Original Article

Simultaneous detection and subtyping of H274Y-positive influenza A (H1N1) using pyrosequencing

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Abstract

Introduction: We investigated the frequency of H274Y-positive swine-origin 2009 A (H1N1) influenza virus outbreak in Thailand during May-August 2009.
Methodology: This study sought to find Oseltamivir resistance mutation H274Y by using pyrosequencing.
Results: From 8,710 real-time RT-PCR swine-origin 2009 A(H1N1) influenza virus-positive specimens, 100 randomly selected samples identified one such virus with H274Y mutation using pyrosequencing.
Conclusions: The patient probably acquired oseltamivir resistance from natural variation, since he had never received that form of treatment before and recovered from influenza-like symptoms without using anti-influenza drugs.

Key words: H274Y; oseltamivir; Influenza A (H1N1); pyrosequencing

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Introduction

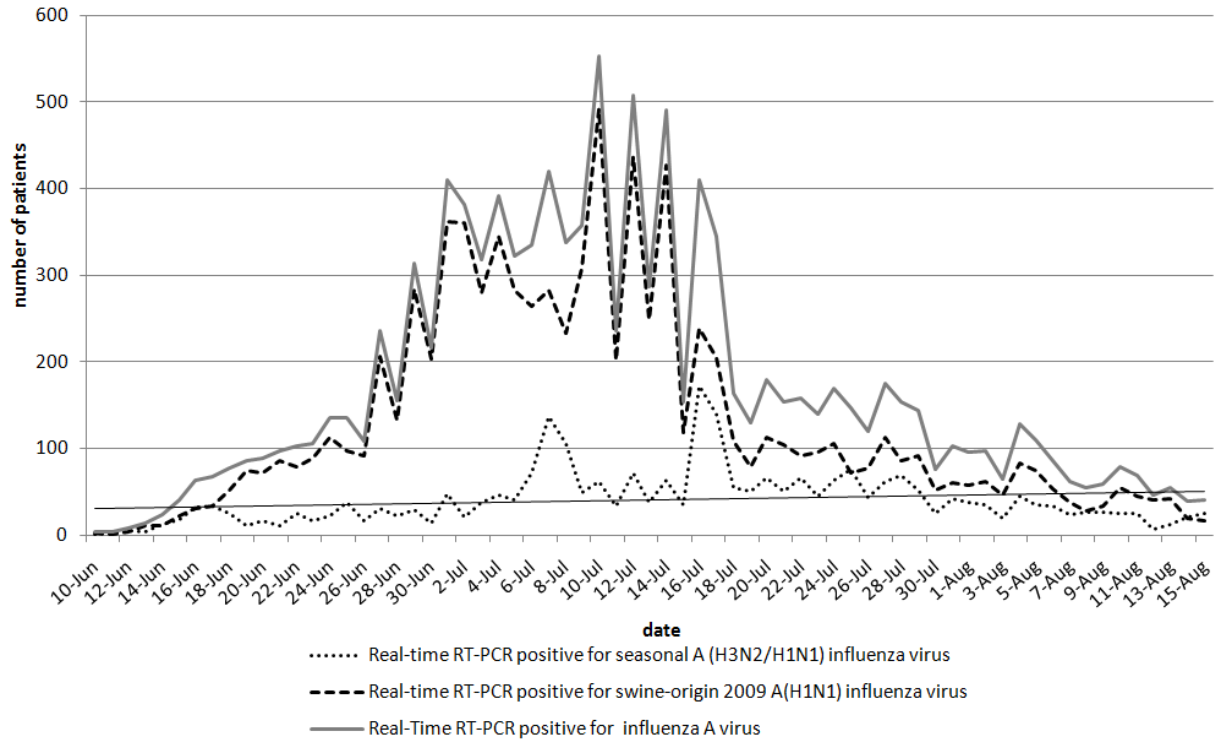
The primary treatments for influenza A infection are the neuraminidase inhibitors (NAI) oseltamivir and zanamivir. They inhibit virus replication by mimicking the natural substrate of the influenza neuraminidase (the sialic acid receptors), and bind to the active site, preventing neuraminidase from cleaving host-cell receptors or releasing new viruses [1]. Generally, resistance of influenza viruses to antiviral drugs can emerge following medication or may result from natural variation [3]. In case of NAIs, different resistant mechanisms have been observed. For example mutant viruses with H274Y mutation in neuraminidase (NA) gene demonstrated on average a 1,466-fold reduction in oseltamivir susceptibility and a 527-fold reduction in peramivir sensitivity compared to wild-type A (H1N1) viruses. Nevertheless, the mutation had no impact on zanamivir susceptibility [2]. An epidemiological study into NAI resistance has been observed with high incidence not only in the clinical but also in the community setting. However, a low prevalence of NAI resistance has been detected among human influenza viruses circulating worldwide [4]. Conversely, the frequency of the oseltamivir-resistant H274Y mutant increased significantly since oseltamivir was the antiviral drug most prescribed for influenza treatment [5].

