# The Antigenic and Phylogenetic Analysis of Surface Protein Genes of Influenza A Viruses in Taiwan, 2004-2007

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**Abstract**

The genetic mutation of influenza viruses (flu viruses) can produce different effects on viral antigenicity. Moreover, genetic changes in key amino acid of influenza viruses may play a major role in global influenza pandemic. Earlier research had found that different genetic changes on haemagglutinin (HA) and neuraminidase (NA) can affect deeply to the antigenicity of influenza viruses. The information constantly collected through monitoring on genetic and antigenic mutations of influenza viruses is important and crucial, to both the control of epidemic status, and the annual selection of influenza vaccine strain To understand the gene mutations of influenza viruses, evaluate the possibility of influenza pandemic, and explore the correlation between gene sequence evolution and antigenicity changes of the viruses in Taiwan, we sequenced the surface protein genes from local influenza virus isolates and analyzed with bioinformatic techniques. Furthermore, sequence analysis of internal matrix protein gene

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to find out the drug resistance pattern to amantadine, an antiviral drug for influenza A infections, during the surveillance period in Taiwan, and the data can be used as a reference for treatment and medication.

**Keywords: influenza A viruses, antigenicity, sequence analysis, Taiwan**

## Introduction

The influenza viruses comprise A, B, and C types according to the antigenicity and genetic characteristics of nucleoprotein (NP) and matrix protein (MP). Type A influenza viruses have caused several pandemics in human history. They contain envelope and eight segments of negative-stranded RNA. Because lack of proofreading activity, the RNA-dependent RNA polymerase makes gene mutations which produce substitutions in amino acid composition and cause changes of viral antigenicity, drug resistance, or virulence [1]. The resulting rapid change in viral genes and surface antigen generates different prevalent strain of influenza each year. Moreover, the molecular phylogenetic analysis of influenza virus can provide not only the relevant genetic information about seasonal isolated strains, but also the reference for predicting seasonal endemic altitude.

The influenza A viruses are subtyped based on surface proteins HA and NA. The main subtypes causing global pandemic are H1N1 and H3N2, which are also main endemic subtypes in Taiwan in recent years. Antigenicity determination of influenza virus is based on a hemagglutination -inhibition (HI) test which targets on the surface protein HA. If a virus shows as a low reactor to vaccine strain antiserum in HI test, the antigenicity of the virus has changed. As a result, antibody produced from immune system after vaccination may not well recognize the new mutant, the strain

may then increase its pathogenicity and becomes a pandenmic virus. Presently researches in evolution and typing of influenza virus in the world are mainly targeting on HA gene. However, besides mutations in HA gene, other viral genes such as NA and other internal genes have similar viral genetic reassortment frequency as well. Moreover, a single mutation of internal gene possibly increases the pathgenicity and drug resistance of the influenza virus [2-4]. This study using influenza A isolates in Taiwan between 2004-2007, determine gene sequences code for surface antigen HA and NA, our objectives are to understand the evolution of the virus in Taiwan in recent years, and also study its correlation with antigenicity.

## Materials and Methods

### A. Virus culture and antigen identification

There were 902 influenza A virus strains collected in Taiwan from 2004 to 2007, a selection of 54 representative strains for study was done by using a software invented by Taiwan CDC and Academia Sinica [5]. Criteria for selection are antigenicity, isolation time and geographic area. There were 19 A/H1N1 strains and 35 A/H3N2 strains selected. All samples were inoculated in MDCK cells for virus culture, collected viral supernatants in 3-5 days, and proceeded to HI test with WHO Influenza Diagnostic Reagent Kit to determine viral antigenicity. Test procedures followed the protocols provided by the kit [6].

### B. Amplification and sequencing of influenza virus gene fragments

The nucleic acids were extracted from viral supernatants with QIAamp viral RNA kit (Qiagen). Amplification for HA, NA, and MP fragments was done with Qiagen one-step RT-PCR reagent kit followed

the manufacturer's instructions. The initial reaction was incubated at 50°C for 30 minutes then heated at 95°C for 15 minutes, followed by 3-step cycling profiles for 35 cycles, each cycle consisted of 30 seconds denaturation (95°C), 30 seconds annealing (55°C), and extension for 60 seconds at 72°C. The final extension was 72°C for 10 minutes. After the reaction was completed, cycle sequencing was performed with BigDye® Terminator v3.1 Cycle Sequencing kit for 25 cycles of 95°C for 5 seconds and 60°C for 4 minutes. Finally, the products were precipitated with 100% alcohol, air dried, then dissolved in HiDi-formamide before sequencing was carried out with a ABI 3130XL sequencer.

