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Original Article

Evaluation of a Quantitative Real-Time PCR Assay for the Detection of JC Polyomavirus DNA in Cerebrospinal Fluid without Nucleic Acid Extraction

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SUMMARY: The JC polyomavirus (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease. The current diagnostic standard for PML is real-time PCR testing of extracted DNA for assessing the presence of JCV DNA in cerebrospinal fluid (CSF). This study was aimed at evaluating the feasibility of a real-time PCR assay without nucleic acid extraction for the rapid quantification of JCV DNA in CSF. CSF samples were heat-treated or treated with DNAzol Direct, a commercially available reagent for direct PCR, and the performances of the real-time PCR assays using templates obtained by either treatment were compared with that using DNA extracts. JCV DNA was detected in the heat- or DNAzol Direct-treated samples containing only a few copies of the viral genome per reaction, and a linear relationship was noted between the copy number detected and the amount of input virus ascertained by the DNA extraction method. The sensitivities of the assays using the heat and DNAzol Direct treatments were 85.7 and 90.5%, respectively, with the results of the DNA extraction method being used as reference. These data demonstrate that the real-time PCR assay introduced in this study can serve as a rapid and cost-effective method of testing for JCV without DNA extraction and thereby facilitate the assessment of PML.

INTRODUCTION

The JC polyomavirus (JCV) is a small DNA virus belonging to the family *Polyomaviridae*, genus *Polyomavirus* (1). JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the central nervous system (2–4). Although PML is most commonly observed in patients with HIV infection, it is also observed in patients with immunodeficiency due to hematological malignancy, chemotherapy, transplantation, lymphocyte depletion, or autoimmune disorders such as systemic lupus erythematosus treated with immunosuppressive agents (5). In addition, PML has recently been diagnosed in patients receiving immunomodulatory therapies with monoclonal antibodies, such as natalizumab, rituximab, and efalizumab (3,4).

PCR detection of JCV DNA in cerebrospinal fluid (CSF) is a reliable and minimally invasive diagnostic marker of PML, particularly when combined with typical magnetic resonance imaging (MRI) findings (2,6). An increasing number of evidences suggest that the amount of JCV DNA in CSF is closely related to the severity of PML (7). Therefore, CSF testing for JCV DNA using a quantitative PCR technique is the current diagnostic standard (3). Several recent studies have shown that real-time PCR is useful for the rapid and specific quantification of JCV DNA in CSF specimens (8–16).

Real-time PCR testing for viral DNA in CSF is generally performed on DNA extracts prepared either by manual or automated methods using commercially available extraction kits (17), and most previous studies evaluating real-time PCR assays for JCV use DNA templates prepared from CSF samples in this manner (8–14,16). These extraction methods are suitable for the reproducible and accurate quantification of JCV DNA in CSF. However, manual extraction methods involve a number of time-consuming procedures with multiple manipulations that give rise to the possibility of sample-to-sample contamination, while automated extraction systems require special and expensive instruments (17).

The present study was undertaken to develop and evaluate a real-time PCR assay for the quantification of JCV DNA in CSF without the need for the DNA extraction process.

MATERIALS AND METHODS

CSF specimens: All CSF specimens were collected by lumbar puncture for diagnostic or therapeutic purposes from patients suspected of having PML on the basis of neurological symptoms and/or MRI findings. The CSF specimens were then transferred from the respective hospitals to the Department of Virology I, National Institute of Infectious Diseases (NIID), Tokyo, Japan, for real-time PCR testing for JCV DNA (18). The specimens were stored at −80°C until further analyses. The study protocol was approved by the Ethical Committee for Biomedical Science of NIID.

Standard DNA and virus preparation: The recom-

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binant plasmid pJC1-4-＞pJCV containing the complete genome sequence of JCV strain Mad-1 (19) was used as the standard DNA for the absolute quantification of the JCV genome by real-time PCR; the plasmid was supplied by the Health Science Research Resources Bank (Osaka, Japan). Cells of the human neuroblastoma JCI line, which have been previously shown to continuously produce JCV (20,21), were used to prepare the virus suspension (21).

