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Diagnostic Value of a "Wide-Range" Quantitative Nested Real-Time PCR Assay for Varicella Zoster Virus Myelitis

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Myelitis is one of the rarest neurological complications of varicella zoster virus (VZV) infection. In this study, the authors remodeled the "wide-range" quantitative nested real-time (QNRT) polymerase chain reaction (PCR) assay to quantitatively detect a small amount of VZV-DNA in cerebrospinal fluid (CSF). For use as a specific internal control "calibrator," an original mutation-VZV (MZ) plasmid was developed. The initial copy number of VZV-DNA in CSF specimens was measured by the amplification rate of the MZ-plasmid. For 17 consecutive CSF specimens collected from three elderly patients with VZV myelitis, the diagnostic value of the wide-range QNRT-PCR assay was evaluated and compared with other conventional PCR assays and enzyme immunoassay (EIA). The MZ-plasmid demonstrated statistically uniform amplifications ($F = 1.016$) against a wide range (1–100,000) of copy numbers of mimic VZV-DNA. The wide-range QNRT-PCR assay quantitatively and rapidly (within 48 hr) detected 5,863, 3,052, 958, and 6,721 copies/ml of VZV-DNA in the CSF specimens collected from all patients in the acute phase. Additionally, there was a significant difference ($^*P = 0.023$) in the copy number of VZV-DNA between before and after acyclovir treatment. Other conventional single PCR assays all revealed negative results, but were nevertheless time-consuming (7 days). The IgG EIA-value for VZV was continually elevated throughout the clinical course of all patients. The MZ-plasmid was thus regarded as an appropriate "calibrator" in the wide-range QNRT-PCR assay. This assay is a novel, rapid, accurate, quantitative, and highly sensitive technique, and will contribute as a reliable and useful clinical examination for the rapid diagnosis of VZV infection to central nervous system.

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KEY WORDS: varicella zoster virus (VZV); myelitis; cerebrospinal fluid (CSF); wide-range quantitative nested real-time (QNRT) PCR assay; internal control

INTRODUCTION

Myelitis is one of the rarest neurological complications of infection with varicella zoster virus (VZV) [Hogan and Krigman, 1973; Devinsky et al., 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. Focal muscle weakness, that is zoster paresis, with or without sensory disturbance, occurs in approximately 5% of cases after acute VZV infection, with complete recovery occurring in 50% to 70%, and approximately 15% without improvement [Hogan and Krigman, 1973; Devinsky et al., 1991; Gilden et al., 1994, 1998;

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de Silva et al., 1996]. At present, cerebrospinal fluid (CSF) analysis is a key tool in the diagnosis of central nervous system (CNS) infection with VZV [Devinsky et al., 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. The detection of intrathecal synthesis of anti-VZV-specific antibodies by EIA and the amplification of VZV-DNA in the CSF by the polymerase chain reaction (PCR) are the most reliable ways of establishing a diagnosis of VZV infection in the CNS [Devinsky et al., 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b].

In 2008, an internally controlled novel "wide-range" quantitative nested real-time (QNRT) PCR assay was developed for *Mycobacterium tuberculosis* DNA for the rapid diagnosis of CNS tuberculosis [Takahashi et al., 2008a, b]. This assay technique combines the high sensitivity of nested PCR with the accurate quantification of real-time (TaqMan®, Applied Biosystems, Foster City, CA) PCR [Takahashi et al., 2008a, b]. In this study, an attempt was made to remodel the wide-range QNRT-PCR assay for the quantitative detection of a small amount of VZV-DNA in a CSF specimen, and the clinical usefulness of this assay was examined in three elderly patients with VZV myelitis. Additionally, the diagnostic value of this assay is discussed comparing with other clinical tests such as EIA and conventional PCR.

METHODS

This study was approved by the Institutional Review Board of our hospital. Additionally, written informed consent was obtained from all patients.

Enzyme Immunoassay

Specific IgG and IgM antibodies to various viruses including VZV, herpes simplex virus type-1 (HSV-1) and cytomegalovirus (CMV) in clinical specimens (serum and CSF) were quantitatively detected by EIA [Hogan and Krigman, 1973; Devinsky et al., 1991; Reiber and Lange, 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Takasu et al., 2003; Haug et al., 2010; Inukai et al., 2010]. The respective EIA values were determined according to a previously reported protocol (see Supplementary Method for the details) [Reiber and Lange, 1991; Takasu et al., 2003], with the analysis outsourced to a major commercial laboratory (SRL, Inc., Tokyo, Japan).

Evaluation of the antibody index provides evidence of the intrathecal synthesis of anti-virus antibodies [Reiber and Lange, 1991; Takasu et al., 2003; Inukai et al., 2010]. The antibody index is expressed as follows: (CSF EIA value/serum EIA value)/(CSF IgG (mg/dl)/serum IgG (mg/dl) [Reiber and Lange, 1991; Takasu et al., 2003; Inukai et al., 2010]. A value greater than 1.5 or 2.0 suggests antibody production in the CNS [Reiber and Lange, 1991; Takasu et al., 2003; Inukai et al., 2010].

PCR Assays

Conventional clinical single PCR and real-time-PCR assays for three types of viral DNA. In this study, all of the conventional clinical single PCR and real-time-PCR assays for VZV, HSV-1, and CMV DNA in CSF specimens were outsourced to a major commercial laboratory (SRL, Inc.).

Extraction and purification of DNA from CSF specimens. A 500-μl aliquot of original cytolysis buffer containing 20 mM Tris–HCl (pH8.0), 300 mM NaCl, 0.8% (v/v) sodium dodecyl sulfate (SDS) and 0.5 mg of proteinase-K was prepared. This lysis buffer was added to 500 μl of the CSF sample, followed by incubation in a waterbath at 65°C overnight. After incubation, a total suspension of 1,000 μl was divided into two 500-μl aliquots for use in original PCR assays.

The DNA specimens including viral DNA were extracted and purified from the 500-μl aliquots by the previously reported conventional phenol–chloroform method and ethanol precipitation [Liu et al., 1994; Scarpellini et al., 1995; Takahashi and Nakayama, 2006; Takahashi et al., 2005, 2007, 2008a, b]. For efficient extraction of a small amount of viral DNA, a high-molecular-weight carrier, Ethachinmate® (Nippon Gene, Tokyo, Japan), as a coprecipitating agent for the nucleotides in the ethanol precipitation, was used. After complete vacuum desiccation, the extracted DNA specimen was resuspended in 20 μl of pure water, and then stored at −20°C until use.

Assay condition of conventional nested PCR for three types of viral DNA. For use in the two consecutive PCR amplification steps of the nested PCR assay, six pairs of outer forward (F1) and reverse (R1) primers as well as inner forward (F2) and reverse (R2) primers for each of the three different viruses (VZV, HSV-1, and CMV) were prepared [Echevarría et al., 1994; Read and Kurtz, 1999; Johnson et al., 2000; Markoulatos et al., 2001; Gregoire et al., 2006; Franzen-Röhl et al., 2007]. The sequences of these primers for three types of viral DNA are shown in Table IA. In the first-step (single) PCR, 2 μl of the extracted DNA specimen as a template was amplified using each of the outer primers, F1 and R1. In the second-step (nested) PCR, 2 μl of the single PCR product as a template was amplified using each of the inner primers, F2 and R2. All of the PCR procedures were subjected to the protocols shown in Table IB, using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The PCR products were analyzed using Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany).

