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Development of cycling probe based real-time PCR methodology for influenza A viruses possessing the PA/I38T amino acid substitution associated with reduced baloxavir susceptibility

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ABSTRACT

Baloxavir marboxil has been used for influenza treatment since March 2018 in Japan. After baloxavir treatment, the most frequently detected substitution is Ile38Thr in polymerase acidic protein (PA/I38T), and this substitution reduces baloxavir susceptibility in influenza A viruses. To rapidly investigate the frequency of PA/I38T in influenza A (H1N1)pdm09 and A (H3N2) viruses in clinical samples, we established a rapid real-time system to detect single nucleotide polymorphisms in PA, using cycling probe real-time PCR. We designed two sets of probes that were labeled with either 6-carboxyfluorescein (FAM) or 6-carboxy-X-rhodamine (ROX) to identify PA/I38 (wild type strain) or PA/I38T, respectively. The established cycling probe real-time PCR system showed a dynamic linear range of 10^1 to 10^6 copies with high sensitivity in plasmid DNA controls. This real-time PCR system discriminated between PA/I38T and wild type viruses well. During the 2018/19 season, 377 influenza A-positive clinical samples were collected in Japan before antiviral treatment. Using our cycling probe real-time PCR system, we detected no (0/129, 0.0%) influenza A (H1N1)pdm09 viruses with PA/I38T substitutions and four A (H3N2) (4/229, 1.7%) with PA/I38T substitution prior to treatment. In addition, we found PA/I38T variant in siblings who did not received baloxavir treatment during an infection caused by A (H3N2) that afflicted the entire family. Although human-to-human transmission of PA/I38T variant may have occurred in a closed environment, the prevalence of this variant in influenza A viruses was still limited. Our cycling probe-PCR system is thus useful for antiviral surveillance of influenza A viruses possessing PA/I38T.

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1. Introduction

Influenza remains a widespread global public health concern. The Centers for Disease Control and Prevention (CDC) determined that up to 650,000 people die of respiratory diseases linked to seasonal influenza each year worldwide (Iuliano et al., 2018). In Japan, anti-influenza drugs are used for uncomplicated influenza cases because the national medical insurance system covers prescriptions for medication used to treat influenza infections (Zaraket and Saito, 2016). Treatment with anti-influenza drugs in Japan has led to a lower incidence of severe cases and a lower fatality rate compared to those in other countries (Kamigaki and Oshitani, 2009; Sugaya, 2011).

Baloxavir marboxil (BXM) became available for influenza treatment in Japan in March 2018 and in the United States in October 2018 (Takashita, 2020; Takashita et al., 2020). BXM functions as a cap-dependent endonuclease inhibitor. Baloxavir acid, the active form of BXM, inhibits the initiation of mRNA synthesis by binding to the active site of polymerase acidic protein (PA) (Noshi et al., 2018; Takashita, 2020).

Amino acid substitution in PA results in reduced susceptibility to BXM. The most commonly reported substitution is of isoleucine (T) for threonine at position 38, (PA/I38T), followed by isoleucine for methionine (PA/I38M) or phenylalanine (PA/I38F), and glutamic acid for lysine (E23K) at position 23. Collective data from clinical trials in adults and children showed that the overall frequency of PA reduced susceptibility substitutions after BXM treatment was 6 (4.3%) out of 138 for A (H1N1)pdm09 viruses and 73 (13.0%) out of 560 for H3N2 viruses (Ince et al., 2020). The details of each substitution was as follows: 2 (33.3%) of I38T, 2 (33.3%) of I38F, 1 (16.7%) of I38N, and 1 (16.7%) of E23K, for A (H1N1)pdm09 viruses; and 60 (82.2%) of I38T, 2 (2.7%) of I38 T/M mixture, 5 (6.8%)of I38M, 3 of (4.1%) E23 G/K, 2 of (2.7%) A37T, and 1 of (1.4%) E199G, for A/H3N2 viruses. Furthermore, variants of Influenza A virus possessing PA/I38T substitution were associated with 27–57-fold changes in the EC50 *in vitro* and showed the highest reduction in susceptibility to BXM compared with those with other PA/I38X substitutions (Gubareva et al., 2019; Omoto et al., 2018).

Sporadic cases of human to human transmission have been reported in influenza A (H3N2) with PA/I38T in the 2018–2019 season (Imai et al., 2020; Takashita et al., 2019a, 2019b) but the potential for community transmission remains unknown. In this regard, the prevalence of the PA/I38T substitution in pre-treatment samples is an important indicator of community transmission. However, detection of PA variants requires time and is expensive: the standard Sanger sequencing methods are time-consuming, next generation sequencing (NGS) technologies are expensive and produce large amounts of data to analyzed (Koszalka et al., 2019). Thus a high-throughput screening method is needed for the surveillance of PA/I38T for influenza A (H3N2) and A (H1N1)pdm09. Recently, a few groups have reported the development of assays for the detection of PA/I38X substitutions in influenza viruses. Two groups (Koszalka et al., 2020; Patel et al., 2020) have developed screening systems of PA variants using pyrosequencing and next generation sequencing. Nakauchi et al. developed a rapid screening system using RNase H2-dependent real-time PCR (rhPCR) for detecting the PA/I38T substitution in clinical samples (Nakauchi et al., 2020). Previously, we developed a different real-time PCR assay to detect single nucleotide polymorphisms in influenza viruses (Suzuki et al., 2010, 2011). Thus, we decided to use our systems to detect PA/I38T viruses for the rapid screening of clinical samples.