**C. Sequence analysis**

The nucleotide sequences were aligned and analyzed by MEGA 4.1 software. Phylogenetic dendrograms were constructed with neighbor-joining method using 1000- bootstrap replicates algorithm [7].

## Results

**A. H1N1 influenza A viruses**

We used the full-length HA1 subunit of HA gene (1193 bp, nucleotide position 1-1193) and full-length NA gene (1413 bp, nucleotide position 1-1413) of influenza A /H1N1 viruses for phylogenetic analyses. We found that from 2004 to 2007, the genes that code for surface antigen of A/H1N1influenza viruses followed the seasonal evolution pattern. When comparing the amino acid composition of the isolated strains from 2004 to 2005 with A/New Caledonia/20/99 vaccine strain, amino acid changes at 169A, 255R, and 256F, were observed. More amino acid changes were found at 81R, 101H, and 269N from the strains in 2005 to 2006, in

addition to those changes found in previous season. The isolates from 2006 to 2007 were found to have three additional amino acid changes, i.e., 144E, 192M, and 197K. On the other hand, by aligning NA gene sequences, we discovered an amino acid change, V48I, for 2004-2005 isolates, when compared with A/New Caledonia/20/99 vaccine strain. The isolates from 2005 to 2006 had additional 64N and 265T amino acid changes. The isolated strains from 2006-2007 had more amino acid changes, i.e., 130K, 213G, and 266M. The phylogenetic topology was shown in Figure 1.

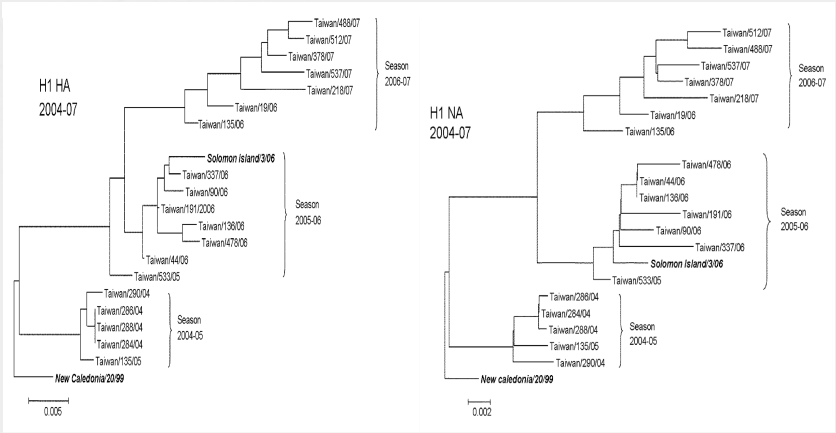


**Figure 1. Phylogenetic tree analysis of surface antigen genes in influenza A/H1N1 viruses from 2004 to 2007(WHO suggested vaccine strains are shown in bold italic font).**

**B. H3N2 Influenza A viruses**

We used the full-length HA1 subunit of HA gene (1178 bp, nucleotide position 7-1184) and full-length NA gene (1407 bp, nucleotide position 1-1407) of influenza A H3N2 viruses for phylogenetic analyses. The results revealed that the surface antigen genes of influenza A/H3N2 viruses from 2004 to 2007 evolved in a time sequential way. The antigenicity of

A/H3N2 influenza viruses isolated from 2004-2005 matched to HA gene sequences. These isolates can be divided into two clades. One clade was similar to the vaccine strain A/Wyoming/3/03 from 2003 to 2004; the other clade was similar to the vaccine strain A/California/7/04 from 2004 to 2005. Further comparison of amino acid composition between two clades, the California-like isolates had an additional 227P change than Wyoming-like isolates. The isolates from 2005 to 2006 had additional 193F and 225N changes, included around one-third of isolates also with 50E change. Compared to the previous flu season, the isolates from 2006 and 2007 had two amino acid changes, 142G and 144D. Some isolates had a position 173E change. The genetic analysis on the NA gene of A/H3N2 influenza viruses from 2006-2007, the isolates could obviously be divided into two clades, included one with 370S and 387S changes, and the other with 86K and 335G changes. The phylogenetic topology was shown in Figure 2.