Wide-range QNRT-PCR assay for VZV-DNA. In the wide-range QNRT-PCR assay, two types of

TABLE IA. Sequences of Primers and TaqMan® Probes for PCR Assays

Assay		Target	Type		Sequence	Nucleotide Position	Product Size
Conventional nested PCR	First-step PCR	Glycoprotein D gene of wild HSV1-DNA[§]	Outer primers	HSV1-F1	5′-CCATACCGACCACACCGACGA-3′	55–75	189
				HSV1-R1	5′-CATACCGGAACGCACCACACA-3′	243–223	
	Second-step PCR		Inner primers	HSV1-F2	5′-TTACGACCAGGAGGGGTATAA-3′	99–119	92
				HSV1-R2	5′-TAGTTGGTCGTTCGCGCTGAA-3′	190–170	
	First-step PCR	IRL-11 (=HCMVTRL-11) gene of wild CMV-DNA[‡]	Outer primers	CMV-F1	5′-GTACACGCACGCTGGTTACCAG-3′	8,868–8,889	185
				CMV-R1	5′-GTACAGCGTCAGATTTTGGCGC-3′	9,052–9,031	
	Second-step PCR		Inner primers	CMV-F2	5′-CGTAGTCTGGCAGCGTTATGAT-3′	8,911–8,932	92
				CMV-R2	5′-GCGTGGTTTCTTTATAGGCGTC-3′	9,002–8,981	
Conventional nested PCR and wide-range QNRT-PCR	First-step PCR	ORF-29 of wild VZV-DNA or WZ-plasmid[¶]	Outer forward primer	WZ-F1	5′-GTCCTAGAGGAGGTTTTATCTGC-3′	53,942–53,964	172
			Outer reverse primer	WZ-R1	Total 23-mer, A4, T8, G7, C4 (GC% 48) 5′-AATGCCATATGTTGTGCTCGAGC-3′	54,113–54,091	
Wide-range QNRT-PCR		Specific internal control “calibrator” = MZ-plasmid	Outer forward primer	MZ-F1	Total 23-mer, A5, T7, G6, C5 (GC% 48) 5′-CGTCTATCGAGTGCTAGAGTGTT-3′		172
			Outer reverse primer	MZ-R1	Total 23-mer, A4, T8, G7, C4 (GC% 48) 5′-TCAGCATATTCCTGCGAGTGATG-3′		
Conventional nested PCR and wide-range QNRT-PCR	Second-step PCR	ORF-29 of wild VZV-DNA or WZ-plasmid	Inner forward primer	WZ-F2	Total 23-mer, A5, T7, G6, C5 (GC% 48) 5′-TCCCTCGTCGGGAAATCGAGAAA-3′	53,976–53,998	88
			Inner reverse primer	WZ-R2	Total 23-mer, A7, T4, G6, C6 (GC% 52) 5′-TATGGCTAATTGAACCATGGCCC-3′	54,063–54,041	
			TaqMan® probe	WZ-VIC	Total 23-mer, A6, T6, G5, C6 (GC% 48) 5′-[VIC]-CGACCAAGTTCGCGGTATAATTG-[TAMURA]-3′		
Wide-range QNRT-PCR		Specific internal control “calibrator” = MZ-plasmid	Inner forward primer	MZ-F2	Total 23-mer, A6, T6, G6, C5 (GC% 48) 5′-CTAGCTCAGGATCAGCAGATGAC-3′	54,009–54,031	88
			Inner reverse primer	MZ-R2	Total 23-mer, A7, T4, G6, C6 (GC% 52) 5′-GGCATCATGTGAGTACTCACCAT-3′		
			TaqMan® probe	MZ-FAM	Total 23-mer, A6, T6, G5, C6 (GC% 48) 5′-[FAM]-AAGTCGACCTCTAGCATTGTGGA-[TAMURA]-3′		
					Total 23-mer, A6, T6, G6, C5 (GC% 48)		

GenBank accession no. [§]NC_001806.1 (J02217), [‡]NC_006273.2 (X17403), [¶]NC_001348.1 (X04370).

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TABLE IB. Conventional Nested PCR Assay Conditions

First-Step PCR		Second-Step PCR	
Initial denaturing	96.0°C: 3 min	Initial denaturing	96.0°C: 3 min
Amplification	35 cycles	Amplification	25 cycles
Denaturing	95.0°C: 30 sec	Denaturing	94.0°C: 30 sec
Annealing	60.0°C: 30 sec	Annealing	55.0°C: 30 sec
Extension	72.0°C: 1 min	Extension	72.0°C: 1 min
Final extension	72.0°C: 10 min	Final extension	72.0°C: 10 min

original plasmid, "wild VZV" (WZ) and "mutation VZV" (MZ), were prepared for the accurate quantitative detection of VZV-DNA, as in previous reports [Takahashi et al., 2008a, b]. The original WZ-plasmid, into which was inserted a 172 base-pair (bp) DNA fragment of the ORF-29 gene of VZV (GenBank Accession No. NC_001348.1 [X04370]) as a vector, was synthesized and purchased from Operon Biotechnologies (Tokyo, Japan) for use as a standard template or a positive control (Fig. 1A). The original MZ-plasmid was designed on the basis of the WZ-plasmid for use as a specific internal control "calibrator" (Fig. 1A). In the MZ-plasmid, a total of five regions, at which the two pairs of outer and inner primers and the TaqMan® probe annealed, were replaced by artificial random nucleotides (Fig. 1A). The sequences of the artificial random nucleotides were set to have the same nucleotide composition as the ORF-29 gene of VZV. The MZ-plasmid was also synthesized by and purchased from Operon Biotechnologies. As in previous reports [Takahashi et al., 2008a, b], 1,000 copies of the MZ-plasmid were adopted as an optimal internal control "calibrator," and added to the CSF specimens before the above-described procedure of extraction and purification for DNA.

For use in the wide-range QNRT-PCR assay, four pairs of primers and two types of TaqMan® probe were prepared, as in previous reports [Takahashi et al., 2008a, b]. The sequences and positions of these primers and probes are shown in Table IA and Figure 1A. These primers and probes were set to have the same nucleotide composition, but different and random sequences. Therefore, the annealing efficiencies of these primers and probes to the wild ORF-29 gene or MZ-plasmid as a template can be regarded as the same. The wide-range QNRT-PCR assay consists of two consecutive PCR amplification steps: conventional PCR as the first step and real-time (TaqMan®) PCR as the second step using the Applied Biosystems 7300 Real-Time PCR system (Foster City, CA). In the first-step PCR, 2 μl of the extracted DNA specimen including "calibrator" MZ-plasmid as a template was amplified by using the outer primers WZ-F1 and WZ-R1 or MZ-F1 and MZ-R1 (Table IA), at 25 amplification cycles. In the second-step PCR, 2 μl of the first-step PCR product was used as a template. The wild ORF-29 gene fragment or WZ-plasmid was amplified by the inner primers WZ-F2 and WZ-R2, and detected by specific TaqMan® probe WZ-VIC (Table IA). The MZ-plasmid was amplified using the inner primers MZ-F2 and MZ-R2, and detected by the specific TaqMan® probe MZ-FAM (Table IA). All of the PCR procedures followed the protocols shown in Table IC.

The initial copy number of VZV-DNA in the CSF specimen was calculated by the amplification ratio relative to 1,000 copies of MZ-plasmid as a "calibrator" using the following equation, as in previous reports [Takahashi et al., 2008a, b].

$$X:W = C:M,\ \therefore X = W \times C/M \quad (1)$$

Here, X is the unknown initial copy number of VZV-DNA per 1 ml of CSF sample, C is the initial copy number of the "calibrator" (=1,000 copies of MZ-plasmid), and W and M are the copy numbers of VZV-DNA and MZ-plasmid, respectively, after passing through the extraction and PCR amplification procedures.

In the WZ genome, it is universally accepted that a single copy of the ORF-29 gene represents a viral particle [Stallings and Silverstein, 2005; Franzen-Röhl et al., 2007]. Therefore, it can be considered that the copy numbers calculated by wide-range QNRT-PCR assay correspond to the viral particle numbers of VZV in the CSF specimen.

