ไวรัสไข้หวัดใหญ่สายพันธุ์ A ที่ดื้อต่อยาต้านไวรัส ชนิดโอเซลทามิเวียร์ในประเทศไทยระหว่าง พ.ศ. 2552-2554

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บทคัดย่อ

เชื้อไวรัสไข้หวัดใหญ่ชนิด A สายพันธุ์ (H1N1) 2009 เริ่มพบในประเทศไทยตั้งแต่เดือนมิถุนายน พ.ศ. 2552 จนถึงปัจจุบัน การศึกษานี้เป็นการนำเสนอข้อมูลความชุกของเชื้อไวรัสไข้หวัดใหญ่ชนิด A ทั้งสายพันธุ์ (H1N1) 2009 และสายพันธุ์ที่มีการระบาดตามฤดูกาล (seasonal H1 และ H3) รวมถึงจำแนกสายพันธุ์ที่ดื้อต่อยาต้านไวรัส oseltamivir ในประเทศไทยในช่วง 3 ปีแรกของการระบาด (พ.ศ. 2552 - 2554) โดยตรวจจีนที่เกี่ยวข้องกับการดื้อยา (genotypic assays) และทดสอบเชื้อไวรัสกับยาต้านไวรัส (phenotypic assays) โดยรวบรวมสิ่งส่งตรวจประเภทน้ำล้างหลังโพรงจมูก จำนวน 7,264 ตัวอย่างจากผู้ป่วยที่มีอาการคล้ายไข้หวัดใหญ่ตั้งแต่เดือนมิถุนายน พ.ศ. 2552 - ธันวาคม พ.ศ. 2554 ผลการศึกษาพบเชื้อไวรัสไข้หวัดใหญ่ชนิด A สายพันธุ์ (H1N1) 2009 ในสิ่งส่งตรวจจำนวน 692 ตัวอย่าง (9.5%) และพบเชื้อไวรัสไข้หวัดใหญ่ชนิด A ที่เป็น seasonal H3 และ H1 ในสิ่งส่งตรวจ จำนวน 322 ตัวอย่าง (4.4%) และ 1 ตัวอย่าง (0.01%) ตามลำดับ ในจำนวน 692 ตัวอย่างที่ตรวจพบเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ A (H1N1) 2009 พบว่ามี 6 ตัวอย่าง (0.86%) ที่ตรวจพบการเปลี่ยนแปลงลำดับเบสของจีนที่เกี่ยวข้องกับการดื้อยา oseltamivir ในจีน NA ที่ตำแหน่ง H275Y ซึ่งการเปลี่ยนแปลงดังกล่าว ไม่พบในเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ A ชนิด seasonal H1 และ H3 ทั้งนี้ผู้ป่วยจำนวน 5 รายที่ตรวจพบการดื้อยา oseltamivir เป็นผู้ที่มีภาวะภูมิคุ้มกันบกพร่อง และต่อมาเสียชีวิต จำนวน 3 ราย โดยตรวจพบการเปลี่ยนแปลงของลำดับเบส D239G ของจีน HA ในผู้เสียชีวิตหนึ่งราย ซึ่งการเปลี่ยนแปลงดังกล่าวอาจส่งผลต่อการดำเนินโรคที่รุนแรง ผลการตรวจ phenotypic assays แสดงค่า IC_{50} ของไวรัสไข้หวัดใหญ่ที่แยกได้ในปี พ.ศ. 2552 ที่ต่ำกว่าไวรัสที่แยกได้ในปี พ.ศ. 2553 โดยพบว่าค่าเฉลี่ยของ IC_{50} ของไวรัสไข้หวัดใหญ่ชนิด A สายพันธุ์ (H1N1) 2009 ที่ดื้อต่อยาต้านไวรัส oseltamivir อยู่ที่ 69.18±18.25 nM ผลการวิเคราะห์ whole genome sequencing ของไวรัสที่ดื้อต่อยาต้านไวรัส oseltamivir ที่แยกได้จากผู้ป่วยจำนวน 6 ราย พบการเปลี่ยนแปลงลำดับเบส E391K ที่จีน HA, V14I และ K716Q ที่จีน PA และ K736E ที่จีน PB1 ซึ่งสอดคล้องกับสายพันธุ์ที่พบในสิงคโปร์ โดยการเปลี่ยนแปลงลำดับเบส E391K และ S200T ที่จีน HA และ I123V ที่จีน NS1 พบเฉพาะในเชื้อไวรัสไข้หวัดใหญ่ชนิด A สายพันธุ์ (H1N1) 2009 ที่แยกได้ในปี พ.ศ. 2553 แต่ไม่พบในไวรัสสายพันธุ์เดียวกันที่แยกได้ในปี พ.ศ. 2552

คำสำคัญ: การดื้อยาต้านไวรัสไข้หวัดใหญ่ oseltamivir ไข้หวัดใหญ่ชนิด A สายพันธุ์ (H1N1) 2009 ไข้หวัดใหญ่ตามฤดูกาล การตรวจทางจีโนทัยป์ การตรวจทางฟีโนทัยป์

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Oseltamivir resistant influenza A viruses in Thailand during 2009-2011

