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Detection of oseltamivir sensitive/resistant strains of pandemic influenza A virus (H1N1) from patients admitted to hospitals in Thailand

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Abstract

Oseltamivir has been used widely for prophylaxis or treatment during outbreaks of the pandemic influenza virus (H1N1) in several countries. The aim of this study was to develop a real-time RT-PCR (reverse transcription-polymerase chain reaction) to be applied for detection and monitoring of the oseltamivir resistant strains of this virus during three outbreaks (May, 2009 to October, 2010) in Thailand. The real-time RT-PCR assay for detecting H275Y proved highly specific for the pandemic influenza virus (H1N1) as no cross-amplification was detected with other respiratory viruses or human total RNA. The assay was also highly sensitive with a detection limit as low as 100 copies/μL for both wild-type and resistant strains. The performance of the assay was evaluated in terms of amplification efficiency (100%). The results obtained by real-time RT-PCR were in complete agreement with direct nucleotide sequencing. However, real-time RT-PCR provided more detail on the relative quantities of ratios between resistant and sensitive strains in each individual. The results revealed that four of 1,288 (0.31%) patients were infected with the oseltamivir resistant strain. The number of patients infected by resistant strains was higher during the third (0.61%) and second (0.24%) waves than during the first (0%) outbreak. In conclusion, the real-time RT-PCR assay for H275Y detection is advantageous because it is specific, sensitive, and provides quantitative data. And it would be useful for large-scale testing and monitoring of oseltamivir resistant strains of the pandemic influenza A virus (H1N1).

Keywords

oseltamivir; pandemic influenza; H1N1; detection

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1. Introduction

The human pandemic influenza A virus (H1N1) continues to be a major public health problem worldwide (Dawood et al., 2009). A large number of patients infected by the virus have been identified. The mortality rate in the general population has been relatively low when compared with that of previous influenza pandemics (Donaldson et al, 2009; Kamigaki and Oshitani, 2009; Vaillant, 2009). In contrast, certain populations including pregnant women, children or morbid obese patients run a high risk of developing severe complications from the pandemic influenza virus (H1N1) infection and have a higher mortality rate than that observed in the general population (Louie et al., 2009; Jamieson et al., 2009; Sachedina and Donaldson, 2010; Morgan et al., 2010).

The recommended treatment for the pandemic influenza virus (H1N1) relies virtually on neuraminidase inhibitors such as oseltamivir (Hersh and Stafford, 2011). The emergence of oseltamivir-resistant strains of the pandemic influenza virus (H1N1) causes significant concern among health care providers all over the world as resistant virus has been isolated from several countries recently (Leung et al., 2009; Le et al., 2010). Most of the oseltamivir-resistant strains harbor the [H275Y] (N1 numbering system) genetic mutation (Histidine to Tyrosine at position 275) in the neuraminidase gene which confers a significant reduction in their sensitivity to oseltamivir. A previous study revealed that oseltamivir-resistant strains were comparable to their oseltamivir-sensitive counterparts in their transmissibility and pathogenicity in animal models (Kiso et al., 2010). The clinical importance and impact of the oseltamivir-resistant strains have yet to be evaluated. However, a number of reports focusing on immunocompromised patients have shown a high mortality rate and unfavorable clinical outcomes when these patients were infected by oseltamivir-resistant strains of the pandemic influenza virus (H1N1) (Antón et al., 2010; Harvala et al., 2010).

Since the first identification of the pandemic influenza virus (H1N1) in April of 2009 (CDC, 2009), Thailand has experienced 3 waves of pandemic influenza virus (H1N1) outbreak including (1) July to December 2009, (2) January to April, 2010 and (3) May to October, 2010. However, oseltamivir-resistant strains of the pandemic influenza virus (H1N1) have not been identified in Thailand previously. In this study, a technique based on real-time RT-PCR for H275Y detection was developed in order to scrutinize oseltamivir resistance of the pandemic influenza virus (H1N1) in Thailand. The demographic data and clinical characteristics of the patients from whom the oseltamivir-resistant strains were isolated are shown along with their clinical outcomes.

