Fukushima J. Med. Sci.,
Vol. 68, No. 3, 2022

[Original article]

Population analysis of oseltamivir-resistant variants for the rapid prediction of drug susceptibility by real-time reverse transcription polymerase chain reaction

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(Received March 31, 2022, accepted August 1, 2022)

Abstract

This study investigated whether quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), using specific probes composed of locked nucleic acids (LNA/qRT-PCR), designed to evaluate H1N1 pdm09 H275Y, H3N2 E119V and R292K variant populations, could replace a neuraminidase (NA) inhibition assay to determine the 50% inhibitory concentration (IC_{50}) of NA activity.

For H1N1 pdm09, when the H275Y variant RNA load was 50% or 70% and the infective H275Y variant load was 40% or 70%, the IC_{50} were >10- and 100-fold higher, respectively, than that of the wild-type (WT) strain. For H3N2, when the E119V RNA load and infective E119V variant load were >90% and >60%, respectively, the IC_{50} of the mixed sample was >10-fold higher than that of the WT strain. The variant-mixed samples with a 70% or 80% R292K variant RNA load and a 60% or 70% infective R292K variant load exhibited >10- and 100-fold decreased susceptibility, respectively, compared with that of the WT. A positive correlation between the variant RNA load and infective variant load populations was observed.

The LNA/qRT-PCR method can be used to improve the treatment and management of patients during antiviral therapy for influenza virus infection.

Key words : influenza, resistant virus, neuraminidase inhibitor, oseltamivir, polymerase chain reaction

Introduction

Influenza is a significant cause of morbidity and mortality in both children and adults and is associated with hospitalization in children aged <5 years[1]. Neuraminidase (NA) inhibitors (NAIs), such as oseltamivir, zanamivir, and laninamivir, have become primary treatment options for patients with influenza virus infection.

Among these NAIs, the dry syrup formulation of oseltamivir is the one mostly prescribed for children. Since the 2009-2010 influenza season and after the seasonal influenza virus H1N1 was replaced by the pandemic A H1N1 in 2009 (H1N1 pdm09), a few oseltamivir-resistant H1N1 pdm09 viruses have been isolated. For the 2009-2010 influenza season, oseltamivir-resistant H1N1 pdm09 viruses represented <1% of viruses detected in the United States, with most cases being isolated from patients who received oseltamivir[2,3]. However, in December 2013, oseltamivir-resistant H1N1 pdm09 with an H275Y substitution in NA (H1N1 pdm09 H275Y) was identified in Japanese patients who had not been exposed to oseltamivir[4,5]. This indicated that the variant would spread globally and that all H1N1 pdm09 cases would be resistant to oseltamivir in the near future based on seasonal H1N1 trends until the 2008-2009 influenza season[5]. Furthermore, an H3N2 virus subtype with an E119V (H3N2 E119V) and R292K substitution (H3N2 R292K) in NA has been identified, with reduced susceptibility to oseltamivir. The susceptibility of the influenza virus to

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NAIs is demonstrated by the 50% inhibitory concentration (IC_{50}) for NA activity ; the methods for measuring IC_{50} and the definition of susceptibility have been standardized by the World Health Organization[6]. However, because the current NA inhibition assay requires virus isolation following cell culture, it cannot provide rapid results to clinicians. Although the variant population was responsible for the apparent increase in IC_{50} values[7], there was a difference in NAI-resistant variant populations between clinical specimens and isolated viruses because of wild-type (WT) strain selection during cell culture[8-10]. Therefore, developing a method for rapid quantitative detection of WT and variant strains using clinical specimens is necessary to predict the IC_{50} values in clinical settings.

Recently, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) using specific probes composed of locked nucleic acids (LNA/qRT-PCR) to quantitatively detect major H1N1 pdm09 and H3N2 oseltamivir-resistant variants was demonstrated[9,11,12]. This PCR method allows for rapid viral susceptibility prediction and is expected to be useful in clinical practice. Therefore, in our study, we investigated whether this novel PCR method can be used to predict the susceptibility of viruses in obtained clinical specimens.