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Abstract

Influenza A (H1N1) pdm09 viruses have been spread in Thailand since June 2009 and have become one of the circulating strains ever since. This study aims to provide the prevalence data of influenza A [pandemic influenza and seasonal influenza (H1/H3)] viruses, including the oseltamivir-resistant strains in Thailand during the first 3-year of pandemic (2009 - 2011) using both genotypic and phenotypic assays. The 7,264 nasopharyngeal wash samples were collected from patients presented with influenza-like illness during June 2009 - December 2011. Influenza A (H1N1) pdm09 viruses was detected in 692 (9.5%) of suspected cases. Seasonal influenza H3 and H1 viruses were detected in 322 (4.4%) and 1 (0.01%) cases, respectively. Among 692 positive Influenza A (H1N1) pdm09 virus samples, 6 (0.86%) viruses contained oseltamivir drug resistant mutation codon (H275Y) in NA gene, which was not found in seasonal influenza H3 and H1 viruses. All 5 oseltamivir-resistant Influenza A (H1N1) pdm09 virus-infected cases were immunocompromised patients; 3 of which were oseltamivir treatment-related and were fatal cases. Mutation at D239G in HA gene was revealed in virus isolated from one dead case, which possibly was related to severe clinical outcome. The phenotypic assay showed that the IC_{50} value of the influenza A viruses isolated in 2009 was lower than that of the viruses isolated in 2010. The mean IC_{50} of the oseltamivir-resistant Influenza A (H1N1) pdm09 virus strain was 69.18±18.25 nM. Whole genome sequence analysis of 6 oseltamivir-resistant isolates showed mutation codons at HA-E391K, PA-V14I, PA-K716Q and PB1-K736E, which was in agreement with the new viral variants reported in Singapore. Only mutations of HA-E391K, HA-S200T and NS1-I123V were commonly found in the oseltamivir-resistant influenza A (H1N1) pdm2009 viruses isolated in 2010, which were absent in the resistant-strain isolated in 2009.

Keywords: oseltamivir resistance, influenza A (H1N1) pdm09 virus, seasonal influenza A virus, genotypic assays, phenotypic assays

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Introduction

Influenza viruses are enveloped RNA viruses of the *Orthomyxoviridae* family. Three types of influenza virus exist, designated A, B and C. The single stranded, negative sense RNA genome comprises eight segments encoding 11-12 proteins.[1,2] Hemagglutinin (HA) and neuraminidase (NA) are the major glycoprotein components on the influenza virus envelope. HA serves as an attachment protein, binding to neuraminic acids on the host cell surface. A highly immunogenic protein HA is very important for stimulation of protective immunity mediated by neutralizing antibody. NA is an enzyme that can digest mucous secretions, allowing efficient spread in the host respiratory tract. Based on the antigenic properties of HA and NA, influenza virus type A is divided into 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11). Six HA subtypes, H1, H2, H3, H5, H7 and H9, have been isolated from humans, but only H1, H2, H3 subtypes along with N1 and N2 are known to be involved in human influenza pandemics.[3] The most recent influenza pandemic was caused by a 2009 H1N1 virus strain, influenza A (H1N1) pdm09, resulting from the emergence of a new subtype of influenza A virus. The novel influenza A (H1N1) pdm09 virus emerged in Mexico and the United States, with many people being naïve to this strain, thus allowing global spread and a resultant influenza-like illness with mild to moderate severity. Approximately 526,060 cases of influenza A (H1N1) pdm09 virus infection and at least 6,770 deaths were reported worldwide by 15 November 2009.[4] The newly emerged influenza A (H1N1) pdm09 virus is a genetic reassortment of six gene segments (PB2, PA, PB1, HA, NP, NS) from the triple reassortant swine-origin (H3N2) virus and two segments (NA and M) from the Eurasian influenza A (H1N1) swine virus lineage. Because of the unexpected origin of this particular strain, there was no vaccine available during the early stages of the pandemic and as such, neuraminidase inhibitor (NAI) antiviral drugs were widely used for treatment and prophylaxis of influenza A (H1N1) pdm09 virus infection. The increased use of antiviral drugs such as oseltamivir, however, carries the risk of facilitating emergence of NAI resistant influenza virus strains.

Early analysis indicated that influenza A (H1N1) pdm09 virus was susceptible to oseltamivir and zanamivir but resistant to amantadine.[5] However, the first case of oseltamivir-resistant virus was documented in June 2009. All of the resistant strains carried the H275Y mutation on the NA gene.[6] This mutation was simultaneously detected in several countries in the Northern Hemisphere and subsequently spread worldwide. The oseltamivir-resistant strains of influenza virus A (H1N1) pdm09 virus during outbreaks in Thailand (May 2009 and October 2010) were documented with a frequency of 0.28%.[6] In 2012-2013, WHO reported surveillance of oseltamivir resistant influenza A (H1N1) pdm09 virus in Europe and demonstrated an infection rate of 1%.[7]

Genetic analysis of oseltamivir-resistant influenza A (H1N1) pdm09 viruses showed that new viruses can arise from different variants with a great diversity under drug pressure.[8] NA and other internal genes that co-evolved with the HA gene were involved in emergence of variants.

Objective

The present study aims to demonstrate the prevalence of influenza A virus and characterize NAI susceptibility of influenza A (H1N1) pdm09 viruses during 2009-2011. Whole genome analysis of oseltamivir-resistant influenza A (H1N1) pdm09 virus was performed to monitor codon mutation in each year.

Materials and Methods

Clinical specimen collection and RNA extractions

From June 2009 through December 2011, 7,264 nasopharyngeal washes from patients presenting with influenza-like illness were collected in viral transport media and sent for influenza A viruses detection. NucliSENS easyMAG (bioMérieux, Marcy l'Etoile, France) was used to extract viral RNA from 200 µl nasopharyngeal secretion. The extracted RNA was eluted with 80 µl elution buffer and examined for pandemic influenza virus.

Detection and subtyping of influenza A virus by real-time polymerase chain reaction (RT-PCR)

Influenza A virus identification by real-time PCR was performed according to Centers for Disease Control and Prevention (USA) protocol for pdm09 identification [WHO, 2009b]. Briefly, total reaction volume was 25 µl, containing 12.5 µl of 2X buffer, 0.8 µM forward primer, 0.8 µM reverse primer, 0.2 µM probe, 0.5 µl of superscript III/ platinum enzyme mix (Invitrogen, USA) and 5.5 µl of DNase/RNase free water. Two sets of primers and probes were used to identify influenza A and influenza A (H1N1) pdm09. All samples showing positivity for influenza A only were further subtyped by using commercial real-time PCR kits for subtype H3 and H1 (Zj Biotech, China).

