

Full Length Research Paper

Surveillance and molecular analysis of pandemic A (H1N1) virus in Shandong province of China during the 2009 to 2010 season

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The objective of the study was to analyze the epidemiologic, demographic and molecular characteristics of pandemic A (H1N1) viruses (A (H1N1) pdm 09) in Shandong Province between May, 2009 and March, 2010. Nasopharyngeal swabs from influenza-like-illness samples were collected and diagnosed by Real Time RT-PCR method. The positive samples were incubated and cultured in MDCK cells. HA and NA genes of isolated A (H1N1) pdm 09 were sequenced, the homology analysis was done with DNASTar Software and the genetic evolution and amino acid substitutions were performed with Mega 4.0 Software. During the period studied, more than 59% of the positive samples were confirmed A (H1N1) pdm 09 infection. Among them, children aged 5 to 14 and young adults aged 15 to 24 showed to be most susceptible to the A (H1N1) pdm 09 infection, whereas people aged ≥ 60 years had the lowest rate. There was no substitution of D239G in HA and H275Y in NA, but there was a D204G mutation. In addition, phylogenetic analysis confirmed that the Shandong strains were on different branch with the vaccine strain A/California/07/2009 (H1N1). Constant epidemiological and molecular surveillance is very important to monitor the pathogenicity of circulating strains and evaluate the efficacy of vaccine strain to them.

Key words: Influenza virus, mutation, surveillance.

INTRODUCTION

The first influenza pandemic of the 21st century was declared with the emergence of a novel influenza A (H1N1) in Mexico and the United States during April 2009 (World Health Organization, 2009). The new virus is a quadruple reassortant virus which was found to be made up of genetic elements from four different flu viruses: North American Mexican influenza, North American avian influenza, human influenza, and swine influenza virus typically found in Asia and Europe: "an unusually

mongrelized mix of genetic sequences" (Smith et al., 2009; Garten et al., 2009). By August 10, 2010, more than 214 countries and overseas territories or communities have reported laboratory confirmed cases of A (H1N1) pdm 09, including over 18449 deaths (World Health Organization. Pandemic (H1N1)-update 112. <http://www.who.int/csr/don/>).

Influenza virus is subject to genetic mutation mainly due to the lack of proof-reading activity of its polymerase.

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Table 1. The primer pairs for the amplification of HA and NA gene of Pandemic Influenza A (H1N1) virus.

Gene	Primer Name	Primers Sequence	Amplified fragment length (bp)
HA	SWHA-F	5'-ATGCGAACAATTCAACAGAC-3'	1590 bp
	SWHA-R	5'-CATCCAGAAACTGATTGCC-3'	
NA	SWN1-F	5'-CCATTGGTTCGGTCTGTA-3'	1390bp
	SWN1-R	5'-AAATGGCAACTCAGCACC-3'	

Mutations in influenza viral genes accumulate over time and are under selection pressure during epidemics or pandemics. The surface glycoprotein HA and NA constitute the main antigen of influenza virus and its continual mutations lead to gradual antigenic changes. Several reports described the site mutations of HA, such as D204 (Garten et al., 2009; Rambaut and Holmes, 2009; Glinsky, 2010; Janies et al., 2010; Maurer-Stroh et al., 2010), S220, D239 (World Health Organization, 2009; Antón et al., 2009), D339 and E391 (Maurer-Stroh et al., 2010). In addition, NA have the H275Y substitution, which are assumed to remain sensitive to zanamivir (Dharan et al., 2009; Hauge et al., 2009).

In this study, we reported the systematic surveillance results of A (H1N1) pdm 09 circulated in Shandong province, China, during 2009 to 2010 season, focusing on the epidemiological, phylogenetic and molecular characteristics of the virus.