2. Materials and Methods

2.1 Patients and specimen collection

Nasopharyngeal (NP) samples were collected from Thai patients with influenza-like illness (fever, headache, sore throat and cough) who had been admitted to Bangpakok Hospital Network (Bangpakok 1, 3, 8 and 9), Chulalongkorn Memorial Hospital (Bangkok), Smitvej Hospital (Bangkok), Chumpare Hospital (Khon Kaen) and Thungsong Hospital (Nakhon Srithamrat). Data were collected in relation to age, sex and clinical symptoms. The samples were collected in 2 mL of virus transport medium with antibiotics (Penicillin G 2×10^6 U/liter and Streptomycin 200 mg/liter) and stored in a biohazard ice box for transportation. All specimens were processed immediately upon arrival. RNA extraction was performed using the Viral Nucleic Acid Extraction Kit (RBC Bioscience Co, Taipei, Taiwan) in a Bio-safety Level 2 plus (BSL2+) laboratory. All investigations were performed at the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University. Demographic and clinical data of every patient were obtained from medical records and kept under an assigned code. The study protocol was approved by the Institutional Review Board

of the Faculty of Medicine, Chulalongkorn University for using anonymous specimens for further investigation upon permission by the director of Chulalongkorn Hospital.

2.2 Detection of the human pandemic influenza A virus (H1N1)

The HA primers and probes used for the detection of novel 2009 H1N1 influenza A virus were modified previously from those described by CDC (WHO, 2009).

2.3 Primers and probes for oseltamivir resistance detection

Real-time RT-PCR based on specific locked nucleic acid (LNA)-TaqMan probes was developed for detection of oseltamivir-resistant strains. The nucleotide sequences (N>200) of the neuraminidase gene of the pandemic influenza virus (H1N1) were taken from the Influenza Virus Resource at NCBI (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) and EpiFluDB at GISAID (<http://platform.gisaid.org/>). The alignments were performed using BioEdit sequence alignment Software version 7.0 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). LNA TaqMan probes were selected from among nucleotides covering the potentially drug resistant region (H275Y) and designed to be specific for both wild type and mutant strains. The wild type (H) and mutant (Y) LNA Taq-Man probes were labeled with FAM and JOE with emission wavelengths at 530 and 548 nm, respectively. Primers and probes were analyzed using the primer design software (OLIGOS Version 9.1 by Ruslan Kalendar, Institute of Biotechnology, University of Helsinki, Finland) and Exiqon Tm prediction 1.1 (<http://lnatm.com/>). The primers and probes used in this study are shown in Table 1.

2.4 Site directed mutagenesis

RNA extracted from a sample identified previously as the pandemic influenza virus (H1N1) [A/Thailand/CU-H340/2009(H1N1), accession no. GQ866938] was used as a template for mutagenesis. The primers shown in Table 1 were used to generate the mutation at amino acid position 275 of the neuraminidase N1 gene which served as a control for oseltamivir resistant strains. Briefly, the primary mutagenesis based on PCR amplification consisted of reaction 1, using the FluA_N1_F671/ PanFlu_275Ymt_R primer pair and reaction 2, utilizing the PanFlu_275Ymt_F/ FluA_N1_R889 primer pair. After the first round of amplification, the PCR products obtained from reaction 1 and reaction 2 were combined and then used as a template for secondary mutagenesis based on PCR amplification with the FluA_N1_F671/ FluA_N1_R889 primer pair. Both primary and secondary mutagenesis reaction mixtures comprised 0.5 µl of DNA template, 0.5 µM each of forward primer and reverse primer, 10 µl of 2.5× MasterMix (Eppendorf, Hamburg, Germany) and nuclease-free water to a final volume of 25 µl. Both amplification processes were performed in a Mastercycler personal (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 94°C for 2 min followed by 35 amplification cycles consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 52°C, 1 min of extension at 72°C and concluded by 7 min of final extension at 72°C.

2.5 Construction of positive controls

PCR products obtained from amplification of the oseltamivir sensitive strain (wild type A/Thailand/CU-H340/2009) and oseltamivir resistant strain (after mutagenesis) were separated by 2% agarose gel electrophoresis and purified using the Perfect Prep Gel Cleanup Kit (Eppendorf, Hamburg, Germany). These purified products were then inserted into the pGEM-T Easy Vector System (Promega, Madison, WI) by TA-cloning strategy according to the manufacturer's specification. The recombinant plasmid was introduced into competent cells (*E. coli* strain DH5α) via heat shock (42°C for 45 sec) transformation. The positive colonies were cultured in 2mL of LB broth containing 100 µg/mL of ampicillin by

overnight incubation at 37°C. The plasmids were extracted and purified using the Fast Plasmid Mini Kit (Eppendorf, Hamburg, Germany) following the manufacturer's recommendation. Both plasmids were confirmed by nucleotide sequencing to ensure the correct target sequences and used as a template for *in vitro* transcription. *In vitro* transcribed RNA was generated by using the RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI) following the recommended protocol. The resulting RNA was extracted with phenol/chloroform followed by ethanol precipitation. These RNAs were used as positive controls for optimization of the real-time RT-PCR assay.

