DISCRIMINATION OF INFLUENZA A(H1N1)PDM09 VIRUS WITH H275Y MUTATION CONFERRING RESISTANCE TO OSELTAMIVIR BY DUPLEX REAL-TIME RT-PCR

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Abstract - Oseltamivir, a selective neuraminidase enzyme inhibitor, is one of the most important drugs for the treatment and prophylaxis of influenza. The oseltamivir resistance of seasonal H1N1 and A(H1N1)pdm09 influenza virus was mainly caused by a histidine (H) to tyrosine (Y) mutation at residue 275 of the neuraminidase (based on the N1 numbering). It is essential to develop a specific molecular method for the identification of H275Y mutation conferring resistance to oseltamivir. Here, a duplex real-time RT-PCR assay, using FAM and HEX dye labeled probes, was established the genotyping method to simultaneously determine the gene sequence coding for H or Y in the 275 site. The discriminative capacity of H275Y using this method was assayed by testing the armored RNA containing wild-typed or mutated target. Specificity was determined using the viral RNA from other influenza viruses. The method was as sensitive as the real-time method in detection of influenza A recommended by WHO. A total number of 923 A(H1N1)pdm09 positive samples collected from May, 2009 to Dec, 2018 were used for the oseltamivir resistance virus screening and one sample with mixed wildtype and mutated viruses was detected, the genotype ratio of oseltamivir resistant and sensitive viruses was 3.5:1.

Keywords - Pandemic Influenza A (H1N1); Oseltamivir Resistance; H275Y; Real-Time RT-PCR

I. INTRODUCTION

Epidemic influenza and occasional pandemic influenza are caused by influenza viruses. Oseltamivir, a neuraminidase inhibitor of the influenza viruses A and B, was introduced into clinical practice between 1999 and 2002 [1]. Administration of oseltamivir following symptom onset reduces the risk of complications and can also improve clinical outcome in patients with severe disease. It is one of the most important drugs for the treatment and prophylaxis of influenza and stockpiling of this drug is part of pandemic-preparedness plans [2]. For the seasonal influenza A (H1N1) virus, the 2007-2008 influenza season marked a significant and sudden increase in resistance to oseltamivir [3, 4]. The proportion of oseltamivir resistant viruses in seasonal influenza A(H1N1) was up to 100% during 2008-2009 in some areas and countries [5, 6]. All of these influenza viruses were found to have a same mutation in neuraminidase (NA) at residue 275 in N1 (residue 274 according to the N2 numbering method), in which a histidine was replaced with a tyrosine to confer resistance to oseltamivir [7].

The 2009 pandemic influenza is a global outbreak of a new strain of H1N1 influenza virus which contains a combination of genes from swine, avian and human influenza viruses [8]. Surveillance of the A(H1N1) pandemic influenza continues to identify viruses that show resistance to oseltamivir. On Aug 2010, WHO announced that the pandemic A(H1N1) influenza has moved into the post-pandemic period. Just before the ending of pandemic, 302 cases of oseltamivir resistant influenza viruses A(H1N1)pdm09 were reported by WHO. Except one sample from an immunocompromised child with reduced susceptibility to oseltamivir due to an amino-acid mutation at position 223 in the neuraminidase, all the other viruses have the H275Y substitution [9].

Based on knowledge about past pandemics, the A(H1N1)pdm09 virus is expected to continue to circulate as a seasonal virus for some years to come [10]. The oseltamivir resistance makes it important for developing a rapid and reliable detection method for the monitoring of antiviral resistant variants. Currently, the gold standard method for antiviral resistance screening is phenotypic resistance assay [11, 12], however, it is time consuming, labor intensive and quite expensive, which includes viral culturing. In the past few years, several detection methods were established in genotypic analyses through the application of molecular technology, such as single nucleotide polymorphism (SNP) genotyping and the rolling circle amplification (RCA) [13]. Sanger dideoxy sequencing or pyrosequencing-based assay [14] of the *NA* gene can be used as reference methods to detect H275Y mutation conferring oseltamivir resistance; however, reverse-transcription PCR and subsequent purification, sequencing reactions and also sequence analyses are needed by using the two methods [15]. It is also time-consuming and labor intensive and increases the risk of cross contamination. van der Vries et al. evaluated locked nucleic acid (LNA) fluorescent probes based real-time RT-PCR for the H275Y discrimination assay and no oseltamivir resistant virus was detected by screening of 121 pandemic H1N1 positive samples [7]. Hindiyeh et al. reported the development of a real-time RT-PCR method for screening of H275Y oseltamivir resistance mutation and no H275Y mutation was detected in 120 pandemic H1N1 positive samples [16]. More recently, two approaches

