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Reliability of direct varicella zoster virus loop-mediated isothermal amplification method for rapid diagnosis of breakthrough varicella

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ABSTRACT

Background: Since patients with breakthrough varicella (BV) have mild symptoms, clinical diagnosis is difficult. In high vaccine coverage area, as BV occurs sporadically, point of care test is required for controlling varicella outbreak. In this study, the reliability of varicella zoster virus (VZV)-loop mediated isothermal amplification (LAMP) was evaluated for the rapid diagnosis of BV.

Study design: A total of 328 swab samples collected from patients with suspected varicella were analyzed. For the laboratory diagnosis of varicella, VZV real-time PCR was carried out using DNA extracted from swab samples. Swab samples without DNA extraction were used for VZV-LAMP(direct-LAMP).

Results: VZV infection was diagnosed by real-time PCR in 285 cases, including 105 natural varicella cases and 180 BV cases. VZV DNA was detected in 250 (87.8%) of the 285 cases by direct-LAMP. The presence and duration of fever, number of skin eruptions, and VZV DNA load were significantly lower in BV than natural varicella. The sensitivity of direct-LAMP for the diagnosis of varicella and BV was 93.3% and 84.4%, respectively.

Conclusions: Direct LAMP was considered to be useful for rapid diagnosis of BV as it has several advantages such as low cost, ease and rapidity, as compared to real time PCR.

1. Background

After the live attenuated varicella vaccine was developed in 1974 [1], it was administered in Japan on a voluntary basis until 2014. Since vaccination was not mandatory, the vaccine coverage rate has been insufficient to control varicella outbreaks, as evidenced by a consistent seasonal epidemiological pattern of varicella infection [2]. However, the implementation of a universal vaccination program of varicella vaccine, in which two doses of varicella vaccine are included, in 2014 has resulted in a remarkable reduction of varicella cases nationwide [3]. Meanwhile, the number of breakthrough varicella (BV) patients has increased gradually, as also seen in other countries that have introduced universal immunization [4]. BV is defined as wild-type varicella zoster virus (VZV) infection occurring in a vaccinated individual more than 42 days after vaccination. The frequency of BV was initially reported as 2–3% of vaccines per year [5]. Although it is well known that BV is characterized by milder symptoms than natural VZV infection, for instance, fewer lesions overall and predominantly maculopapular features (i.e., a lower proportion of vesicular lesions) [6,7], it can spread VZV infection to susceptible individuals, causing outbreaks [8]. Furthermore, BV can cause severe complications not only in immunocompromised individuals, but also, in rare cases, in those who are immunocompetent [9–12]. Thus, while the precise diagnosis of BV has become very important for controlling varicella outbreaks since the implementation of universal vaccination [10,11], this is difficult due to the lack of typical clinical manifestations. While VZV DNA detection and the paired serum antibody assay have been examined as diagnostic methods for BV [13–16], more reliable, rapid, and convenient techniques are strongly desired from clinical point of view.

The loop-mediated isothermal amplification (LAMP) method can amplify template DNA under isothermal conditions with high efficiency and specificity as a nested polymerase chain reaction (PCR) [17]. This method has been widely used in Japan as a point-of-care test for detection of various infectious pathogens, such as *Legionella*, *Mycoplasma* pneumoniae, and *Mycobacterium tuberculosis* [18–20]. Our institutes were the first to develop LAMP assays for the detection of the eight

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human herpesviruses, including VZV [21–28]. Additionally, this method can directly amplify template DNA in swab samples without requiring DNA extraction, in a process called direct LAMP [27,29]. Although the real-time PCR method is superior for high throughput analysis of large numbers of samples, the direct LAMP method is more convenient for a rapid analysis of a few samples, because it's low cost, easiness, and rapidity.

2. Objectives

Therefore, in this study, we sought to evaluate the reliability of the VZV direct LAMP assay for the rapid diagnosis of BV.