Statistical Analysis

The statistical analysis was conducted using the data analysis software program SPSS 13.0 for Windows. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Reliability of Wide-Range QNRT-PCR Assay

The precision of a specific standard curve is one of the most important factors for the accurate quantitative detection of target DNA [Takahashi et al., 2007, 2008a, b]. In the wide-range QNRT-PCR assay, two specific standard curves for quantitative detection of VZV-DNA and the MZ-plasmid are needed, as described in previous reports [Takahashi et al., 2007, 2008a, b]. The two specific standard curves are shown in Figure 1B and C. In simple regression analysis, both of these standard curves demonstrated a

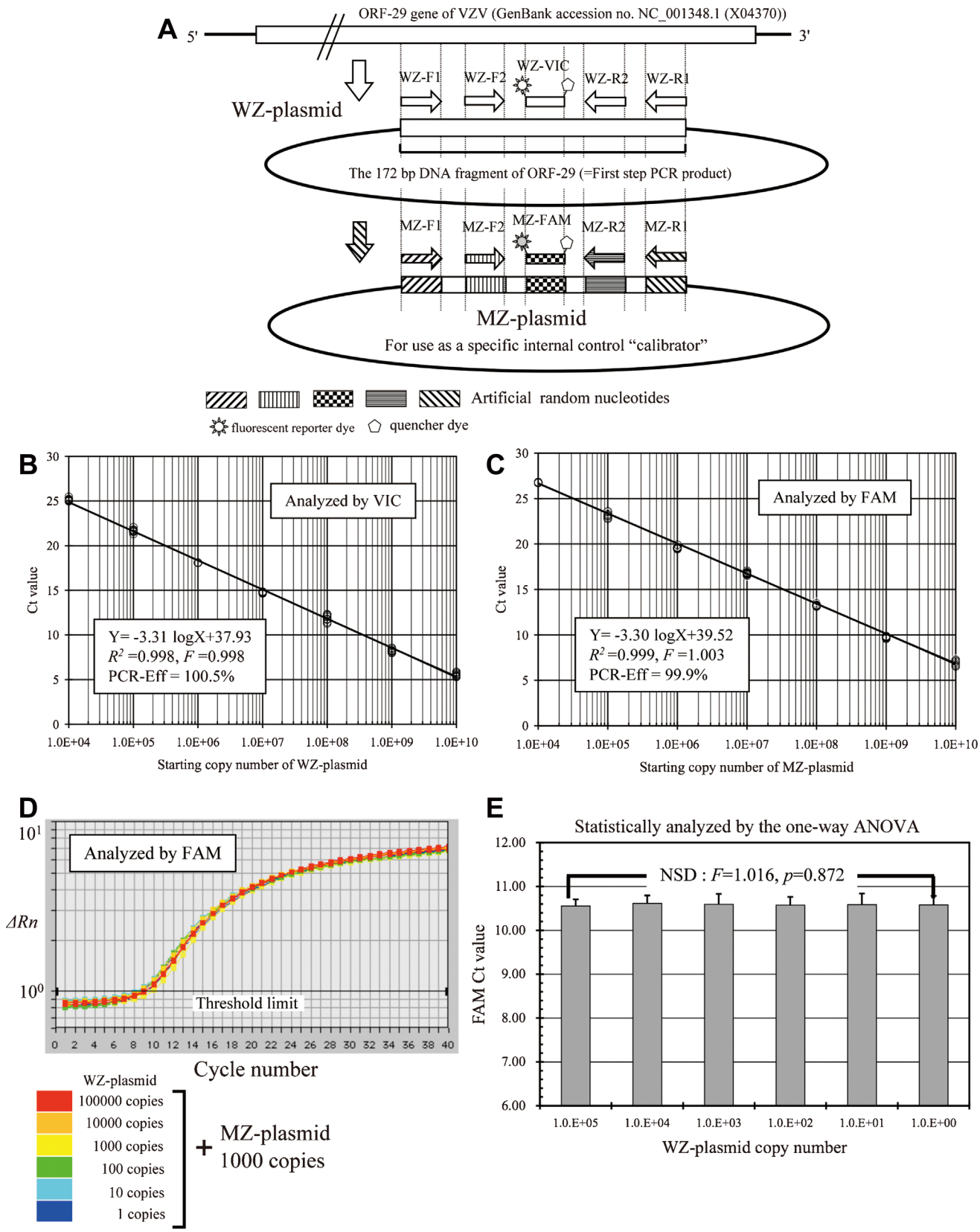
A
ORF-29 gene of VZV (GenBank accession no. NC_001348.1 (X04370))
5'
3'
WZ-plasmid
WZ-F1
WZ-F2
WZ-VIC
WZ-R2
WZ-R1
The 172 bp DNA fragment of ORF-29 (=First step PCR product)
MZ-F1
MZ-F2
MZ-FAM
MZ-R2
MZ-R1
MZ-plasmid
For use as a specific internal control "calibrator"
Artificial random nucleotides
fluorescent reporter dye
quencher dye
B
Analyzed by VIC
Ct value
Y= -3.31 logX+37.93
R² =0.998, F =0.998
PCR-Eff = 100.5%
Starting copy number of WZ-plasmid
C
Analyzed by FAM
Ct value
Y= -3.30 logX+39.52
R² =0.999, F =1.003
PCR-Eff = 99.9%
Starting copy number of MZ-plasmid
D
Analyzed by FAM
ΔRn
Threshold limit
Cycle number
WZ-plasmid
100000 copies
10000 copies
1000 copies
100 copies
10 copies
1 copies
+ MZ-plasmid 1000 copies
E
Statistically analyzed by the one-way ANOVA
NSD : F=1.016, p=0.872
FAM Ct value
WZ-plasmid copy number

Fig. 1.

TABLE IC. Wide-Range QNRT-PCR Assay Conditions

First-Step PCR		Second-Step PCR	
Initial denaturing	96.0°C: 3 min	Incubation	50.0°C: 2 min
Amplification	35 cycles	Initial denaturing	95.0°C: 10 min
Denaturing	95.0°C: 30 sec	Amplification	40 cycles
Annealing	60.0°C: 30 sec	Denaturing	95.0°C: 15 sec
Extension	72.0°C: 1 min	Annealing-extension	60.0°C: 1 min
Final extension	72.0°C: 10 min		

significant linear relationship ($R^2 > 0.99$) between the threshold cycle number (Ct value) (y-axis) and the log of the starting copy number for each standard template (x-axis). In both standard curves, no significant differences were found among the plots in repeated trials ($n = 10$; $F = 0.998$, $P = 0.73$ and $F = 1.003$, $P = 0.68$) by two-way ANOVA. The efficiency of real-time PCR (PCR-Eff) can be calculated by the slope of the standard curve using the following equation: $\text{PCR-Eff} = 10^{(-1/\text{slope})} - 1$ [Pfaffl et al., 2002]. The values of PCR-Eff calculated using this equation based on the slopes (−3.31 or −3.30) of two standard curves were 100.5% and 99.9%. Thus, both of the specific standard curves demonstrated statistically significant precision. Therefore, any overall errors relating to the dilution procedure or within each experiment can be disregarded among the plots of the two specific standard curves.

In wide-range QNRT-PCR assay, the reliability of a specific internal control "calibrator" is also the most important factor for the accurate quantitative detection of the target DNA [Takahashi et al., 2007, 2008a, b]. The Ct values of MZ-plasmids against five consecutive sets of 10-fold-diluted WZ-plasmids (1–100,000 copies) as a mimic template instead of actual VZV-DNA were strictly evaluated by statistical analysis, according to a previously reported preliminary trial protocol [Takahashi et al., 2007, 2008a, b]. When 1,000 copies of the MZ-plasmid were set as an optimal copy number of "calibrator," the amplification curves of the MZ-plasmid revealed extremely uniform patterns at all starting copy numbers (1–100,000) of WZ-plasmids as a mimic of VZV-DNA (Fig. 1D). The Ct values for 1,000 copies of the MZ-plasmid also revealed statistically significant uniform variance between all starting copy numbers of WZ-plasmids ($F = 1.016$, $P = 0.872$) by one-way ANOVA (Fig. 1E). Therefore, 1,000 copies of MZ-plasmid were adopted as the reliable and optimal copy number of "calibrator."