The cycling probe real-time PCR system is a useful tool to detect single nucleotide polymorphisms (SNPs) using fluorescence-labeled chimeric RNA-DNA probes cleaved by RNase H during PCR cycles (Cloney et al., 1999). Previously, we established the cycling probe method for the rapid detection of SNPs in virus genes that cause drug resistance such as S31N in the M2 gene of influenza A viruses, and H275Y in the NA gene of influenza A (H1N1)pdm09 viruses (Suzuki et al., 2010, 2011). Using the cycling probe real-time PCR system, we have also been investigating the drug resistant influenza viruses in Japan, Myanmar and Lebanon since 2008 (Chon et al., 2019; Dapat et al., 2010, 2012, 2013a, 2013b; Hibino et al., 2016; Htwe et al., 2019; Kondo et al., 2016; Kyaw Win et al., 2020; Mawatari et al., 2019; Oguma et al., 2011; Saito et al., 2010, 2016, 2020; Shobugawa et al., 2012; Zaraket et al., 2010a, 2010b, 2010c, 2011, 2014, 2016).

Herein, we established a new set of cycling probe real-time PCR systems to detect influenza A viruses possessing PA/I38T and investigated the frequency of variant detection in pre-antiviral treatment samples during the 2018/19 influenza season in Japan to evaluate whether these mutant viruses could become widespread. We also analyzed the clinical information of patients with influenza A viruses harboring PA/I38T prior to antiviral treatment to determine whether this variant could affect the clinical course when patients were treated with anti-influenza drugs, BXM, or neuraminidase inhibitors.

2. Materials and methods

2.1. Sample collection and clinical records of patients

We collected samples in Japan during the 2018–2019 season from out-patients with influenza-like illness, presenting with symptoms, such as fever, cough, sore throat, general fatigue, or sneeze, who visited clinics of pediatrics and internal medicine in Hokkaido, Gunma, Niigata, Chiba, Kyoto, Nagasaki, Kumamoto and Okinawa (Saito et al., 2020). Patients were enrolled in the study if the Quick Navi™–Flu + RSV rapid diagnostic test (RDT) (Denka Company Limited, Tokyo, Japan) was positive for influenza A and written informed consent was obtained. Nasopharyngeal aspirates or nasal swabs were collected from patients before antiviral treatment with BXM or neuraminidase inhibitors such as oseltamivir, zanamivir, laninamivir or peramivir.

Samples were suspended in viral transport medium (Dapat et al., 2009) and stored at −20 °C at each clinic until they were transported to Niigata University for further virological investigation. Along with sample collection at the first clinic visit, clinical data of the patients, which included age, sex, body temperature during clinical visit, and clinical background, were recorded by clinicians. After the first sample collection, BXM or neuraminidase inhibitors were administered following the standard prescriptive course in Japan (Mawatari et al., 2019; Saito et al., 2020). Daily records including axillary body temperature measurements three times daily were kept by patients or their caregivers for a maximum of 8 days. The duration of fever was defined as the time from the first clinic visit to the last record $\geq$37.5 °C.

Ethical approval for this study was obtained from the Ethics Committee at Niigata University (approval number 2018–0317).

2.2. RNA extraction and reverse transcription

Viral RNA was extracted from 100 μL of clinical samples in viral transport medium using RNA EXTRAGEN II (Tosoh Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. The details of the RNA extraction procedure are described in the Supplemental Materials and Methods. RNA pellets were resuspended in 10 μL of Nuclease-Free Water (Promega, Madison, USA), mixed with 0.5 μL of 20 μM Uni 12 primer (5′-AGCAAAAGCAGG-3′) for influenza A (Hoffmann et al., 2001), 0.5 μL of 20 μM Uni 11 primer (5′-AGCAGAAGCRS -3′) for influenza B (Dapat et al., 2009), 5 μL of 5 × first-strand buffer (Invitrogen, Carlsbad, USA), 5 μL of 2 mM of each deoxynucleoside triphosphate (Promega, Madison, USA), 2 μL of 0.1 mM dithiothreitol (Invitrogen), 1 μL of 10 U/μL RNase inhibitor (Invitrogen), and 1 μL of Moloney murine leukemia virus reverse transcriptase (Invitrogen); samples were incubated at 37 °C for 2 h for cDNA synthesis. To exclude influenza B infection, we performed an additional real time PCR using segment-specific influenza B primers targeting the haemagglutinin gene; these were included at the time of cDNA synthesis as reported previously (Htwe et al., 2019; Saito et al., 2020).

