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Detection of neuraminidase inhibitor-resistant influenza A (H1N1)pdm09 viruses obtained from influenza surveillance in Indonesia

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Abstract

Background: Influenza antiviral resistance has been shown to occur in many countries and is commonly found in influenza A(H1N1)pdm09 and A(H3N2). In this study, we monitored and investigated the neuraminidase inhibitor resistance of influenza A(H1N1)pdm09 viruses through the influenza surveillance system in Indonesia.

Methods: A total of 4752 clinical specimens were collected from patients with influenza-like illness and severe acute respiratory infection during the year 2016. An allelic discrimination assay was conducted by a single base substitution or a single-nucleotide polymorphism that is specific to the H275 wild-type and Y275 mutant. Sequencing was performed to confirm the H275Y mutations, and we analysed the phylogenetic relationship.

Results: The first occurrence of oseltamivir-resistant influenza A(H1N1)pdm09 was observed in the samples from the influenza-like illness surveillance. Two H275Y oseltamivir-resistant viruses (0.74%) out of 272 influenza A(H1N1)pdm09 positives were found. Both of them were collected from untreated patients.

Conclusion: The number of oseltamivir-resistant influenza A(H1N1)pdm09 viruses in Indonesia is very low. However, it is necessary to continue with active surveillance for oseltamivir resistance in severe and mild cases.

Keywords

Neuraminidase inhibitor resistant, influenza A(H1N1)pdm09, surveillance

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Background

Influenza viruses cause substantial morbidity and mortality in humans worldwide, especially in high-risk groups such as persons at both extremes of age, hospitalized, and immunocompromised patients.[1,2] These risk groups benefit from prompt institution of antiviral treatment for influenza A and B. At present, two classes of anti-influenza drugs are available: inhibitors of the viral matrix protein 2 ion channel (adamantanes: amantadine, rimantadine) and inhibitors of the viral neuraminidase (oseltamivir, zanamivir, peramivir).[3] However, antiviral resistance development has been shown to occur during and in the absence of antiviral treatment.[4]

Neuraminidase inhibitor resistance has been reported in several countries.[5] Global surveillance data have shown that neuraminidase inhibitor resistance among influenza viral strains is very low (<1%). These data also indicate that there is a larger cluster of resistant influenza strains among influenza previous seasonal A(H1N1) than A(H3N2).[3] Although the influenza former strain A(H1N1) was replaced by influenza A(H1N1)pdm09 in 2009, the risk of a neuraminidase inhibitor-resistant strain developing is of concern.[4]

The resistance in influenza A(H1N1)pdm09 has been reported to be correlated with the H275Y mutation in the neuraminidase (NA) gene.[6] This resistant influenza A(H1N1) pdm09 virus can possibly spread globally and become a major clinical and public health issue. Therefore, continuous

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monitoring for NA inhibitor resistant viruses is very important to anticipate any increases in the number of resistant viruses since NA inhibitors are used broadly for treatment and outbreak control.

Influenza surveillance activity in Indonesia has been conducted since 1999 in hospitals and primary health care.[7,8] Since 2014, we have conducted monitoring for NA inhibitor resistance through influenza surveillance among severe acute respiratory infection (SARI) cases in six hospitals. Between 2014 and 2015, we collected a total of 1823 SARI cases, and none of the influenza A(H1N1)pdm09 strains had the H275Y mutation.[9] The monitoring for the H275Y mutation among circulating influenza A(H1N1)pdm09 viruses through influenza surveillance is still ongoing. In this study, we monitored and investigated the NA inhibitor resistance among circulating influenza viruses in Indonesia through influenza surveillance of both influenza-like illness (ILI) and SARI cases during 2016.

Methods

Clinical specimens

We collected nasopharyngeal and/or oropharyngeal specimens from ILI and SARI surveillance cases throughout Indonesia. We defined ILI cases to include fever ⩾38°C, cough, and symptom onset within the last 10 days, while SARI cases included acute respiratory infection symptoms within 10 days of onset of cough, a measured/history of fever >38°C, and hospitalization.[10] Specimen collection was conducted from January to December of 2016 among cases admitted to the 27 public health centres and 6 hospitals as sentinels of ILI and SARI in 27 out of 34 provinces in Indonesia. All of the specimens collected were then stored and analysed at the National Influenza Centre (NIC) of Indonesia.