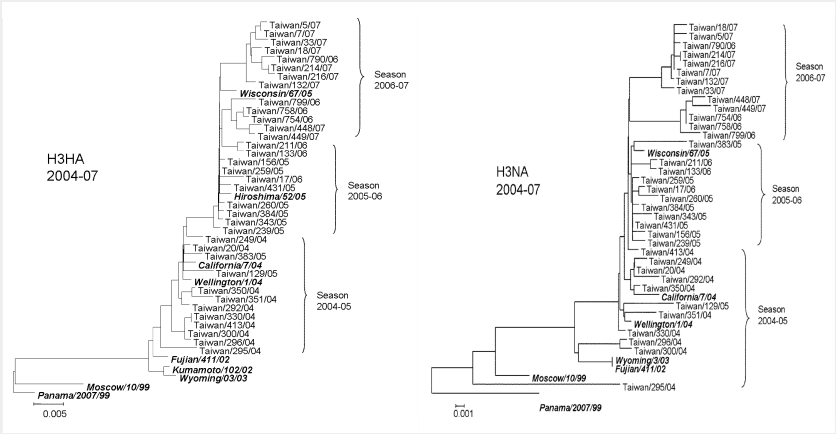


**Figure 2. Phylogenetic tree analysis of surface antigen genes in A/H3N2 influenza viruses from 2004 to 2007(WHO suggested vaccine strains are shown in bold italic font)**

## Discussion

Because the change of antigenicity of influenza A viruses from 2004 to 2007, WHO has recommended the selection of vaccine strain for northern hemisphere from A/Wyoming/3/03 to A/California/7/04, then A/Wisconsin/67/05, and has finally become A/Brisbane/10/07-like. For selection of A/H1N1 vaccine strain during 1999-2006, A/New Caledonia/20/99 was suggested, then A/Solomon Island/3/06 for 2006 to 2007. Now the vaccine strain has changed to A/Brisbane/59/07-like from 2008 to 2009. In conclusion, it is obvious that mutation in both A/H1N1 and A/H3N2 influenza viruses are frequent in recent years. However, some gene mutations do not induce change in the composition of amino acids, so the viral antigenicity is not influenced obviously. These so-called "silent mutation" does not change antigenicity apparently. However, detecting variations in viral nucleotide sequence is very important in the study of viral evolution.

**A. Influenza A /H1N1 viruses in Taiwan from 2004 to 2007**

The main type of influenza isolates during flu epidemic season in Taiwan from 2005 to 2006 was A/H1N1. Based on the antigenicity testing, these were grouped as A/New Caledonia/20/99-like and those with 4-fold or greater HI titer differences from A/New Caledonia/20/99. The first group of isolates had two amino acid changes, 81R and 169A, in comparison with A/New Caledonia/20/99 strain. Whereas the group which has at least a 4-fold difference in HI titer from A/New Caledonia/20/99 had additional amino acid changes at neutralizing epitope site, i.e., 92 S and 94 G. In previous studies, a change of antigenicity in influenza virus would need at least two amino acid changes at neutralizing epitope site in HA protein [8].

Therefore, the isolates which had at least 4-fold differences in HI titer from A/New Caledonia/20/99, the mutation in amino acid may play an important role in antigen determination.

Although influenza H3N2 was the major type in Taiwan from 2006 to 2007, there were still about 30 strains of influenza A/H1N1 isolated. About 85% of them had 4-fold or greater antigenic differences from A/New Caledonia/20/99. These isolates and A/Solomon Island/3/06 vaccine strain had amino acid change 144E. Amino acid 144 was near the antigen epitope site Ca2, mutation occurs at this position might influence the antigenicity. Furthermore, when compared to A/Solomon Island/3/06 strain we discovered that some isolates had additional changes, 192M and 197K. The amino acid at position 192 was in the neutralizing epitope site, and amino acid position 197 was near receptor binding site, mutation in both sites might influence viral antigenicity.

Although the antigenicity between the two clades of viral strains isolated from 2005 to 2006 was different, but the sequences on NA gene of influenza A/H1N1 viruses were the same. When compared to the isolates from 2004-2005, three additional substitutions in amino acid were discovered in these strains. Moreover, the sequence of NA protein from 2005-2006 isolates was similar to 2008 vaccine strain A/Solomon Island/3/06. This phenomenon indicates that appearance of viral strain in Taiwan was earlier than that of WHO vaccine strain for 2-3 years. In recent years, an anti-influenza virus medicine called Tamiflu, which targets at NA protein of both influenza A and B viruses, is effective and has been recommended as medication for influenza H5N1 outbreak by WHO. Studies have discovered that virus becomes resistant to Tamiflu

when amino acid of NA protein at position 274 was substituted by Y [9]. We did not find mutation with 274Y substitution on NA protein from A/H1N1 isolates in Taiwan during 2004-2007. Nor resistance to Tamiflu was found during surveillance period from the isolates either. In conclusion, the drug-resistant influenza strain that cause international concern recently was not appeared in Taiwan until 2008.