2.3. Cycling probe real-time PCR assay to screen PA/I38T substitution

For the rapid detection of PA/I38T, which confers reduced susceptibility to BXM in influenza A (H1N1)pdm09 and A (H3N2) (Omoto et al., 2018), we established a cycling probe real-time PCR method using fluorescent-labeled chimeric RNA-DNA probes, and the commercially available cycling probe real-time PCR kit, Cycleave PCR® Reaction Mix (TaKaRa Bio Inc., Ohtsu, Japan). The two probes were designed in the reverse-complement direction to mRNA and were labeled with either 6-carboxyfluorescein (FAM) or 6-carboxy-X-rhodamine (ROX). Respective probe and primer sets were designed for A (H1N1)pdm09 and A (H3N2) due to sequence differences in the subtypes. The FAM probe matched with the nucleotides encoding isoleucine (*ATT*) for A (H1N1) pdm09 and (*ATA*) for A (H3N2), and the ROX probe matched with threonine (*ACT*) for A (H1N1)pdm09 and (*ACA*) for A (H3N2), respectively, to detect the SNP (T to C substitution) corresponding to PA/I38T at nucleotide 113 in segment 3, PA subunit. The reference sequence used for influenza A (H1N1)pdm09 virus was A/Kyoto/18FS209_1/2019 (GISAID accession no. **EPI584627**) and that for the A (H3N2) virus was A/Nagasaki/18FS251_1/2019 (**EPI1585537**). The sequences and locations of the primers and probes used for the cycling probe real-time PCR are listed in Table 1.

The Cycleave PCR® Reaction Mix (Takara Bio Inc.) was used for cycling probe real-time PCR following the manufacturer's instructions. Briefly, real-time PCR was carried out in a total volume of 25 μL. The reaction mix contained 9 μL of Nuclease-Free Water (Promega), 12.5 μL of 2 × CycleavePCR Reaction Mix, 0.25 μL of 20 μM of each PCR primer (forward and reverse), and 1 μL of 5 μM of each probe (FAM- and ROX-labeled probe). One microliter of cDNA was added to the reaction mix as a DNA template. The PCR conditions were as follows: initial denaturation for 10 s at 95 °C and 45 cycles of denaturation for 5 s at 95 °C, primer annealing for 10 s at 59 °C and for 10 s at 59.5 °C, and extension/detection of fluorescence for 20 s at 72 °C. Individual runs for A (H1N1) pdm09 and A (H3N2) were required. Both PCRs were carried out using the Thermal Cycler Dice® Real Time System II (Takara Bio Inc.).

2.4. Control plasmids

For the positive control of the cycling probe real-time PCR, two types of DNA plasmids containing the nucleotide coding either for isoleucine or threonine at position 38 in the PA gene were constructed for each subtype. The sequence of the sensitive control plasmids was based on A/Kyoto/18FS209_1/2019 (EPI1584627) for A (H1N1)pdm09, and A/Nagasaki/18FS251_1/2019 (EPI1585537) for A (H3N2), flanked by respective forward and reverse primer sets of the cycling probe assay. For the mutant controls, a nucleotide relevant to amino acid position 38 was changed from T to C at the 2nd nucleotide of the triplet codon at residue 38. The generation of control plasmids was entrusted to Takara Bio Inc. It is optimal to use *in vitro* transcribed RNA (Nakauchi et al., 2020) rather than a control DNA plasmid in this study, because the efficiency of reverse transcription varies with the primers used, temperature, and the amount of reverse transcription enzyme (Sigma-Aldrich (2020)). However, we used DNA plasmids as a surrogate for *in vitro* transcribed RNA as PCR controls for the reason of stability and ease in routine examinations.

To determine the detection limit of the cycling probe real-time PCR, we included six replicates in the PCR runs for each of the five different amounts of plasmid DNA (50, 25, 10, 1, and 0 copies per reaction); and the samples were classified according to the variant status and the subtype, namely, wild type or PA/I38T for A (H1N1)pdm09; and wild type or PA/I38T for A (H3N2) (Nakauchi et al., 2020). Probit analysis was conducted using Analyse-it (Analyse-it Software, Ltd., Leeds, UK) to determine the limit of detection according to the results (positive or negative) of the six replicates of the five different DNA amounts for the above four sets.

To evaluate the detection range of PA/I38 (wild type) and PA/I38T in mixture conditions, wild type and PA/I38T plasmids were mixed in a total amount of DNA of 10^3 at ratios of 99:1, 98:2, 95:5, 90:10, 50:50, 10:90, 5:95, 2:98, and 1:99. Each mixture was tested six times for A (H1N1)pdm09 and A (H3N2).

2.5. Sequence analysis of PA gene

To identify substitutions in the PA subunit that confer reduced susceptibility to baloxavir, genetic sequencing of PA gene was conducted using the Sanger method. The PA gene was amplified using outer primer sets (1st PCR forward primer and reverse primer) for the first PCR and inner primer sets (2nd PCR forward primer and reverse primer) for nested PCR as reported elsewhere (Saito et al., 2020). Both PCRs were conducted using PrimeSTAR HS DNA Polymerase (Takara Bio Inc.). The nested PCR amplicons were purified with the QIA quick PCR Purification Kit (QIAGEN, Germany) and sequenced using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Bio Systems, Carlsbad, USA). Sequences were resolved with an ABI Prism 3130xl Genetic Analyzer. The nucleotide sequences of the PA gene were edited and assembled using DNASTAR Lasergene (DNASTAR, Inc., Madison, USA). To verify the results of cycling probe real-time PCR, the amino acid substitutions in PA were evaluated using MEGA X (Kumar et al., 2018). In addition, the sensitivity and specificity of cycling probe real-time PCR (positivity or negativity) against genetic sequencing (successful sequencing or not-available sequences) were calculated.