2.6 Optimized condition for real-time RT-PCR detection

The real-time RT-PCR reaction was performed using the SuperScript III Platinum One-Step RT-PCR system (Invitrogen, Carlsbad, CA) in a Rotor Gene RG-3000 (Corbett Research, New South Wales, Australia). The conditions were tested and optimized in terms of primer/probe concentrations, magnesium concentration and thermal profile. The optimized real-time RT-PCR reaction mixture comprised 3 µL of RNA, 0.75 µM final concentration of each primer, 0.25 µM final concentration of each probe, 5 µL of 2× reaction buffer (Invitrogen, Carlsbad, CA), additional 2.5mM MgSO₄, 0.2 µL of SuperScript III RT Platinum® Taq Mix (Invitrogen, Carlsbad, CA) and DPCE-treated water to a final volume of 10 µL. The optimized thermal profile included a reverse transcription step at 50°C for 45 min. After an initial denaturation step at 95°C for 10 min, amplification was performed during 50 cycles including denaturation (95°C for 15 sec) and annealing/extension (60°C for 40 sec). Fluorescent signals (FAM and JOE) were obtained once per cycle upon completion of the extension step. Data acquired by the real-time RT-PCR assay was analyzed using the Rotor-Gene data analysis software, Version 6.0 (Corbett research, New South Wales, Australia).

2.7 Limit of detection and specificity test

The concentration of the *in vitro* transcribed RNAs was calculated by measuring A₂₆₀. The copy number of transcript was calculated by the formula: Numbers of transcript (copy number/µl) = [RNA concentration (g/µl) × 6.02 × 10²³] / [length of *in vitro* transcribed RNA (bp) × 340]. RNAs were then serially diluted 10-fold (ranging from 10⁸ to 10 copies/µl) for the sensitivity test. The specificity of the real-time RT-PCR assay was evaluated by cross-reaction tests with RNA extracted from nasopharyngeal suction containing H1N1 seasonal influenza A virus [n = 10], H3N2 seasonal influenza A virus [n=10] and H5N1 avian influenza A virus [n = 10], influenza B virus [n = 10], parainfluenza virus [n = 3], respiratory syncytial virus [n = 3], human rhinovirus [n = 3] and human metapneumovirus [n = 3] as well as human total RNA [n = 10].

3. Results

3.1 Interpretation of the real-time RT-PCR for oseltamivir resistance detection

The result of oseltamivir resistance detection based on real-time RT-PCR was obtained by using two LNA TaqMan probes labeled with the FAM and JOE fluorescent signal for oseltamivir sensitivity and oseltamivir resistance, respectively. The fluorescent signal from real-time RT-PCR can be interpreted as shown in Figures 1A and 1B. A sample containing only the oseltamivir sensitive strain will emit the fluorescent signal entirely via the FAM channel (530 nm) whereas a sample containing the oseltamivir-resistant strain with a nucleotide substitution at position 275 of the neuraminidase gene will emit the fluorescent signal in the JOE channel (548 nm). In addition, a sample containing mixed populations of sensitive and resistant strains will emit both FAM and JOE fluorescent signals.

3.2 Specificity test

To test the specificity of this technique, the assay was evaluated by cross-amplification tests with RNA extracted from nasopharyngeal suction specimens containing other subtypes of influenza A virus or other respiratory virus. The result revealed that no cross-amplification was detected with any of these specimens (data not shown) indicating that the technique is highly specific for the detection of the neuraminidase gene of the pandemic influenza virus (H1N1).

3.3 Limit of detection and real-time RT-PCR performance

The results obtained from 3 independent experiments indicated clearly that the detection limit was as low as 100 copies / μ L for both oseltamivir sensitive and oseltamivir resistant strains (Figures 1C and 1D). With the standard curves obtained from this study, the slopes were -3.32 (100% efficiency) and R^2 values were 0.999 for both oseltamivir sensitivity and oseltamivir resistance indicating excellent amplification efficiency and overall performance of the real-time RT-PCR assay.