Preparation of PCR templates: Total DNA was extracted from 200 μL of samples using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, Calif., USA) and eluted in 60 μL of AE buffer (Qiagen), according to the manufacturer's protocol. For real-time PCR assay without nucleic acid extraction, 40 μL of each sample was heat-treated at 98°C for 5 min, cleared in a low-speed centrifuge, and used as a PCR template. Alternatively, 20 μL of each sample was mixed with an equal volume of DNAzol Direct reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA), incubated at 98°C for 5 min, and directly subjected to real-time PCR. The manufacturer recommends that fluid specimens, such as serum and saliva, be mixed with a 10-fold volume of this reagent and incubated for 15 min at room temperature. However, preliminary pilot studies showed that the above-described condition was also the most suitable protocol for CSF specimens, with respect to the incubation time of samples and the detection limit of real-time PCR. All PCR templates were stored at −30°C until real-time PCR analyses.

Real-time PCR analysis: A pair of primers, P149 (5′-AAG TAC ATG CCC ATA AGC AA-3′) and P85 (5′-AGA CAG CCA TAT GCA GTA G-3′), and a TaqMan probe (5′-AAA CCT GCT TAG TTT CTT CTG GTT CTT-3′) were designed to detect a highly conserved region within the JCV large T gene. The 5′ and 3′ ends of the probe were labeled with 6-carboxyfluorescein and Black Hole Quencher-1, respectively. Real-time PCR was performed in a total volume of 20 μL containing 10 μL of the LightCycler 480 Probes Master (Roche, Penzberg, Germany) and 1 μL each of the 10 μM forward and reverse primers, and the 3.2 μM probe, and 2 μL of the DNA template prepared as described above. Amplification and real-time fluorescence detection were performed using LightCycler ST300 (Roche), and the cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 1 s. The results were quantified by interpolation on the standard curve of serial 5-fold dilutions of the above-described standard DNA using the LightCycler software (Roche).

Nested PCR analysis: Primers for nested PCR targeting the non-coding control region within the JCV genome have been described previously (22). The first-round PCR was carried out in a 50-μL reaction mixture containing the Blend Taq Plus reagent (Toyobo, Tokyo, Japan), outer primers, and 2 μL of the DNA template, according to the manufacturer's protocol. Amplification was performed in a thermal cycler (PCR Thermal Cycler Dice; Takara, Shiga, Japan) with initial denaturation at 98°C for 1 min, followed by 25 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The second-round amplification was performed using the first-round product and inner primers in the manner described above, with the exception of the PCR amplification cycles being 30 instead of 25. The PCR products were separated by 2% agarose gel electrophoresis and visualized using SYBR Green I nucleic acid gel stain (Invitrogen, Carlsbad, Calif., USA).

Statistics: The correlation between each pair of variables was analyzed by Pearson's correlation coefficient test.

RESULTS

Optimization of the real-time PCR assay: With the view to achieve rapid and sensitive quantification of JCV DNA, a real-time PCR assay was developed based on the LightCycler platform, which involves rapid thermal cycling in glass capillaries. Database analysis of the primer and probe sequences indicated that they were 100% identical to the corresponding region of over 360 isolates of various origins from GenBank (data not shown). The feasibility of the real-time PCR assay was first examined using serial 5-fold dilutions of the plasmid pJC1-4-＞pJCV as the standard DNA. Figure 1A