Table 1
Primers and probes used for cycling probe real-time PCR.

Subtype	Primers or probes	Direction	Sequence (5′–3′)	Location[a]
A (H1N1) pdm09	Forward primer	Sense	5′-CAATCCAATGATCGTCGAG-3′	27–45
	Reverse primer	Antisense	5′-TGGAAATCCGAATACATGAAAC-3′	134–155
	PA-Ile38 FAM probe[b]	Antisense	5′-(Eclipse[c])-GTGTGCAA[d]T-(FAM[e])-3′	112–121
	PA-Thr38 ROX probe[b]	Antisense	5′-(Eclipse[c])-TGTGCAA[d]TT-(ROX[e])-3′	111–120
A (H3N2)	Forward primer	Sense	5′-ATGATTGTCGAACTTGCAGA-3′	34–53
	Reverse primer	Antisense	5′-TTCCAGCTCCAGTAGTGTTG-3′	285–304
	PA-Ile38 FAM probe[b]	Antisense	5′ -(Eclipse[c])-ATA[d]TTGCTGCA-(FAM[e])-3′	105–115
	PA-Thr38 ROX probe[b]	Antisense	5′ -(Eclipse[c])-ATG[d]TTGCTGC-(ROX[e])-3′	106–115

[a] Location of primers and probes in the PA gene, segment 3 of A/Michigan/45/2015 (H1N1)pdm09 (GISAID accession no. **EPI662590**) and A/Hong Kong/4801/2014 (H3N2) (GISAID accession no. **EPI578426**).
[b] Quencher and fluorescent dye labeled DNA/RNA chimeric probe.
[c] Quenching molecule.
[d] The nucleotides replaced by RNA are underlined.
[e] Fluorescent molecules, FAM: 6-carboxyfluorescein, ROX: 6-carboxy-X-rhodamine.

2.6. Real-time PCR for generic influenza A

To see the match between the conventional quantitative real-time PCR and cycling probe real-time PCR assay, TaqMan probe real-time PCR targeted at M gene of generic influenza A was performed using the same clinical samples with the methods reported previously (Kondo et al., 2016), and the results of viral cDNA copies were compared between the two methods.

2.7. Data availability

Respective nucleotide sequences analyzed in this study are available via the Global Initiative on Sharing All Influenza Data, GISAID (https://www.gisaid.org), and the list of samples and GISAID accession numbers are provided in the supplemental material (Tables S1 and S2).

3. Results

3.1. Establishment of cycling probe real-time PCR

To evaluate the cycling probe real-time PCR for PA/I38T, we used 10-fold serial dilutions of the positive control plasmids from 10^1 to 10^6 copies/reaction (Fig. 1). The results for each primer/probe set showed that the cycling probe real-time PCR had high linearity ($R^2 > 0.99$) (Fig. 1) and the FAM probe reacted only to the wild type plasmid, and the ROX probe reacted only to the PA/I38T for both A (H1N1)pdm09 and A (H3N2), showing successful differentiation of the SNPs by the cycling-probe PCR method. The Probit analysis was performed six times using five different DNA amounts (Table 2), according to the results of the PCR reactions, and it showed that the limit of detection (LOD) for wild type and PA/I38T in A (H1N1) pdm09 was 2.1 and 2.1 copies/reaction, and that in A (H3N2) was 2.1 and 1.1 copies/reaction, respectively. However, we set a cut-off value as 10 copies/reaction for routine examinations because it is the minimum copies of plasmid DNA that we used as the positive controls, ranging from 10^6 to 10^1 copies.

Table 2
Detection limits of cycling probe real-time PCR.

Template cDNA copies (copies/reaction)	A (H1N1)pdm09		A (H3N2)	
	WT[a]	I38T[b]	WT	I38T
50	6/6	6/6	6/6	6/6
25	6/6	6/6	6/6	6/6
10	6/6	6/6	6/6	6/6
5	6/6	6/6	6/6	6/6
1	4/6	4/6	4/6	5/6
NC	0/6	0/6	0/6	0/6

[a] WT: wild type (PA/I38).
[b] I38T: PA/I38T.