3. Study design

3.1. Patients and samples

Four hundred twenty patients with suspected varicella were enrolled between September 2015 and August 2017. Swab samples were collected from skin eruptions using a cotton-tipped swab, and the swab was then immersed in 1 mL of isotonic saline. Ninety-two patients were excluded because of missing data regarding, for instance, background, clinical symptoms, and past history of varicella. Therefore, the samples of 328 patients with suspected varicella underwent virological analysis (Fig. 1). Written informed consent was obtained from the guardians of patients in the study because patients in this study were all under fourteen years old. This study was approved by the Institutional Review Board of our university (No. 15-247).

3.2. Patient background and clinical symptoms

A self-report questionnaire was used to obtain patient background information, such as number of siblings, whether they attended day care or school, whether they lived with grandparents, and clinical symptoms, including incidence of febrile episodes, duration of febrile period, numbers of skin eruptions at the first visit to the clinic, complications, and recent VZV exposures. The history of VZV vaccination was confirmed by the immunization record.

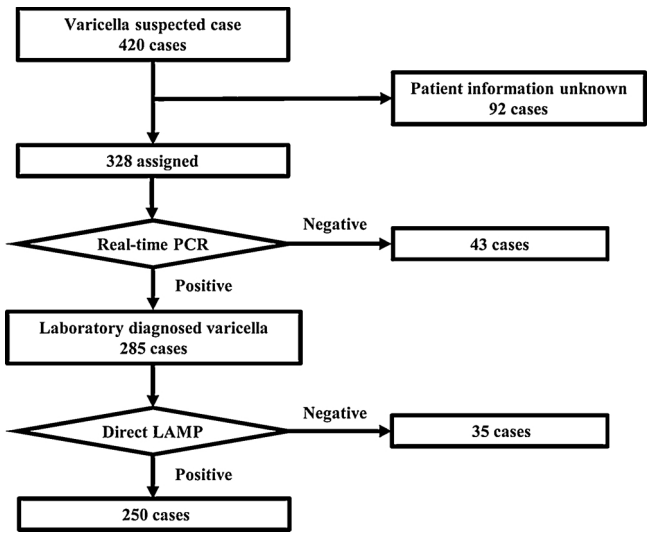

Fig. 1. Results of virological examinations in suspected varicella cases.
Results of real-time PCR using DNA extracted from patients' swab samples are shown. The swab samples were used directly (without DNA extraction) for loop-mediated isothermal amplification (LAMP).

3.3. DNA extraction and real-time PCR

DNA was extracted from the swab samples using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA, USA). After DNA extraction, the DNA was eluted in 50 μL buffer and stored at −20 °C until PCR analysis. The primers and probe of the real-time PCR were previously described [30]. The reaction mixture (30 μL total volume) of the real-time PCR contained 15 μL of 2× Fast Universal PCR Master mixture (Applied Biosystems, Foster City, CA, USA), 5 μL of template DNA, 5 pM probe, 10 pM primers, and nuclease-free water. The PCR reaction was performed using the StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in fast mode. PCR conditions were as follows: denaturation for 20 s at 95 °C, 50 cycles of melt for 1 s at 95 °C, and primer annealing and extension/emission of fluorescence for 20 s at 60 °C. The lower detection limit of this real-time PCR assay was 500 copies/mL. Therefore, the real-time PCR was used as the gold standard for evaluation of diagnosis of varicella.

3.4. Direct LAMP assay

Swab samples, which were immersed in 1 mL of isotonic saline, were diluted five-fold in distilled water (without DNA extraction). These were used for the direct LAMP assays. Details of the LAMP assay were described in a previous report [27]. The LAMP reaction was carried out using the Loopamp DNA amplification kit (Eiken Chemical, Tokyo, Japan). The LAMP mixture (25 μL total volume) contained 0.6 μM of the FIP and BIP primers, 0.1 μM of each outer primer (F3 and B3 primers), 0.3 μM of each loop primer (LPF and LPB primers), 12.5 μl of 2× reaction mixture, 5.0 μl of diluted samples, 1.0 μL (8U) of *Bst* DNA polymerase, and nuclease-free water. In order to increase the assay sensitivity, a heat denaturation step (incubation at 96 °C for 30 s) was carried out before adding *Bst* DNA polymerase. Finally, this mixture was incubated at 65 °C for 60 min. The LAMP products were detected turbidometrically using a LA-200 device (Teramecs, Kyoto, Japan). The cutoff value for discrimination between positive and negative samples was 0.1. All steps of this assay were completed within 80 min. The limit of detection of VZV DNA for direct LAMP assay was 5000 copies/mL.

