

# Isolation of novel triple-reassortant swine H3N2 influenza viruses possessing the hemagglutinin and neuraminidase genes of a seasonal influenza virus in Vietnam in 2010

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Surveillance of swine influenza viruses (SIVs) in 31 pig farms in northern and southern parts of Vietnam was conducted. Six H3N2 influenza A viruses were isolated from a pig farm in southern Vietnam. They were novel genetic reassortants between a triple-reassortant SIV and a human seasonal H3N2 virus. Their hemagglutinin and neuraminidase genes were derived from a human virus circulating around 2004–2006 and the remaining

genes from a triple-reassortant SIV that originated in North America. This is the first report describing the isolation of a novel triple-reassortant SIV in Vietnam.

**Keywords** H3N2 subtype, influenza virus, surveillance, swine, triple reassortant.

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A novel H3N2 swine influenza A virus emerged in 1998 and was called “triple-reassortant swine influenza virus (SIV)”.<sup>1</sup> Gene constellation of the “triple reassortant” consisted of hemagglutinin (HA), neuraminidase (NA), and PB1 genes from a human strain, PB2 and PA genes from an avian strain, and M, NP, and NS genes from a classical swine strain. Since then the viruses with six internal genes of the so-called triple reassortant internal gene (TRIG) cassette<sup>2</sup> have been isolated from pig populations in North America, Korea, and China.<sup>2–4</sup> They were accompanied by subsequent reassortment with the classical swine H1N1 or human H1N1 virus.<sup>2</sup> The pandemic (H1N1) 2009 virus that has spread worldwide in humans emerged from a reassortment between a “triple reassortant” and an avian-like swine influenza virus.<sup>5</sup>

Little is known about the prevalence of SIVs and their genetic characteristics in Vietnam, although Vietnam is one of the major pig meat-producing countries, the 5th largest in the world in 2008 (FAOSTAT). In the present study, we carried out a virological surveillance of SIVs in Vietnam, and H3N2 SIVs were isolated from a farm in the southern part. They possess a novel genetic constellation in combi-

nation with surface antigen genes from a seasonal human strain and the TRIG cassette. Thus, this is the first report, to our knowledge, of novel H3N2 reassortant viruses circulating in the Vietnamese pig population.

Sixteen farms in two provinces in northern and 15 farms in three provinces in southern Vietnam were visited from February to March 2010. A total of 759 nasal swab samples were collected from sows, weaning pigs, fattening pigs, nursery pigs, and boars. In a farm located in Binh Duong Province, where the viruses were isolated in this report, 25 nasal swab samples were collected from five sows (older than 1 year), 10 weaning pigs (4–8 weeks), and 10 fattening pigs (older than 8 weeks) on February 2010. Pigs did not show any clinical signs when the nasal swab samples were collected. Two of five pooled swab samples were positive for the influenza A virus by a SYBER green real-time PCR using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Takara Bio Inc., Shiga, Japan) with primers targeting the M gene of the influenza A viruses, M33F (5'-TTCTAACCGAGGTCGAAACG-3') and M264R2 (5'-ACAAAGCGTCTACGCTGCAG-3'). Individual swabs, constituting the positive pools, were inoculated on MDCK cells for virus isolation. Six influenza A

**Table 1.** Genetic homology of the A/swine/Binh Duong/03\_09/2010 in Vietnam in this study

Segment	Region compared (nt)	Lineage	Virus with greatest homology	Homology (%)	
PB2	1–2280	Avian	Triple reassortant	A/mallard/South Dakota/Sg-00128/2007 (H3N2)	97
PB1	1–2274	Human	Triple reassortant	A/Wisconsin/10/1998 (H1N1)	97
PA	1–2151	Avian	Triple reassortant	A/swine/Korea/CY05/2007(H3N2)	96
HA	1–1701	Human	Seasonal human	A/New York/365/2004 (H3N2)	97
NP	1–1515	Swine	Triple reassortant	A/mallard/South Dakota/Sg-00128/2007 (H3N2)	97
NA	1–1410	Human	Seasonal human	A/Denmark/201/2005 (H3N2)	96
M	1–982	Swine	Triple reassortant	A/Wisconsin/10/1998 (H1N1)	97
NS	1–838	Swine	Triple reassortant	A/Turkey/MO/24093/1999 (H1N2)	96

nt, nucleotide.

viruses were, then, isolated from swabs collected from weaning pigs. They were designated as A/swine/Binh Duong/03\_06/2010, A/swine/Binh Duong/03\_08/2010, A/swine/Binh Duong/03\_09/2010, A/swine/Binh Duong/03\_10/2010, A/swine/Binh Duong/03\_13/2010, and A/swine/Binh Duong/03\_14/2010.

Subtypes of the six swine isolates were identified as H3N2 by conventional RT-PCR.<sup>6,7</sup> The sequences of the full-length protein-coding regions of all isolates, determined as previously described,<sup>7</sup> revealed that the six isolates were similar to each other with nucleotide and amino acid identities ranging from 99.9% to 100% and from 99.7% to 100%, respectively. Previously known strains in GenBank of the highest similarity to the representative isolate, A/swine/Binh Duong/03\_09/2010, in eight viral gene segments were determined by means of BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Table 1). They were triple-reassortant swine viruses isolated in the United States and Korea (96–97% similarity) for the six internal genes. On the other hand, the HA and NA genes of the virus are most similar to the seasonal human viruses isolated in 2004 (97% similarity) and 2005 (96% similarity), respectively.