3.4 Detection of oseltamivir resistant strains from patients in Thailand

From April, 2009 to October, 2010, three waves of the outbreak were observed in Thailand. Totally, 1,288 specimens were found positive for the pandemic influenza virus (H1N1). All positive specimens were further tested to identify the oseltamivir-resistant strain by our in-house real-time RT-PCR assay. The number of patients confirmed in each period in addition to the number and percentage of patients with oseltamivir resistant strains of the pandemic influenza virus (H1N1) are summarized in Figure 2. No oseltamivir resistant strain (0%) has been found during the first wave of outbreak (April-December, 2009) in Thailand. Subsequently, the numbers of oseltamivir resistant strains were 0.24% and 0.61%, respectively during the second wave (January-April, 2010) and third wave (May-October, 2010). Totally, 4 of the 1,288 specimens (0.31%) were positive for oseltamivir resistant strains as detected by real-time RT-PCR and further confirmed by direct nucleotide sequencing (Table 2).

3.5 Relative quantities by real-time RT-PCR

The wild type strain and mutant strain were identified simultaneously in these four patients by real-time RT-PCR. Therefore, Ct values obtained from both channels (FAM and JOE) can be used to calculate the relative quantities. The calculation was performed based on the delta Ct method (Ct of JOE – Ct of FAM) and the result has been summarized in table 2. The population ratios between oseltamivir resistant and oseltamivir sensitive strains were approximately 9-, 6841-, 2487- and 13-fold in sample H1222r, H2283, H2358 and H2389, respectively.

3.6 Sequencing results

The result of oseltamivir resistance detection was confirmed by direct nucleotide sequencing. All 4 patients with oseltamivir-resistant strains harbored the H275Y mutation (CAC $\rightarrow$ TAC) of the neuraminidase gene (table 2). The minor sequencing signal of oseltamivir sensitive strains was partially identified in 2 patients (H1222r and H2389) as well but only in a trivial proportion. All sequences of oseltamivir resistant strains were submitted to the GenBank database and assigned accession numbers (CY082964-CY082967). Moreover, the NA sequences were further analyzed in conjunction with other previously described mutations associated with resistance to oseltamivir such as D199G, I223V, N295S and S334N residues (Pizzorno et al., 2011). The result revealed that all four patients harbored amino acid residues D199, I223, N295 and S334 similar to those found in

wild-type strains, indicating that the H275Y mutation was mainly responsible for the oseltamivir resistant characteristics observed in the 4 patients.

Furthermore, specimens from patients with oseltamivir sensitive strains were randomly selected for direct nucleotide sequencing of the NA gene (N=67) and assigned accession numbers (CY039988, CY074976, CY074984, CY074992, CY075000, CY075008, CY075016, CY075673, CY075679, CY075691, CY075697, CY075703, CY075709, CY075715, CY075721, CY075727, CY075733, CY075739, CY075745, CY075751, CY075757, CY075762, CY075768, CY075774, CY075780, CY075786, CY075792, CY075798, CY075804, CY075810, CY075816, CY075822, CY075828, CY075834, CY075840, CY075846, CY080301, CY080309, CY080317, CY080325, CY080333, CY080341, CY081109, CY081115, CY081121, CY081127, CY081133, CY081139, CY081145, CY081151, CY081157, CY081163, CY081169, CY081175, CY081181, CY081187, CY081193, CY081199, CY081205, CY081211, CY081217, GQ866936, GQ866937, GQ866938, GQ866953, GQ866961 and HM446344). The result showed that all selected samples harbored amino acid residues similar to the oseltamivir sensitive strain.

3.7 Clinical data of the patients with oseltamivir resistant strains

The clinical data of each patient with oseltamivir resistant strains of the pandemic influenza virus (H1N1) are shown in Table 2 along with the treatment outcomes. The three patients (H1222r, H2358 and H2389) had mild symptoms including fever, headache, sore throat and cough, and recovered after hospitalization. However, one patient (H2283) was a 14-year old girl with an immunocompromised condition (SLE, on corticosteroid administration). She had severe symptoms including pneumonia, respiratory failure and finally died during hospitalization.