3.5. Definition of natural varicella and BV

The diagnosis of varicella was confirmed by positive VZV DNA in swab samples by real-time PCR. Natural varicella was defined as varicella without a past history of VZV vaccination. BV was defined as varicella occurring more than 42 days after inoculation with the varicella vaccine.

3.6. Statistics analysis

Patients' characteristics (age, gender, presence of siblings, number of siblings, living with grandparents, and being at home daytime), clinical symptoms (fever, febrile period, and number of skin eruptions), and copy numbers of VZV DNA in swab samples determined by real-time PCR were compared between natural varicella and BV patients. Additionally, VZV DNA copy numbers were compared between LAMP positive samples and false negative samples in both natural varicella and BV patients to clarify the reason of the false negative results. Demographic factors and clinical data were compared by Pearson's chi-square test or Wilcoxon signed-rank test. $P < 0.05$ was considered to be statistically significant. The statistical analysis was performed with JMP statistical analysis software Version 7 (SAS Institute Inc, Cary, NC).

4. Results

4.1. Virological examination in suspected varicella cases

The results of molecular diagnosis of varicella are shown in Fig. 1. Two hundred eighty-five patients (86.9%), including 105 with natural

Table 1
Characteristics of natural and breakthrough varicella patients.

	Natural varicella n = 105 n (%)	Breakthrough varicella n = 180 n (%)	*P* value
Age, years (mean ± SD)	4.6 ± 2.8	5.4 ± 2.5	0.143
Gender			0.264
Male	62 (59.0)	94 (52.2)	
Female	43 (41.0)	86 (47.8)	
Presence of siblings			0.249
Yes	87 (82.9)	158 (87.8)	
No	18 (17.1)	22 (12.2)	
Number of siblings (mean ± SD)	1.6 ± 1.1	1.5 ± 1.0	0.516
Living with grandparents			0.595
Yes	15 (14.3)	30 (16.7)	
No	90 (85.7)	150 (83.3)	
Being at home daytime			0.003
Yes	25 (23.8)	19 (10.6)	
No	70 (76.2)	161 (89.4)	

SD, standard deviation.

varicella and 180 with BV, were diagnosed with varicella by real-time PCR. The direct VZV LAMP assay was positive in 250 (87.7%) of the 285 cases. None of the 43 real-time PCR-negative samples were direct LAMP positive.

4.2. Comparison of natural varicella and breakthrough varicella

Characteristics of natural varicella and BV patients are summarized in Table 1. These were no significant differences between the two groups in terms of age, gender, presence and number of siblings, and whether or not patients lived with their grandparents. The proportion of patients attending nursery or elementary school was statistically higher in those with BV (161/180, 89.4%) than those with natural varicella (80/105, 76.2%) ($P = 0.003$).

The participants' clinical symptoms are shown in Table 2. Forty (38.1%) of the 105 natural varicella patients had a significantly higher febrile episode than that of the BV patients (40/180, 22.2%) ($P = 0.004$). Additionally, the fever duration was significantly longer in natural varicella patients (mean ± SD; 0.6 ± 1.1 days) than in BV patients (0.4 ± 0.7 days) ($P = 0.007$). The number of skin eruptions was also significantly higher in natural varicella patients (median: 20, IQR: 9–30) than in BV patients (median: 10, IQR: 6–20) ($P < 0.001$). As shown in Fig. 2 A, VZV DNA copy number in the swab samples of patients with natural varicella (Median: 4.6×10^8 copies/mL, Min: 3.3×10^3, Max: 2.2×10^{10}, IQR: $5.5 \times 10^6 - 2.6 \times 10^9$) was significantly higher than that in BV patients (Median: 1.2×10^7 copies/mL, Min: 6.5×10^2, Max: 1.0×10^{10}, IQR: $6.1 \times 10^5 - 1.4 \times 10^8$) ($P < 0.001$).

Table 2
Patient's clinical symptoms and viral DNA load in swab sample.

