Genetic variability makes influenza virus to escape the immunity and causes yearly epidemics. Monitoring those changes is necessary for vaccine selection. In addition, H3N2 viruses were considered to be seeded from Southeast Asia before spreading globally. This study described the molecular epidemiology of influenza A during the post-pandemic season 2010–2011 in Vietnam. Nasopharyngeal samples were collected from children with respiratory infections at Children’s Hospital 2, Ho Chi Minh City. The HA, NA, M genes were amplified, sequenced and analyzed. Thirty-five of 1,082 (3.2%) patients were positive for influenza A, including 14 pandemic H1N1 2009 (H1N1pdm09) and 21 H3N2 infections. H3N2 was dominant in the rainy season (May–October 2010) while H1N1pdm09 was dominant in the dry season (November 2010–April 2011). Phylogenetic analysis showed that Vietnamese H1N1pdm09 sequences in 2010–2011 formed the distinct cluster, with other contemporary Asian and 2012-American sequences, suggesting a possible common ancestor. All were oseltamivir-sensitive except two strains carrying S247N and D199N in NA which reduced the neuraminidase inhibitor susceptibility. The Vietnamese H3N2 viruses in mid-2010 belonged to the emerging subclade Perth10/2010, which then spread worldwide in 2011. The Vietnamese influenza viruses were well matched with the Southern Hemisphere vaccine formulation. Mutations at antigenic sites were also identified in these viruses. Surveillance of influenza viruses in tropical countries is important not only for development of their prevention and control strategies but also for earlier identification of the newly emerged strains that may be selected for future vaccine. 

**KEY WORDS:** epidemiological; molecular characteristics; influenza A; Vietnam

**INTRODUCTION**

Influenza virus infection is the major cause of morbidity and mortality in humans worldwide. The influenza epidemics lead to approximately 3 to 5 million severe illness cases and 250,000 to 500,000 deaths every year [World Health Organization, 2014]. The ability to acquire genetic changes makes the virus to overcome the immunity from previous infections and causes yearly epidemics. The hemagglutinin (HA) and neuraminidase (NA) genes of influenza A viruses usually mutate at high frequencies [Peter et al., 2013]. As a result, the accumulation of these mutations at critical positions may lead to alter the antigenic characteristics of surface glycoproteins and the antiviral drug susceptibility. Therefore,

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monitoring these genetic changes of influenza virus is important for vaccine strain selection as well as identification of drug resistant strains.

In addition, the influenza activity has been well described in temperate countries with outbreaks usually occurring during winter seasons [Peter et al., 2013]. Thus, influenza vaccine is delivered annually to protect against the newly emerged influenza strains. In contrast, influenza virus circulates throughout the year with no clear seasonality in tropical regions. Moreover, little epidemiological information about influenza makes it more difficult to develop the prevention and control programs in these settings [Viboud et al., 2006]. It also has been proposed that Southeast and East Asia are regions where influenza virus A/H3N2 first evolved before spreading worldwide [Rambaut et al., 2008; Russell et al., 2008a]. Therefore, influenza virologic surveillance in these areas is necessary not only to understand the circulation of influenza viruses but also to identify the virus strains which may be included in the annual influenza vaccine composition.

In Vietnam, the national surveillance has shown influenza infection peaks 1–2 times per year [Nguyen et al., 2013]. The annual influenza incidence is 17–26% based on serological studies [Horby et al., 2012]. The Northern Hemisphere influenza vaccine is available in the private sector but is not commonly used [Members of the Western Pacific Region Global Influenza Surveillance Response System et al., 2013]. Oseltamivir-resistant strains of pandemic H1N1 2009 (H1N1pdm09) were reported even on individuals not being received oseltamivir [Le et al., 2010]. In this study, the molecular and epidemiological characteristics of influenza A viruses circulating during the post-pandemic season 2010–2011 were described. These data may improve the understanding of the temporal and geographic circulation of influenza viruses and provide important information to develop the public health vaccination policies throughout the region.