4. Discussion

Oseltamivir (Tamiflu) has been used for prophylaxis and treatment of influenza A virus infection widely during outbreaks of the pandemic influenza virus (H1N1) especially in urban areas of Thailand. There were three outbreaks reported in Thailand between May, 2009 and October, 2010 with increasing numbers of infected patients. The isolation of oseltamivir-resistant strains of the pandemic influenza virus (H1N1) has been reported with increasing frequency. The situation of oseltamivir-resistant strains in Thailand displays a similar prevalence to that observed in other countries (WHO, 2010; Ujike et al., 2011; Ramirez-Gonzalez et al., 2011; Duwe et al., 2011; Yang et al., 2010; Harvala et al., 2010; Chen et al., 2009). In this study showed that the number of patients infected by oseltamivir resistant strains was higher in the course of the second (0.24%) and third (0.61%) waves than during the first (0%) outbreak. All four patients infected by resistant strains belonged to the group of patients admitted to hospitals in urban areas where oseltamivir was used for treatment of influenza virus infection usually, implying that oseltamivir exposure may increase the probability of viral genetic mutation and thus, resistance to the anti-viral drug. The clinical symptoms observed in three patients (H1222r, H2358 and H2389) were flu-like illness and low severity including fever, headache, sore throat and cough. However, more severe and atypical symptoms including pneumonia, and respiratory failure were observed in one patient (H2283) whose condition had been immunocompromised. This patient was a 14-year-old girl who had been diagnosed with systemic lupus erythematosus and received immunosuppressants to control the underlying disease. The other three patients were immunocompetent with no significant co-morbidities. These three patients recovered fully from oseltamivir resistant H1N1 infection. None of the four patients had received oseltamivir as a prophylactic medication prior to the isolation of oseltamivir resistant H1N1 strains.

Previously, several real-time RT-PCR assays for oseltamivir resistance detection were developed based on TaqMan MGB (minor groove binder) chemistry (Wong et al., 2011; Suzuki et al., 2011; Hindiye et al., 2010; Chidlow et al., 2011). In this study, the in-house assay based on real-time RT-PCR using specific LNA (locked nucleic acids) TaqMan probes was developed for the detection and determination of relative quantities of the oseltamivir resistant and oseltamivir sensitive strains of the pandemic influenza virus (H1N1). The locked nucleic acids (LNAs) are nucleotide analogues that reduce conformational flexibility and increase the thermal stability of oligonucleotides (about 3–8 °C per modified base in the probe). This can be used to increase the sensitivity and specificity of TaqMan probes. The assay provided clear and interpretable detection results without any noise or non-specific signal. In addition, the assay revealed high specificity against the pandemic influenza virus (H1N1) without cross-amplification of other subtypes of influenza virus or other respiratory viruses found in the human respiratory tract. Moreover, the limit of detection was as low as 100 copies / μ L for both oseltamivir sensitive and oseltamivir resistant strains, indicating an acceptable limit of detection by real-time RT-PCR comparable to previous studies (Chutinimitkul et al., 2007; Wong et al., 2011). The slope of the standard curves is used to determine the amplification efficiency, which in theory should be 100%. It is remarkable that standard curves with a slope between –3.1 and –3.6 are typically acceptable for amplification efficiency between 90 and 110%. The R^2 value indicates how well all data fit the standard curve. An absolutely linear standard curve has an R^2 value equal to 1 whereas R^2 0.985 indicates that the data of the standard curve are acceptable (Payungporn et al., 2008). According to the standard curves obtained from this study, the slopes were –3.32 (100% efficiency) and R^2 values were 0.999 for both oseltamivir sensitive and oseltamivir resistant strain detection, implying excellent amplification efficiency and overall performance of the real-time RT-PCR assay.

There were a few shortcomings of this study including 1) the positive control for oseltamivir resistant strains was generated by mutagenesis instead of naturally occurring oseltamivir-resistant virus due to lack of oseltamivir-resistant isolates at the start of the epidemic. 2) No phenotypic testing of the mutated positive control since the mutated positive control was a cloning vector containing a partial sequence of the neuraminidase (NA) gene. Therefore, this vector could not be used for expression of the functional NA protein for the phenotypic assay. However, the nucleotide sequences inserted in the cloning vector were confirmed by sequencing. The mutated NA sequence was exactly the same as those found in oseltamivir resistant strains of pandemic influenza virus (H1N1) reported from other countries implying the same characteristics of oseltamivir resistance. 3) No antiviral susceptibility testing was performed on the virus isolated from patients with the H275Y mutation to confirm the resistant phenotype due to unsuccessful virus isolation from those samples. Nevertheless, the oseltamivir resistant characteristics were detected by real-time RT-PCR and then confirmed by nucleotide sequencing.