Influenza typing and sub-typing assays

We identified all of the collected clinical specimens of ILI and SARI using the centres for Disease Control (CDC) (Atlanta, United States) real-time *reverse transcription polymerase chain reaction* (RT-PCR) typing and sub-typing assay.[11] This assay is based on TaqMan chemistry and includes a panel of oligonucleotide primers and dual-labelled hydrolysis probe sets for universal influenza A and B, H1pdm09, H3, H5, and RNP (ribonucleoprotein). Viral ribonucleic acid (RNA) was extracted from 140 μL of the nasopharyngeal specimens using a QIAamp Viral Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amplification was performed using the SuperScript™ III Platinum Taq one-step quantitative kit (Invitrogen, Carlsbad, CA, USA) in the IQ5 quantitative PCR system (Bio-Rad, Hercules, CA, USA). The total volume of the amplification reaction was 25 μL, consisting of 12.5 mL of 2× buffer, 0.5 μL of enzyme mix, 0.5 μL of both forward and reverse primers (40 mM), and 0.5 μL of probe (10 mM) and Diethylpyrocarbonate (DEPC)-treated water each, which added to a total volume of 20 μL. Finally, 5 μL of viral RNA extracted from the clinical samples was added to the real-time RT-PCR assay mix.

Allelic discrimination assay

All of the ILI and SARI clinical specimens that were identified as influenza A(H1N1)pdm09 was then subjected to an allelic discrimination assay by a single nucleotide polymorphism (SNP) that is specific for influenza A(H1N1)pdm09 viruses. The assay is based on multiplex one-step real-time RT-PCR, which uses a pair of primers with two fluorogenic TaqMan Minor Groove Binder probes (MGB). One of the probe is specific for cytosine (C) nucleotide at the codon of CAC, which is represent for histidine at the position 275 (H275) wild-type (labelled with FAM). While the other probe is specific for thymine (T) of the TAC of Tyrosine (Y275) mutant which is labelled with VIC. The primers and probes were designed by the Regional Influenza Reference Laboratory of the SEA Region (*RIRL*, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand).[12] The total volume reaction mix was 20 μL, consisting of 10 μL of 2× reaction mix, 0.4 μL of enzyme mix, 0.4 μL of both primers forward and reverse, 0.4 μL of both probe mutant and wild type (10 mM), and 0.4 μL nuclease free water. Finally, 5 μL of viral extracted RNA from clinical samples was added to the assay mix. The amplification was performed with a pre-read at 60°C for 1 min, a reverse transcriptase at 50°C for 15 min, initial PCR activation step for 95°C for 2 min, 40 cycles of amplification including denaturation at 95°C for 15 s and annealing at 59°C for 1 min. The final step post-read collect data were 60°C for 1 min. A substantial increase in FAM-labelled probe fluorescence indicates the presence of the wild-type H275, whereas a substantial increase in VIC-labelled probe fluorescence indicates the presence of the Y275 oseltamivir resistance mutation.

Sequencing assay and phylogenetic analysis

We amplified and sequenced the extracted RNA using a specific primer for the hemagglutinin (HA) and NA genes of the influenza A(H1N1)pdm09 as described previously.[13] The assembly process of sequences from the HA and NA segments was performed using Sequencher 5.0 software (Gene Codes, USA), then aligned, and compared with reference viruses available from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ Database[14] and the National Centre for Biotechnology Information – Influenza Virus Resources (NCBI-IVR).[15] A residue analysis was generated using BioEdit version 7.0.8.0[16] and phylogenetic analyses conducted using the neighbour-joining (N-J) method. The tree was constructed using Kimura's two-parameter

Table 1. Demographic data and GISAID accession number of influenza A(H1N1)pdm09 used in this study.