	Natural varicella n = 105 n (%)	Breakthrough varicella n = 180 n (%)	*P* value
Fever (temperature, ≥38.0 °C)			0.004
Yes	40 (38.1)	40 (22.2)	
No	65 (61.9)	140 (77.8)	
Febrile period (days): mean ± SD	0.6 ± 1.1	0.4 ± 0.7	0.007
Number of skin eruptions: median (IQR)	20 (9 – 30)	10 (6 – 20)	< 0.001

SD, standard deviation; VZV, varicella zoster virus; IQR, interquartile range.

4.3. Comparison of sensitivity and positive predictive value of direct LAMP in natural varicella and BV

As the results of real-time PCR assay are regarded as a golden standard in diagnosis of varicella, the sensitivity of the direct LAMP method for diagnosing natural varicella and BV was 93.3% (98 direct LAMP-positive results out of 105 real-time PCR-positive results) and 84.4% (152 direct LAMP-positive results out of 180 real-time PCR-positive results), respectively (Fig. 3). The positive predictive value of the direct LAMP method was 100% for diagnosing both natural varicella and BV. A comparison of VZV DNA copy numbers between the false-negative and LAMP-positive samples is shown in Fig. 2B. In the natural varicella patients, the VZV DNA copy number by real-time PCR assay in the false-negative swab samples (Median: 4.9×10^3 copies/mL, Min: 3.3×10^3, Max: 1.2×10^6, IQR: $4.5 \times 10^3 - 1.5 \times 10^5$), which means that the result was positive for real-time PCR assay and negative for LAMP assay, was significantly lower than that in the LAMP-positive samples (Median: 5.4×10^8 copies/mL, Min: 5.0×10^4, Max: 6.3×10^9, IQR: $1.2 \times 10^7 - 2.8 \times 10^9$) ($P < 0.001$). In BV patients, the VZV DNA copy number in the false-negative swab samples (Median: 1.4×10^4 copies/mL, Min: 6.5×10^2, Max: 1.1×10^6, IQR: $4.4 \times 10^3 - 7.2 \times 10^4$) was significantly lower than that in the LAMP-positive samples (Median: 2.6×10^7 copies/mL, Min: 2.3×10^3, Max: 1.0×10^{10}, IQR: $2.3 \times 10^6 - 3.4 \times 10^8$) ($P < 0.001$).

5. Discussion

Cases of BV were reported soon after the introduction of the varicella vaccine [6,15]. However, as the incidence was low and only a few patients developed severe BV, though some had fatal outcomes [10–12], rapid diagnosis of BV has not been emphasized. After the implementation of the universal varicella vaccination, there was a marked reduction in the number of natural varicella patients; as a result, those with BV accounted for the majority of varicella patients overall, and this became a major public health problem in many countries [8,9,31]. Since BV patients demonstrate mild, atypical clinical symptoms, the diagnosis of the disease is challenging. Additionally, in high vaccine coverage area, since BV occurs sporadically, we have to respond quickly small number of BV suspected cases. In this study, varicella was diagnosed in the laboratory based on detection of VZV DNA in skin swab samples using real-time PCR, which is considered to have the highest assay sensitivity and is widely used for high-throughput analysis of large number of samples. Among 285 laboratory-confirmed varicella patients, 250 (87.8%) were positive for VZV by direct LAMP, with no false-positive samples. Furthermore, the assay sensitivity of the direct LAMP method in natural varicella and BV patients was 93.3% and 84.4%, respectively suggesting high reliability of the LAMP method.

Although the number of analyzed samples was limited, only one previous study compared the reliability of several diagnostic methods, including PCR, in natural varicella and BV patients [32]. They demonstrated that the sensitivity of macular and/or papular swab PCR was 100% in both natural varicella and BV patients compared to vesicular and/or scab swab PCR. In this study, the amount of VZV DNA was significantly lower in the swab samples obtained from BV patients than in those obtained from natural varicella patients (Fig. 2A), which is consistent with the mild clinical symptoms of BV patients (Table 1). Additionally, the copy number of VZV DNA was significantly lower in the false-negative samples than in the LAMP-positive samples ($P < 0.001$) (Fig. 2B). The lower detection limit of direct LAMP is slightly higher than that of real-time PCR due to differences in assay sensitivity and the lack of DNA extraction in direct LAMP [27,29]. These findings suggest that the low copy numbers of VZV DNA in the swab samples obtained from BV patients contributed to the lower detection rate in these patients compared with natural varicella patients. Although the direct VZV LAMP assay is inexpensive and quick and easy to perform, lower detection limit of the assay compared to the real-time