Clinical Features of the Patients

The clinical features of the three patients are summarized in Table II and Figure 3. The patients consisted of a woman and two men with unremarkable medical and familial histories, and were all elderly and HIV-negative. Patients 1 and 2 presented with focal skin rash of zoster with pain at disease onset and at the time of myelitis diagnosis, but patient 3 had no rash, namely, "zoster sine herpete." In the neurological examinations upon admission, all three patients demonstrated various degrees of muscle weakness of their lower limbs, whereas sensory disturbance was found only in patient 1. Additionally, urinary retention was shown in patients 2 and 3. Spinal cord MRI showed various degrees of high-signal-intensity lesions on T_2-weighted images without gadolinium enhancement and on T_1-weighted images in all three patients (Fig. 2A–F). The initial routine CSF studies showed moderate lymphocytic pleocytosis, elevation of protein and a negative result for bacterial culture in all patients (Table II). These authors diagnosed patients 1 and 3 with myelitis, and patient 2 with myelitis and radiculopathy caused by VZV infection on the basis of the clinical findings, including PCR assays and EIA results (described below in detail). All three patients were treated with acyclovir at 10 mg/kg intravenously three times daily for 2 weeks, and additionally treated with various doses of corticosteroids. In parallel with these drug treatments, rehabilitation was performed. The treatment and rehabilitation were generally successful, and the neurological symptoms and CSF findings gradually improved. Although all patients exhibited sequelae of various degrees of severity, they almost

Fig. 1. The principle of wide-range quantitative nested real-time (QNRT) PCR assay. **A**: A schema of the position of primers and probes in the ORF-29 gene of VZV, and the construction of "wild VZV" (WZ) and "mutation VZV" (MZ) plasmids. The MZ-plasmid was developed for use as a specific internal control "calibrator." **B**: The specific standard curve for use in the quantitative detection of VZV-DNA or WZ-plasmid. VIC (TaqMan® probe WZ-VIC) was used for analysis. **C**: The specific standard curve for use in the quantitative detection of the MZ-plasmid as a specific internal control "calibrator." FAM (TaqMan® probe MZ-FAM) was used for analysis. **D**: Amplification curves for 1,000 (10^3) copies of the MZ-plasmids as a new internal control. **E**: One-way ANOVA against Ct values for 1,000 (10^3) copies of the MZ-plasmid.

TABLE II. Clinical Features of the Patients on Admission

Case	Patient 1	Patient 2	Patient 3	
Admission date	March 11, 2010	April 13, 2011	January 13, 2012	
Age/sex	79/female	73/male	84/male	
Immunosetting/HIV	Normal/–	Normal/—	Normal/—	
General findings				
Temperature	37.6°C	36.8°C	36.2°C	
Skin lesion (focal rash) distribution	+ Sacral 2–3 region of gluteus	+ Dorsal thoracic 5–8 region	—	
Neurological findings				
Motor system	Moderate left dominant paraplegia mild right-hand weakness	Moderate flaccid right leg weakness gait disturbance	Severe left dominant paraplegia gait disturbance	
Reflexes	Bilateral hyperreflexia of lower limbs	Hyperreflexia of right leg slight right Babinsky sign	Bilateral hyperreflexia of lower limbs marked left Babinski sign	
Sensory system	Numbness with decreasing sensation in left arm and bilateral trunk and legs below thoracic −10	— (No sensory disturbance)	— (No sensory disturbance)	
Urinary retention	—	+	+	
Other symptoms	—	Severe anorexia	—	
Routine clinical CSF findings				
CSF collection date	March 11	April 13	January 13	January 16
RBC (/μl)	2	0	1	2
Cell (WBC) (/μl)	156	110	18	191
M:P	460:9	320:11	9:2	61:20
Protein (mg/dl)	155	93	108	204
Glucose (mg/dl)	51	51	46	62
Bacterial culture	—	—	—	—
CSF IgG (mg/dl)	34.0	22.5	21.5	37.5
Serum IgG (mg/dl)	1,115	1,855	1,467	2,031
Original PCR assays				
Nested PCR assays				
VZV-DNA	+	+	+	+
HSV1-DNA	—	—	—	—
CMV-DNA	—	—	—	—
Wide-range QNRT-PCR Assays: copy numbers of VZV DNA (copies/ml)	5,863	3,052	958	6,721
Reported date	Within 48 hr (March 12)	Within 48 hr (April 14)	Within 48 hr (January 17)	
Routine commercial clinical single-PCR assays (SRL, Inc.)				
VZV-DNA	—	—	—	—
HSV1-DNA	—	—	—	—
CMV-DNA	—	—	—	—
Reported date	7 days later (March 18)	7 days later (April 20)	7 days later (January 20)	7 days later (January 23)
IgM EIA value (negative < 0.8; 1.2 < positive)				
Anti-VZV				
CSF	0.51	0.24	0.47	0.36
Serum	1.26*	0.18	0.56	0.25
Anti-HSV-1				
CSF	0.62	0.30	0.37	0.24
Serum	0.71	0.46	0.41	0.33

Anti-CMV				
CSF	0.44	0.20	0.24	0.18
Serum	0.34	0.37	0.28	0.26
IgG EIA value (CSF: negative < 0.20, 0.40 < positive; serum: negative < 2.0, 4.0 < positive)				
Anti-VZV				
CSF	>12.8*	>12.8*	1.16*	6.73*
Serum	>128.0*	>128*	36.9*	68.4*
Antibody index	3.28†	8.24	2.14†	5.33†
Anti-HSV-1				
CSF	8.8*	7.3*	<0.20	<0.20
Serum	>128.0*	>128*	<2.0	<2.0
Antibody index	2.25†	4.70†	—	—
Anti-CMV				
CSF	2.5*	2.0*	<0.20	<0.20
Serum	84.5*	32*	<2.0	<2.0
Antibody index	0.97	5.15†	—	—
Reported date	7 days later (March 18)	7 days later (April 20)	7 days later (January 20)	7 days later (January 23)
Treatment				
Anti-viral drugs	Acyclovir 10 mg/kg intravenously three times daily for 2 weeks	Acyclovir 10 mg/kg intravenously three times daily for 2 weeks	Acyclovir 10 mg/kg intravenously three times daily for 2 weeks	
Additional use of corticosteroids	Methylprednisolone 500 mg intravenously daily for 3 days	Methylprednisolone 1,000 mg intravenously daily for 3 days + Dexamethasone 10 mg intravenously daily, tapering off by 2 mg every 4 days	Methylprednisolone 1,000 mg intravenously daily for 3 days + Prednisolone 50 mg orally daily, tapering off by 10 mg every 7 days	
Outcome	Recovery Discharge home	Recovery Discharge to rehabilitation hospital	Recovery Discharge to rehabilitation hospital	

CSF, cerebrospinal fluid; PCR, polymerase chain reaction; M, mononuclear cell; P, polymorphonuclear cell; EIA, enzyme immunoassay; *, positive EIA value; †, positive antibody index.