MATERIALS AND METHODS

Sample collection for surveillance

The nasal or throat swabs which are suspected cases of A(H1N1) pdm 09 or outbreak cases of influenza-like-illness (ILI) were collected from 27 national surveillance hospitals located in Shandong Province, during the period from May 2009 to March 2010 (weeks 20/2009-13/2010). All samples were sent to the National Surveillance Influenza Laboratory in commercial transport medium within 2 days after collection. All the information of patients was input in Chinese Influenza Surveillance Information System. Patients' information including age, gender, work place, school, and city of residence, phone number, and clinical findings were recorded in a form prepared and sent to the laboratory with the clinical specimens.

RNA extraction and real-time RT-PCR for sub-typing

RNeasy Mini Kit (Qiagen, Cat no: 74104) was used to extract RNA from the nasal or throat swabs as described by the manufacturer. Real-time RT-PCR was done by Quantitanti™ Probe Real-Time RT-PCR Kit (Qiagen, Cat no: 204443, Germany) with pandemic A (H1N1) virus, influenza virus B, seasonal influenza A (H3N2) and seasonal influenza A (H1N1) primer-probe pairs and the protocols recommended by Chinese National Influenza Centre (CNIC).

Amplification of influenza A (H1N1) virus gene

The partial positive samples of pandemic A (H1N1) virus were used

for isolation. The isolated virus was sequenced following the RNA extraction by RNeasy Mini Kit (Qiagen, Cat no: 74104), and RT-PCR was done by One-Step RT-PCR Kit (Qiagen, Cat no: 210212, Germany) with specified HA and NA primer pairs designed by our laboratory. Details of the primers used in this study are given in Table 1. Primers were designed with Primer 5.0 software based on sequences available in public domain (www.ncbi.nlm.nih.gov).

Sequencing and phylogenetic analysis

RT-PCR products of HA gene and NA gene were sent to HuaDa Gene Biotechnology Company (Beijing, China) for sequencing. Phylogenetic trees were constructed with the Neighbor-Joining method, using Clustal W compared with the vaccine strain A/California/07/2009 (H1N1). Genetic distances and pair wise distances were calculated using MEGA 4.0.1. Confidence values for the tree clades were provided by bootstrap analysis of 1000 datasets.

RESULTS

Identification of influenza virus by real-time RT-PCR

The information of a total of 17,126 samples was input to the Chinese Influenza Surveillance Information System. Among them, 6733 (39.31%) were positive by Real-Time RT-PCR detection including 4031 cases of pandemic A (H1N1) virus, 1075 cases of influenza virus B virus, 526 cases of seasonal influenza A (H3N2) virus, 26 cases of seasonal influenza A (H1N1) virus and 1075 cases of un-subtyped influenza A virus respectively. The predominant subtype was pandemic influenza A (H1N1) virus, which accounted for 59.87% of all the positive samples followed by influenza A virus un-subtype (16.03%) and type B (15.97%). The time distribution of positive samples of influenza virus subtypes was different (Figure 1). The prevalent influenza virus in different months was influenza virus B from May to June in 2009 and from January to March in 2010, seasonal influenza A (H3N2) from August to September in 2009, and pandemic A (H1N1) virus from October to December in 2009. The relationship between the time distribution and the prevalent influenza virus is shown in Figure 1.

Demographic data was available for the 4031 confirmed cases analyzed during the study period. More than half of these cases (69.34%) belonged to two age groups; 5-14 years old (n=1213, 30.09%) and 15-24 years old (n=1582, 39.25%). The 25 to 59 year age group included 23.64% (n=953) of the confirmed cases.