We further examined the performance of this PCR method to recognize the clinically identified viruses with reduced BXM susceptibility analyzed in our previous study: PA/I38T (9), I38K (1), I38M (1), I38S (1), and E23K (1) in A (H1N1)pdm09 and A (H3N2) (Saito et al., 2020). Our results showed that the cycling probe real-time PCR successfully identified PA/I38T in all clinical samples previously identified as positive for PA/I38T viruses. Next, as expected, positive reaction was observed only with the wild type probe but not with PA/I38T probe for samples containing PA/I38K, I38M, and E23K viruses. However, the PA/I38S tested in our samples also tested positive using wild type and PA/I38T probes, the reason behind this has not been elucidated yet (data not shown). Since we only had one clinical sample carrying PA/I38S virus, further investigation is needed to clarify whether this reaction can occur across the PA/I38S viruses or if it is restricted to this particular

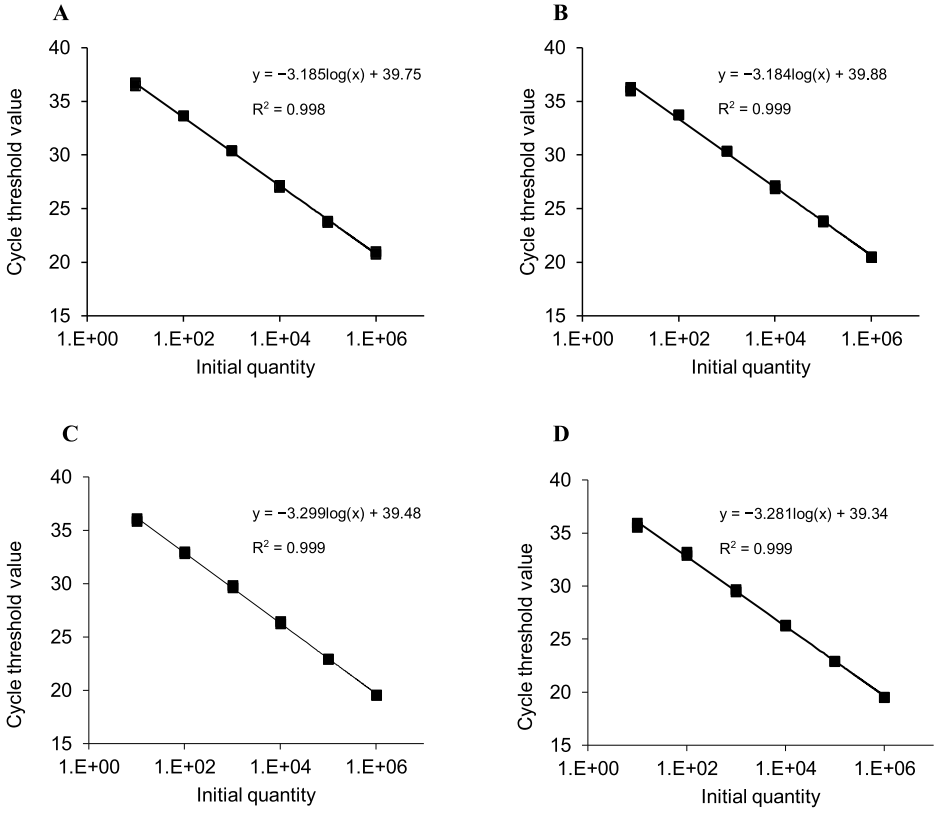

Fig. 1. Standard curves for influenza A viruses possessing PA/I38 and PA/I38T based on the specific cycling probe real-time PCR. The cycling probe real-time PCR was evaluated by testing 10-fold serial dilutions of positive control plasmids (A) pA (H1N1)pdm09 P A/I38, (B) pA (H1N1)pdm09 P A/I38T, (C) pA (H3N2)PA/I38, and (D) pA (H3N2) PA/I38T from 10 to 10^6 copies per reaction. The slopes and R^2 values are shown in the graphs.

sample.

3.2. Assessment of the PA/I38T detection level in mixture conditions

The minimum range of detection in the mixed condition was found to be 90:10 or 10:90 (wild type and PA/I38T) for both A (H1N1)pdm09 and A (H3N2) after six replicates of each PCR (Table 3).

3.3. Frequency of PA/I38T substitutions in pre-treatment samples

To evaluate the prevalence of influenza A viruses in the community with reduced susceptibility to BXM, we investigated the PA/I38T substitution in viruses from pre-treatment samples using the established cycling probe real-time PCR. We tested 377 pre-treatment samples that were influenza A positive with RDT during the 2018–2019 season, by cycling probe real-time PCR for PA/I38T detection. We identified 129 (34.2%) influenza A (H1N1)pdm09 virus, 229 (60.7%) influenza A (H3N2), and 19 (5.0%) negatives; meanwhile, we detected no (0.0%) PA/I38T substitution in 129 A (H1N1)pdm09, and 4 (1.7%) PA/I38T in 229 A (H3N2) samples collected prior to antiviral treatment. These 4 pre-treatment PA/I38T viruses reacted only to the PA/I38T (ROX) probe but not to the wild type (FAM) probe.