Oseltamivir drug resistant influenza A detection by pyrosequencing

For oseltamivir-resistant influenza A (H1N1) pdm09 detection, the mutated H275Y residue on the NA gene was used as a marker. The NA gene was amplified by using a one-step RT-PCR kit (Bioline, UK) with WHO-published primers, and pyrosequencing was performed on PyroMark ID system (Biotage) with a WHO-published sequencing primer.[9]

For oseltamivir-resistant seasonal influenza A (H3N2), E119, D151, R292 and N294 residues of the NA gene were used

as a marker for detection. As previously described, the NA gene was amplified by using one-step RT-PCR kit (Bioline, UK) and pyrosequencing was performed on Pyromark ID system (Biotage) according to Deyde et al.[10]

Neuraminidase inhibition assay

All oseltamivir-resistant influenza A viruses were isolated from clinical samples inoculated onto Madin–Darby canine kidney (MDCK) cells. Briefly, MDCK cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum (Gibco, USA) and inoculated with clinical samples. MDCK cells were passaged three times or until the virus reached sufficient titer for the neuraminidase inhibition assay (NAI). NAI assay was conducted by using a commercial kit, NA-star (Applied Biosystems, Foster City, CA). Briefly, 25 µl of each oseltamivir dilution was added to wells and 25 µl of each virus dilution was then added. After incubation at room temperature for 10 to 30 minutes, 10 µl of 10 mM of NA-star substrate was added to each well and incubated at room temperature for a further 10 to 30 minutes. Sixty microliters of NA-star accelerator was added to each well and luminescence signal was measured by luminometer Victor 3 (Perkin-Elmer, Shelton, CT). Fifty percent inhibition concentrations (IC_{50}) were calculated using nonlinear curve fitting with GraphPad Prism 5.01 software.

Full-length genome of oseltamivir-resistant influenza A viruses

All eight segments of influenza A virus were amplified by using PrimeScript one-step RT-PCR kit with primers MBTuni-12 (5'-ACGCGTGATCAGCAAAAGCAGG-3') and MBTuni-13 (5'-ACGCGTGATCAGTAGAAAC AAGG-3').[11] Briefly, total reaction volume was 50 µl, containing 25 µl of 2X one-step buffer, 20 µM MBTuni-12, 20 µM MBTuni-12, 2 µl of PrimeScript one-step enzyme mix (Takara, Japan), 11 µl of DNase/RNase free water and 10 µl of extracted RNA from clinical samples or cultures. The temperature cycle was 50°C for 30 min; 94°C for 2 min, 5 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 3 min, then 31 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 3 min. PCR products were purified by Purelink PCR purification kit (Invitrogen, USA) and quantified prior to using the Ion Xpress fragment library kit (Life Technologies, Carlsbad, CA.) for sequencing on an Ion Personal Genome Machine (PGM) (Life Technologies).

All purified DNAs were sheared and blunt ends repaired by using Ion Shear plus reagent (Life Technologies), followed by P1 adaptation and barcode ligation (one barcode corresponds to one patient sample), and DNA purified again using Agencourt®AMPure®XP (Beckman Coulter). DNA fragments were then separated by size with E-Gel SizeSelect 2% agarose (Life Technologies) and purified again.

One hundred nanograms of each purified barcoded DNA was pooled and enriched using an Ion One Touch 200 template kit v2 DL in Ion One Touch 2 system (Life Technologies). Templates were quantified with Agilent High Sensitivity DNA kit (Agilent Technologies) and loaded into an Ion 314 chip (Life Technologies) for sequencing on the Ion PGM (Life Technologies). Base calling and variant calling were performed with the Torrent analysis suite version 4.0.2 and Torrent variant caller version 4.0 (Life Technologies), respectively.

Nucleotide analysis and accession number

A maximum likelihood tree was constructed using Mega software (version 6.0). The alignment of nucleotide sequences was conducted by using DNAStar Lasergene (version 12). The nucleotide and deduced amino acid sequences of all gene segments were compared with those of the influenza A virus strains available in the GenBank database. The full-length sequences of oseltamivir-resistant influenza A (H1N1) pdm09 viruses were deposited in GenBank under accession numbers KM013714–KM013801.

Statistical analysis

GraphPad Prism 5.01 software was used to analyze the IC_{50} for oseltamivir determined by chemiluminescence NAI and Robosage software was used for the fluorometric NA inhibition assay data. Student's paired T-test and Pearson's correlation were used to determine the difference and correlation between the IC_{50} of these two assays. The statistical cut off value for oseltamivir-resistant influenza virus was set by using the receiver operating characteristic curve analysis in the SPSS program.

Ethical statement

The study protocol was approved by the Siriraj Institutional Review Board (COA: si257/2012).

Results

Prevalence of influenza A virus

A total of 7,264 nasopharyngeal washes were obtained from patients with clinically suspected respiratory infections from June 2009 through December 2011. RT PCR was used to screen for influenza A and influenza A (H1N1) pdm09. Influenza A viruses were found in 13.9% (1,015/7,264) of patients with influenza-like illness, while influenza A (H1N1) pdm09 viruses were found in 9.5% (692/7,264). Of these cases, 329 (47.5%) were male and 363 (52.5%) were female. The seasonal distribution of influenza A (H1N1) pdm09 was predominant in June 2009, February 2010 and August 2010 (Fig. 1). Patients were aged from 1 month to 92 years: mean, 25.8 years; and median, 21 years. The positivity rate of influenza A (H1N1) pdm09 during 2009-2011 was highest among young children (Fig. 2).