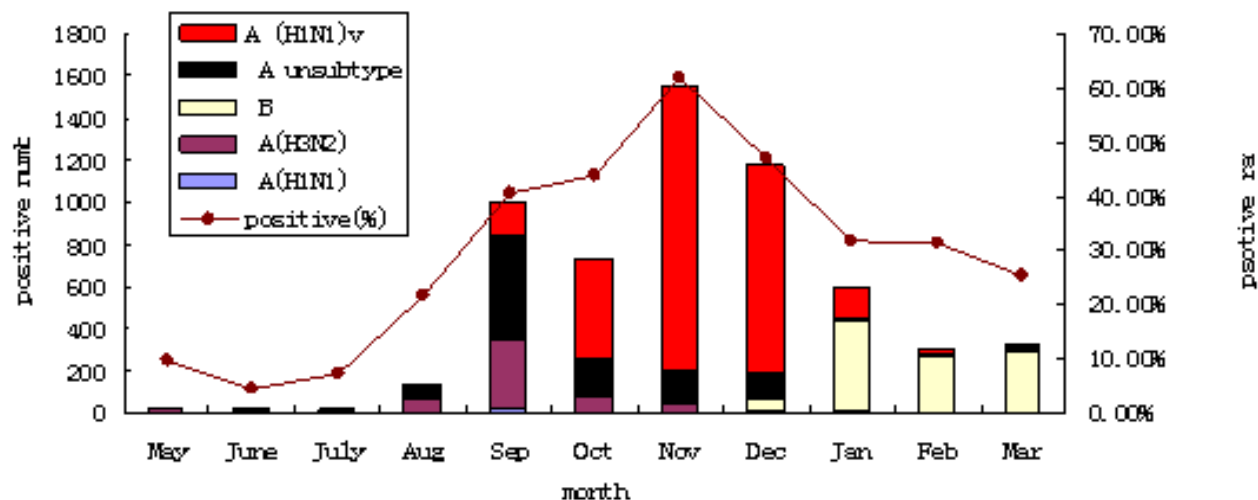


Figure 1. The relationship between the time distribution and the prevalent influenza virus in 2009 to 2010 season.

Phylogenetic analysis

25 isolated pandemic A (H1N1) virus strains were sequenced for further molecular and phylogenetic analysis. Of these 25 strains, 1 was from patients in May 2009, 8 from ones in September 2009, three from the positive cases in October to December 2009, and three from patients in January 2010. Of above 25 isolates, 16 were isolated from males and 9 from females. Three of these pandemic virus isolates were from the severe patients. The nucleotides identity within these sequences for HA and NA were in the range of 96.4~99.6% and 99.1~100%, while that between these sequences and the vaccine strain A/California/07/2009 were in the range of 96.9~99.3% and 99.1%~99.6%. The phylogenetic trees of HA genes and NA genes were constructed and presented in Figures 2 and 3 respectively. The trees showed that none of the strains analyzed here clustered together with the vaccine strain A/California/07/2009 (H1N1).

Molecular characteristics

In comparison to the vaccine strain A/California/07/2009 (H1N1), HA amino acid sequence of Shandong strains revealed 18 mutations, which were 38 (E-V), 40 (N-D), 56 (G-E), 87 (L-F), 90 (A-S), 100 (P-S), 103 (D-G), 145 (S-P), 172 (G-R), 173 (N-K), 204 (D-G), 212 (A-T), 220 (S-T), 288 (P-L), 300 (K-N), 303 (I-L), 325 (K-R) and 338 (I-V). Compared with the vaccine strain, the most common variations were P100S, S220T and I338V, which were actually found in all of the Shandong strains.

Analysis of the NA amino acid sequence showed 13 mutations, which included 60(T-N), 73 (N-H), 100 (Y-S), 106 (V-I), 122 (I-V), 241 (V-L), 248 (N-D), 298 (G-V), 351 (Y-F/S), 369 (N-T), 386 (N-D), 394 (V-A) and 399 (W-L). Compared with the vaccine strain, the most common variations in NA were N248D and Y351F/S, which were found in all of the Shandong strain.

DISCUSSION

This study was conducted to analyze the epidemiological, phylogenetic and molecular characteristics of A (H1N1) pdm 09 in Shandong Province between May, 2009 and March, 2010. During the study period, more than 59% of the positive samples had confirmed A (H1N1) pdm 09 infections. Only a few cases were imported cases in Shandong province, and mostly were reported in summer. By the start of the education period, outbreaks occurred in elementary schools and the positive rate of the laboratory-confirmed cases increased tremendously. The number of cases began to increase in September 2009, reaching its peak in November 2009, and then continually decreased from December 2009. The confirmed cases had only 2.2% of the positive samples in March 2010. Children aged 5 to 14 and young adults aged 15 to 24 appeared most susceptible to the A (H1N1) pdm 09 infection; whereas people aged ≥ 60 years had the lowest rate. Gender was not considered to be a significant risk factor for A (H1N1) pdm 09 infections.