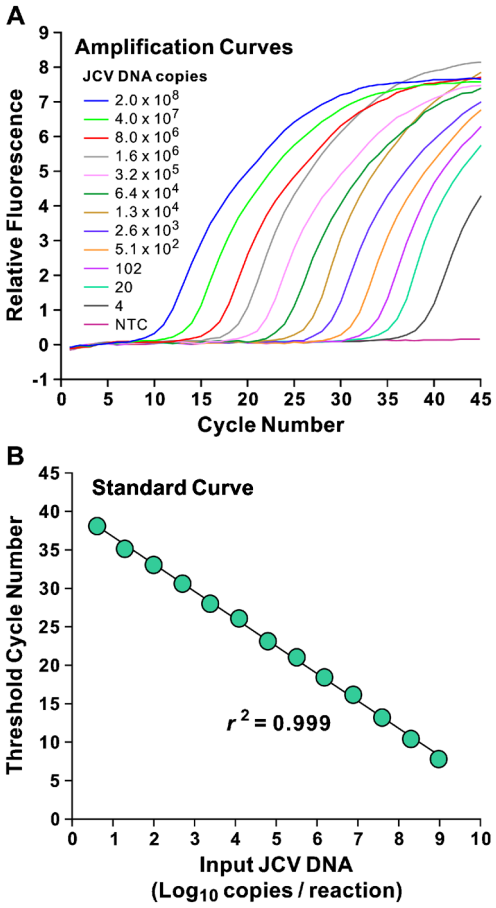

Fig. 1. Development of a real-time PCR assay for the quantification of JCV DNA. (A) Amplification curves. The reactions were performed in the absence or presence of standard DNA (2.0×10^8 to 4 copies per reaction). Relative fluorescence is plotted against cycle number. (B) Standard curve from the same experiment shown in panel A. The threshold cycle number is plotted against the $\log_{10}$ of the starting copy number. NTC indicates "no template control." These data are representative of three independent experiments.

Table 1. Detection of JCV genome in DNA extracts from CSF specimens by the nested PCR and real-time PCR assays

Nested PCR	Real-time PCR		Total
	Positive	Negative	
Positive	21	0	21
Negative	1	157	158
Total	22	157	179

shows the amplification plots of fluorescence intensity against the PCR cycle. Target DNA was detected in a range from 2×10^8 to 4 copies per reaction. A linear relationship was observed between the threshold cycle number and the log copy number of standard DNA ($r^2 = 0.999$) (Fig. 1B). In addition, no amplification signal was detected even in the presence of high concentrations (over 10^7 copies) of other polyomaviruses, including BK polyomavirus, simian virus 40, and murine polyomavirus (data not shown).

Validation of the real-time PCR assay: To evaluate the reliability of the developed real-time PCR for clinical testing, the performance of this assay was compared to that of nested PCR using CSF specimens (Table 1). In the preliminary experiments, the nested PCR described in an earlier study (22) was found to be capable of detecting at least 20 copies of JCV DNA per reaction (data not shown). Total DNA extracts were prepared from CSF specimens collected from patients using a QIAamp DNA Blood Mini Kit and subjected to each PCR assay. All 21 nested PCR-positive specimens showed a positive reaction in the real-time PCR, while 157 of the 158 nested PCR-negative samples exhibited a negative reaction. In the real-time PCR-positive specimen of one patient diagnosed with PML (23), JCV DNA was not amplified by nested PCR; this was probably due to a low copy number (approximately 5 copies per reaction). Thus, the sensitivity and specificity of the real-time PCR were 100% (21/21) and 99.4% (157/158), respectively, in comparison with nested PCR.

Quantitative detection of JCV DNA in virus suspensions using a real-time PCR assay without DNA extraction: To assess the performance of the real-time PCR assay using templates prepared without DNA extraction, known amounts of JCV isolated from persistently infected cells were heat-treated or treated with a lysis reagent and used as PCR templates. The amounts of JCV DNA in the virus suspensions were determined by the real-time PCR assay, with the use of DNA extracts for the preparation of a standard virus suspension. The virus suspension containing 10^8 genome copies (144.8 hemagglutination units) per mL of JCV was serially diluted with PBS and subjected to DNA extraction, heat treatment, or treatment with DNAzol Direct reagent, as described above. The JCV DNA was detected in the DNAs extracted from the virus suspensions containing 10^8 to 200 genome copies per mL (Fig. 2). When the heat- or DNAzol Direct-treated suspensions were used as PCR templates, the target DNA was detected in samples containing at least 10^3 viral genome copies per mL (2 or 1 copy equivalents per reaction, respectively), but no amplification signal was observed in the presence of 200 viral genome copies per mL (0.4 or 0.2 copy equivalents per reaction, respectively) (Fig. 2). For both the heat- and DNAzol Direct-treated samples, a linear relationship was noted between the copy number detected and the amount of input virus in the range of 10^8–10^3 copies per mL ($r^2 = 0.983$ and 0.997, respectively).