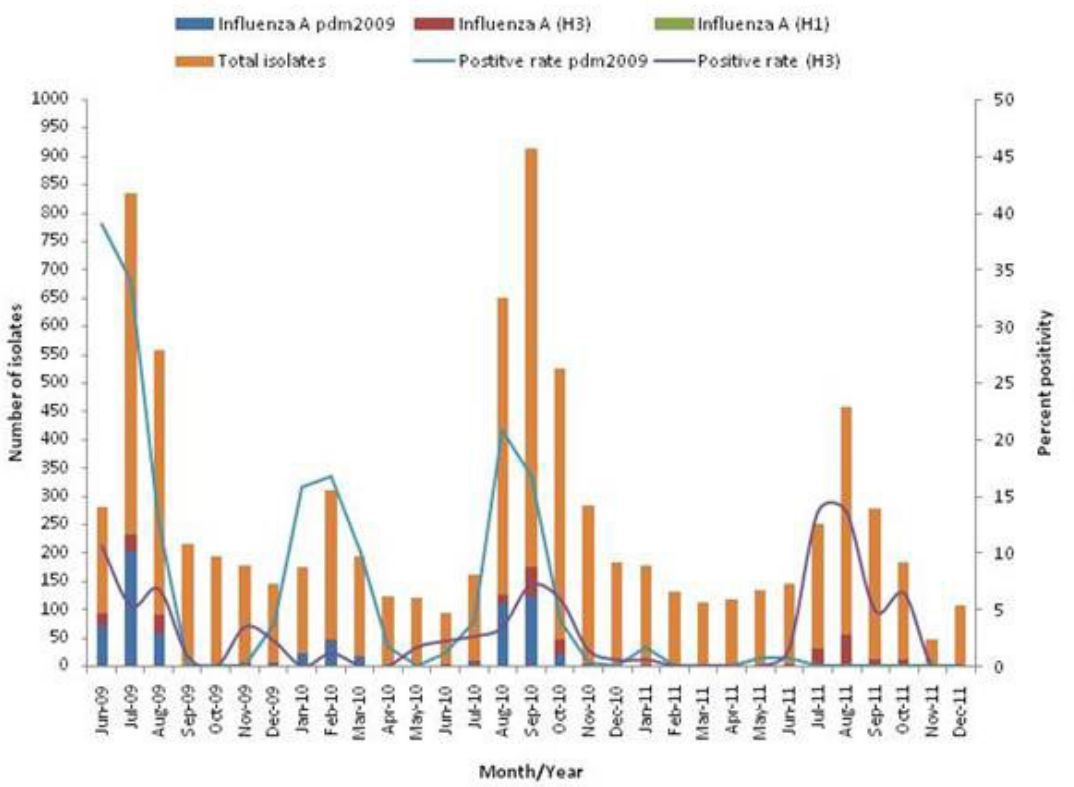

Fig. 1 Monthly distribution of influenza A viruses in Thailand during June 2009-December 2011. Each virus is indicated by a colored bar and positivity rate is shown as a line.

Three hundred and twenty-three samples showing positive for influenza A viruses and negative for influenza A (H1N1) pdm09, were further subtyped for seasonal influenza A H3 and H1 viruses. These seasonal strains were found in 4.4% (322/7,264) and 0.01% (1/7,264) of patients, respectively, and were predominantly detected in September 2010 and August 2011 (Fig. 1). Similar to the demographic of those infected with pandemic virus, there were 143 (44.4%) male and 179 (55.6%) female patients and these 322 patients infected with the H3 seasonal virus were aged from 1 month to 95 years: mean 42.2 years; and median, 42.5 years. The positive rate of influenza A (H3) during 2009-2011 was also highest among young children aged 0-10 years (30.1%) and lowest among adults over 91 years of age (0.9%) (Fig. 2).

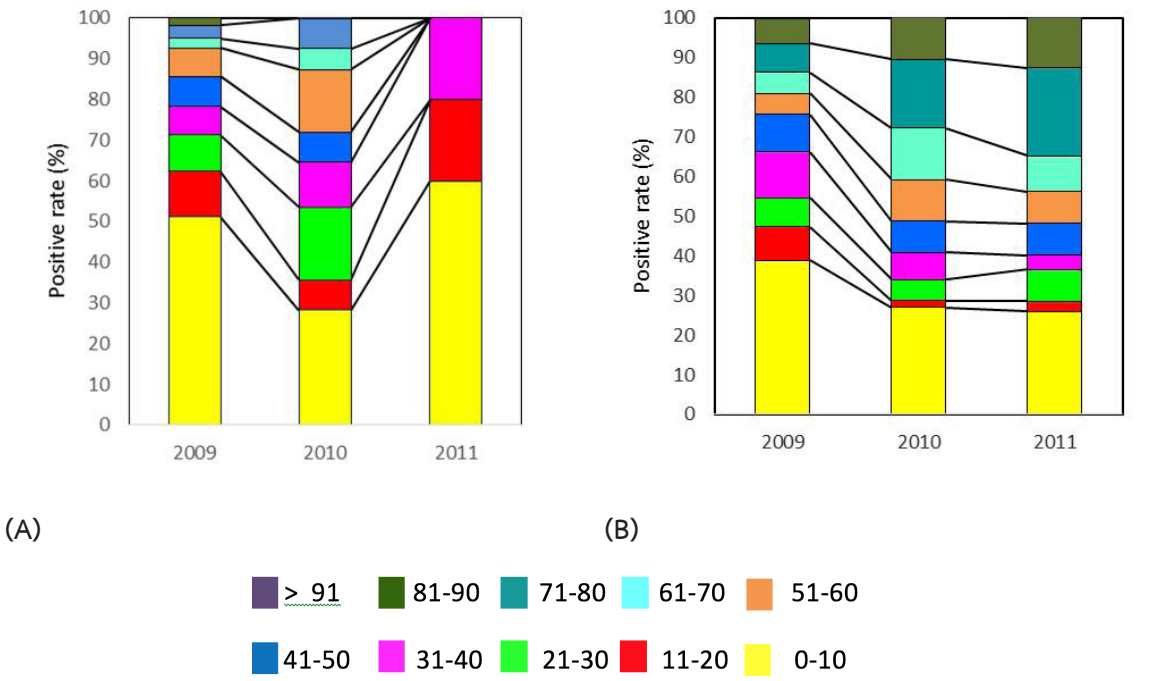

Fig. 2 Distribution of Influenza A in each age group, based on detection by real-time PCR
A) The proportion of patients infected with influenza A (H1N1) pdm09 viruses
B) The proportion of patients infected with seasonal influenza A (H3) viruses