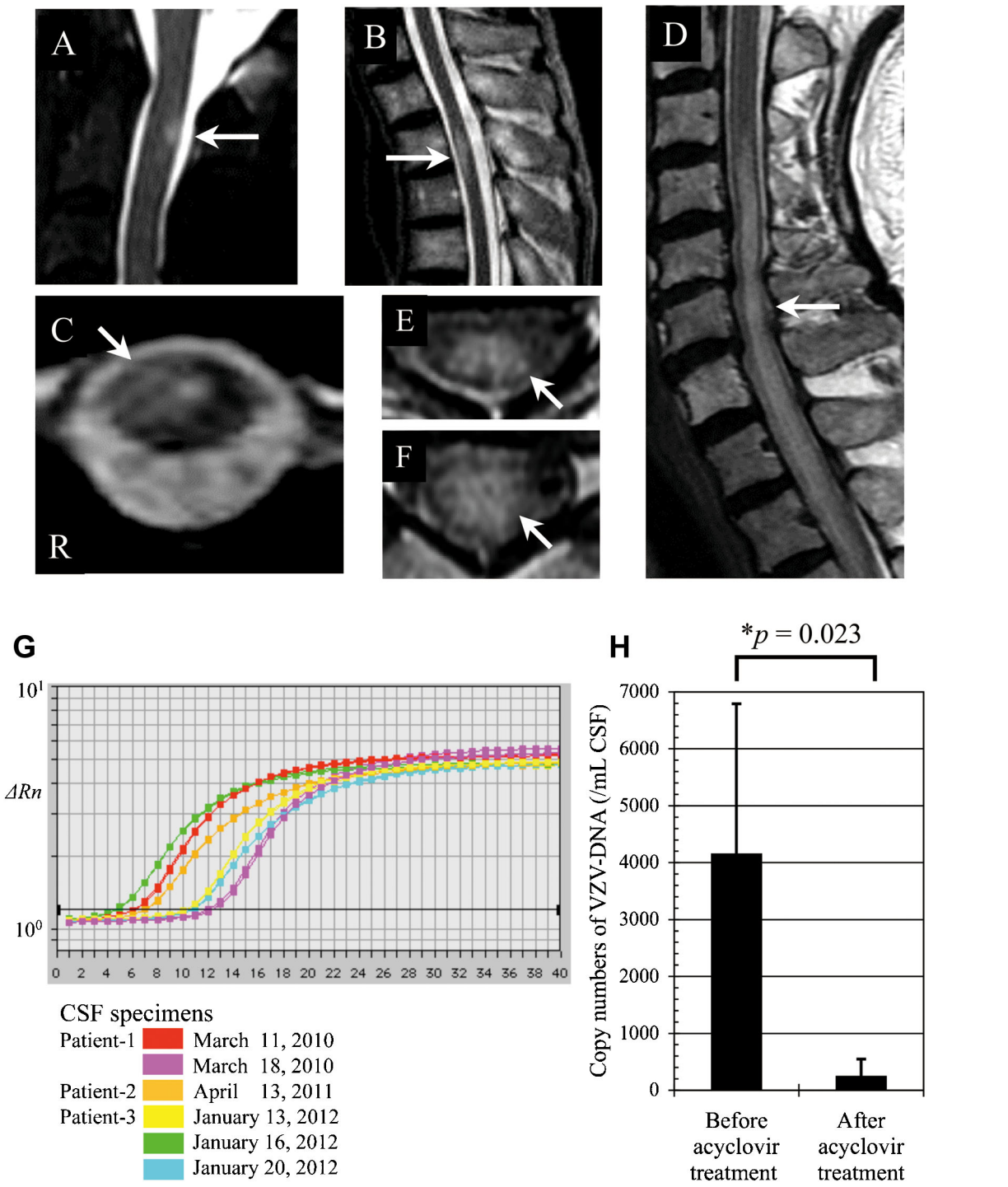

Fig. 2. The clinical data of the three patients. **A–F**: Spinal cord MRI findings. **A**: Sagittal T_2-weighted MRI (TR 3809, TE110) of cervical (**C**) spine in patient 1. A high-signal-intensity lesion at C-2 is shown. **B**: Sagittal T_2-weighted MRI (TR 3797, TE 110) of thoracic (Th) spine in patient 2. **C**: Axial T_2-weighted MRI (TR 539, TE 18) of Th spine in patient 2. A high-signal-intensity lesion on the right side of Th3-4 is shown. **D**: Sagittal T_2-weighted MRI (TR 3100, TE 26) of C-Th spine in patient 3. **E**: Axial T_2-weighted MRI (TR 3100, TE 26) of C-4 spine in patient 3. **F**: Axial T_2-weighted MRI (TR 3100, TE 26) of C-7 spine in patient 3. A diffuse high-signal-intensity lesion at C4-Th2 is shown. **G**: Amplification curves of VZV-DNA by the wide-range QNRT-PCR assay in the three patients (analyzed by VIC). **H**: The result of Student's *t*-test for the copy numbers of VZV-DNA between before and after acyclovir treatment.