Oseltamivir is the anti-influenza drug currently used in Thailand. Due to rapid emergence of oseltamivir resistance in seasonal H1N1 virus isolates globally, there is vast concern that an oseltamivir-resistant variant of pandemic (H1N1) 2009 virus may emerge. Here we report the first case of positive selective pressure for oseltamivir resistance conferred by a genotype of known mutation, H274Y, in NA swine-origin 2009 A(H1N1) influenza virus in Thailand.

Methodology

Since the outbreak of swine-origin 2009 A(H1N1) influenza virus took place in Thailand, and the first real-time RT-PCR positive case was found at the Molecular Virology Laboratory, Ramathibodi Hospital, on 10 June 10 2009, with subsequent cases peaking on 10 July, 2009 (Figure 1A), the consumption of oseltamivir has risen dramatically. The hospital's Virology Laboratory Unit has

Figure 1. Specimens from nasal swabs were sent to the Molecular Virology Laboratory, Ramathibodi Hospital, between June 10 and Aug15, 2009. Among 11,417 real-time RT-PCR influenza A positive specimens, 8,710 (76.3%) were swine-origin 2009 A (H1N1) influenza viruses and 2,707 (23.7%) were seasonal A (H3N2/H1N1) influenza viruses respectively.

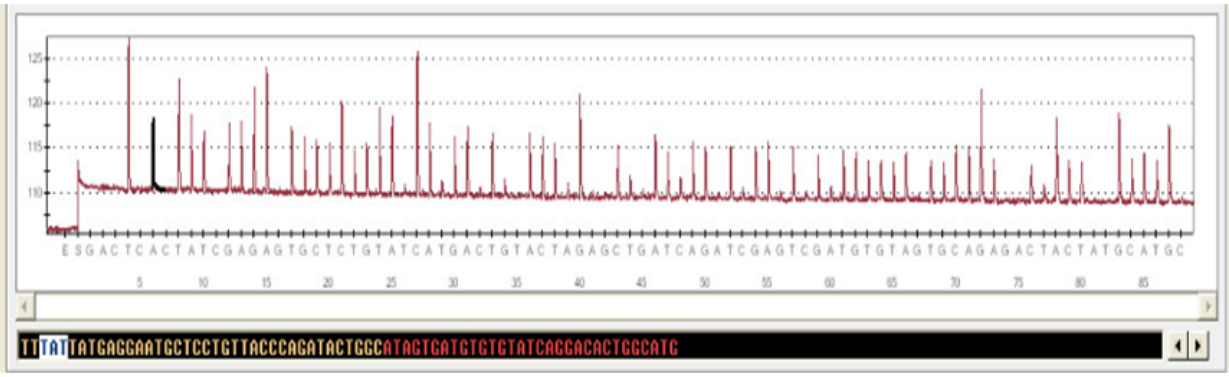


conducted routine nasal swab services to collect samples from patients for its own hospital and 54 others in Bangkok and nearby provinces, using real-time RT-PCR as the first screening assay for this influenza virus (The Real-time RT-PCR Kit for Swine H1N1 Influenza Human Pandemic Strain, Primer Design Ltd, (Southampton, Hants, United Kingdom), followed by customizing for influenza sub-typing and anti-influenza drug resistance testing using pyrosequencing in a timely and cost-efficient manner. The lower limit of detection of the assay was 500 copies of genome equivalent/ml. Using RT-PCR and sequencing primers of the M2 gene, the assay could differentiate Influenza A subtype, Seasonal H1N1 2008, Swine origin H1N1 2009, Seasonal H3N2 2008, and avian influenza A H5N1. Amantadine and rimantadine resistance resulting from the change of normal serine (S) at position 31 to asparagine (N) also could be detected by this primer set. RT-PCR and sequencing primers of the NA gene could differentiate Influenza A subtype, Seasonal H1N1 2008, Swine origin H1N1 2009, and Swine H1N1 2008, as well as detect oseltamivir resistance caused by changing of Histidine (H) at position 274 to Tyrosine (Y).

The NA and M2 sequences from pandemic H1N1 viruses available from the database in the public domain were aligned by using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA).

