culture to 20% (36/177) by a real-time PCR assay in FN children with cancer.

The present study showed that we significantly improved the detection rate from 11.8% by blood culture to 25.0% by a real-time PCR assay in patients with FNEs.

On the other hand, a sensitivity of the real-time PCR assay among blood culture positive cases was only 67%. In 3 blood culture-positive cases (No.20, 35, 46), the real-time PCR assay could not detect any bacteria. We tried the detection of bacteria for

these 3 cases by increase of DNA quantity in the template, however any bacteria were not detected. It may be considered that quantity of bacteria was lower than the detection limit of the real-time PCR assay in these cases. Santolaya et al also reported the low sensitivity of the PCR assay as 56%, which is similar to the present study.

Lilienfeld-Toal M *et al*.[24] reported an increase in positivity from 3% with blood culture to 15% with a multiplex real-time PCR assay in 119 FNEs episodes of adult patients during antibiotic therapy. In this study, two patients (No.7, 12) were detected causative bacteria during the preventive antibiotic therapy. It was thought that a real-time PCR assay was a useful and strong tool for the pathogen detection during antibiotic therapy.

In this study, fungi were not detected in 76 FNEs by blood culture and the real-time PCR assay, although several reports have shown a real-time PCR assay was useful for early detection of fungi[6-9]. However fungi are rarely detected, because antifungal therapy is often given preventively. No detection of fungi in this study may be due to antifungal prophylaxis with micafungin or fluconazole in all cases during ANC of <500 cells/ mm^3.

In this study, viruses were detected in 6 of 76 FNE (7.9%) by the LAMP methods and real-time PCR. EBV was detected with No.15 and 18 in the same patient, but NK/T cell lymphoma was known to be related to EBV infection. EBV detected in No.15 and 18 might be due to primary disease and not be due to FNEs. HHV6 was detected in No.31, 32, 61 and 62 from the same ALL girl, because she was at extra high risk group of ALL and, the intensity of chemotherapy was very high. Therefore, her immunosuppression was very strong, and it may be considered that she was infected with a reactivation of HHV6. In this study, the specimens from patients undergoing hematopoietic stem cell transplantation (HSCT) was only two. If the specimens from HSCT patients increase, the rate of detection of virus may rise. Because cell-mediated immune deficiency occur by HSCT, T cell depletion increases the risk of viral reactivation and virus-related disease[25].

We conclude a real-time PCR assay could give its higher detection rate, and shorter turnaround time, and the smaller volume of blood sample than blood culture. A real-time PCR assay along with blood culture improve microbiological documentation in FNEs. We may choose early optimal antibiotics to patients with FNEs by a real-time PCR assay.

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