Materials and methods

1. *Viruses*

H1N1 pdm09 WT (A/Perth/265/2009), H275Y variant (A/Perth/261/2009), H3N2 WT (A/Fukui/20/2004), and E119V (A/Fukui/45/2004) variant were provided by Dr. Aeron Hurt (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia), and the R292K (A/Kagoshima/2/2012) variant was provided by Drs. Mutsuyo Gokuden (Kagoshima Prefectural Institute for Environmental Research and Public Health, Kagoshima, Japan), Takato Odagiri, and Emi Takashita (Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan). These viruses were propagated by cell culture five times using Madin-Darby canine kidney (MDCK) cells provided by Fukushima Institute of Public Health, Fukushima, Japan and were recovered from the supernatant of the cell culture.

2. *Plaque assay*

The infective viral load in each cell culture supernatant was determined using a plaque assay as described previously[13,14]. Briefly, 100 μL of viral supernatant was inoculated onto MDCK cells that had become monolayer-confluent in a 12-well plate, and the cells were washed after 1 h incubation at room temperature. The MDCK cells were incubated in 2 mL of Eagle's minimum essential medium (MEM) containing 0.6% of Avicel (FMC BioPolymer, Philadelphia, PA USA), MEM vitamin and MEM amino acid solutions (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.2% bovine serum albumin (Fraction V) (Calbiochem, La Jolla, CA, USA), 2 μg/mL of trypsin (Sigma Aldrich, St. Louis, MO, USA), 0.6 mg/mL of L-glutamine, and penicillin-streptomycin solution (Wako Pure Chemical Industries, Osaka, Japan) for 3 days at 34°C in 5% CO_2/95% air. The cells were fixed with 4% paraformaldehyde solution in MEM for 30 min at 4°C, washed with phosphate-buffered saline, and stained with hematoxylin and eosin solution.

The number of plaques of each virus was counted, and each infective viral load was determined.

3. *qRT-PCR*

Viral RNAs were extracted from the supernatant of cell cultures using the QIAamp MiniElute Virus Spin Kit (Qiagen, Inc., Valencia, CA, USA). PCR mixtures contained 4×TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), 900 nM of each primer[9,11,12], 270 nM of each probe[9,11,12] (Table 1), and 2 μL of target RNA, with the volume increased to 20 μL using nuclease-free water. PCR amplification was performed using the following conditions : reverse transcription of cDNA at 50°C for 5 min, followed by reverse transcriptase inactivation at 95°C for 20 s and PCR amplification by 40 two-step cycles (3 s at 95°C for denaturation and 30 s at 60°C for annealing and extension) using the QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The limit of detection for each qRT-PCR was four copies per reaction. The viral copy numbers of all WT and variant strains were determined by comparison with a serially diluted plasmid standard of a known concentration.

4. *NA inhibition assay*

Before performing the NA inhibition assay, 1×10^5 pfu/mL each of the infectious WT and variant strains as well as 1×10^7 copies/mL of each RNA of the WT and variant strains were prepared to produce 11 different variant-mixed samples in which the population level of resistant variants was 0%-100%. These variant-mixed samples were used to conduct the NA inhibition assay, which was per-

formed using a NA-XTD chemiluminescence-based NA inhibition assay kit (Thermo Fisher Scientific), and the IC_{50} values were calculated using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA), as previously described[9]. Oseltamivir carboxylate was provided by F. Hoffmann-La Roche, Ltd. (Basel, Switzerland).

The plaque assay, measurement of viral RNA load, and NA inhibition assay were all performed three times in duplicate.

Results

The IC_{50} values of the H1N1 pdm09 WT and H275Y variant strains were 0.17-0.35 and 37.2-45.7 nM, respectively. The variant-mixed samples with 50% and 70% H275Y variant RNA loads demonstrated 10.4- and 157.3-fold lower susceptibility than the H1N1 pdm09 WT samples, respectively (Figure 1a). Moreover, when the infective H275Y variant load was 40% or 70%, the IC_{50} values were 23.5- or 100.3-fold higher than that of the H1N1 pdm09 WT

Table 1. Primers and probes for the influenza PCR assays[11]