using high-resolution melting to identification and quantification of H275Y mutant in influenza virus A/H1N1pdm09 were developed to discriminate mutants by different melting temperature with low detection cost [17, 18]. Here, we report a one-step duplex real-time RT-PCR method that can be used to monitor the genetic mutation of H275Y in the *NA* gene. Using this method, the oseltamivir susceptible virus strain will produce HEX fluorescence, while the oseltamivir resistant virus will produce FAM fluorescence. The detection specificity and sensitivity were evaluated, and the method was also applied in the screening of H275Y mutation in 923 samples collected from May 2009 to Dec, 2018.

II. MATERIALS AND METHODS

Virus isolates, samples and viral RNA extraction

15 A(H1N1)pdm09 viruses isolated by culturing of clinical samples (collected from May to Aug, 2009) in MDCK cells and a total of 908 influenza A(H1N1)pdm09 samples (collected from Sep, 2009 to Dec, 2018), were used in this study. All the samples above were A(H1N1)pdm09 positive confirmed by real-time RT-PCR following the diagnostic instructions of WHO [19] and a real-time RT-PCR detection kit co-designed by our laboratory [20]. Other influenza virus isolates, including seasonal H1N1, H3N2 and influenza B, H5N1 high pathogenic avian influenza virus from human case and an avian-derived H9N2 were used for the evaluation of assay specificity of the duplex real-time RT-PCR. Viral RNA was extracted using Qiagen RNeasy Mini Kit following the manufacturer's instruction. DNA purification kit and *Pfu* DNA polymerase were purchased from Promega (Madison, WI). Roche Titan One Tube RT-PCR Kit was used for amplifying the ORF region of *NA* gene. TaKaRa One Step PrimeScript RT-PCR (Perfect Real-time) kit (Dalian, China) was used for real-time detection on AB 7500 real-time PCR system.

Design of primers and probes

Based on the *NA* gene sequences from GenBank and the alignment result, primers and TaqMan minor groove binding (MGB) probes for allelic discrimination were designed using the Primer Express 3.0 software to distinguish CAC or TAC corresponding to the position 823 to 825 of *NA* ORF. HEX (hexachloro-6-carboxyfluorescein) fluorescence was labeled at 5' end of probe 1 to detect the sequence of oseltamivir-sensitive virus; FAM (6-carboxy-fluorescein) fluorescence was labeled at 5' end of probe 2 to detect the oseltamivir-resistant virus. Non-fluorescent quencher and MGB were labeled at 3' end of each probe. The sequences of primers and probes were as follows:

PN1H275Y-F: AGGCCTCATACAAGATCTTCAGAATA;
PN1H275Y-R: AAGACACCCACGGTCGATTC;
PN1H275-Pb1: HEX-ATGCCCCTAATTATCACTA-MGBNFQ;
PN1Y275-Pb2: FAM-AATGCCCCTAATTATTACTA-MGBNFQ.

The point mutation was positioned 7 bases from the 3' end in the probe (indicated by underlined character). The amplified fragment was 168 base pairs in length. Primers and probes were synthesized by Shanghai Chaoshi BioTechnologies Co. Ltd.