Detection of oseltamivir resistance-associated mutations in the NA gene

During June 2009 and December 2011, 1,015 patients were diagnosed with influenza A virus infection. All of these patients were further tested for the oseltamivir-resistant genotype in the NA gene by pyrosequencing. The H275Y mutation codon was selected to represent oseltamivir-resistant influenza A (H1N1) pdm09 virus while E119V, D151V/A, R292K and N294S represented oseltamivir resistant influenza A (H3) viruses. Among the 692 patients infected with the pdm09 virus, 10 patients (1.45%, 10/692) were found to harbor virus with the H275Y mutation codon. The monthly detection ratio of H275Y mutation was calculated and shown in Figure 3. The pattern showed three peaks: the first peak in July 2009 (0.9%), the second peak in March 2010 (5.6%) and the third peak in September 2010 (3.3%). This three peak pattern was similar to the epidemic peak of influenza A (H1N1) pdm09 virus. For the 323 patients infected with seasonal viruses, no instances of oseltamivir resistance-related mutations E119, D151, R292 and N294 were detected.

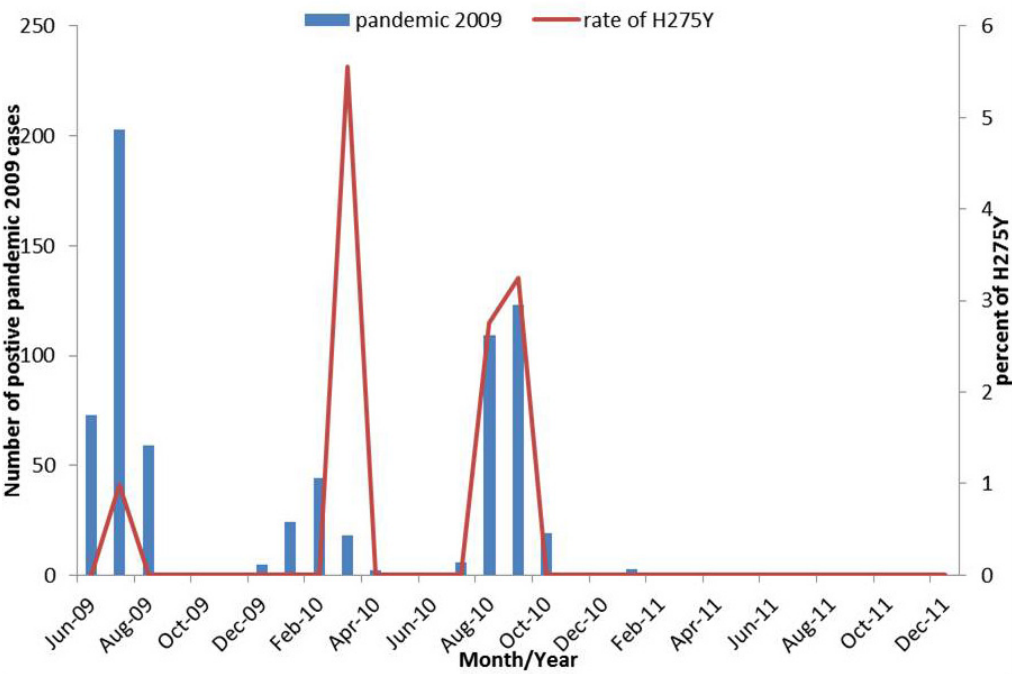

Fig. 3 Monthly percent distribution of oseltamivir resistant influenza A (H1N1) pdm09 during 2009–2011

IC_{50} of oseltamivir resistant influenza A (H1N1) pdm09 viruses

A chemiluminescent substrate-based assay was used to determined IC_{50} of oseltamivir against influenza A (H1N1) pdm09 virus. Isolates from six of the 10 patients with H275Y-containing virus were used for oseltamivir resistant influenza A (H1N1) pdm09 viruses detection. In 2009, the oseltamivir IC_{50} value for the H275Y virus isolates, belonging to clade 6, was 37.18 nM; in 2010, the oseltamivir IC_{50} values for the H275Y virus isolates, belonging to clade 8, were in the range 69.96±8.94 nM, while the oseltamivir IC_{50} values for the remaining H275Y virus isolates, belonging to clades 11-2, were in the range 79.31±11.20 nM. The mean oseltamivir IC_{50} value for all isolates was 69.18±18.25 nM.

Characteristics of patients with resistant virus

The majority of patients suffering infection with oseltamivir-resistant virus were also immunocompromised, in the age range of 9 months to 75 years (mean age, 31.9 years). There were four male and two female patients. Underlying diseases were recorded in five (83.3%) of the six patients, and three cases ultimately passed away. The course of oseltamivir treatment was 8 to 28 days. Demographic data for all six patients are presented in Table 1.