Influenza H1N1 wild-type and H275Y variant assay		Label (5'/3')	Position
Forward primer	5'-CAGTCGAAATGAATGCCCCTAA-3'		776-805
Reverse primer	5'-TGCACACACATGTGATTTCACTAG-3'		833-856
WT probe	5'-TTAt**c**aCTaTGaGGAATG -3'	FAM/BHQ-1	812-827
H275Y probe	5'- TTAt**t**aCTaTGaGGAATG -3'	Yakima Yellow/BHQ-2	812-827
Influenza H3N2 wild-type and E119V variant assay			
Forward primer	5'-AGGACAATTCGATTAGGCTTTCC-3'		305-327
Reverse primer	5'-CTGTCCAAGGGCAAATTGGTA-3'		388-408
WT probe	5'-ACaAGaG**a**AcCTTaTG-3'	FAM/BHQ-1	349-364
E119V probe	5'-ACaAGAG**t**AcCTTaTG-3'	Yakima Yellow /BHQ-2	349-364
Influenza H3N2 wild-type and R292K variant assay			
Forward primer	5'-GTGCTCCTGCTATCCTCGATATC-3'		831-853
Reverse primer	5'-TTTATATCTACGATGGGCCTATTGG-3'		893-917
WT probe	5'-CCaGttGtCt**c**TGC-3'	FAM/BHQ-1	872-885
R292K probe	5'-CTGCA**A**AGACAACTGG-3'	VIC/MGB	870-885

DNA nucleotides are denoted in standard upper case, locked nucleic acid nucleotides are denoted in lower case, and nucleotides complementary to the single nucleotide polymorphism are in bold font and underlined. WT, wild-type

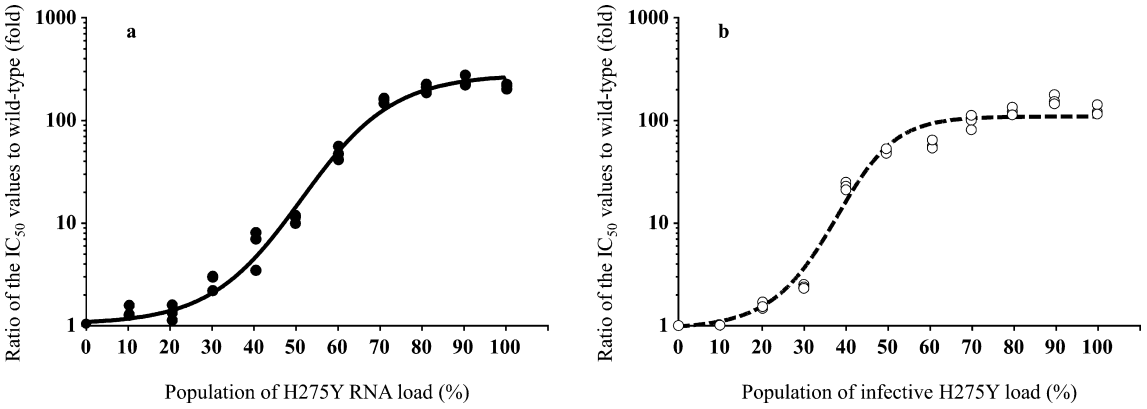

Fig. 1. IC_{50} values of mixed H1N1 pdm09 virus, including WT and H275Y variant. The variant-mixed samples with >50% H275Y variant RNA load (a) and >40% infective H275Y variant load (b) exhibited >10-fold higher IC_{50} values than that of the H1N1 pdm09 WT strain.
All data points represent the means of duplicate determinations. A sigmoid curve was determined by non-linear least squares fitting with GraphPad Prism 6 software. IC_{50}, 50% inhibitory concentration for neuraminidase activity ; WT, wild-type.

strain, respectively (Figure 1b). The IC_{50} values of the H3N2 WT, E119V, and R292K variant strains were 0.24–0.27, 3.3–5.9, and 268.9–437.6 nM, respectively. When the E119V RNA load and infective E119V variant load were 90% and 60%, respectively, the IC_{50} values of this mixed sample were 12.1- and 10.3-fold higher than that of the H3N2 WT strain, respectively (Figure 2a, 2b). The variant-mixed samples with 70% and 80% R292K variant RNA loads exhibited 15.8- and 114.5-fold lower susceptibility than that of the H3N2 WT, respectively (Figure 3a). Moreover, when the infective R292K variant loads were 60% or 70%, the IC_{50} values were 56.5- and 1,444.8-fold higher than that of the H3N2 WT strain, respectively (Figure 3b).