Production of armored RNA for wild-type or mutated variant

To produce the armored RNA templates, two plasmids, which contain the sequence coding for wild-type (H275) or resistant variant (Y275), were constructed. The fragment containing H275 coding sequence was amplified from a clinical sample using primers F-B1 (GGATCCAGGCCTCATACAAGATCTTCAGAATA) and R-H3 (AAGCTTAAGACACCCACGGTCGATTC). The amplified fragment was cloned into pGEM-T easy vector and sequenced by M13 primer, and then the sequence confirmed plasmid was designated as Teasy-H275. The mutated target was completed by PCR-mediated overlap extension method [21]. Primers F-B1 and 275Rm (GCATTCCTCATAGTAATAATTAGGG), 275Y-Fm (CCCTAATTATTACTATGAGGAATGC) and R-H3 were employed to generate intermediate PCR products using Teasy-H275 as template in the first round PCR using *Pfu* DNA polymerase, then the two products were purified and denatured as template for the second round PCR in which F-B1 and R-H3 were used for amplification. Final product was inserted into pGEM-T easy vector and sequenced to ensure the presence of the desired mutation. The sequence confirmed plasmid, containing mutated target was named Teasy-Y275. Armored RNA was produced according to the method described by Pasloske and Zhao [22, 23]. Briefly, the wild-type and mutated targets were separated from plasmids, Teasy-H275 and Teasy-Y275, by *Bam*HI and *Hin*dIII digestion, then subcloned into the same sites of pET28a-MS2 vector and transformed into competent *E. coli* DH10B cells. DNA of the resultant pET28a-MS2-H275 and pET28a-MS2-Y275 plasmids were transformed into the competent *E. coli* BL21 (DE3) strain for expressing, respectively. Single colony was inoculated into 3 ml LB medium and allowed to grow at 37 °C overnight. 100 µl inoculant was transferred into fresh LB and allowed to grow for about 3 h, and then IPTG (final concentration of 1.0 mM) was added to induce expressing for about 16 h at 37 °C. Based on the OD600 value, equivalent number of cells were pelleted and lysed by sonication, the supernatant was incubated with RNase A and DNase I at 37 °C for 4 h. RNA was extracted from the nuclease treated samples using Qiagen RNeasy Mini Kit.

Determination of specificity of the real-time RT-PCR

For the specificity of discrimination of H275 and Y275, 10^4-fold diluted armored RNA of H275 or Y275 genotype was used as template in the specificity assays. The mixture of 10^4-fold diluted RNA of both genotypes was also included to evaluate the performance of duplex real-time RT-PCR reaction to detect a possible heterozygote. The nucleic acids from other influenza viruses, including seasonal influenza H1N1, H3N2 and influenza B, H5N1 high pathogenic avian influenza virus from human case and avian-derived H9N2, were used for the evaluation of assay specificity. RNA was extracted from nuclease treated armored RNA or infected MDCK cells of each virus, then 5 µl RNA was used in real-time RT-PCR reaction, the final concentration of each primer was 0.4 µM and 0.2 µM for each probe, ROX dye was added in reaction as passive reference fluorescence, 12.5 µl of 2×One Step RT-PCR Buffer, 0.5 µl TaKaRa Ex Taq™ HS and 0.5 µl PrimeScript™ RT Enzyme Mix were added following manufacturer's instruction. DEPC-treated water was added to obtain 25 µl of reaction volume. The thermal profile was as follows: 42 °C reverse transcription for 15 min, an initial denaturation for 1 min at 95 °C and 40 cycles of amplification including 5 s of denaturation at 95 °C, 40 s of annealing and extension at 57 °C, fluorescence collection in FAM and VIC (equivalent to HEX fluorescence collection in ABI 7500 system) channels was included in the annealing and extension step of each cycle.

Evaluation of detection sensitivity of the duplex real-time RT-PCR

Relative sensitivity of the duplex real-time RT-PCR was compared in parallel with the sensitivity of real-time method for detection of influenza A (targeting the *matrix* gene) recommended by WHO [19] and the real-time method for detection of A(H1N1)pdm09 (targeting the *hemagglutinin* gene) [20]. Ten-fold serial dilutions of viral RNA extracted from MDCK isolated A(H1N1)pdm09 virus were employed as template. Reaction condition and thermal program were the same with the specificity assays, reactions for each diluted RNA contained 3 repeats.

Evaluation of oseltamivir resistant virus detection ability in a mixed population

Compare with the oseltamivir sensitive viruses, the proportion of oseltamivir resistant viruses are very small. To evaluate the detection ability for oseltamivir resistant virus in a possible mixed population of Y275 among H275, 10^9-fold diluted armored RNA of Y275 was included in every mixed sample, and then different amount of armored RNA of H275 was added to obtain allele ratios of H:Y to 1%, 2%, 3%, 4%, 5%, 10%, 20% and 50%, respectively, the control reaction without Y275 template was also included. Reaction condition and thermal program were the same with the specificity assays, reactions for each diluted RNA contained 3 repeats.

Application in sample screening

Oropharyngeal washes or swabs were collected from influenza-like illness (ILI) patients or patients with lower respiratory tract infections including pneumonia and severe acute respiratory illness (SARI) from Sep, 2009 to Dec, 2018. All A(H1N1)pdm09 influenza viruses positive samples were subjected to the screening of H275Y mutation. Besides the amplification step to generate real-time data, the scatter plot could also be obtained by the AB 7500 system by adding pre-read and post-read steps at 60 °C for 1 min before and after the amplification step. The software plotted the results of the allelic discrimination run on a scatter plot of Allele X versus Allele Y by variation of differences in the extent of reporter dye fluorescent intensity before and after PCR amplification.