MATERIALS AND METHODS

Patients and Samples

Children younger than 15 years old who were hospitalized with acute respiratory infections at the Children’s Hospital 2, Ho Chi Minh City, Vietnam from April 2010 to May 2011, were enrolled in the study. The study was approved by the Scientific and Ethical Committee of the Children’s Hospital 2. Informed consent was obtained from the parent or legal guardian of the patients before sample collection. Acute respiratory infection was defined as any child presenting with cough or/and difficulty in breathing [World Health Organization, 2005]. Nasopharyngeal flocked swabs (MicroRheologics, Brescia, Italy) were collected from all enrolled children within 24 hr after hospitalization. The samples were immediately kept at −20°C until further analysis at the laboratory.

Influenza Virus Detection and Subtyping

Viral nucleic acids were extracted directly from the clinical samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and stored at −80°C. Thirteen respiratory viruses (influenza virus A and B, human respiratory syncytial virus, human metapneumovirus, parainfluenza virus types 1 to 4, human rhinoviruses, human coronaviruses (229E and OC43), adenovirus and human bocavirus) were screened on each specimen by multiplex hemi-nested (RT)-PCR as described previously [Tran et al., 2014]. Samples positive for influenza A were further subtyped as seasonal H1N1, seasonal H3N2, or H1N1pdm09 by multiplex nested PCR [Furuse et al., 2010].

Sequencing and Phylogenetic Analysis

The HA, NA, and M genes of H1N1pdm09 and H3N2 viruses were amplified using gene-specific primers recommended by the World Health Organization [World Health Organization, 2012]. PCR products were then sequenced by the commercial company (Macrogen Japan Corp., Tokyo, Japan). Sequences were assembled by using Cap3 Sequence Assembly program [Huang and Madan, 1999]. Vaccine strain and reference sequences used for the phylogenetic trees were obtained from the NCBI GenBank Database (www.ncbi.nlm.nih.gov/genbank) and GISAID EpiFluTM (www.gisaid.org). The sequence data and the phylogeny were analyzed using BioEdit v.7.0.5 [Hall, 1999]. Phylogenetic analysis was performed using MEGA 5.0.5 [Tamura et al., 2011]. The neighbor-joining method [Saitou and Nei, 1987] with maximum composite likelihood model [Tamura et al., 2004] and 1,000 bootstrap replicates was used to construct the phylogenetic trees. Deduced amino acid sequences were analyzed and compared with the vaccine strains A/California/7/2009 (H1N1pdm09) and A/Perth/16/2009 (H3N2). HA and NA numbering is accordant with the respective subtype.

The HA, NA and M sequences in this study have been deposited in the GenBank database under accession numbers KJ955501-KJ955605.

Antigenic Site Mapping of HA and NA

The HA and NA protein sequences of influenza virus in this study were compared with the vaccine strains. The amino acid changes were mapped to the previously reported HA [Wiley et al., 1981; Lee and Chen, 2004; Xu et al., 2010] and NA antigenic sites [Fanning et al., 2000; Gulati et al., 2002]. Protein structures were rendered by PyMol software v1.3 (http://www.pymol.org) using the HA (PDB: 3LZG for H1, 1MQL for H3) and NA (PDB: 3NSS for N1, 1IVG for N2) structures from Protein Data Bank (RCSB PDB, http://www.pdb.org) [Berman et al., 2000].
RESULTS

Between April 2010 and May 2011, 1,082 children with acute respiratory infections were enrolled. Influenza A virus was found in 35 samples (3.2%), in which 14 were H1N1pdm09 and 21 were H3N2. The median age of children infected with influenza A virus was 15 months (range from 1 to 60 months). Children younger than 6 months had the lowest influenza A infection rate (1.4%) while those aged from 24 to 59 months had the highest infection rate (7.1%). It is worth mentioning that among children less than 5 years old, there is a progressive increase in influenza A positive rates with increasing age (Fig. 1). Of note, influenza A virus caused infections in both seasons of this tropical country, with H3N2 in the rainy season (May–October) and H1N1pdm09 viruses in the following dry season (November–April) (Fig. 2). No influenza-positive cases in this study were severe enough to require mechanical ventilation or become fatal. None of them had been vaccinated against influenza before or received any antiviral treatment.