To confirm the PA/I38T substitution identified by cycling probe real-time PCR and to assess the sensitivity and specificity of the cycling probe assay, we performed genetic sequencing of PA by the Sanger method. Among the 377 pre-treated samples tested by cycling probe real-time PCR, 288 were selected for genetic sequencing, and 267 samples were successfully sequenced. Within these samples, we found 4 PA/I38T - A (H3N2), 119 wild type - A (H1N1)pdm09, and 149 wild type - A (H3N2) positive samples and 16 cycling probe real-time PCR negative samples. Among the 267 successfully sequenced samples, the 4 PA/I38T- A (H3N2) samples were detected positive using the cycling probe assay were verified by Sanger sequencings. Of note, these 4 viruses showed only the existence of PA/I38T but not a mixture of PA/I38 by both methods. In addition, one A (H1N1)pdm09 case associated with a substitution at position 36 of PA from alanine (A) to valine (V) was found by genetic sequencing. The sample showed that the wild type was positive but PA/I38T negative, showing no cross-reactivity to this substitution by cycling probe real-time PCR. No other known mutations related to reduced BXM susceptibility were identified in the sequenced samples.

The overall sensitivity and specificity of the cycling probe real-time PCR assay based on genetic sequencing was 97.4% and 42.9%, respectively; which indicates that the cycling probe PCR assay has high sensitivity but low specificity (Table S3). The Ct-values for 12 positive cycling probe real-time PCR results in negative samples are listed in Table S4.

Table 3
Detection level of PA/I38 and PA/I38T in mixture conditions.

WT[a]:I38T[b]	No. of positive results/No. of tests			
	A (H1N1)pdm09		A (H3N2)	
	WT	I38T	WT	I38T
100:0[c]	6/6	0/6	6/6	0/6
99:1	6/6	0/6	6/6	0/6
98:2	6/6	0/6	6/6	0/6
95:5	6/6	2/6	6/6	5/6
90:10	6/6	6/6	6/6	6/6
50:50	6/6	6/6	6/6	6/6
10:90	6/6	6/6	6/6	6/6
5:95	4/6	6/6	5/6	6/6
2:98	0/6	6/6	3/6	6/6
1:99	0/6	6/6	0/6	6/6
0:100	0/6	6/6	0/6	6/6

[a] WT: wild type (PA/I38).
[b] I38T: PA/I38T.
[c] Total 10^3 copies/reaction.

To evaluate the reliability of the cycling probe real-time PCR, we compared the Ct-values for 51 A (H1N1)pdm09 and 104 A (H3N2) samples tested by the wild type (FAM) probe cycling probe real-time PCR and the corresponding values of quantitative TaqMan probe real-time PCR for generic influenza A (Kondo et al., 2016). The Ct-values between the two methods showed strong correlations in A (H1N1) pdm09 and A (H3N2) ($R^2 = 0.7494, 0.8367$), respectively (Fig. S1 & S2), showing the reliability on quantification with the cycling probe PCR assay.

3.4. Clinical background of patients harboring the virus with PA/I38T substitution in pre-treatment samples

The clinical information of four A (H3N2) patients harboring viruses with the PA/I38T substitution prior to treatment showed that all were less than 10 years of age (Table 4). Two of the four patients (case no. 1 and 3) were administered BXM after sample collection, and the durations of fever were 2.0 days and 1.1 days, respectively (Table 4). Other two patients (case no. 2 and 4) were given oseltamivir and zanamivir, and their duration of fever was 0.3 and 0.9 days respectively.

3.5. Clinical timeline of case no. 2 and family

We obtained a nasopharyngeal aspirate from the older sibling of case No. 2, who was treated with oseltamivir. Surprisingly, the sibling also possessed PA/I38T without BXM treatment. The 5-year old sibling developed fever 4 days before the case No.2, and was given oseltamivir without sample collection then (Fig. 2). When the older sibling visited the clinic again on the 5th day of fever onset, a sample was collected and the virus showed PA/I38T by cycling probe real-time PCR and direct sequencing. Their parents also developed influenza-like symptoms between the disease onset in their two children. The parents were treated with BXM but their samples were not obtained. For the two sibling A (H3N2) cases, genetic sequencing of HA and NA segments was performed using the Sanger method as previously reported (Dapat et al., 2009b). HA segment of the two cases showed an identical match, suggesting a family infection. NA segment sequencing showed that the viruses had no reported amino acid substitutions conferring resistance to oseltamivir as shown in the supplemental material (Table S2). From the timing of onset, the PA/I38T virus detection, and oseltamivir use in case No. 2 as well as in the older sibling, we assessed that this family was infected with PA/I38T viruses. The possible source was outside the family, but this remained unknown. This family infection suggested that

Table 4
Clinical background of patients infected with influenza A (H3N2) viruses possessing PA/I38T prior to treatment.

Case	Strain name	Age (years)	Presumed infection source	Onset to first visit (day)	Drug[a]	Duration of fever[b] (day)
1	A/Niigata/18FS005/2019	7	School	1.2	BXM	2.0
2	A/Niigata/18FS011/2019	3	Family	0.5	OS	0.3
3	A/Chiba/18FS163_1/2019	6	School	0.5	BXM	1.1
4	A/Nagasaki/18FS252_1/2019	9	School	1.0	ZA	0.9

[a] BXM: baloxavir marboxil, OS: oseltamivir, ZA: zanamivir. All drugs were administered after sample collection.
[b] Duration of fever was defined from the first visit to the clinic to the last record of a temperature ≥37.5 °C.