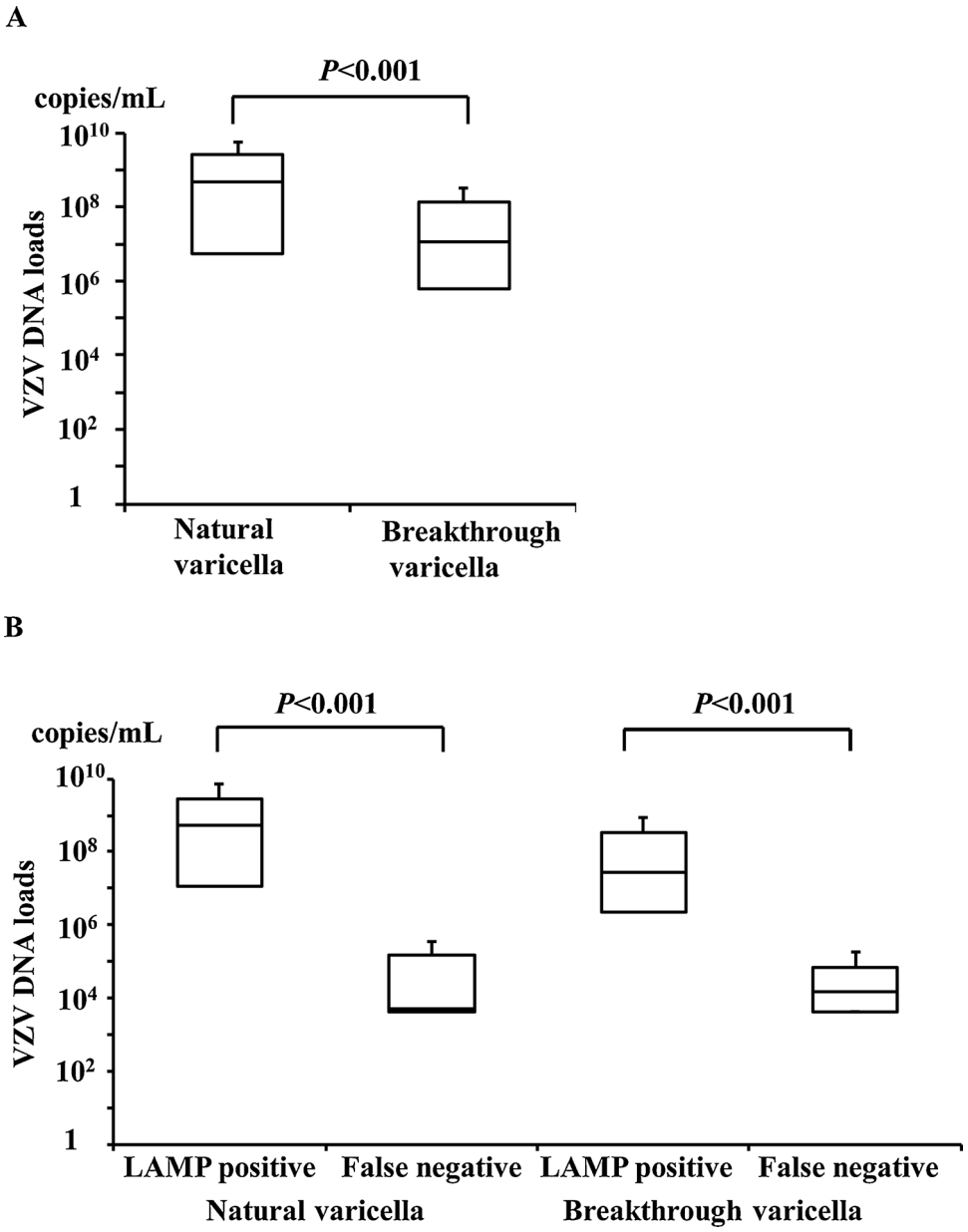

Fig. 2. A: Comparison of VZV DNA copy numbers between natural varicella and breakthrough varicella patients. B: Comparison of VZV DNA copy numbers between false-negative and false-positive samples. The boxes represent the first through third quartiles, with the lines within these boxes representing the median and the lines outside the boxes representing the 75th percentiles. The Y-axis indicates copy number of VZV DNA for real-time PCR assay.