Table 1 Demographic data of oseltamivir influenza A (H1N1) pdm09 isolates during 2009-2011

Sample	Date of specimen collection	Specimen	Age (years)	Gender	Date of disease onset	Duration of oseltamivir/ zanamivir treatment (days)	Underlying disease	IC_{50} of oseltamivir (nM)
simi501	30/07/09	NP Wash	16	Male	22/07/09	28**	SLE	37.18
simi508*	23/03/10	NP Wash	37	Male	16/03/10	24	Chronic myelogenous leukemia, HBV	63.63
simi504	30/08/10	NP Wash	27	Female	Unknown	Unknown	Unknown	91
simi505*	01/09/10	NP Wash	75	Female	22/08/10	8	Urethral injury, Hypertension neuromuscular dysfunction of bladder	68.65
simi502	08/08/10	NP Wash	9 months	Male	Unknown	10**	TB, pneumonia, RSV and secondary bacterial infection	78.3
simi503*	14/08/10	Sputum	36	Male	Unknown	10**	TB, HIV	76.28

*Deceased

**Specimen collected after oseltamivir treatment

Genetic characteristics of oseltamivir-resistant influenza A (H1N1) pdm09 virus

Full-length sequences of the eight gene segments in all six samples of oseltamivir-resistant influenza A (H1N1) pdm09 virus were determined, followed by phylogenetic analysis. To compare the similarity of sequences from cultured and direct specimens, four samples of full-length sequence that were obtained directly from specimens or from culture were compared and percent identity determined. The result showed that percent identity ranged between 99.1-100%. These data indicate that full-length sequences from specimens can be used for studying oseltamivir drug resistance and viral genetic diversity.

Analysis of the *HA gene*

Based on the HA gene, influenza A (H1N1) pdm09 virus can be classified into 11 clades.[8] To identify virus clade, a phylogenetic tree of six HA sequences samples was generated and clustered into three clades, which were clade 6 (n=1), clade 8 (n=2), and clade 11-1 (n=3), as shown in Figure 4. The HA protein of influenza A (H1N1) pdm09 virus contains five major antibodies binding sites; Sa, Sb, Ca, Cb and Pa.[12,13] Mutational changes in these sites can generate novel strains allowing immunological escape. Amino acid mutations of oseltamivir-resistant influenza A (H1N1) pdm09 virus are shown in Table 2. The result showed amino acid substitutions at position 202 and 220 of Sb and Ca sites, respectively. One sample, SIMI503, showed an amino acid substitution at position D239G of Ca site, which might contribute to clinical severity.[14]

Table 2 Antigenic epitope of oseltamivir-resistant influenza A (H1N1) pdm09 viruses during 2009-2011

Influenza A virus strains	Antigenic epitope																		
	Cb						Sa		Pa	Ca						Sa			
	87	88	89	90	91	92	141	142	147	154	155	156	157	158	159	170	171	172	173
A/California/07/2009	L	S	T	A	S	S	P	N	K	P	H	A	G	A	K	K	K	G	N
A/Bangkok/SIMI501/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI502/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI503/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI504/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI505/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI508/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Sa							Ca					Sb						
	174	176	177	178	179	180	181	183	184	185	186	187	201	202	203	204	205	206	207
A/California/07/2009	S	P	K	L	S	K	S	I	N	D	K	G	T	S	A	D	Q	Q	S
A/Bangkok/SIMI501/2009	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI502/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
A/Bangkok/SIMI503/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Bangkok/SIMI504/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
A/Bangkok/SIMI505/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
A/Bangkok/SIMI508/2010	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Sb					Ca													
	208	209	210	211	212	220	221	222	238	239	252	253	254						
A/California/07/2009	L	Y	Q	N	A	S	S	R	R	D	E	P	G						
A/Bangkok/SIMI501/2009	.	.	.	.	.	.	.	.	.	.	.	.	.						
A/Bangkok/SIMI502/2010	.	.	.	.	.	T	.	.	.	.	.	.	.						
A/Bangkok/SIMI503/2010	.	.	.	.	.	T	.	.	.	G	.	.	.						
A/Bangkok/SIMI504/2010	.	.	.	.	.	T	.	.	.	.	.	.	.						
A/Bangkok/SIMI505/2010	.	.	.	.	.	T	.	.	.	.	.	.	.						
A/Bangkok/SIMI508/2010	.	.	.	.	.	T	.	.	.	.	.	.	.						

Analysis of the *NA gene*

All six isolates were shown to possess an amino acid substitution at position H275Y, which is related to oseltamivir drug resistance. Moreover, all isolates were found to contain additional mutations at positions V106I and N248D, which were similar to previously reported studies.[15] Other mutations are shown in Table 3. Moreover, amino acid substitutions at position S247N, I223V or I223R, which are known to decrease susceptibility to oseltamivir, were not found in these isolates.[16] Furthermore, a zanamivir resistance-associated mutation, R152K, was also absent in these isolates.[17]

Table 3 Amino acid changes in oseltamivir-resistant influenza A (H1N1) pdm09 isolates during 2009-2011.

Gene	Mutation Position	Number of isolates with amino acid changes
PB2	V344M	V(3), M(3)
	I354L	I(3), L(3%)
	G509E	G(5), E(1)
	M570I	M(5), I(1)
	A624S/V	A(4), S(1), V(1)
PB1	I364V	I(5), V(1)
	K386R	K(5), R(1)
	I397M	I(3), M(3)
	I637V	I(5), V(1)
	K736E	K(5), E(1)
PA	C8S	C(5), S(1)
	V14I	V(5), I(1)
	P224S	P(1), S(5)
	K256Q	K(5), Q(1)
	N321K	N(3), K(3)
	A343T	A(3), T(3)
	N373S	N(5), S(1)
	I423V	I(4), V(2)
	K716Q	K(5), Q(1)

Table 3 (Con.)