III. RESULTS

Production of armored RNA as templates for real-time detection standards

The wild-type (H275) amplified from a clinical sample and the oseltamivir resistant variant (Y275) generated by PCR-mediated overlap extension were sequence confirmed for further use in the armored RNA technology. After induction, the target RNA of wild-type and resistant variant was encapsidated by the expressed MS2 bacteriophage coat protein which was highly resistant to ribonuclease digestion. After nuclease treatment and appropriate dilution, the recombinant target RNA in its packaged form generated ideal standards for the discrimination of H275Y mutation in the *NA* gene of A(H1N1)pdm09 influenza virus.

Specificity of the real-time detection

The specificity of the method was tested for cross-reactions with other respiratory pathogens. Viruses, which have been tested positive for seasonal H1N1, H3N2 and influenza B, H5N1 high pathogenic avian influenza virus from human case and avian-derived H9N2, were used for the cross reactivity analysis. The results were shown in Fig. 1. No cross-reaction was observed among other influenza viruses detected. FAM or HEX fluorescence increasing was also not detected in the no template (negative) control. For the detection using armored RNA, increasing of HEX fluorescence was only observed from H275 genotype and increasing of FAM fluorescence was only observed from Y275 genotype. For the mixture of H275 and Y275, increasing from both fluorescences was detected in one-tube. This result suggested that the duplex TaqMan-MGB real-time RT-PCR could discriminate H275Y of the pandemic A (H1N1)

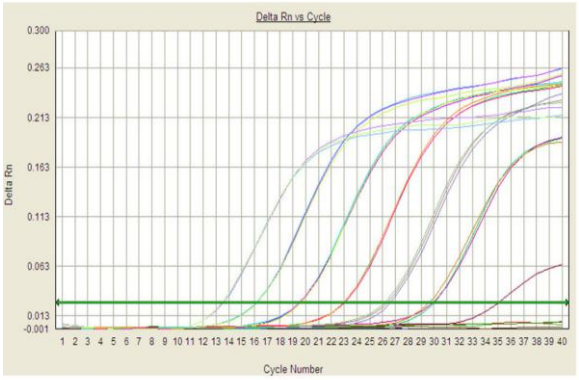
influenza virus with high specificity.



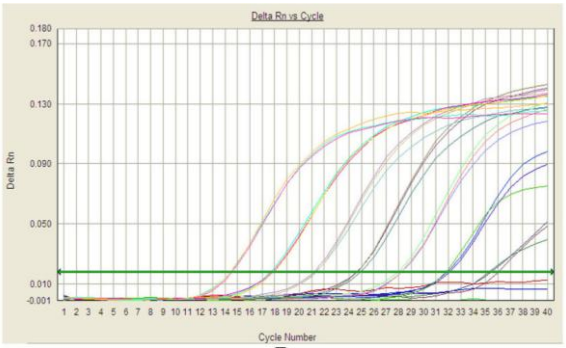
Fig. 1. Determination of reaction specificity using the duplex real-time RT-PCR.

Detection sensitivity of the duplex real-time RT-PCR

Serial 10-fold dilutions of viral RNA extracted from MDCK isolated A(H1N1)pdm09 were used in this experiment. The relative detection sensitivity was evaluated by comparing the sensitivity of real-time method recommended by WHO and the real-time method for detection of A(H1N1)pdm09. The detection limit was determined by positive amplification in all 3 independent repeat reactions. As shown in Fig. 2, using the same serially diluted template, the real-time detection for A(H1N1) pdm09 targeting the *hemagglutinin* gene gave the best sensitivity, the duplex real-time for discrimination of H275Y gave the same sensitivity as the method of detection of influenza A targeting the *matrix* gene recommended by WHO.