Molecular Characterization and Phylogenetic Analysis

Sequences in this study were blasted searched in the NCBI's influenza virus sequence database and the GISAID EpiFlu database in order to find the similar sequences. Other strains from Vietnam, reference and vaccine strains were also analyzed together. Genetic groups in this study were named according to the WHO Influenza Centre London classification [WHO Influenza Centre London. National Institute for Medical Research, 2013].

**H1N1pdm09 virus.** Phylogenetic analysis showed the simultaneous co-circulation of three influenza virus groups in Vietnam (Fig. 3). In detail, one Vietnamese strain (A/HoChiMinh/962.11/2010) clustered with other strains from Vietnam, Cambodia, Singapore, and Australia in 2009–2010. These strains were characterized by the double mutations A197T and S203T and formed an additional genetic group which had not been named in the WHO Influenza Centre London classification [WHO Influenza Centre London. National Institute for Medical Research, 2013]. Another strain (A/HoChiMinh/1137.2/2011) belonged to genetic group 6, characterized by the mutations D97N, S185T in the HA1 region and E374K, S451N in the HA2 region. This strain clustered with the European and North American strains in 2011. Finally, the rest of Vietnamese strains in this study formed a distinct cluster within genetic group 7, characterized by the mutations S143G, S185T, A197T, E374K and S451N. They clustered with strains isolated in East and Southeast Asia, Australia, Europe in 2010–2011 and strains isolated in North and Central America in 2011–2012. Of note, all the Vietnamese strains in this study (except A/HoChiMinh/1137.2/2011 carried only L420V) carried all the substitutions L420V, D436E, and S539F that had not been reported in other influenza viruses before. These changes formed a unique signature for the 2010–2011 Vietnamese H1N1pdm09 sequences.

The HA protein of Vietnamese H1N1pdm09 showed some amino acid mutations with respect to the vaccine strain A/California/7/2009. Some of them located within the major antigenic sites Ca (H138Q, S203T), Cb (S71A), Sa (L161I), and Sb (S185T) of the HA molecule (Supplemental Figure 1). Moreover, other mutations S143G and A197T were also found in the vicinity of the Ca and Sb antigenic sites.

**H3N2 virus.** The phylogenetic analysis of the HA gene of H3N2 virus indicated that the 2010 Vietnamese sequences formed a distinct cluster, most closely related to the vaccine strain A/Perth/10/2010 subclade of the A/Victoria/208/2009 clade (Fig. 4). They did not group with the A/Perth/16/2009 clade (the vaccine strain). However, these two clades had similar antigenicity. These Vietnamese strains shared some similar key amino acid substitutions with the genetic groups 5 and 6 (D53N, Y94H, I230V, and E280A). They also carried the substitutions N389I, T485I, which had not been reported before. These mutations made the Vietnamese strains form the separate cluster from group 5 and 6.

When compared with the vaccine strain (A/Perth/16/2009), the Vietnamese strains had some mutations within the antigenic sites A (K144N, R150I), C...
(D53N, S54R, N278Y, E280A, N310H), D (R208K, T212A, S214I, I230V), and E (K62E, G78D, Y94H) on the HA protein (Supplemental Figure 1).

The NA phylogenetic tree was generally in agreement with that of the HA (Fig. 4). The NA protein also showed some mutations compared with the vaccine strain, in which 6 located at 3 antigenic sites F’ (D339N), I’ (S367N, K369T), L’ (I464L, L466F, L466I) (Supplemental Figure 1).

The M gene analysis showed that all strains were resistant to Amantadine due to possession of the S31N substitution in the M2 ion-channel protein.

Interestingly, one patient was infected with the swine influenza H3N2 variant (A/HoChiMinh/459.6/2010). This variant clustered together with other swine influenza viruses from Binh Duong province (Vietnam), Hong Kong and China. The full genome characterization of this variant is beyond the scope of this paper and will be described in another one.