Genetic relationships of the HA genes and NA genes showed the Shandong isolates were relevant to the WHO-recommended vaccine strain, A/California/07/2009 (H1N1), with sequence identity of 96.4~99.6% and 99.1~100% respectively. As shown in Figures 1 and 2, the phylogenetic analysis revealed that the HA gene and NA gene of Shandong isolates are all closely related to each other and derived from the vaccine strain.

Several studies had been performed to evaluate the mutations which may result in resistant and/or more virulent strains during the spread of this new virus among human population. The surface glycoprotein HA is the major target of neutralizing antibodies. Sequence analysis showed that mutations have been predominately observed in HA1 domain and mostly clustered in five main antigenic regions. Mutations P100S, S220T and I338V of HA were observed in all the Shandong isolates and mutation

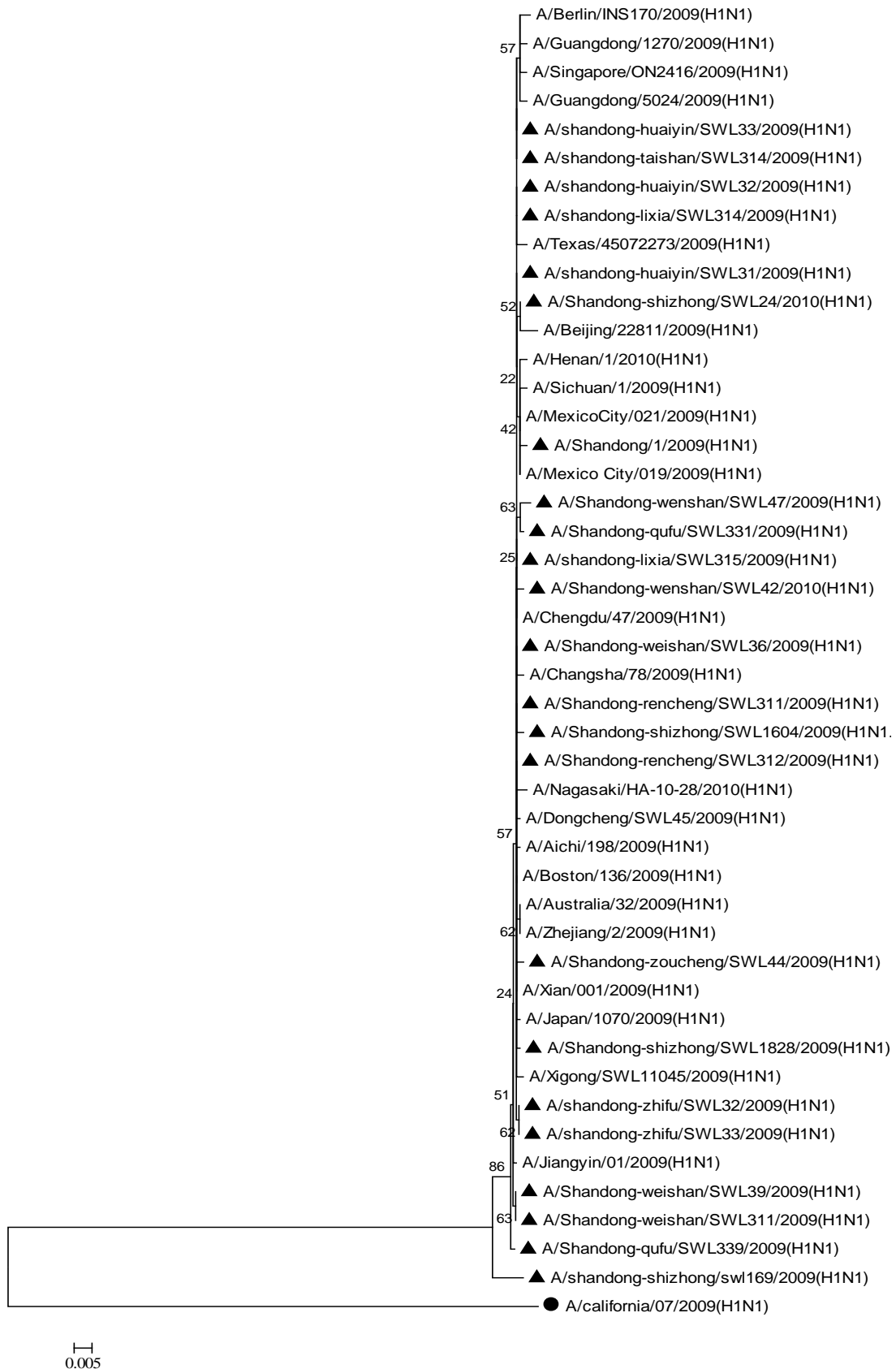


Figure 2. Phylogenetic tree of the hemagglutinin (HA) gene of Shandong pandemic A (H1N1) virus isolates (▲) and the vaccine strain (●).