For Influenza A sub-typing and H274Y mutation detection, the pyrosequencing RT-PCR and sequencing primers were designed by using pyrosequencing assay design software (Biotage, Uppsala, Sweden) from the consensus sequences of M2 (M2-Forward: 5'-CARaTg CaRcGaTTcAagTGA-3'; M2-Reverse-biotin: 5'-TCCTBYCGRTAYTCTTCCCTC-3'; M2-sequencing: 5'-TTGCACYTGATATTGTGGAT-3') and NA (NA-Forward: 5'-TTYACYDT AATGACYGATGG-3', NA-Reverse-biotin: 5'-CCaCTGCAKATgTAYCCTAT-3''; NA-sequencing: 5'-RWTGAATGCMCCYAATT-3') respectively. The M2 primer set was used for sub-typing for influenza A, namely, swine-origin influenza A (H1N1) 2009 viruses, seasonal influenza A (H1N1) viruses, seasonal Influenza A (H3N2) viruses, and avian Influenza A (H5N1) viruses. The

Fig.ure 2A. Sequence data of the influenza neuraminidase gene of subtype N1 obtained from the patient could be used to both identify influenza subtypes and detect H274Y mutation (CAT to TAT). BLAST showed the highest scores using the partial sequence of NA; TTTATTATGAGGAATGCTCCTGTTACCCAGATACTGGCATAGTGATGTGTGTATCAGGACAACTGGCATGG.



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ423419.1	Influenza A virus (A/Iowa/01/2009(H1N1)) segment 6 neuraminidase	97.1	97.1	100%	4e-18	100%
GQ423407.1	Influenza A virus (A/Thailand/1035/2008(H1N1)) segment 6 neuramin	97.1	97.1	100%	4e-18	100%
GQ423406.1	Influenza A virus (A/Iowa/01/2009(H1N1)) segment 6 neuraminidase	97.1	97.1	100%	4e-18	100%
GQ397512.1	Influenza A virus (A/Andalucia/RR3681/2009(H1N1)) segment 6 neura	97.1	97.1	100%	4e-18	100%
GQ397505.1	Influenza A virus (A/PaisVasco/RR3343/2009(H1N1)) segment 6 neur	97.1	97.1	100%	4e-18	100%
GQ397498.1	Influenza A virus (A/PaisVasco/RR3174/2008(H1N1)) segment 6 neur	97.1	97.1	100%	4e-18	100%
AB510223.1	Influenza A virus (A/Aichi/89/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510222.1	Influenza A virus (A/Aichi/88/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510221.1	Influenza A virus (A/Aichi/86/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510220.1	Influenza A virus (A/Aichi/84/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510219.1	Influenza A virus (A/Aichi/83/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510218.1	Influenza A virus (A/Aichi/49/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510217.1	Influenza A virus (A/Aichi/38/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510216.1	Influenza A virus (A/Aichi/37/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510215.1	Influenza A virus (A/Aichi/36/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510214.1	Influenza A virus (A/Aichi/29/2009(H1N1)) NA gene for neuraminidasa	97.1	97.1	100%	4e-18	100%
AB510213.1	Influenza A virus (A/Aichi/9/2009(H1N1)) NA gene for neuraminidasa,	97.1	97.1	100%	4e-18	100%
AB510211.1	Influenza A virus (A/Aichi/6/2009(H1N1)) NA gene for neuraminidasa,	97.1	97.1	100%	4e-18	100%