PCR is major problem for precise diagnosis of BV. Therefore, improvement of assay sensitivity of the direct VZV LAMP method is necessary for clinical use of this method as a rapid diagnostic method for BV. Additionally, drying the reagents leads to a faster and easier diagnosis [33], and further improvement of the reagents is now underway.

As mentioned above, a previous study demonstrated that there was no difference in the sensitivity of PCR between vesicular swab samples (gold standard) and macular and/or papular swab samples [32]. However, the amount of VZV DNA is thought to be lower in macular and papular swab samples than in vesicular swab samples. The type of skin eruption was not examined in this study. Since false-negative swab samples contained less VZV DNA than LAMP-positive samples (Fig. 2B), these samples showed false-negative in direct VZV LAMP analysis in this study may have been collected from macular or papular skin eruptions. Therefore, future studies are necessary to determine what types of skin eruptions are most suitable for direct VZV LAMP analysis.

Other clinical specimens, such as saliva and peripheral blood, have been examined for PCR analysis [34]. The collection of peripheral blood is invasive, and the VZV DNA detection rate in blood specimens was found to be insufficient for the diagnosis of BV [32,34]. Leung et al. demonstrated that in BV patients, the VZV DNA detection rates based on cheek swabs, throat swabs, and oral fluid were 62%, 70%, and 85%, respectively [32]. Meanwhile, Watanabe et al. demonstrated that the sensitivity of saliva PCR was 87.1% for the diagnosis of BV [34]. Therefore, in the future, the method which is diagnosing BV in saliva samples by the direct VZV LAMP method also should be evaluated. Direct fluorescent antibody detection (DFA) is considered to be reliable as a rapid diagnostic method for BV because of its convenience and rapidity. Although Watanabe et al. demonstrated that this assay had high sensitivity [34], comparable to PCR, another study found that adequate sampling was difficult and the sensitivity of the assay was low [35]. Since the frequency of vesicular skin eruptions that are suitable for DFA analysis is not high [32], DFA may be useful only for patients with vesicle.

A : Natural varicella

		Real-time PCR positive	negative	Total
Direct LAMP	positive	98	0	98
	negative	7	0	7
Total		105	0	105

B : Breakthrough varicella

		Real-time PCR positive	negative	Total
Direct LAMP	positive	152	0	152
	negative	28	0	28
Total		180	0	180

Fig. 3. Comparison of the sensitivity and positive predictive value of direct LAMP for natural varicella versus breakthrough varicella. A: Natural varicella, B: Breakthrough varicella.

In conclusion, the VZV direct LAMP method (without DNA extraction) is highly sensitive and specific for the laboratory diagnosis of BV. While its sensitivity is slightly lower than that of real-time PCR, its low cost, rapidity, and convenience are major advantages for the rapid diagnosis of BV. As BV occurs sporadically in the highly vaccinated developed countries, the direct LAMP method is considered to be superior to the real-time PCR method in terms of rapid analysis of a small number of samples. The development of dry reagents may allow for further improvement in the feasibility of this assay. As universal varicella vaccination expands worldwide, the importance of the laboratory diagnosis of BV is expected to further increase.

Declaration of competing interest

All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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CRediT authorship contribution statement

Yuki Higashimoto: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Yoshiki Kawamura:** Project administration, Supervision, Validation, Writing - original draft, Writing - review & editing. **Ayumi Kuboshiki:** Methodology. **Fumihiko Hattori:** Conceptualization, Data curation, Investigation. **Hiroki Miura:** Investigation. **Naoko Nishimura:** Investigation. **Takao Ozaki:** Investigation, Writing - review & editing. **Masaru Ihira:** Methodology. **Tetsushi Yoshikawa:** Conceptualization, Funding acquisition, Supervision, Validation, Writing - review & editing.

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