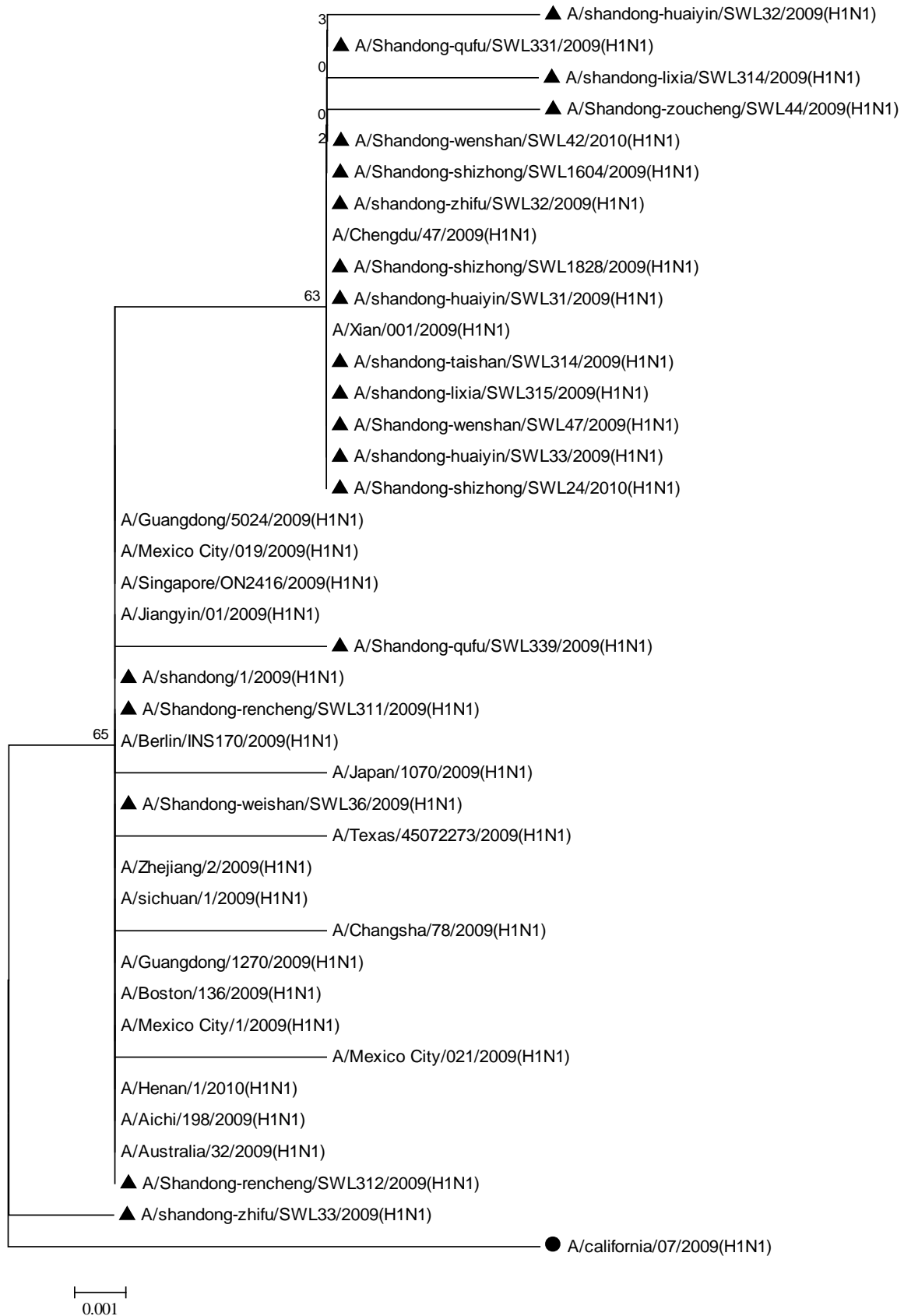


Figure 3. Phylogenetic tree of the neuraminidase (NA) gene of Shandong pandemic A (H1N1) virus (▲) and the vaccine strain (●).

S220T was located in the antigenic site Ca (Igarashi et al., 2010). Both the mutation L87F and A90S of A/Shandong-rencheng/SWL312/2009 were located in site Cb, while the mutations G172E/R and N173K of A/Shandong-weishan/SWL36/2009 were located in site Sa. Mutations D204G (A/Shandong-shizhong/SWL169/2009) and A212T (A/Shandong-zhifu/SWL32/2009, A/Shandong-zhifu/SWL33/2009) were located in site Sb. The other mutations were synonymous.

Nucleotide alignment of the HA amino acid revealed that residues that make contact with sialic acid (Y112, S150, W167 and H197) (Potdar et al., 2010) and residues in cleavage site (PSIQSR 353-358) (Potdar et al., 2010) were conserved among all Shandong isolates. Amino acids at positions 204 and 239 in the pandemic influenza A virus, HA appear to determine its receptor binding specificity because changes at these positions caused a switch in preference from the $\alpha 2, 6$ SA to the $\alpha 2, 3$ SA. It was reported that the D239G mutation allows the virus to bind to receptors on cells lining the lung. Although the mutation of D239G/N/E have been reported from different countries, such as Norway, China, Japan, Ukraine, the United States, India, and the Netherlands, no mutations of D204 and D239 were detected in Shandong isolates except for the strain A/Shandong-shizhong/SWL169/2009 having a mutation in site 204. This mutation may explain the severe case caused by the strain. Besides, seven potential glycosylation sites (position 28, 40, 104, 293, 304, 498 and 557) were found in the Shandong isolates expect A/shandong-qufu/SWL331/2009 and A/shandong-weishan/SWL311/2009, which were mutated at position 40.

Similar to HA, molecular analysis of NA showed the expected conserved residues in both catalytic sites and framework sites for all isolates. Mutations of V106I, N248D (Bashir et al., 2012) and Y351F were found in all the Shandong isolates and the first two mutations were also found in isolated from other countries. Furthermore, all other glycosylation sites were found in the NA of the other Shandong isolates except N386D (A/Shandong-qufu/SWL339/2009). And all the Shandong isolates had residue H274Y a known marker for sensitivity to the Oseltamivir—a neuraminidase inhibitor.

As more epidemiological and sequencing data on influenza viruses in Shandong province becomes available, a better understanding of their continuing evolution will be achieved. In particular, it will be established whether any reassortment events with local seasonal influenza viruses may have occurred. The continued surveillance of A(H1N1)pdm09 viruses ensure early detection of new antigenic or drug resistant variants. This will facilitate better pandemic planning and response capacity at national as well as global levels.

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