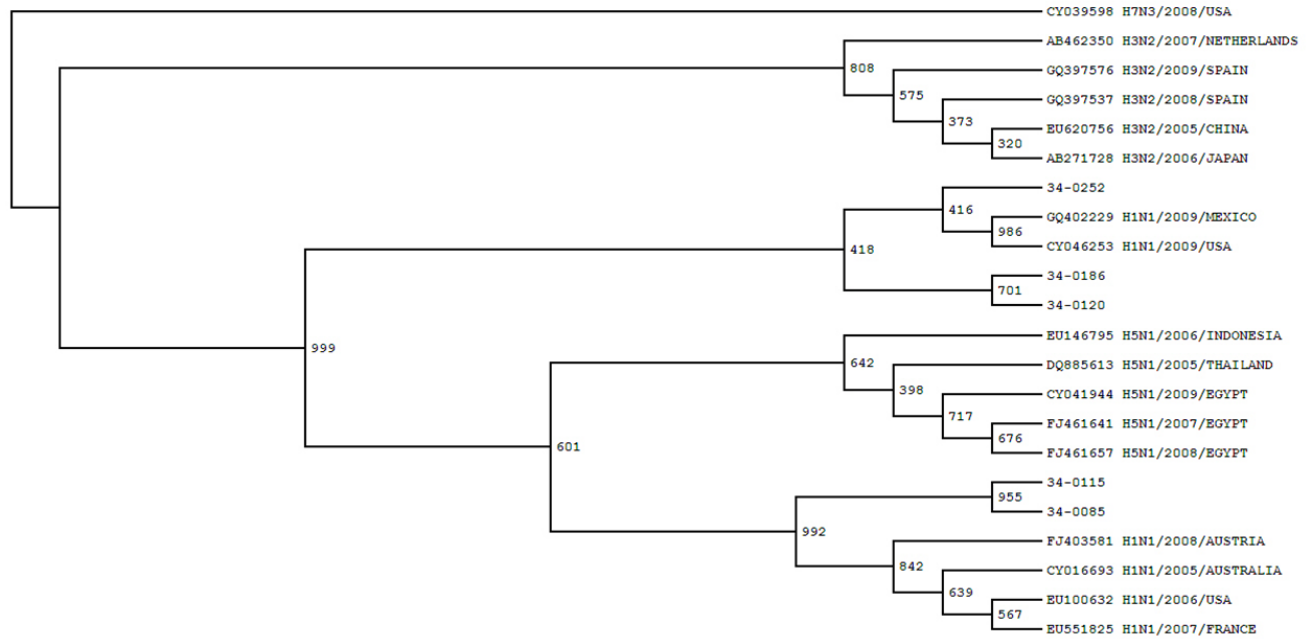
NA primer set was utilized for H274Y mutation screening from Influenza A real-time RT-PCR positive samples. The lower limit of detection of this pyrosequencing assay was 100 genome equivalents of influenza A viruses per reaction.