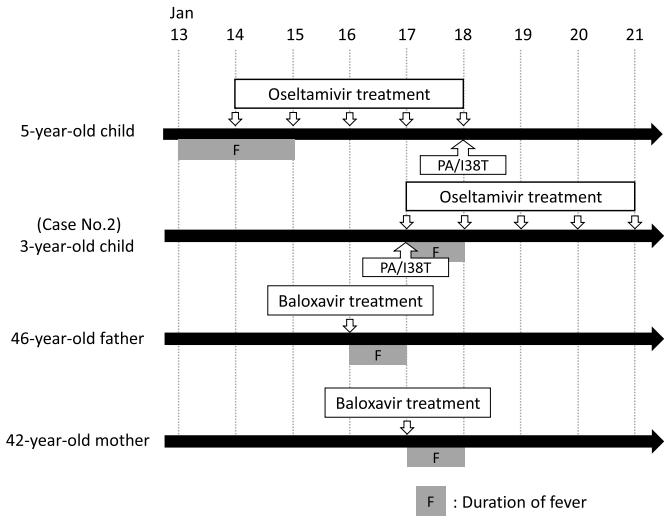

Fig. 2. Clinical timeline of case No. 2 infected with influenza A (H3N2) virus possessing PA/I38T without baloxavir treatment and that of his or her family.

human-to-human transmission of the PA/I38T virus sporadically occurs in A (H3N2) in closed environments.

4. Discussion

We developed a rapid screening method using cycling probe real-time PCR systems to detect influenza A (H1N1)pdm09 and A (H3N2) viruses possessing PA/I38T and investigated the PA/I38T substituted virus in the pre-treatment samples during the 2018/19 influenza season in Japan. Our results showed that this cycling probe real-time PCR system functioned well, and found four influenza A (H3N2) viruses possessing PA/I38T (4/229, 1.7%) in pre-treatment samples. In addition, our findings suggested that the transmission of PA/I38T viruses could occur sporadically in A (H3N2).

In this study, the cycling probe real-time PCR can detect at least 2.1 copies/reaction for PA/I38 and I38T, and differentiated well in a mixture condition with at least 90:10 or 10:90 for each primer/probe set using DNA plasmids. The other real-time PCR method, rhPCR, to discriminate PA/I38T viruses (Nakauchi et al.), was reported to have a detection limit of approximately 30 copies per reaction, which is 10 times lower than the limit of our method, using the synthesized RNA; nonetheless, they obtained better differentiation of mixed population, at least 5% of either wild type or PA/I38T in the mixture, compared to the 10% obtained in our cycling probe assay. These observations show that the results of our cycling probe assay are comparable with those of the rhPCR real-time PCR assay.

In addition, there was no discrepancy in discriminating influenza A (H1N1)pdm09 and A (H3N2) viruses possessing PA/I38T between the results of cycling probe real-time PCR and direct sequencing in clinical samples. Therefore, these cycling probe real-time PCR systems are useful and reliable to identify PA/I38T viruses from clinical samples. The cycling-PCR system is speedy and economical to detect SNPs compared to the standard Sanger method and next generation sequencing. Further, the cycling-PCR system is more beneficial than pyrosequencing as since the pyrosequencing assay equipment is not commonly available (Koszalka et al., 2019). A pitfall of the cycling probe real-time PCR is that the substitutions other than PA/I38T cannot be identified due to the design and mechanism of the PCR system. However, the treatment emergent variant viruses were largely reported to be PA/I38T following BXM administration. Ince et al. reported that 33.3% of viruses with substitutions in PA in A (H1N1)pdm09, and 89.2% in A (H3N2) were PA/I38T (Ince et al., 2020). We have recently reported that PA/I38T attributed to 50.0% (2/4) of treatment emergent PA substitutions in A (H1N1)pdm09, and 77.7% (7/9) in A/(H3N2) in a pediatric observational study (Saito et al., 2020). As expected, our PA/I38T specific probe (ROX) did not react with other substitutions such as PA/I38K, I38M, and E23K. However, PA/I38S viruses showed slight reaction with the ROX probe, suggesting that cross reaction occurred due to an unknown reason. To this end, our cycling probe assay showed higher sensitivity (97.4%) than the Sanger genetic sequencing method, and had an excellent match with the quantitative PCR with regards of to the Ct values corresponding to the RNA titer in the samples, suggesting that our cycling probe is useful for quick screening of PA/I38T in clinical samples, but further genetic analysis by Sanger sequencing, pyrosequencing or NGS is needed to determine other PA substitutions associated with reduced susceptibility to BXM.