Gene	Mutation Position	Number of isolates with amino acid changes
HA	K2E	K(5), E(1)
	F12L	F(5), L(1)
	N55D	N(5), D(1)
	S202T	S(3), T(3)
	A214T	A(3), T(3)
	S220T	S(1), T(5)
	D239G	D(5), G(1)
	R276K	R(5), K(1)
	T294A	T(5), A(1)
	E391K	E(1), K(5)
	F432L	F(5), L(1)
	V466A	V(5), A(1)
	S468N	S(3), A(3)
	I564V	I(5), V(1)
NA	T9I	T(5), I(1)
	I26V	I(5), V(1)
	Q43K	Q(5), K(1)
	N44S	N(3), S(3)
	I46V	I(5), V(1)
	V62L	V(5), L(1)
	V106I	I(6)
	V234I	V(5), L(1)
	V241I	V(3), L(3)
	N248D	D(6)
	H275Y	Y(6)
	T332K	T(5), K(1)
	N369K	N(3), K(3)
NP	V100I	I(6)
	L122Q	Q(6)
NS1	V65M	V(5), M(1)
	T76I	T(5), I(1)
	L90I	L(3), I(3)
	R108M	R(5), M(1)
	I156V	I(5), V(1)
	D207N	D(5), N(1)
M1	F79L	F(3), L(3)
	D88E	D(5), E(1)
	A200E	E(6)
	N207T	N(5), T(1)

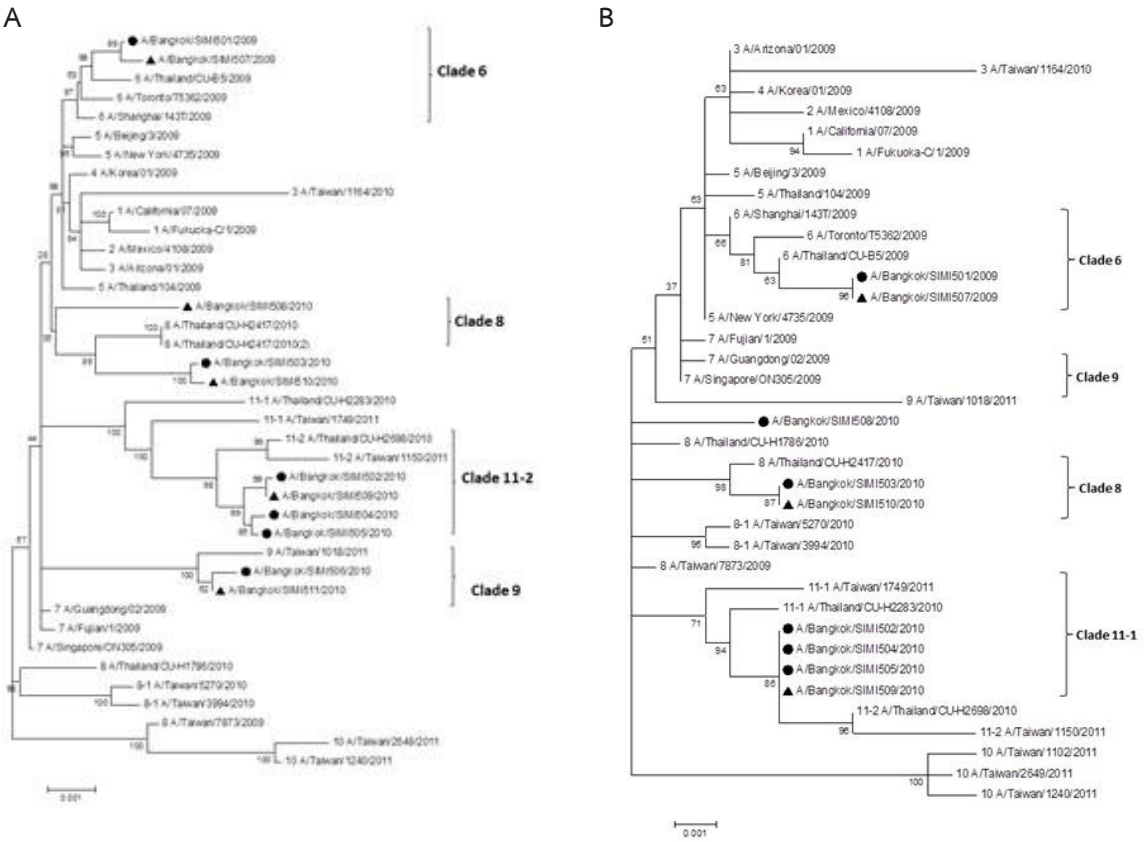

Fig. 4 Phylogenetic relationships of A) PB2-PB1-PA-HA-NP-NA-M-NS concatenated sequences, B) Full-length HA in oseltamivir-resistant influenza A (H1N1) pdm09 viruses circulating during June 2009-December 2011. The phylogenetic tree was constructed using the maximum-likelihood method, with 1000 bootstrap replications. ● Cultured sample, ▲ Original specimen.

Analysis of *other genes*

It has recently been reported that mutations in PB2 and PB1-F2 could affect virulence by interacting with mitochondrial antiviral signaling protein and inhibiting beta interferon expression.[18] However, these known virulence-associated mutations in the PB2 gene at amino acid positions E158G/T271A/T588I/E627K/E677G/D701N were found to be absent in all six pdm09 isolates. The PA gene of influenza virus is also known to contain virulence-associated mutations; the P224S mutation is located in the nuclear localization signal domain and may influence the transport and accumulation of PA in the nuclei of virus-infected cells. The PA-A70V mutation was identified in the mouse-adapted H1N1/09 virus, and on its own on a A/CA/04 virus background it did not cause lethality or more than 10% weight loss.

However, we have previously demonstrated that PA-A70V combined with PA-P224S significantly boosted the lethality and lung pathology of PA-P224S alone.[19] In the present study, we only found PA-P224S in five of our six oseltamivir-resistant pdm09 isolates (83%). Finally, all six isolates were found to be negative for the NS1-I123V mutation, which may play a role in enhancing viral gene expression.[20]

Discussion

In 2009, a novel influenza virus was recognized as the causative agent for an outbreak of respiratory illness in Mexico and the United States. The virus spread rapidly and reached other countries within several weeks. In Thailand, two confirmed infected cases were first reported on 12th May 2009. The first wave of the outbreak attacked young, school aged children, with an attack rate of 20%, and peaked in July-August 2009. The second wave was documented in adults over the age of 25 years during the winter period, with the rate of attack at 10% and peaked in February 2010. The third wave was found in the elderly during the Thai rainy season and peaked in September 2010. The attack rate for the third wave was 15% of the entire Thai population.