	Strain	
	A/Indonesia/NIHRD_PLK135/2016	A/Indonesia/NIHRD_RIU263/2016
Age (year old)	18	3
Sex	Male	Male
Collection date	1 January 2016	16 June 2016
Location	Central Kalimantan	Riau
GISAID Accession no.	HA: EPI1123265, NA: EPI1123266	HA: EPI1123267, NA: EPI1123268

HA: Hemagglutinin; NA: neuraminidase; GISAID: Global Initiative on Sharing All Influenza Data.

distance model with 1000 bootstrap replicates implemented in MEGA 7 software.[17]

Results

A total of 4752 clinical specimens were collected through ILI and SARI surveillance during the year 2016. Two hundred and seventy-two of them (5.72%) were identified as influenza A(H1N1)pdm09. All of the 272 specimens were derived from patients who had not been treated by oseltamivir antivirals. They were screened for H and Y amino acid SNPs at 275 amino acid positions by allelic discrimination real-time RT-PCR assay. Out of the 272 specimens, two (0.74%) specimens were found to have a homozygous allele 1 (275Y), while 270 specimens were found to have a homozygous allele 2 (H275). The homozygous allele 1 has been correlated with oseltamivir resistance and was identified in two outpatient specimens from ILI cases.

Full-length sequencing of the HA and NA genes was conducted directly from the clinical specimens. The chromatogram clearly shows the mutant wild-type 275Y (TAC) population for two of these viruses. Confirmative analysis of the results obtained from Sanger sequencing revealed the specimens are resistant to oseltamivir antiviral. The NA sequence of the A/Indonesia/NIHRD_PLK135 has been identified in almost 99% of cases during the same period in the oseltamivir-resistant virus A/Tokyo/NCCHD2/2016 (GenBank accession number: LC270884.1), A/Georgia/31/2016 (GenBank accession number: KX409453.1), A/NewYork/36/2016 (GenBank accession number: KX409541.1), and A/Czech Republic/14/2016 (GenBank accession number: KX014856.1). The NA gene of A/Indonesia/NIHRD_RIU263 is 99% similar to the A/TOYAMA/86/2016 (GISAID Accession number: EPI841478), A/Linkou/0029/2015 (GISAID Accession number: EPI997891), and A/Sydney/185/2015 (GISAID Accession number: EPI704165). In this study, the resistant Indonesian influenza A(H1N1)pdm09 demographic patients and sequences data were deposited in the GISAID database (Table 1).

Phylogenetic tree analyses based on the full-length HA and NA genes showed that the circulating influenza A(H1N1) pdm09 viruses during the 2016 season in Indonesia were distinct from the 2009 season viruses. Even though the Indonesian resistant viruses belong to the same clade of 6B, they were cluster separated into the clade 6B.1 for A/Indonesia/NIHRD_PLK135/2016 and clade 6B.2 for A/Indonesia/NIHRD_RIU262/2016 (Figure 1(a)). Furthermore, the NA genes of the Indonesian H275Y oseltamivir-resistant viruses were clustered together with other oseltamivir-sensitive viruses collected during 2015 and 2016 (Figure 1(b)). They were not specifically from the oseltamivir-resistance group.

Discussion

The Global Influenza Surveillance and Response System of World Health Organization (GISRS-WHO) was developed to reduce the burden of disease associated with influenza.[19] By collecting and sharing information on influenza activity, the participating countries are contributing to the annual determination of the influenza vaccine content and influenza pandemic preparedness activities.[20] The GISRS, through an antiviral susceptibility expert working group (WHO-AVWG), also serves as a global alert mechanism for antiviral resistance and susceptibility data and as an early warning of the emergence of influenza viruses with pandemic potential.[21] More global surveillance data are needed to understand and predict the development of resistance. In addition, real-time detection of resistance in high-risk populations such as children and immunocompromised patients warrants attention in view of their treatment decisions.[22] One of the strategies used to support the antiviral surveillance programme and clinical management of patients is real-time analysis of antiviral resistance mutations in a population and in treated patients. Next-generation sequencing, Sanger sequencing, and allelic discrimination assay real-time RT-PCR are standard genotypic assays that can be used to detect antiviral resistance.[23]