When the variant load was estimated using the RNA load in the infective variant-mixed samples, a positive correlation was observed between the variant RNA load and infective variant load using the GraphPad Prism 6 software (H1N1 pdm09 H275Y: $r^2 = 0.94$, $p < 0.0001$; H3N2 E119V: $r^2 = 0.87$, $p < 0.0001$; H3N2 R292K: $r^2 = 0.94$, $p < 0.0001$) (Figure 4).

Discussion

In our study, the IC_{50} values increased when the infective H1N1 pdm09 H275Y variant load was >40%. A previous study reported that the IC_{50} values of the variant-mixed samples increased when the infective H275Y variant load was >20%[7]. This difference may be attributable to the use of different

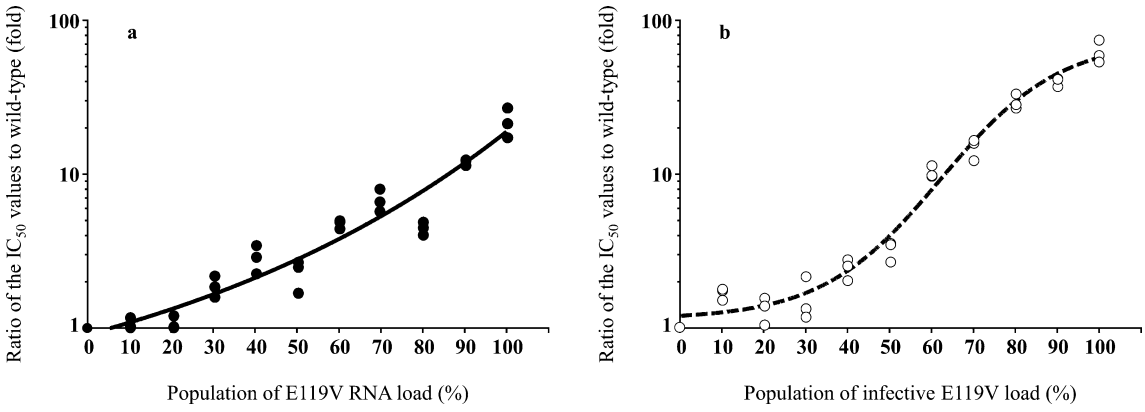

Fig. 2. IC_{50} values of mixed H3N2 virus with WT and E119V variant. The variant-mixed samples with >90% E119V variant RNA load (a) and >60% infective E119V variant load (b) exhibited >10-fold higher IC_{50} values than that of the H3N2 WT strain.
All data points represent the means of duplicate determinations. A sigmoid curve was determined by non-linear least squares fitting with GraphPad Prism 6 software. IC_{50}, 50% inhibitory concentration for neuraminidase activity; WT, wild-type.

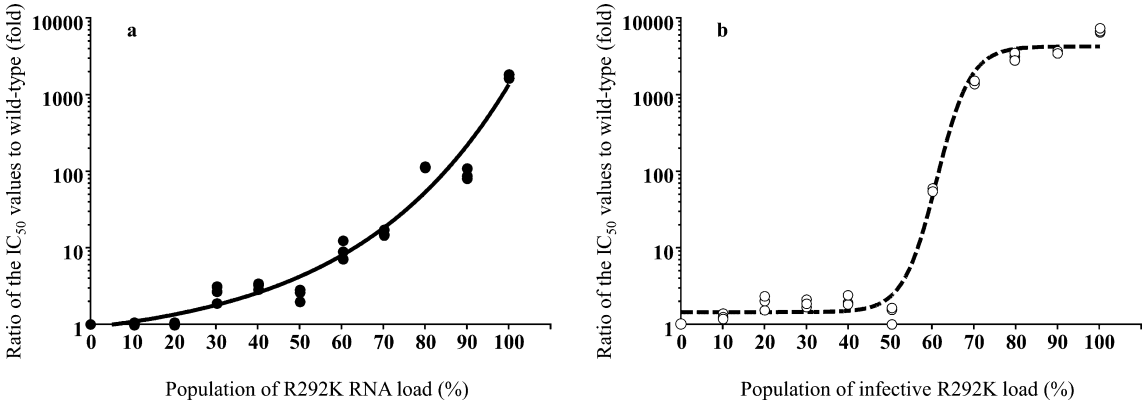

Fig. 3. IC_{50} values of mixed H3N2 virus with WT and R292K variant. The variant-mixed samples with >70% R292K variant RNA load (a) and >60% infective R292K variant load (b) exhibited >10-fold higher IC_{50} values than that of the H3N2 WT strain.
All data points represent the means of duplicate determinations. A sigmoid curve was determined by non-linear least squares fitting with GraphPad Prism 6 software. IC_{50}, 50% inhibitory concentration for neuraminidase activity; WT, wild-type.