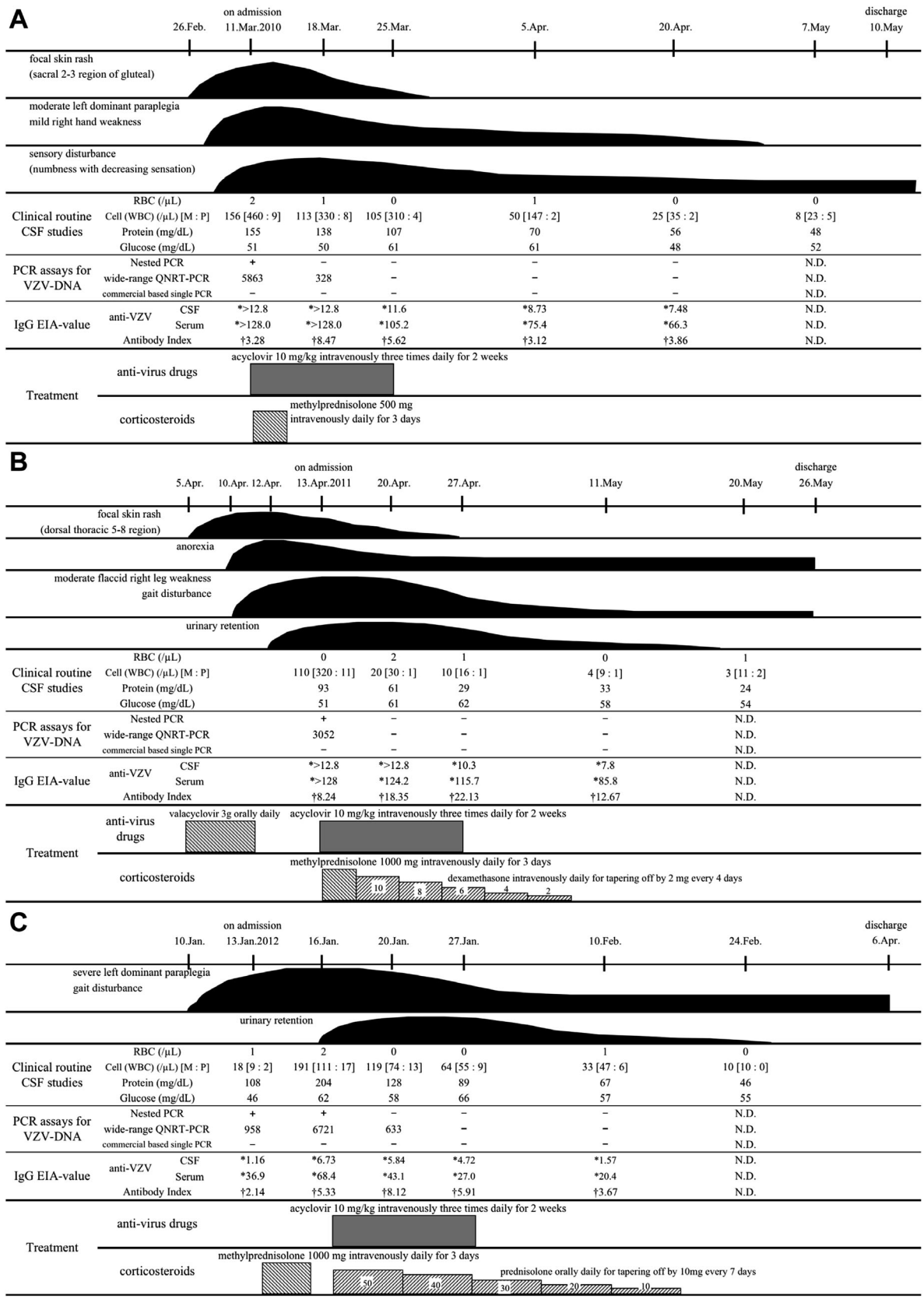
A
26.Feb.
on admission
11.Mar.2010
18.Mar.
25.Mar.
5.Apr.
20.Apr.
7.May
discharge
10.May
focal skin rash
(sacral 2-3 region of gluteal)
moderate left dominant paraplegia
mild right hand weakness
sensory disturbance
(numbness with decreasing sensation)
RBC (/μL) 2 1 0 1 0 0
Clinical routine CSF studies
Cell (WBC) (/μL) [M : P] 156 [460 : 9] 113 [330 : 8] 105 [310 : 4] 50 [147 : 2] 25 [35 : 2] 8 [23 : 5]
Protein (mg/dL) 155 138 107 70 56 48
Glucose (mg/dL) 51 50 61 61 48 52
PCR assays for VZV-DNA
Nested PCR + − − − − N.D.
wide-range QNRT-PCR 5863 328 − − − N.D.
commercial based single PCR − − − − − N.D.
IgG EIA-value
anti-VZV CSF *>12.8 *>12.8 *11.6 *8.73 *7.48 N.D.
Serum *>128.0 *>128.0 *105.2 *75.4 *66.3 N.D.
Antibody Index †3.28 †8.47 †5.62 †3.12 †3.86 N.D.
Treatment
anti-virus drugs
acyclovir 10 mg/kg intravenously three times daily for 2 weeks
corticosteroids
methylprednisolone 500 mg
intravenously daily for 3 days
B
5.Apr.
10.Apr.
12.Apr.
on admission
13.Apr.2011
20.Apr.
27.Apr.
11.May
20.May
discharge
26.May
focal skin rash
(dorsal thoracic 5-8 region)
anorexia
moderate flaccid right leg weakness
gait disturbance
urinary retention
RBC (/μL) 0 2 1 0 1
Clinical routine CSF studies
Cell (WBC) (/μL) [M : P] 110 [320 : 11] 20 [30 : 1] 10 [16 : 1] 4 [9 : 1] 3 [11 : 2]
Protein (mg/dL) 93 61 29 33 24
Glucose (mg/dL) 51 61 62 58 54
PCR assays for VZV-DNA
Nested PCR + − − − N.D.
wide-range QNRT-PCR 3052 − − − N.D.
commercial based single PCR − − − − N.D.
IgG EIA-value
anti-VZV CSF *>12.8 *>12.8 *10.3 *7.8 N.D.
Serum *>128 *124.2 *115.7 *85.8 N.D.
Antibody Index †8.24 †18.35 †22.13 †12.67 N.D.
Treatment
anti-virus drugs
valacyclovir 3g orally daily
acyclovir 10 mg/kg intravenously three times daily for 2 weeks
corticosteroids
methylprednisolone 1000 mg intravenously daily for 3 days
dexamethasone intravenously daily for tapering off by 2 mg every 4 days
10
8
6
4
2
C
10.Jan.
on admission
13.Jan.2012
16.Jan.
20.Jan.
27.Jan.
10.Feb.
24.Feb.
discharge
6.Apr.
severe left dominant paraplegia
gait disturbance
urinary retention
RBC (/μL) 1 2 0 0 1 0
Clinical routine CSF studies
Cell (WBC) (/μL) [M : P] 18 [9 : 2] 191 [111 : 17] 119 [74 : 13] 64 [55 : 9] 33 [47 : 6] 10 [10 : 0]
Protein (mg/dL) 108 204 128 89 67 46
Glucose (mg/dL) 46 62 58 66 57 55
PCR assays for VZV-DNA
Nested PCR + + − − − N.D.
wide-range QNRT-PCR 958 6721 633 − − N.D.
commercial based single PCR − − − − − N.D.
IgG EIA-value
anti-VZV CSF *1.16 *6.73 *5.84 *4.72 *1.57 N.D.
Serum *36.9 *68.4 *43.1 *27.0 *20.4 N.D.
Antibody Index †2.14 †5.33 †8.12 †5.91 †3.67 N.D.
Treatment
anti-virus drugs
acyclovir 10 mg/kg intravenously three times daily for 2 weeks
corticosteroids
methylprednisolone 1000 mg intravenously daily for 3 days
prednisolone orally daily for tapering off by 10mg every 7 days
50
40
30
20
10

Fig. 3.

recovered and were discharged home (patient 1) or to a rehabilitation hospital (patients 2 and 3).

PCR Assays and EIA Results

In this study, the 17 consecutive CSF specimens collected from three patients with VZV myelitis throughout their clinical courses were tested. The EIA and PCR results in the three patients are summarized in Table II and Figure 3. The original PCR assays, nested-PCR and the wide-range QNRT-PCR were all positive for VZV-DNA in the initial CSF specimens collected from the three patients during the acute phase of disease (before acyclovir treatment) (Figs. 2G and S1A–C). In addition, the wide-range QNRT-PCR assay detected quantitatively 5,863, 3,052, 958, and 6,721 copies/ml of VZV-DNA in the CSF specimens collected before acyclovir treatment. The copy numbers of VZV-DNA measured by the wide-range QNRT-PCR assay declined rapidly after starting acyclovir treatment. There was a significant difference ($^{*}P = 0.023$) in the copy numbers of VZV-DNA between before and after acyclovir treatment by Student's *t*-test (Fig. 2H). The positive