A



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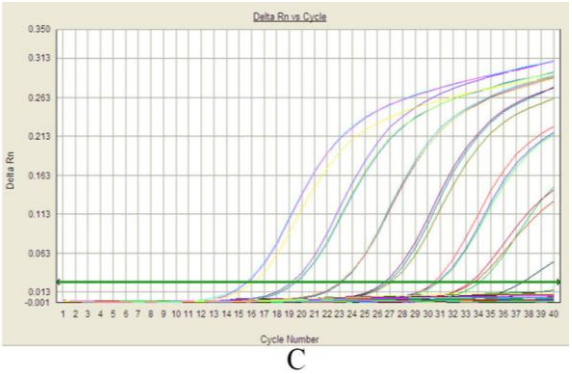

C

Fig. 2. Determination of the sensitivity of the duplex real-time RT-PCR detection.
(A), WHO's real-time method for detection of influenza A (targeting *M* gene); (B), Real-time method for detection of A(H1N1)pdm09 (targeting *HA* gene); (C), duplex real-time RT-PCR for genotype discrimination of H275Y mutation of A(H1N1)pdm09 (targeting *NA* gene).

Evaluation of oseltamivir resistant virus detection ability in a mixed population

By using the duplex real-time RT-PCR method, the detection ability could be clearly figured out by plotting the amplification curves using logarithmic scale. The amplification was dominated by the armored RNA template from H275 when only 0.5% or 1% Y275 was presented in a mixed population. With the increasing of Y275 template in allele ratio to 2% or more, the fluorescence from amplification of Y275 could be detected in these reactions.

Screening of patient samples using the method

RNA was extracted from a total of 923 samples as template for the detecting of H275Y mutation. Amplification curve showed that HEX fluorescence was positive in samples and the FAM fluorescence was positive only in one sample. By using the end-point assay, the post-read run automatically subtracted the baseline fluorescence determined during the pre-read run process, a scatter plot was generated by variation of reporter dye fluorescent intensity. As shown in Fig. 3, the dots represented viruses with H275 genotype, the triangle represented heterozygotes sample of Haishu-110 which gave increase of both FAM and HEX fluorescences.

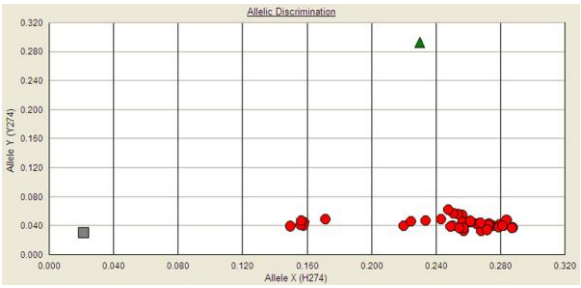

Fig. 3. Allelic discrimination for genotyping of H275Y in A(H1N1)pdm09 samples.

RNA from A(H1N1)pdm09 positive samples was used as template for the detecting of H275Y mutation and scatter plot was plotted for genotype calling of

each sample distinctly. ●: H275 positive sample with increasing of HEX fluorescence; ▲: A both H275 and Y275 positive sample with increasing of FAM and HEX fluorescence; ■: no template control.

This sample was collected at Jan 4, 2010 from a 29-year-old immunocompetent female outpatient with mild symptom. She did not receive oseltamivir prophylaxis and got infected without any traveling history before and recovered uneventfully from mild symptoms after taking some Chinese traditional patent medicines. For the confirmation of H275Y mutation, the *NA* gene was amplified using two primers as follows, NA-F: GACCAGCAAAAGCAGGAGTT and NA-R: AGTAGAAACAAGGAGTTTTTTGAAC. The 1.4 kb amplified fragment was cloned into pGEM-T easy vector, by blue-white screening, 69 white colonies were discriminated by the real-time PCR method, 15 were H275 positive and 54 were Y275 positive. 5 H275 positive and 5 Y275 positive colonies were selected randomly and cultured overnight and further sequenced by M13F and M13R primers, all the sequencing results were consistent with the real-time PCR method. The sample from this patient had mixed wildtype and mutated viruses and the genotype ratio of oseltamivir resistant and sensitive viruses was about 3.5:1. The *NA* gene sequence with H275Y mutation was deposited in GenBank database under accession number HM145748.