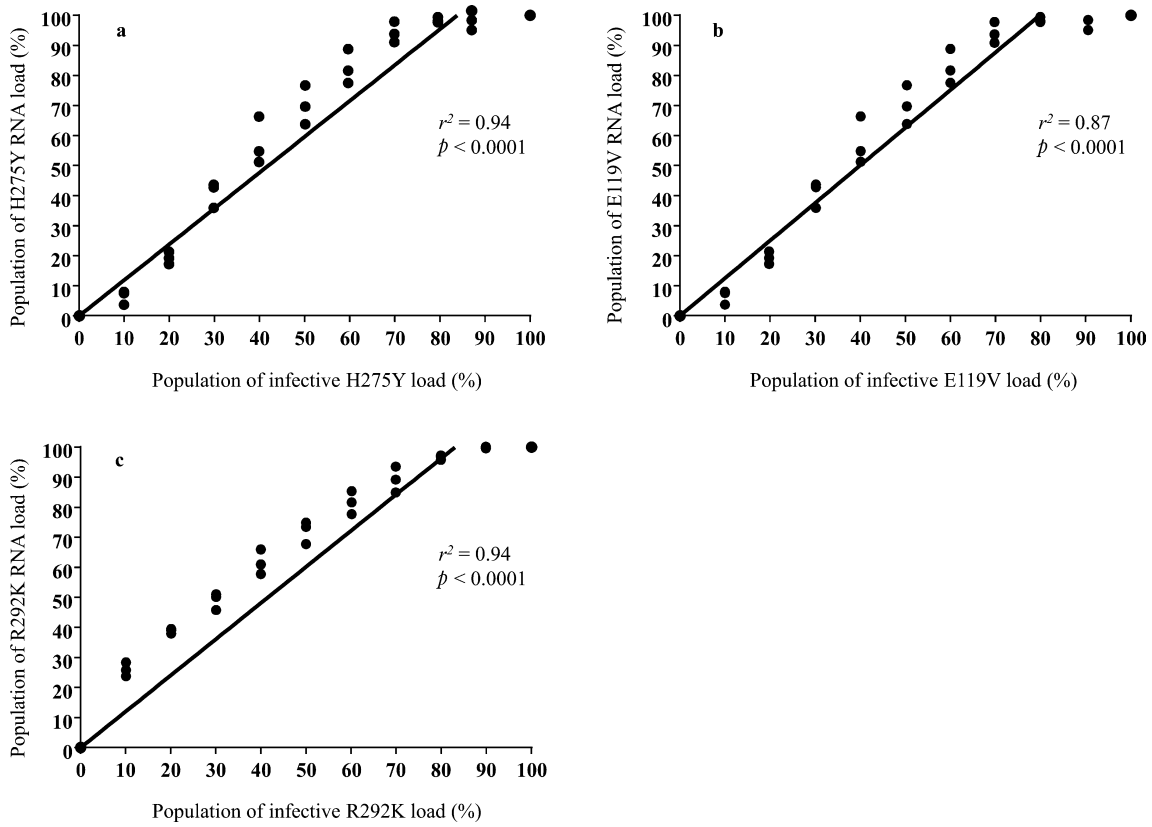

Fig. 4. The correlation between variant RNA load and infective variant load. A positive correlation was observed between H1N1 pdm09 WT and H275Y variant (a) ; H3N2 WT and E119V variant (b) ; and H3N2 WT and R292K variant (c).
All data points represent the means of duplicate determinations. A fitness curve and Pearson's correlation coefficient were calculated with GraphPad Prism 6 software. IC_{50}, 50% inhibitory concentration for neuraminidase activity ; WT, wild-type.