This study shows the first occurrence of oseltamivir-resistant influenza A (H1N1)pdm09 viruses in Indonesia during surveillance of ILI. Two (0.74%) oseltamivir-resistant viruses from 272 cases were detected in respiratory samples taken from outpatients at the primary health centre. Both of the patients were not treated with antivirals. In Indonesia, oseltamivir is not used for prevention and treatment of seasonal influenza. It is only used as a drug for the management of avian influenza H5N1 cases due to the policy of the Ministry of Health.[24]

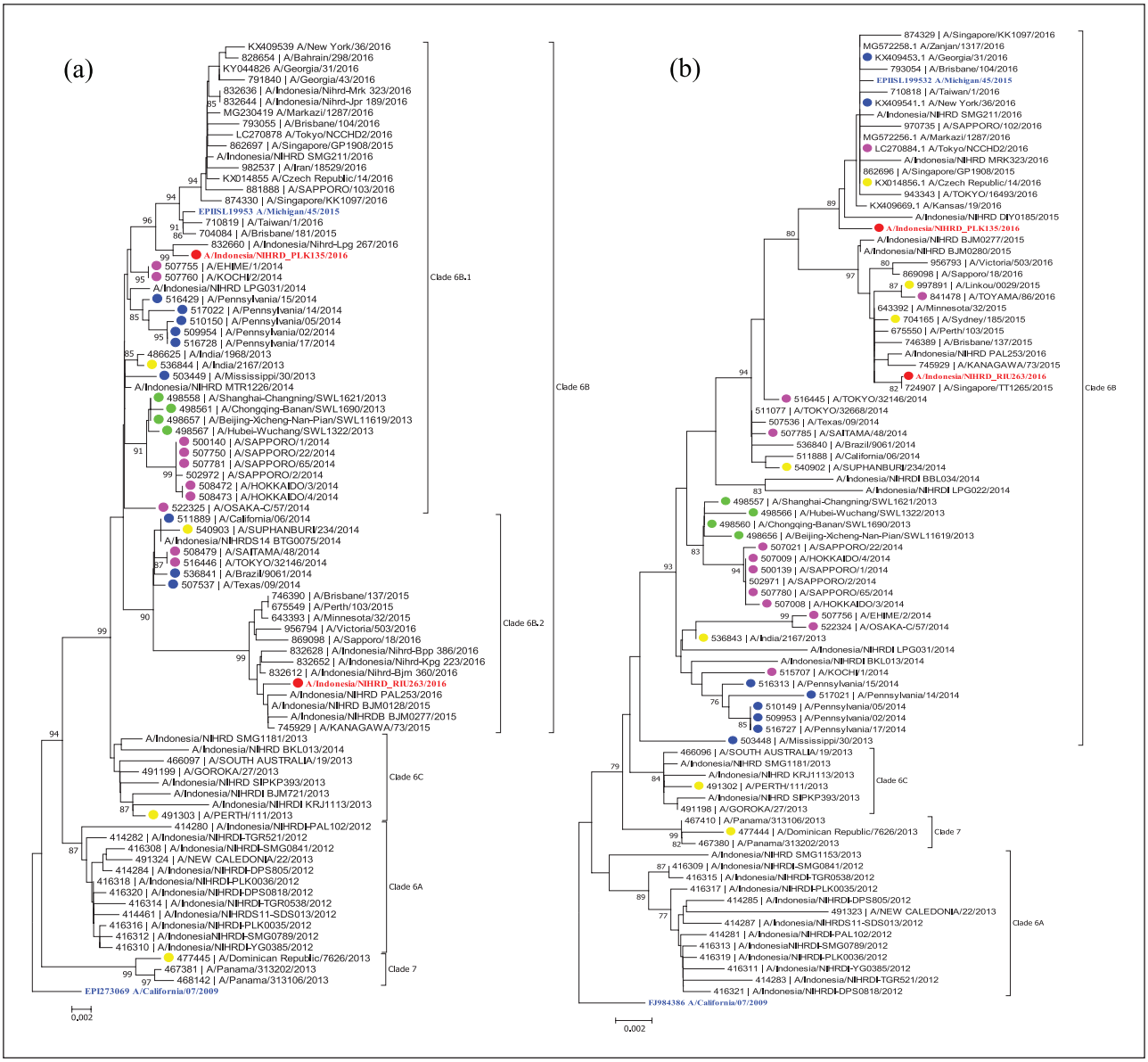

Figure 1. Phylogenetic tree for the HA (a) and NA (b) sequences. The evolutionary history was inferred using the neighbour-joining method, and the evolutionary distances were computed using the Kimura 2-parameter method. Evolutionary analyses were conducted in MEGA7. Red circle and sequences indicate the Indonesian oseltamivir H275Y-resistant influenza A(H1N1)pdm09 virus. Viruses carrying the mutation H275Y found in resistant influenza A(H1N1)pdm09 viruses from other country are marked with coloured circles (magenta circles from Japan, blue circle from United States, green circles from China, and yellow circles from the rest of the country).[18] The blue sequences indicate the vaccine virus 2016/2017 and 2018/2019. All of the sequences are rooted to the vaccine virus 2016/2017 as a reference.

Resistant H275Y influenza A(H1N1)pdm09 viruses in people not treated with oseltamivir have also been reported. As described in a study conducted in Sapporo Japan, a cluster of influenza A(H1N1)pdm09 viruses with H275Y mutation was detected in 39 (29%) of 135 samples collected from community with no antiviral treatment.[18] In Brazil, two (0.96%) out of 208 cases were carrying the H275Y mutation without prior antiviral treatment.[25] As part of their global monitoring of antiviral resistant and susceptibility programme from different regions of the world, the WHO also reported 74 H275Y mutant viruses; 21 (28.4%) cases were from untreated patients.[20] In a recent publication by WHO, 4 out of the 7 H275Y influenza A(H1N1)pdm09 cases were not received oseltamivir before samples collection.[26]