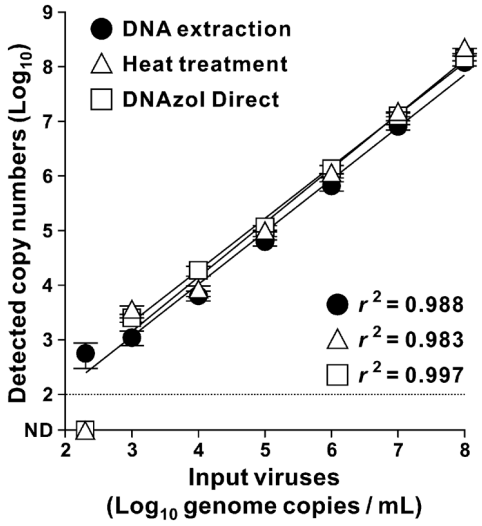

Fig. 2. Quantification of JCV DNA in virus suspensions using real-time PCR assay with or without DNA extraction. The virus suspensions in PBS containing the indicated amounts of JCV were subjected to DNA extraction, heat treatment, or DNAzol Direct treatment to obtain PCR templates. The copy numbers of the JCV DNA in each template were quantified using the real-time PCR assay and here are plotted against the amount of input virus (genome copy equivalents per mL). Data are shown as means ± standard errors of the means from three separate experiments. ND indicates "not detected."

Quantification of JCV DNA in CSF specimens using a real-time PCR assay without DNA extraction: The next set of experiments was conducted to examine whether the real-time PCR assay using templates without DNA extraction can be applied to the quantification of JCV DNA in CSF. The amount of JCV genome in the CSF specimen from a PML patient was determined by real-time PCR using a DNA extract as the template. This sample was serially diluted with CFS negative for JCV DNA, and the real-time PCR assays were performed on different templates, as described above (Fig. 3). JCV DNA was detected in the heat- or DNAzol Direct-treated CSF samples containing at least 10^3 viral genome copies per mL (2 or 1 copy equivalents per reaction, respectively). A linear relationship was observed between the copy number detected and the amount of input virus, in both the heat- and DNAzol Direct-treated samples containing 10^7 to 10^3 copies per mL of JCV ($r^2 = 0.999$ or 0.994, respectively). The detected copy numbers of the JCV genome in the DNAzol Direct-treated CSF samples were between those in the extracted DNA samples and the heat-treated samples, for 10^7–10^4 copies per mL of input virus.

Real-time PCR testing for JCV DNA in CSF specimens without DNA extraction: The performance of the real-time PCR test for JCV DNA using templates prepared without DNA extraction was evaluated in clinical CSF specimens obtained from patients (Table 2). Twenty-one of 66 specimens were positive for JCV DNA according to real-time PCR assay using DNA ex-

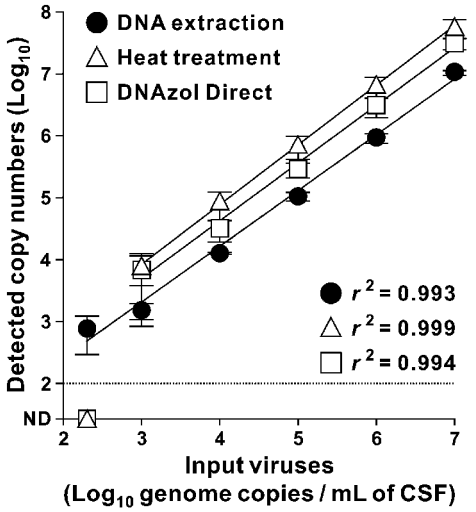

Fig. 3. Quantification of JCV DNA in standard CSF samples using real-time PCR assay with or without DNA extraction. The JCV DNA-positive CSF samples containing the indicated amounts of JCV were subjected to DNA extraction, heat treatment, or DNAzol Direct treatment to obtain PCR templates. The copy numbers of JCV DNA in each template were quantified using the real-time PCR assay and are here plotted against the amount of input virus (genome copy equivalents per mL). Data are shown as means ± standard errors of the means from three separate experiments. ND indicates "not detected."