NA inhibition assays (chemiluminescence-based or fluorescence-based NA inhibition assays). The chemiluminescence-based NA inhibition assay is more sensitive than the fluorescence-based assay ; therefore, a higher H275Y variant load may be required to increase the IC_{50} values compared to that when using a chemiluminescence-based NA inhibition assay.

In this study, when the variant RNA population in the infective variant-mixed samples was estimated, a positive correlation was observed between the variant RNA and infective viral loads, which suggests that the LNA/qRT-PCR method facilitates the prediction of the infective variant load. However, the variant RNA load in the infective variant-mixed samples was higher than that of the infective variant load because qRT-PCR methods might be more sensitive than plaque assays. Therefore, a variant RNA population larger than the infective variant population was required to increase the IC_{50} values. When the infective H1N1 pdm09 H275Y variant load was 40% or 70%, the IC_{50} value decreased by >10- and 100-fold, respectively, compared with to that of the WT strain. The variant RNA loads in these mixed samples were calculated as 58% and 94%, respectively. In addition, the H3N2 E119V and R292K variant RNA loads in the 60% infective variant-mixed samples, which decreased the IC_{50} value by >10-fold compared with the WT strain, were calculated as 90% and 81%, respectively. These variant RNA populations were similar to those in the variant-mixed samples prepared on the RNA load (Figures 1-4).

Our study suggests that mere detection of a variant strain in a sample does not indicate a decrease in the IC_{50}, and that determining the variant population remains important in evaluating the IC_{50}[7]. Furthermore, measuring the variant and WT RNA loads in clinical samples using the LNA/qRT-PCR method facilitates NAI susceptibility estimation in clinical specimens, and clinical samples may have clinically low susceptibility when the H1N1 pdm09 H275Y, H3N2 E119V, and R292K variant RNA populations are >50%, >90%, and >60%, respectively.

The absolute variant load after antiviral therapy is usually much lower than that before treatment[9,14,15], and a lower variant viral load has no impact on the clinical course of illness, even if the variant virus population increases during antiviral therapy[9,16,17]. Therefore, it is important to monitor the absolute viral load over time. Hence, deep sequencing methods are the most ideal to estimate the population of variants in the clinical specimens[18,19]. However, these methods are clinically impractical as they are expensive and time-consuming. In contrast, the LNA/qRT-PCR method used in this study provides results within hours after clinical sample collection ; thus, this is a practical method for management of patients with influenza virus infection, particularly when a patient is infected with H1N1 pdm09 H275Y variants with decreased susceptibility to oseltamivir[20]. The primers and probes used in the present study were made by ordering gene sequences from a third-party vendor, and the PCR reagents are expensive. Therefore, the PCR method used in this study was practical only at a limited number of facilities, such as some university laboratories. However, with the recent outbreak of severe acute respiratory syndrome coronavirus 2, many hospitals have acquired real-time PCR equipment ; moreover, the reagents are becoming less expensive, enabling this method to be more widely adopted in the future.

In conclusion, a qRT-PCR using specific probes composed of locked nucleic acids is clinically useful for estimating drug susceptibility in clinical settings.

Funding

This work was supported in part by JSPS KAKENHI (grant number : 20K08210).

Acknowledgments

We thank Dr. Aeron Hurt (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia) for providing reference H1N1 pdm09 viruses, two WT strains (A/Perth/265/2009 and A/Fukui/20/2004), the H275Y variant (A/Perth/261/2009), H3N2 viruses, and an E119V variant (A/Fukui/45/2004). We thank Drs. Mutsuyo Gokuden (Kagoshima Prefectural Institute for Environmental Research and Public Health, Kagoshima, Japan), Takato Odagiri, and Emi Takashita (Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan) for providing the R292K variant (A/Kagoshima/2/2012).

The authors would like to thank Enago (www.enago.jp) for English language review of early manuscript text.

Conflict of interest disclosure

All authors declare no personal conflicts of interest.

The Department of Pediatrics, Fukushima Medical University, has received research funding through the research promotion division of the Fukushima Medical University from Otsuka Pharmaceutical Co., Ltd. and Shiongi & Co., Ltd.

Oseltamivir carboxylate was provided by F. Hoffmann-La Roche, Ltd. (Basel, Switzerland).

Author contribution statement

Masatoki Sato : Conceptualization, methodology, writing-original draft. Koichi Hashimoto and Mitsuaki Hosoya : writing-review and editing.

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