IV. DISCUSSION

In Apr 2009, infections caused by a novel influenza A(H1N1)pdm09 virus were reported in Mexico and USA and the most countries and overseas territories or communities have reported laboratory confirmed cases of this pandemic influenza H1N1 [9]. Since localized outbreaks are likely to continue in the post-pandemic period, WHO encouraged to detect the genetic markers for oseltamivir resistance of the A(H1N1)pdm09 virus. Current methods for monitoring drug resistance include phenotypic and genotypic analyses of neuraminidase or *NA* gene [11], measure of HA titers in presence of drug [24], detection of adamantine resistance using microarray [25], and some molecular detection methods [7, 26, 27]. Here, we developed a duplex real-time RT-PCR method for genotyping the H275Y mutation conferring oseltamivir resistance in the A(H1N1)pdm09 virus. The one-step real-time RT-PCR reaction used two TaqMan-MGB probes specific for both resistance and sensitive viruses and reacted in one tube. MGB molecule forms a stable complex with the minor groove of single-stranded DNA and then effectively raise the melting temperature of probes, allowing use of shorter probes [28]. A single base mismatch between the template and the MGB probe results more destabilization than non-MGB TaqMan probe and makes it more accurate in mutation detection. Real-time PCR method makes it possible to detect the nucleic acids in the PCR reaction without opening of the reaction vessels and post-PCR processing steps, which eliminates the risk of product contamination. However, the TaqMan probe based real-time PCR method can only detect known mutations and only discriminate one site mutation in a single reaction. Any other point mutation conferring oseltamivir resistance in A(H1N1)pdm09 needs for further analyzing.

The presence of two different dye-labeled probes in the reaction allows genotyping of the wild-type and possible variant in one-tube. HEX-labeled probe is a perfect match to the wild type (H275) and the FAM-labeled probe is a perfect match to the mutated sequence (Y275), during the real-time PCR reaction, the HEX positive while FAM negative amplification indicated the virus is sensitive to oseltamivir, otherwise it is oseltamivir resistance. The reaction can be carried out directly from clinical samples like throat swabs or oropharyngeal washes, without the propagation or culture of virus, to obtain a rapid and high-throughput genotyping of H275Y mutation.

The use of armored RNA facilitates the determination of the assay specificity and also can be used in sensitivity evaluation or quantitative purpose. The armored target RNA is ideal for use as detection standard or positive controls because of its high resistance to ribonuclease digestion. The specificity of the method was tested for cross-reactions with other influenza viral pathogens and the result implied that there was no amplification from the control influenza viruses tested. The relative detection sensitivity of the duplex real-time for discrimination of H275Y was the same as the method of real-time detection of influenza A recommended by WHO.

To date, a very small proportion of A(H1N1)pdm09 influenza virus strains are oseltamivir resistant by H275Y mutation in the neuraminidase protein and the majority of mutant viruses were generated as a result of drug treatment. However, many individuals infected with resistant strains had not received antivirals. No H275Y oseltamivir resistant mutant virus was screened from 121 and 120 A(H1N1)pdm09 samples also suggested that infection with H275Y mutated virus was apparently still a rare event at this time [7, 16]. In our monitoring of 923 samples, one sample with mixed population of oseltamivir resistant and sensitive viruses was recorded. The H275Y resistance virus had a ratio of 0.108% in populations without drug treatment or prophylaxis, it was comparable to the result in which H275Y mutation of oseltamivir-resistant virus was detected in about 0.127% (2 in 1570 cases) in the absence of oseltamivir drug treatment patients [14]. According to the WHO's report, the risk of resistance is considered higher in patients with severely compromised or suppressed immune systems, and the risk is also considered higher in people who have

received oseltamivir treatment or in people who receive oseltamivir for so-called "post-exposure prophylaxis" following exposure to another person with influenza.

A(H1N1)pdm09 virus has been circulating in humans since 2009 and now becomes a seasonal human influenza virus. In most countries in the northern temperate regions, influenza activity continues to increase this year. Many countries of North America, Europe, north Africa, eastern Mediterranean and temperate Asia have reported increasing influenza activity recently and Influenza A(H1N1)pdm09, A(H3N2) and B viruses co-circulated some regions. The newly identified A(H1N1)pdm09 still remains sensitivity to oseltamivir at present, but systematic surveillance of the spread of viruses with H275Y mutation is very important for the patient treatment, control of flu epidemics or to conduct a retrospective epidemiological survey.

V. CONCLUSIONS

The duplex real-time RT-PCR assay was developed for rapid and sensitive detection of H275Y mutation within the *neuraminidase* gene of A(H1N1)pdm09 virus and was useful in rapid screening or monitoring this mutation conferring oseltamivir resistance. The detection method could be used to screen not only culture stocks but also large numbers of clinical samples rapidly. Since the advantages of single-step, closed-tube detection and low cost, the assay could be helpful in the surveillance of H275Y mutation of A(H1N1)pdm09 influenza viruses.

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DISCLOSURE POLICY

The authors declare no conflicts of interest.

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