Table 2. Detection of JCV genome in CSF specimens from patients by the real-time PCR assays with or without DNA extraction

DNA extraction	Heat treatment		DNAzol Direct		Total
	Positive	Negative	Positive	Negative	
Positive	18	3	19	2	21
Negative	0	45	0	45	45
Total	18	48	19	47	66

tracts as templates. Among the heat-treated specimens, 18 of the 66 samples showed a positive reaction. The sensitivity and specificity of the real-time PCR assay using the heat-treated specimens were 85.7% (18/21) and 100% (45/45), respectively, with the results of assays of DNA extracts used as reference. When the DNAzol Direct-treated specimens were used as templates, 19 of the 66 samples were positive for JCV DNA, and the sensitivity and specificity of the assay were 90.5% (19/21) and 100% (45/45), respectively, with reference to the results of the assays of DNA extracts. To assess whether heat- or DNAzol Direct-treated CSF specimens are suitable for quantitative testing of JCV, the absolute copy numbers of the JCV genome in each sample were determined as described above (Fig. 4). In the heat-treated specimens, a linear relationship was observed between the copy number detected and the amount of input virus (r^2 = 0.906) (Fig. 4A). This correlation improved when CSF samples were treated with DNAzol Direct (r^2 = 0.982) (Fig. 4B).

DISCUSSION

The current study aimed to develop a real-time PCR assay for the rapid and sensitive quantification of JCV DNA in CSF specimens without the need for nucleic acid extraction. Previous reports have indicated that CSF specimens could be used as templates for conventional, nested, or real-time PCR assays for JCV DNA (15,24–26). Among these assays, real-time PCR was reported to detect even low amounts of JCV DNA in heat-treated CSF specimens (15). However, in the aforementioned study, the detection limit and quantitative ability of the real-time PCR assay of heat-treated samples were not evaluated using a conventional DNA extraction method, which has been considered the golden standard for CSF testing for JCV DNA (15). On the contrary, a series of experiments was performed to exa-

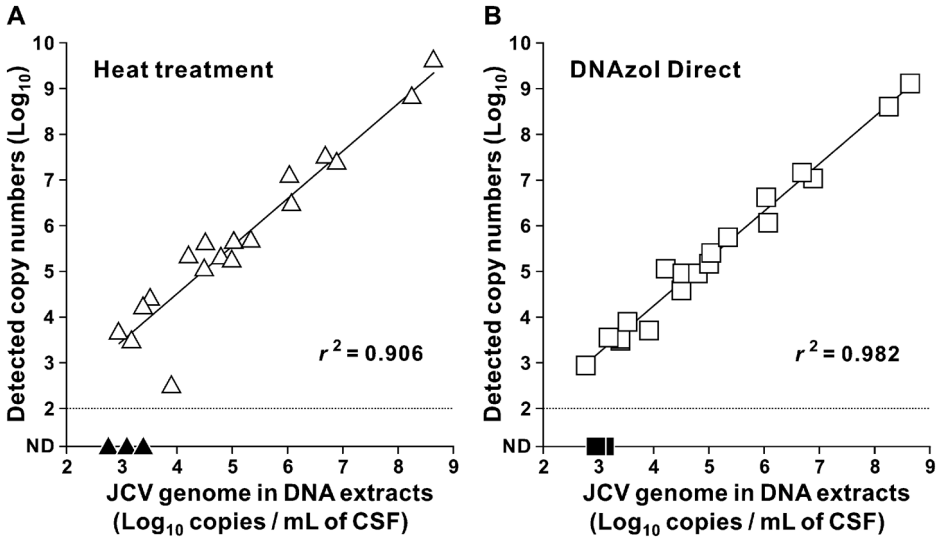

Fig. 4. Relationships between the JCV genome copies in CSF specimens determined by real-time PCR assay with or without DNA extraction. The CSF specimens from patients were heat-treated (A) or treated with DNAzol Direct (B) to obtain PCR templates. The copy numbers of JCV DNA in each template were quantified using the real-time PCR analysis and are here plotted against the results obtained by the assay using DNA extraction. ND indicates "not detected." The CSF specimens, which showed a negative reaction by the assay using the heat or DNAzol Direct treatments, are indicated by the closed triangle or square symbols, respectively.

mine the performance of the real-time PCR assay using heat- or DNAzol Direct-treated samples in comparison with that using a DNA extraction method on the same platform as that used in the present study.