Fig. 3. The schema of the clinical course in three patients. CSF, cerebrospinal fluid; PCR, polymerase chain reaction; M, mononuclear cell; P, polymorphonuclear cell; EIA, enzyme immunoassay; *, positive EIA value; †, positive antibody index; N.D., not done. **A**: Patient 1: On February 26, 2010, a previously healthy 79-year-old woman developed mild fever and focal skin rash with pain in her gluteal region. Moreover, on March 9, she developed gradually progressive gait disturbance. Upon admission on March 11, her temperature was 37.6°C and focal rash of zoster was distributed in the sacral two to three gluteal region. Upon neurological examination, she demonstrated mild right-hand weakness, moderate left dominant paraplegia with bilateral hyperreflexia of the lower limbs, and numbness with a decreasing sensation in the left arm and the bilateral trunk and legs below thoracic (Th) 10. Both plantar responses were absent. Initial routine CSF studies showed lymphocytic pleocytosis (156 cells/μl) and elevation of protein (155 mg/dl). Original PCR assays (nested PCR and wide-range QNRT-PCR) revealed positive results for VZV-DNA in the initial CSF specimen. In particular, 5,863 copies/ml of CSF of VZV-DNA were quantitatively detected by the wide-range QNRT-PCR assay. Anti-VZV IgG EIA values were positive in serum (>128) and CSF (>12.8), and the IgG antibody index was also elevated (3.28), which suggested intrathecal synthesis of IgG antibody for VZV. The conventional commercial clinical PCR assays (single PCR and real-time PCR) all revealed negative results. After admission, she was diagnosed with VZV myelitis, and immediately treated with acyclovir at 10 mg/kg intravenously three times daily for 2 weeks and methylprednisolone at 500 mg intravenously daily for 3 days, in parallel with rehabilitation. The treatment and rehabilitation were successful, and her symptoms and routine CSF findings gradually improved. The copy number of VZV-DNA declined rapidly after the start of treatment, whereas the IgG EIA value and the antibody index for VZV were continually elevated throughout the course of clinical treatment. Although numbness in the distal parts of both lower limbs remained as a sequela, she recovered to the extent that she was able to walk without assistance. She was discharged home on May 10, 2010. **B**: Patient 2: On April 5, 2011, a 73-year-old man developed zoster rash on the right chest and dorsum, and was started on treatment with valacyclovir at 1 g orally three times daily. Despite this treatment, he developed severe anorexia, gait disturbance (on April 10) and urinary retention (on April 12), one after another. Upon admission on April 13, he was afebrile (36.8°C) and focal rash of zoster was distributed in the dorsal Th5-8 region. Upon neurological examination, he demonstrated moderate flaccid right-leg weakness with hyporeflexia, slight right Babinsky sign and severe difficulty urinating, but no sensory disturbance. Except for a surgical operation for gastric cancer at the age of 60, his medical and familial histories were unremarkable. Initial routine CSF studies showed lymphocytic pleocytosis (110 cells/μl) and elevation of protein (93 mg/dl). Original PCR assays revealed positive results for VZV-DNA in the initial CSF specimen. In particular, 3,052 copies/ml of CSF of VZV-DNA were quantitatively detected by the wide-range QNRT-PCR assay. Anti-VZV IgG EIA results were positive in serum (>128) and CSF (>12.8), and the IgG antibody index was also elevated (8.24). The commercial single PCR assays all revealed negative results. After admission, he was diagnosed with VZV myelitis and radiculopathy, and immediately treated with acyclovir at 10 mg/kg intravenously three times daily for 2 weeks and methylprednisolone at 1,000 mg intravenously daily for 3 days, and then dexamethasone at 10 mg intravenously daily, tapering off by 2 mg every 4 days, in parallel with rehabilitation. The treatment and rehabilitation succeeded, and his symptoms and routine CSF findings gradually improved. The copy numbers of VZV-DNA declined rapidly after the start of treatment, whereas the IgG EIA value and the antibody index for VZV were continually elevated throughout the course of clinical treatment. Although mild anorexia remained as a sequela, his urinary retention recovered completely, and he recovered to the extent that he could walk with a walker. He was discharged to a rehabilitation hospital on May 26, 2011. **C**: Patient 3: On January 10, 2012, a previously healthy 84-year-old man suddenly developed left-leg weakness. His muscle weakness deteriorated gradually and expanded to his right leg. It became difficult for him to walk, and he was hospitalized on January 13. Upon admission, he was afebrile (36.2°C), and had no dermatomal rash. Upon neurological examination, he demonstrated severe left dominant paraplegia with bilateral hyperreflexia of the lower limbs and marked left Babinski sign, but no sensory disturbance. The initial routine CSF studies showed slight lymphocytic pleocytosis (18 cells/μl) and elevation of protein (108 mg/dl). After admission, he was immediately treated with methylprednisolone at 1,000 mg intravenously daily for 3 days. Despite this treatment, he developed urinary retention on January 16. Second routine CSF studies on January 16 showed prominent lymphocytic pleocytosis (191 cells/μl) and elevation of protein (204 mg/dl). The original PCR assays revealed positive results for VZV-DNA in the initial and second CSF specimens. In particular, 958 and 6,721 copies/ml of CSF of VZV-DNA were quantitatively detected by the wide-range QNRT-PCR assay, respectively. Anti-VZV IgG EIA result were positive in serum (36.9 and 68.4) and CSF (1.16 and 6.73), and the IgG antibody index was also elevated (2.14 and 5.33) in the initial and second studies. The commercial single PCR assays all revealed negative results. From January 17, he was treated with acyclovir at 10 mg/kg intravenously three times daily for 2 weeks and prednisolone at 50 mg orally daily, tapering off by 10 mg every 7 days, in parallel with rehabilitation. The treatment and rehabilitation succeeded, and his symptoms and routine CSF findings gradually improved. The copy numbers of VZV-DNA declined rapidly after starting acyclovir, whereas the IgG EIA value and antibody index for VZV were continually elevated throughout the clinical treatment course. Although his urinary retention and right-leg weakness improved, severe left-leg weakness remained as a sequela, and thus he could not walk. He was discharged to a rehabilitation hospital on April 6, 2012.

results of wide-range QNRT-PCR assay during the subacute and chronic phases of disease (after acyclovir treatment) were 2 out of 13 serial CSF specimens (15.4%) collected throughout the course of the clinical treatment of the three patients. In contrast, the nested PCR assays were all negative for VZV-DNA in the 13 serial CSF specimens collected after starting acyclovir treatment, and for HSV-1 and CMV DNA in all 17 CSF specimens (data not shown). In addition, it was noteworthy that these original PCR assay results were obtained within 48 hr of the CSF collection.

The EIA values of IgG antibodies were positive for VZV in both serum and CSF specimens collected from all three patients. The EIA value of IgM antibody was only positive for VZV in the initial serum specimen of patient 1. The IgG antibody indexes for VZV were elevated in all three patients. These elevations suggested the intrathecal synthesis of IgG antibodies for VZV. In addition, the IgG EIA value and the IgG antibody index for VZV were continually elevated throughout the courses of clinical treatment in the three patients. In contrast, the conventional commercial clinical PCR assays, single PCR and real-time PCR, all revealed negative results for the three types of viral DNA (data not shown). Regrettably, these EIA and commercial clinical routine PCR assay results were from 7 days after the respective CSF collection.

DISCUSSION

Generally, the immune setting of a patient affects the clinical presentation of VZV-related neurological complications [de Silva et al., 1996; Gilden et al., 1998; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. In previous studies, VZV myelitis was found to occur frequently in immunocompromised patients without dermatomal rash, whereas disease is rare in immunocompetent patients who usually present with rash [de Silva et al., 1996; Gilden et al., 1998; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. In this study, the authors described three rare cases of elderly and HIV-negative patients with VZV myelitis. Two patients (patients 1 and 2) presented with dermatomal rash, but the other one (patient 3) was without rash. These three patients were probably at least transiently in an age-related immunodeficient state at disease onset.

Yet, the isolation or detection of VZV from CSF specimens is rare and difficult in cases of VZV infection of the CNS owing to the poor yield of VZV in the CSF [Devinsky et al., 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. In addition, virus presence closely depends on the timing of the CSF collection during the clinical course of CNS viral infection [Echevarría et al., 1994; Gregoire et al., 2006; Franzen-Röhl et al., 2007; Inukai et al., 2010]. However, the appropriate timing of CSF collection is very difficult because VZV infection of the CNS has various clinical courses: acute, subacute or chronic [Echevarría et al., 1994; Gilden et al., 1998; Gregoire et al., 2006; Franzen-Röhl et al., 2007; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. Previously, in CSF specimens collected during the acute phase within the first 7–10 days after the onset of a rash, the rate of VZV-DNA detection by the conventional single PCR assay was reported to be 61–76%, and the rate of anti-VZV IgG antibody elevation by EIA was reported to be 0–43% [Echevarría et al., 1994; Gregoire et al., 2006; Inukai et al., 2010]. In contrast, during the subacute phase, more than 1 week after onset, the rate of positivity by the single PCR assay was 25–47%, but the rate of anti-VZV IgG antibody elevation was 83–100% [Echevarría et al., 1994; Gregoire et al., 2006; Inukai et al., 2010]. Also, in the chronic phase, more than 20 days after onset, the former was 0% and the latter was 100% [Echevarría et al., 1994; Gregoire et al., 2006; Inukai et al., 2010]. Thus, the rates of VZV-DNA detection previously reported by the single PCR assay in CSF specimens tended to decline rapidly within 7–10 days after the onset of rash, whereas the rate of anti-VZV IgG antibody elevation tended to increase and then be maintained during the clinical course [Echevarría et al., 1994; Gregoire et al., 2006; Inukai et al., 2010].