The results of oseltamivir resistance detection were also confirmed by direct nucleotide sequencing, which revealed the 275Y mutation based on a CAC to TAC nucleotide substitution in all four patients. The results obtained by real-time RT-PCR were in perfect agreement with direct nucleotide sequencing. However, real-time RTPCR provided more detail on relative quantities for ratios between resistant and sensitive strains in each individual whereas the chromatogram obtained from direct sequencing revealed unclear signals of the minor strains. In two patients (H2283 and H2358) treated with oseltamivir for five days prior to detection, the ratios between resistant strains and sensitive strains were as high as 6841- and 2487- fold, respectively. On the other hand, the ratios found in two patients (H1222r and H2389) not treated with oseltamivir were as low as 9- and 13-fold, respectively. This implied that oseltamivir treatment can inhibit the replication of the sensitive strain but not of the resistant strain.

In conclusion, this study described the methods based on real-time RT-PCR for detection of oseltamivir resistant strains. This method was also applied for detection of oseltamivir resistant strains in patients with ILI admitted to hospitals in Thailand. The real-time RT-PCR described here is advantageous because it is specific, sensitive, provides excellent amplification efficiency and quantitative data. Therefore, it would be useful for large-scale testing and monitoring of oseltamivir resistant strains of the pandemic influenza virus (H1N1) without the requirement for virus isolation and phenotypic assay.

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Figure 1. Representative results of amplification plots (A and B) and standard curves (C and D) obtained from real-time RT-PCR assay for detection of oseltamivir sensitive and resistant strains of the human pandemic influenza A virus (H1N1). The R^2 values, slope of the standard curve and amplification efficiency obtained from this study were appropriate for the detection.

Figure 2.

Surveillance of oseltamivir resistant strains. Amounts of samples positive for the pandemic influenza virus (H1N1) 2009 and amounts of oseltamivir resistant strains detected in each outbreak in Thailand during May, 2009 to October, 2010.

Table 1

Primers and probes for oseltamivir resistance detection.

Primer/Probe	Sequence (5' → 3') *	Strand	Position **	Application
pFlu_N1-275H	FAM- ttcCtc r TagtGataAttAggg-BHQ1	AS	834-813	Detection
pFlu_N1-275Y	JOE- ttcCtc r TagtAataAttaGgg-BHQ1	AS	834-813	
pFlu_N1_F	5'-tragrachcargagtctgaatgtg-3'	SS	671-694	Detection & mutagenesis
pFlu_N1_R	5'-gccarttrtcyctgcacacaca-3'	AS	889-868	
pFlu_275Ymt_F	5'-gaaatgaatgccctaattattactatgaggaatgctcctgtt-3'	SS	802-884	Mutagenesis
pFlu_275Ymt_R	5'-aacaggagcattcctcatagtaataattagggcattcatttc-3'	AS	884-802	

* Bold and capital letters represent LNA residues

** The neuraminidase gene of A/California/04/2009(H1N1) GISAID accession number EPI176472 served as reference

Clinical data and sequencing results of each patient with oseltamivir resistant strains of the pandemic influenza virus (H1N1) 2009 observed in this study.

Table 2

Code	Date	Sex	Age	Clinical symptoms	Oseltamivir Treatment Before testing	Outcomes	Relative Quantitation 275Y: 275H (folds)	Nucleotides coding for 275Y	Accession no.
H1222r	Feb 10, 2010	M	28	Fever Headache Sore throat Cough	No	Recovery	9	T A C	CY082964
H2283	Aug 31, 2010	F	14	Immunocompromised (SLE, on corticosteroids) Pneumonia Respiratory failure	Yes (5 days)	Death	6841	T A C	CY082965
H2358	Sep 7, 2010	F	6	Fever Headache Running nose Cough	Yes (5 days)	Recovery	2487	T A C	CY082966
H2389	Sep 10, 2010	M	6	Fever Headache Cough Soar throat	No	Recovery	13	T A C	CY082967