Results

From 10 June through 15 August 2009, a total of 11,417 confirmed real-time RT-PCR influenza A-positive cases were observed. Along with pandemic 2009 A(H1N1) influenza virus, 8,710 (76.3%) were swine-origin 2009 A (H1N1) influenza viruses while 2,707 (23.7%) were seasonal A (H3N2/H1N1) influenza viruses. Of the 8,710 swine-origin 2009 A (H1N1) influenza virus-purified RNA positive samples, 100 samples were randomly selected for pyrosequencing. M2 and NA genes were sequenced using amplification and sequencing primers specific for these genes. One of the samples was identified with H274Y mutation by pyrosequencing. However, oseltamivir resistance could not be confirmed by in vitro assay because of lack of space for storing such a large number of nasal swab specimens (more than 700 samples/day) for viral isolation and cultivation.

Pyrosequencing is a suitable technology to identify NA mutations for larger numbers of isolates because it provides a simplified and faster preparative and analytical workflow compared to conventional sequencing. Pyrosequencing could sequence the NA region of 96 samples within one hour.

Figure 2B. Phylogenetic trees for NA sequence reveal that the clinical sample (34-0186) contained S-OIV is in the same group of GQ402229 H1N1/2009/MEXICO and CY46253 H1N1/2009/USA



Case Report

The oseltamivir resistant (H274Y-positive) swine-origin 2009 A (H1N1) influenza virus was detected in a 26-year-old Thai man who came to the Family Medicine Clinic of Ramathibodi Hospital on 11 June 2009, because of sore throat, cough, malaise, and fever. His illness began shortly after he returned from a southern province where he had gone to crowded areas. His body temperature was 39ºC and only mildly injected pharynx was found on physical examination. No laboratory test other than nasal swab for PCR was done because systemic complication was not suspected. He received antipyretic as symptomatic treatment. One week later, he returned for a follow-up, reporting that he was already defervesce but still had some cough. He recovered fully about two weeks of the onset without receiving oseltamivir.

Discussion

Using phylogenetic analysis of neuraminidase sequence, this study shows the presence of H274Y mutation in swine-origin 2009 A (H1N1) influenza virus, conferring oseltamivir resistance (Figures 2A and 2B). Sequencing data from M2 and NA genes was also used to identify influenza sub-types by running an alignment search through the Influenza Sequence Database from GenBank [6]. In addition, the H274Y mutation (CAT to TAT) could be easily detected from the NA sequence. Phylogenetic analysis of the NA and M2 gene sequences also showed that the clinical sample containing H274Y mutant swine origin 2009 A(H1N1) influenza virus is in the same group as GQ402229 H1N1/2009/MEXICO and CY46253 H1N1/2009/USA.

Recently, oseltamivir resistant A (H1N1) viruses have emerged in many countries [7-10]; however, this study reports the first case of swine origin influenza in Thailand that is resistant to oseltamivir. Oseltamivir has been widely prescribed for persons infected with pandemic (H1N1) 2009 virus in Thailand [10]. The widespread use of oseltamivir can lead to the emergence of a resistant strain of influenza virus [11]. This report raises strong concerns that an H274Y resistant mutation in the pandemic (H1N1) 2009 virus might emerge and circulate in our country. The infected patient described here was not treated and had no history of anti-influenza treatment before the pyrosequencing assay; therefore, it is doubtful that the H274Y mutation was drug-induced.

In conclusion, this study describes the design of pyrosequencing assays for the detection of H274Y-positive swine-origin 2009 A (H1N1) influenza virus in Thailand. Influenza strains with H274Y

neuraminidase mutation must be closely monitored because the virus containing the H274Y mutation is easily transmissible in the absence of O-SIV pressure. Moreover, the pyrosequencing methods will be necessary for rapid detection and high-throughput during the outbreak and re-emergence of swine-origin 2009 A (H1N1) influenza viruses.

Acknowledgements

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Conflict of interests: No conflict of interests is declared.