In this study, the remodeled wide-range QNRT-PCR assay was developed for quantitative detection of VZV-DNA in CSF specimens. Therefore, two types of the original plasmid, WZ- and MZ-plasmids, have been newly prepared. In particular, for use as a specific internal control "calibrator," the MZ-plasmid was designed to have amplification and detection efficiency against actual VZV-DNA equivalent to those in previous reports [Takahashi et al., 2007, 2008a, b]. The data of Ct values in repeated trials demonstrated statistically significant precision and equivalence. Therefore, the remodeled wide-range QNRT-PCR for VZV-DNA is a reliable assay for clinical test. In this study, although the conventional commercial single PCR assays all yielded negative results, the original PCR assays, nested-PCR and the wide-range QNRT-PCR, could detect VZV-DNA in CSF specimens collected from all three patients before acyclovir treatment. The original PCR assays are thus regarded as much more sensitive than the conventional commercial single PCR assays. In previous studies, the sensitivity of the nested-PCR was reported to be 1,000–10,000 times greater than that of a conventional single PCR assay [Liu et al., 1994; Scarpellini et al., 1995; Takahashi et al., 2005]. In order to improve both the sensitivity and the specificity of the PCR assay, efficient procedures for the extraction and purification of a small amount of VZV-DNA and elimination of the amplification inhibitors, such as protein, in the CSF specimens are the most important factors [Liu et al., 1994; Scarpellini et al.,

1995; Takahashi et al., 2005]. In the present study, the CSF specimens were treated with original cytolysis buffer containing proteinase-K and SDS as a surface-active agent, and then VZV-DNA was purified by a phenol-chloroform method. Using these procedures on the CSF specimens, the cell components were almost completely dissolved and the proteins that interfered in the PCR amplification were also completely eliminated. Also, a small amount of VZV-DNA was extracted efficiently by using a high-molecular-weight carrier as a coprecipitating agent for the nucleotides together with the ethanol precipitation. In addition, the wide-range QNRT-PCR assay could detect the copy numbers of VZV-DNA in CSF specimens accurately, quantitatively and rapidly (within 48 hr of CSF collection). During the clinical course of the three patients, the copy numbers of VZV-DNA declined rapidly after the start of acyclovir treatment. A significant difference ($^{*}P = 0.023$) was observed in the copy numbers of VZV-DNA between before and after acyclovir treatment. It is considered that, initially, accurate and quantitative analysis by the wide-range QNRT-PCR assay may provide a significantly reliable foundation for appropriate clinical decisions, such as starting intravenous acyclovir treatment, changing the dose of acyclovir, and the additional use of corticosteroids and other anti-inflammatory or immune-modulatory adjunctive treatments, in patients with VZV infection of the CNS. In particular, the wide-range QNRT-PCR assay played a more definitive role for the diagnosis in the case of "zoster sine herpete" in patient 3 than in the cases with a skin rash. The positive results in the acute phase by wide-range QNRT-PCR assay are also beneficial for discrimination from other inflammatory diseases, such as autoimmune or allergic myelitis and radiculopathy. The rapidity of clinical laboratory test is often vitally important in clinical practice because a delay in making an appropriate clinical decision, including when to start intravenous acyclovir treatment, can have a serious impact on the prognosis of VZV infection of the CNS. Rapid results by the wide-range QNRT-PCR assay affected markedly the clinical management of the three patients with VZV myelitis in this study compared with the commercial EIA and conventional PCR assay results that were reported 7 days later. On the other hand, the results of the wide-range QNRT-PCR assay did not influence the duration of acyclovir treatment because it was considered that it was necessary to continue the standard acyclovir treatment for two weeks in order to eradicate VZV in the CNS, even if the wide-range QNRT-PCR assay results became negative rapidly.

At present, whether the etiology of VZV myelitis is directly and exclusively viral, secondary immunopathological, or both is still unknown. In previous studies, pathological and virological tests of progressive fetal VZV myelitis revealed that the disease is caused by direct viral invasion of the spinal cord [Hogan and Krigman, 1973; Devinsky et al., 1991; Gilden et al., 1994, 1998; de Silva et al., 1996; Tavazzi et al., 2008; Haug et al., 2010; Inukai et al., 2010; Corti et al., 2011a, b]. Tavazzi et al. [2008] reported a case of VZV-related meningio-encephalomyelitis without rash in an elderly and HIV-negative patient (85-year-old woman). In this case, 3.05×10^6 copies/ml of VZV-DNA were detected in a CSF specimen by conventional single-step real-time PCR assay during the acute phase of meningio-encephalomyelitis. The patient died of systemic complications of disseminated VZV infection [Tavazzi et al., 2008]. Histopathologically, this meningio-encephalo-myelitis was characterized by severe diffuse small vessel vasculopathy with inflammation, demyelination and necrosis caused by direct viral invasion with the concurrent involvement of meninges, brain and spinal cord [Tavazzi et al., 2008]. These histopathological findings indicated multifocal and severe CNS damage [Tavazzi et al., 2008]. In this study, the copy numbers of VZV-DNA in CSF specimens detected by the wide-range QNRT-PCR assay were all below 10,000 copies/ml in the acute phase of all three patients. In addition, the copy numbers of VZV-DNA rapidly decreased after the start of acyclovir treatment. These results suggest that the myelitis of these three patients was milder than in the patient with meningio-encephalo-myelitis reported by Tavazzi et al. [2008]. However, elevation in the value of intrathecal anti-VZV IgG EIA continued throughout the clinical course of treatment in three patients. This dissociation between EIA and PCR assay results may suggest that, despite the eradication of VZV, overactive immune responses against VZV continue in the CNS of the host. Fortunately, because all three patients recovered as various degrees as a result of treatment, there were no histopathological data based on autopsy material in this study. However, it is speculated that both direct VZV invasion to the spinal cord as a trigger and continuous overactive immune responses in the host may be closely related to the etiology of these three myelitis cases. The combined treatment with high-dose acyclovir and corticosteroid, for example, methylprednisolone, of these three patients was appropriate and effective for improvement of their outcomes.

In conclusion, the wide-range QNRT-PCR assay is a novel, rapid, accurate, specific, quantitative and highly sensitive assay technique, and is reliable and useful for the rapid diagnosis of VZV infection of the CNS, such as myelitis. In particular, the wide-range QNRT-PCR assay has been shown to be applicable for rapid and accurate diagnosis of difficult cases in which the conventional assay methods, such as commercial PCR assays, cannot detect VZV-DNA or can only do so after a delay. To our knowledge, there have been no previous studies that measured the copy numbers of VZV DNA in CSF specimens that could not be detected by conventional single-step real-time-PCR assay. Additionally, the versatility of

this assay technique for various targets has been evidenced in this study, as asserted by the authors in a previous report in 2008 [Takahashi et al., 2008a, b]. It is considered that, if the wide-range QNRT-PCR assay is adopted appropriately in clinical practice, it will contribute as a powerful tool to the rapid and accurate diagnosis of VZV infection to CNS.

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Authors' contribution: (1) Teruyuki Takahashi is the corresponding author, and was in charge of the three patients. (2) Masato Tamura is the president of Nagaoka-Nishi Hospital, and was in charge of the three patients. (3) Satoshi Kamei is a Professor of the Division of Neurology, Department of Medicine, Nihon University School of Medicine, and reviewed this manuscript. (4) Toshiaki Takasu is an Emeritus Professor of the Division of Neurology, Department of Medicine, Nihon University School of Medicine, and the Chief Consultant of Nagaoka-Nishi Hospital. He reviewed this manuscript.

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