In this study, samples were collected from the beginning of the outbreak of pandemic influenza in Thailand through the post-pandemic period (2009-2011), first wave through third wave. Influenza A was detected in 13.9% (1,015/7,264) of patients during this time by real-time PCR. Influenza A (H1N1) pdm09 virus was detected in 9.5% (692/7,264), while seasonal influenza A viruses of H3 and H1 genotype were detected in 4.4% (322/7,264) and 0.01% (1/7,264), respectively. Seasonal influenza A/H1 virus was found in the early stage of the outbreak but was outcompeted by the pandemic strain and disappeared during the post-pandemic period.[21,22] Peaks of influenza A (H1N1) pdm09 virus infection in this study were found in June 2009, February 2010 and August 2010, which concurs with surveillance center findings at the Bureau of Epidemiology (BOE), Ministry of Public Health, Thailand.[23]

During the emergence and subsequent pandemic of this novel strain of influenza virus, antiviral agents, especially oseltamivir, were widely used for treatment in Thailand. Commonly Thai physicians prescribe oseltamivir for treatment of influenza, which may contribute to an increase of oseltamivir-resistant influenza virus strains.[24] The emergence of noticeable resistance to oseltamivir among seasonal influenza A viruses during late 2007 to early 2008 was identified with a high prevalence worldwide, so it is necessary to conduct antiviral drug susceptibility surveillance among circulating influenza viruses worldwide.[25] The detection of oseltamivir-resistant influenza A in this study was performed in 692 positive influenza A samples. The appearance of oseltamivir-resistant influenza A (H1N1) pdm09 viruses was shown in 1.45% (10/692) and was

associated with mutation at H275Y of the NA gene, which is in agreement with reports from other countries (0.31-1.3%).[26,27,28] Moreover, the prevalence of oseltamivir-resistant influenza A (H1N1) viruses was found to be (0.9%), (5.6%), (3.3%), during the first, the second and the third waves of the pandemic, respectively. Interestingly, the highest number of oseltamivir-resistant influenza A (H1N1) viruses was found in the third wave of outbreak, which was similar to other reports from the UK.[29] In addition, reports from the United States showed the incidence of oseltamivir-resistant influenza A (H1N1) viruses in 2009-2010 and 2010-2011 to be 0–5% and 0.9% [Roberts et.al., 2012). To determine susceptibility of oseltamivir for influenza A (H1N1) pdm09 virus, phenotypic assays were performed to identify the IC_{50} values for the six available samples (Table 1) with the mutation H275Y. The IC_{50} range was 69.18±18.25 nM, approximately 364-fold higher than that of oseltamivir sensitive virus (data not shown). Furthermore, the demographic data showed that most oseltamivir-resistant influenza A (H1N1) pdm09 viruses were detected in immunocompromised patients, in agreement with other reports.[30]

The genetic characterization of full-length oseltamivir-resistant influenza A (H1N1) pdm09 viruses was performed in six samples by using next generation sequencing. To evaluate the type of samples which were suitable for genetic study, four pairs of full-length nucleotide sequences from culture and direct samples were compared. The results showed that each pair were between 99.1-100% percent identical, implying that specimens can be used directly for investigating viral genetic diversity and antiviral drug resistance. To classify influenza A (H1N1) pdm09 viruses into 11 clades, HA genes was used.[31,32] In this study, oseltamivir-resistant influenza A (H1N1) pdm09 viruses were classified into clade 6, clade 8, clade 9 and clade 11-2. The majority of oseltamivir-resistant influenza A (H1N1) pdm09 viruses belonged to clade 6, clade 8 and 9, and clade 11-12 for the first, second and third wave of epidemic, respectively. This result was in agreement with the findings of Yang J et al. who showed that viruses belonged to clade 1-8 in the first period, while later viruses belonged to clade 11.8 Analysis of whole genome sequencing of the oseltamivir-resistant influenza A (H1N1) pdm09 viruses in this study showed the presence of substitutions including HA-E391K, PA-V14I, PA-K716Q, PB1-K736G, which were in concordance with the study of new variant pandemic 2009 viruses in Singapore.[33] However, no further mutations were found at any other antigenic site. Most studies propose that the mutation at position 222 in the HA gene was related to the severe morbidity and mortality of the pandemic 2009 H1N1 virus.[34] In addition, we found the D239G mutated residue to be present in the virus isolated from one mortality case. This mutation also was reported related to severe disease.[14] Moreover, this case also

contained many mutated codons found including HA-E391K, PA-V14I, PA-K716Q and PB1-K736E. A report from Maurer-Stroh et al. showed that mutation at HA-E391K could affect the stability of membrane fusion but the roles of the other mutations remain unknown [Maurer-Stroh et.al., 2010]. Interestingly, only mutations at position HA-E391K, HA-S200T and NS1-I123V were found in oseltamivir-resistant influenza A (H1N1) pdm09 viruses isolated in 2010 and contained an IC_{50} value 2-fold higher than that of 2009 isolates.

Conclusion

Results obtained from this study will provide insight into the characteristics of the oseltamivir-resistant influenza A (H1N1) pdm09 viruses that caused an outbreak in Thailand during 2009-2011. This is the first report of full-length genome analysis of oseltamivir-resistant influenza A (H1N1) pdm09 viruses related to clinical outcome in Thailand. Although the number of infected pdm09 patients has now declined, the new variants of these viruses remain in circulation and may increase in the future. Thus, surveillance of influenza A (H1N1) pdm09 virus and drug resistance should be performed annually.

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Conflict of interest: There are no conflicts of interest with regard to the present study.

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