Importantly, all of the Indonesian viruses contained two other NA mutations, V241I and N369K together, since 2012. The virus with single mutation of H275Y causes a

detrimental effect on stability of NA gene.[27] However, those negative effect conferred into positive effect by two V241I and N369K mutations on virus having an H275Y mutation. This will enhance the NA expression and improve the fitness of the virus[28,29] and increase the risk of spreading and circulating the H275Y viruses into the larger community.[30] These two NA mutations have also been found in a community cluster in Japan and Australia.[30,31]

Phylogenetic analysis of HA and NA (Figure 1) showed that the Indonesia 2016 viruses are phylogenetically close to the viral vaccine 2017/2018 A/Michigan/45/2015. The viruses also clustered in a difference branch with the 2009 viruses. This suggests a positive selection result and better replication in the host. The 2016 Indonesian oseltamivir viruses have a close relationship with other oseltamivir viruses from Japan, the United States, and Europe in the same period. However, it is also grouped with other sensitive-viruses.

Our data have shown that the allelic discrimination real-time RT-PCR assay in this study is a simple and accurate way to detect H275Y oseltamivir-resistant mutants among influenza A(H1N1)pdm09 viruses. However, it is only detecting the amino acid change in the 275 NA gene position. Although the H275Y mutation is the most common mutation for oseltamivir resistant, other mutations in the NA gene can also affect drug susceptibility. Therefore, new allelic discrimination real-time RT-PCR detection will be necessary to detect other known or novel mutations conferring resistance to oseltamivir.

Conclusion

In conclusion, although the incidence of oseltamivir-resistant pandemic influenza A (H1N1) 2009 viruses in Indonesia is currently very low, it is necessary to continue with active surveillance for oseltamivir resistance in severe and mild cases because of the possibility of future widespread oseltamivir-resistant viruses in the general population.

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