**DISCUSSION**

Unlike in temperate countries where a single peak of influenza infections is usually observed during winter seasons, in this study, influenza viruses circulated throughout the year with 2 peaks occurring in the rainy and dry seasons. Not as expected, during the rainy season immediately after the pandemic period (May–October, coincide with the Southern Hemisphere winter season), no H1N1pdm09 virus was detected. Only H3N2 virus was detected. During the following dry season (November–April, coincide with the Northern Hemisphere winter season), H3N2 virus was replaced completely by H1N1pdm09. The national surveillance in Vietnam from 2006 to 2009 also revealed two peaks of seasonal influenza each year, one between June and October, and an additional minor peak during the winter time of the Northern Hemisphere [Nguyen et al., 2009, 2013]. Similar bimodal seasonal pattern of influenza activity was also reported in Thailand, Indonesia and India [Saha et al., 2014]. Determining the onset and the duration of influenza epidemics in the community is critical in guiding the appropriate timing for influenza vaccination, especially for high risk individuals, as well as guiding the use of antiviral agents.

Vietnam has been using the Northern Hemisphere formulation influenza vaccine in the private sector [Members of the Western Pacific Region Global Influenza Surveillance Response System et al., 2013]. Due to limitation of data on influenza seasonality, the usage of influenza vaccine composition was historically based on its hemispheric location. However, the phylogenetic analysis showed that the mid-2010 Vietnamese H3N2 viruses grouped with viruses from other countries in the winter 2010–2011 and belonged to the A/Perth/10/2010 subclade (Fig. 4). This subclade was antigenically similar to vaccine strain A/Perth/16/2009 in the 2010 winter season of the Southern Hemisphere and in the 2010–2011 winter season of the Northern Hemisphere. The Vietnamese H3N2 viruses did not group with the vaccine strain A/Brisbane/10/2007 in 2009–2010. These data proved that the influenza activity in Vietnam appeared earlier than in other temperate countries in the Northern Hemisphere, and the current Northern Hemisphere vaccine usage may be too late to provide the optimal protection. These findings are consistent with the recent genetic relatedness analysis of seasonal influenza A viruses circulating in Vietnam from 2001 to 2009, which showed the high match with the Southern Hemisphere vaccine strains [Vuong et al., 2013].

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Therefore, taken together with previous studies [Nguyen et al., 2009, 2013; Vuong et al., 2013; Saha et al., 2014], these results suggest that the Southern Hemisphere formulation and timing should be considered for use in Vietnam instead of the current Northern Hemisphere one. This is in accordance with reports from Brazil emphasizing that the adoption of seasonal influenza vaccine based on hemispheric location may not apply to tropical countries [de Mello et al., 2009]. It is also noted that the sub-clade A/Perth/10/2010, to which the strains in this study belonged, emerged and spread worldwide in the winter season of 2010–2011 [WHO Influenza Centre London. National Institute for Medical Research, 2011].

Regarding H1N1pdm09, there was co-circulation of different genetic groups in Vietnam during the post-pandemic period. None of these genetic groups were considered antigenically distinct from the vaccine strain. Most of the Vietnamese strains belonged

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Fig. 4. Phylogenetic analysis of HA (A) and NA (B) genes of influenza H3N2 viruses circulating in southern Vietnam during 2010–2011. Phylogenetic trees were constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values of greater than 70% are shown at the branch nodes. Characteristic mutations of a particular branch are indicated on the left side nodes. The genetic group assignment is indicated by the brackets on the right. The influenza strains in this study are marked with solid round. The influenza strains from Vietnam are marked with solid triangles. The Perth/10/2010 sub-clade is boldfaced. Vaccine strains for the Northern (N) and Southern (S) Hemisphere are shown in bold and italic and corresponding years are enclosed in boxes.