When a virus suspension in PBS was subjected to the real-time PCR assay, the detected copy numbers of JCV DNA in the heat- and DNAzol Direct-treated samples were almost identical to those detected in the DNA extracts, for 10^8–10^3 viral genome copies per mL. These results suggest that the developed real-time PCR assay without DNA extraction is feasible for the quantification of the JCV genome. Unlike the case with methods involving DNA extraction, total DNA could not be isolated with either heat or DNAzol Direct treatment. Thus, it is reasonable that the detection limits of these methods are 10^3 viral genome copies per mL (2 or 1 copy equivalents per reaction, respectively) and that no amplification signal is found in the presence of 200 genome copies per mL (0.4 or 0.2 copy equivalents per reaction, respectively).

The real-time PCR assays of the heat- and DNAzol Direct-treated samples could quantitatively detect the viral genome both in the virus suspension and in CSF specimens containing known amounts of JCV. The detection limit of these methods was around 10^3 genome copies per mL (2 or 1 copy equivalents per reaction, respectively) using standard CSF samples. The detected copy numbers of JCV DNA in the heat-treated samples were slightly higher than those in the DNA extracts, although statistical differences were observed only in the samples containing 10^6 and 10^7 viral genome copies ($P = 0.0381$ and 0.0386, respectively). Since these differences were not observed for the experiments using a virus suspension in PBS, it is likely that either the amplification of the target gene or the fluorescence intensity of probe was affected to some extent by heat treatment of the CSF samples.

DNAzol Direct is a lysis reagent for direct PCR assay of biological materials. Although the exact chemical composition has not been disclosed, this reagent is an alkaline solution containing polyethylene glycol (PEG), chaotropic ions, and other additives for the rapid lysis of samples and the inactivation of PCR inhibitors, as described in the manufacturer's protocol. DNAzol Direct-treated samples can be directly subjected to PCR without the need for clarification or neutralization. In addition, it has been suggested that an alkaline PEG solution inactivates common pathogens, such as bacteria and viruses (27). Hence, the preparation of PCR templates using this reagent is thought to be beneficial for the safe processing of clinical specimens that may contain infectious agents. While some reports have described the use of DNAzol Direct-based PCR assay to monitor bacterial or parasitic infections (28–30), the utility of this reagent for virus detection has hitherto remained unclear. Therefore, this study is believed to be the first to apply the DNAzol Direct method to PCR testing for viral DNA.

In the present study, quantification of viral DNA using the DNAzol Direct method appeared to be more accurate than that using the heat treatment method, as demonstrated by the assays of standard CSF containing known amounts of JCV and clinical specimens collected from patients. Notably, this difference in the quantitative performance was not observed when the assays were performed with virus suspentions in PBS. Since experiments using standard CSF revealed that the detection limit of the DNAzol Direct method was similar to that of the heat treatment method, it is unlikely that the release of viral DNA from the capsid was enhanced by this reagent. In fact, it is possible that this reagent weakened the influence of undefined CSF factors, thereby improving the stability of PCR amplification and/or detection. Considering its superior quantitative performance and the ability to inactivate infectious agents by virtue of being an alkaline PEG solution, the DNAzol Direct method is thought to be more suitable than the heat treatment for the real-time PCR testing of JCV DNA in CSF specimens.

Since total DNA cannot be concentrated by heat or DNAzol Direct treatment, the real-time PCR assay for the JCV genome without nucleic acid extraction is less sensitive than that using a DNA extraction procedure. Some CSF specimens showed a negative reaction in the real-time PCR using the heat and DNAzol Direct treatments, but relatively low levels of viral DNA were detected by using DNA extraction method. Thus, with respect to the sensitivity of PCR testing, the assay involving DNA extraction is more suitable for the diagnosis of PML than the assays without DNA extraction. However, the present methods are advantageous in that the PCR templates can be prepared rapidly from extremely small amounts of CSF. This feature is valuable for high-throughput and less-invasive testing for JCV DNA during the follow-up of PML patients and the clinical trials of potential therapeutic interventions, both of which require sequential monitoring of viral load in CSF. Moreover, the possibility of sample-to-sample contamination is minimized in the present protocol through the omission of the multiple manipulations required for manual or automated extraction. The false-positive detection of JCV in CSF is a serious concern, particularly for patients receiving immunosuppressive therapies, because it may lead to a change in therapeutic strategy to improve the quality of life of the individuals involved. Therefore, the protocols proposed in this study may be useful to confirm the results of PCR assay involving DNA extraction, when a relatively high level of JCV DNA is detected.

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Conflict of interest None to declare.

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