**B. Influenza A /H3N2 viruses in Taiwan from 2004 to 2007**

Influenza A/H3N2 had been active in Taiwan during 2004-2007 and was isolated all year round. Influenza A/H3N2 was the major viral strain in 2004-2005 [10]. According to antigenicity testing, the isolated strains were divided into two groups, a group similar to 2003-2004 vaccine strain A/Wyoming/3/03, the other group similar to 2004-2005 vaccine strain A/California/7/04. Although there were a 4-fold difference in HI titer between A/Wyoming/3/03 strains and A/California/7/04 strains, cross-protection between the two strains still exist. The isolates obtained from 2005 to 2006 had additional changes of 193F and 225N than those isolates from previous season. Moreover, one-third of isolates revealed a change of 50E. All of these amino acid substitutions occurred in receptor binding site and antigen epitope site. These isolates showed a 4-fold antigenic difference in HI titer against A/California/7/04 strain. However, antiserum to A/Hiroshima/52/05 vaccine strain in 2006/2007 had high HI titer meant that the three amino acid substitutions could affect antigenicity of virus. Compared to A/Hiroshima/52/05, sixty-two (62) percent of the isolates from 2006 to 2007 had at least a 4-fold difference in HI titer and additional amino acid substitutions with 142G and 144D, some of which had position 173E substitution. The three amino acid substitutions are located in antigen

epitope site. In general, influenza A/H3N2 viruses in Taiwan from 2004 to 2007 are continuing evolution, and most changes happened on antigen epitopes. Previous studies indicated some amino acids on HA gene of the influenza A/H3N2 viruses are highly unstable and will undergo change in few years [11]. The study on the correlation between the substitution in amino acid and the changes in antigenicity will be beneficial to the future investigation on molecular evolution of virus and antigenicity appearance.

In this research, by analyzing NA gene of influenza A/H3N2 viruses, the evolutionary pattern of isolates between 2004 and 2005 was quite similar to that of influenza A/H1N1 strains isolated during 2005-2006 in Taiwan. Although the antigenicity of both strains was different, the amino acid sequence of NA protein was the same which revealed that the evolution of HA and NA genes was proceeding independently. The genetic instability in influenza A viruses was a result of their unique genetic structure, therefore the pace for antiviral drug development cannot keep up with the speed of viral mutation. Tamiflu resistance in H3N2 viruses was a result of amino acid substitution occurred as 274Y, and 119G/A/D/V in NA gene [9]. During 2004-2007, we did not find any A/H3N2 isolate resistant to Tamiflu in Taiwan. However, resistance to Tamiflu was observed from the isolates in many countries near Taiwan, causing treatment difficulties. Nowadays, international travel is more frequent, which might provide chances for gene reassortment of viruses and increase drug resistance.

Besides, we discovered an increasing of 31N amino acid substitution in M2 protein from A/H3N2 isolates in Taiwan between 2004 and 2007, and the mutation reached almost to 100% in 2006, the trend pattern was similar to the data from global surveillance [12]. Formation of ion channel

by M2 protein provides transporting of genetic material from virus to host cell. Amantadine interferes with the uncoating of the virus inside the cell through blocking the ion channel formed by the M2 protein that spans the viral membrane. Influenza A virus could convert to amantadine resistant strain when mutations of M2 like 27A, 30T, 31N occurred. The observed data about site mutations of viruses cause drug resistance was proved in recent studies [12,13]. Many isolates in Taiwan had 31N mutation, implying that some A/H3N2 strains in Taiwan might have converted to drug resistant. Alignment of the sequences on internal genes of these drug resistant strains, we discovered amino acid substitutions, 101G, 256Q, and 421V on PA gene. More studies are needed to elucidate the relationship between MP and PA genes of A/H3N2 viruses.

## Conclusion

In the study on antigenicity and phylogenetic analysis of influenza A viruses in Taiwan during 2004 to 2007, we discovered the high correlation between antigenicity and amino acid substitution in HA protein. The mutation positions discovered in this study deserves further monitoring. Although the influenza A virus isolates from 2004 to 2007 did not become resistant to Tamiflu, but the resistant rate to amantadine was getting higher. Furthermore, the correlation between MP and other genes of these drug resistant strains, the viral protein structure and the virulence, should be analyzed further. For prevention purpose, U.S. CDC has issued an alert to the public not to use amantadine derivatives [13] temporarily. Taiwan's policy on this issue relies on the discussion among the public health authority, medical professionals, and scientists.

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