Four cases of influenza A (H3N2) viruses carrying PA/I38T were detected in pre-treatment samples in this study. They were all under 10 years of age, and their presumed infection sources were either at school or from family. Takashita et al. detected 4 A (H3N2) viruses possessing PA/I38T isolated from children < 12 years of age without prior BXM treatment during the 2018–2019 season in Japan (Takashita et al., 2019a). They assumed that the children were infected with PA variant viruses acquired from other influenza patients who were previously treated with BXM. In addition, the past viruses deposit in the public genetic database showed that A (H3N2) viruses with PA/I38T had never been reported before BXM was launched (Gubareva et al., 2019; Takashita et al., 2020). They also reported family clusters of PA/I38T in A (H3N2) just like our family cluster (Takashita et al., 2019a, 2019c). Following their results, Takashita et al. recommended that close monitoring is needed for patients <12 years of age because the frequency of PA variants was higher than that in patients of 12–64 years of age. Interestingly, it may be a unique characteristic of human-to-human transmitted reduced susceptibility viruses that PA/I38T viruses in pre-treatment samples both in our and Takashita's studies showed genuine PA/I38T and not a mixture of wild type PA/I38 viruses (Takashita et al., 2019a). Takashita's group has further confirmed the sequence by NGS but we have not done this.

The clinical information of the two patients who possessed PA/I38T viruses prior to treatment and were subsequently treated with BXM suggested that there was no difference in fever duration between patients harboring PA/I38T and non-substituted I38 viruses. Recently our group demonstrated that the duration of fever for patients between PA/I38T and non-substituted viruses showed no statistical difference, on average 24.6 h (standard deviation, 28.2 h) and 25.7 h (standard deviation, 24.1 h), respectively < 19 years of age (Saito et al., 2020). Thus, the duration of fever of BXM treated patients with PA/I38T prior to treatment was within the range of 95% confidence interval in the previous study, showing no prolongation of fever was observed in this study. Meanwhile, the impact of influenza viruses harboring the PA/I38T substitution on the clinical effectiveness of BXM is still controversial as both positive and negative effects have been reported, and the evidence in this matter remains insufficient (Hayden et al., 2018; Hirotsu et al., 2020; Ikematsu et al., 2020; Ison et al., 2020; Sato et al., 2020; Uehara et al., 2020).

Interestingly, in pre-treatment samples we found no influenza A (H1N1)pdm09 viruses carrying PA/I38T. This may be attributed to the lower emergence of PA/I38X in A (H1N1)pdm09 compared to that of A (H3N2) viruses after BXM treatment (Hirotsu et al., 2020; Ikematsu et al., 2020; Sato et al., 2020; Uehara et al., 2020). It has been reported that PA/I38T viruses in influenza A (H1N1)pdm09 and A (H3N2) tend to retain a significant level of viral replication *in vitro* with retained fitness in animal models (Checkmahomed et al., 2020; Imai et al., 2020). However, a recent study showed mild *in vitro* attenuation of seasonal viruses carrying PA/I38T in A (H1N1)pdm09 and A (H3N2), while in a competitive growth experiment in ferrets inoculated with a mixed population, the same research group reported that the wild type virus presented a growth advantage over PA/I38T containing A (H3N2) virus, although this advantage was limited (Chesnokov et al., 2020). Anyway,

there is a possibility that the fitness of PA/I38X viruses may differ between the subtypes of influenza A in humans.

Our study has some limitations. In this study, we used plasmid DNA to evaluate the limit of detection and linearity for the real-time PCR instead of RNA. Nakauchi et al. have used cDNA synthesized from *in vitro*-transcribed PA gene as RNA controls to evaluate their RNase H2-dependent PCR (rhPCR), developed for screening of PA/I38T in A (H1N1)pdm09 and A (H3N2) viruses from clinical samples (Nakauchi et al., 2020). It is thus optimal to use cDNA synthesized from *in vitro* transcribed PA gene RNA from control DNA plasmids as in their study. In that sense, our real-time PCR method quantitates cDNA copies but not RNA copies. However, we used plasmid DNA as controls for routine examinations due to reasons of stability and ease. We further plan to verify our results with cDNA using *in vitro* transcribed PA gene RNA. Second, the sensitivity of our real-time PCR test against genetic sequences of known identity was not 100%. We applied nested PCR to analyse the sequence of PA gene by Sanger method, which probably amplified less than 10 copies of the target gene per reaction. Third, our ROX probe should detect only PA/I38T but it reacted with PA/I38S, this needs further investigation. Thus, a polymorphism at amino acid position 36 or 37 might affect the probe binding and give false results.

In conclusion, the prevalence of the community transmitted influenza A viruses possessing PA/I38T remained low in Japan during the 2018–2019 season and is almost the same as that of influenza A viruses conferring resistance to oseltamivir and peramivir (National Institute of Infectious Diseases Japan, 2019). Still there may have been limited human-to-human transmission cases in Japan; however, this might not have occurred widely as our study demonstrated a low frequency of influenza A viruses possessing PA/I38T mutation in the pre-treatment samples, suggesting that there is a low possibility of these mutant viruses spreading. Nevertheless, we should continue to monitor influenza viruses harboring PA/I38T as a surrogate marker for community prevalence from a public health standpoint. We are currently developing cycling probe real-time PCR systems to detect PA/I38T in the influenza B virus, Yamagata and Victoria lineages. Our cycling-probe PCR will thus contribute to the rapid surveillance of PA/I38T mutant viruses that may affect the treatment guidelines for influenza virus infections.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2021.105036.

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