to group 7, clustered with contemporary strains from East and Southeast Asia, Australia during 2010–2011. They also tended to group with the North and Central American strains during the 2011–2012 season, suggesting that the American influenza viruses in 2011–2012 may have an Asian origin. Some of the amino acid substitutions were observed to locate within the major antigenic sites of the HA globular head. The S203T located within the Ca antigenic site but its buried position near the...
monomer-monomer interface makes its role unclear [Xu et al., 2010]. The S185T located within the receptor binding site and the A197T located nearby may affect the binding of HA with its receptor [Yang et al., 2010]. The E374K located in the HA oligomerization interface may have role in membrane fusion [Russell et al., 2008b]. The mutation D222G associated with more virulent genotype was not present in these samples [Liu et al., 2010]. Recent studies have also found some important antigenic sites in the stem of HA, of which, the 2 unique mutations L420V and D436E in Vietnamese strains were located in the R1 and R2 antigenic sites [Xu et al., 2011]. However, the antigenic properties of these viruses against the vaccine strain were not examined in this study. Therefore, whether these changes affect the vaccine effectiveness needs to be elucidated. In addition, the mutation associated with oseltamivir-resistant H275Y was not detected. Instead, 2 mutations D199N and S247N on the NA, which reduced the neuraminidase inhibitor susceptibility, were found. It should be noted that none of these patients were treated with antiviral drugs. The S247N was observed in viruses from Australia and Southeast Asia with mild reduction in oseltamivir and zanamivir susceptibility [Hurt et al., 2011]. The D199N mutation has recently detected from patients with oseltamivir resistance [Deyde et al., 2010; Ghedin et al., 2011] and exhibited to reduce susceptibility to oseltamivir and zanamivir [Okomo-Adhiambo et al., 2013]. Although the D199E/G/N mutations have respectively shown to reduce susceptibility to oseltamivir in seasonal H1N1, H5N1, and B influenza viruses, the effect of D199N mutation among H1N1pdm09 needs further investigation [Ghedin et al., 2011]. It should be noted that the viruses in this study were not cultured to avoid the generation of adaptive mutations in vitro.

In this study, the infection rates of influenza A rose with increasing age implies that maternal antibodies may provide protection against influenza during infancy. Previous research has demonstrated that this protection lasted up to 1 year after maternal influenza immunization [Zaman et al., 2008]. When the children become older, the involved influenza virus strains have drifted far apart and therefore the previous protection could not cross-protect against the new strains. However, influenza vaccination for pregnant women is not recommended in Vietnam. The data reported in this study have several limitations. Firstly, only 14-month data were available. Additional surveillance data are needed to confirm the seasonality and the circulation of influenza viruses. Secondly, a small number of influenza cases was identified. According to the hospital’s triage process, patients with suspected influenza infections will be referred to Infectious Diseases ward for isolation and treatment. As only samples from Respiratory ward were collected, the number of influenza infections in this study may be underrepresented. However, this result was in line with the finding of recent systematic review indicating that influenza infection rate was 3% (2.2–4.0%) among children under 5 years of age hospitalized with acute respiratory infection [Luksic et al., 2013]. Moreover, the infection rate might depend on different types of influenza virus affecting individuals of different ages, with school-aged children were the most commonly affected [Olson et al., 2007; Nguyen et al., 2013]. Most of patients in this study were <2 years of age might explain for the low influenza infection rate. Thirdly, the data were only collected on patients seeking hospital care, thus the results may not reflect episodes of milder disease occurring simultaneously in the community. Finally, the study was lack of the antigenic assay against vaccine strains and the antiviral drug susceptibility tests. Therefore, the inferences regarding the protection of vaccine strains or of antiviral drugs are limited. However, the genetic data are also useful since amino acid mutations in some important regions usually correlate with significant antigenic changes [Smith et al., 2004].

In conclusion, the findings in this study may help to understand better the seasonal influenza virus circulation, which are essential for deciding the optimal time for vaccination as well as the suitable vaccine formulation. These data may help policymakers to inform and develop the influenza prevention and control strategies, not only in Vietnam but may also be applied throughout the region with similar influenza seasonal pattern. Continuing surveillance and characterization of circulating viruses in order to identify the emergence of genetic, antigenic, and drug-sensitivity changes is critical to sustain the prevention and control policy.

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