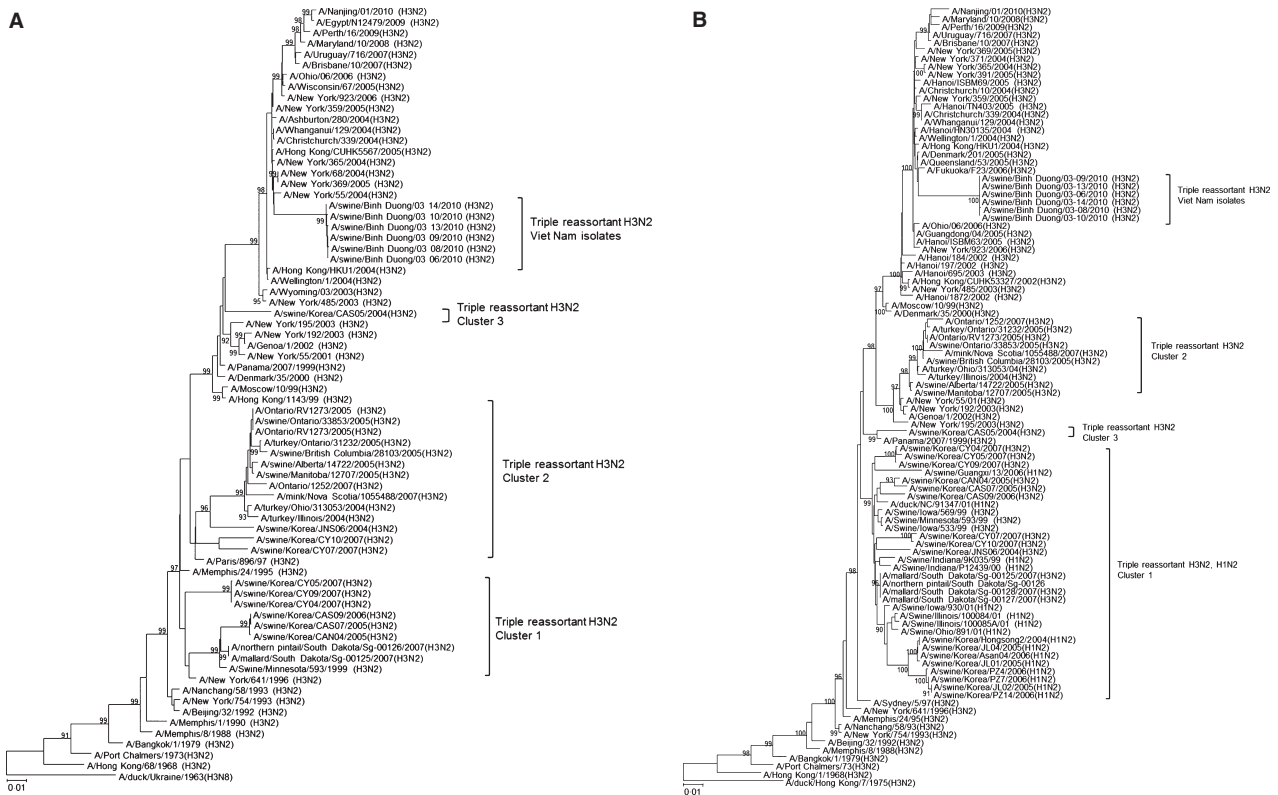
Phylogenetic analysis revealed that the HA and NA genes of the Vietnamese isolates had originated from the human H3N2 viruses isolated from 2004 to 2006. Vietnamese isolates obtained in this study formed a distinct cluster of those three other clusters of triple-reassortant viruses isolated from pigs in the United States, Canada, Korea, and China (Figure 1). The six internal genes of the Vietnamese isolates are closely related to the swine triple-reassortant viruses isolated in China, Korea, and United States (data not shown) as determined by the BLAST search, indicating that the Vietnamese isolates are the novel reassortants between a seasonal human influenza virus and the triple-reassortant SIV.

To determine the antigenic relationship between the putative ancestral seasonal human H3N2 viruses and the Vietnamese swine isolates, the HI test was performed using post-infection ferret antisera to the seasonal human H3N2 strains, A/Panama/2007/99, A/Wyoming/3/2003, A/New York/55/2004, and A/Hiroshima/52/2005 (Table 2). The Vietnamese isolates were antigenically similar to each other with a fourfold to eightfold titer reduction compared with the 1999 and 2003 seasonal H3 strains, while a 32- to 64-fold titer reduction to the 2004 and 2005 seasonal H3 strains was observed in the Vietnamese isolates.

When deduced amino acid of the HA1 region of A/swine/Binh Duong/03\_09/2010 was compared with those of A/New York/55/2004 and A/Hiroshima/52/2005 (Table 3), 23 and 24 amino acid differences, respectively, were found. Fourteen of them were found in the predicted antigenic sites of the H3 HA protein and three at residues 122, 135, and 144 resulted in the loss of potential glycosylation sites. Some of those could confer the antigenic differences observed by the HI analysis.

The NA inhibitors' susceptibility test suggested that the Vietnamese isolates were sensitive to the neuraminidase inhibitors (Table 4), and the NA protein of the Vietnamese isolates did not possess the amino acid substitutions known to confer resistance to neuraminidase inhibitors.<sup>9</sup>

In 1998, a triple-reassortant H3N2 virus was first isolated from a pig in the United States that possessed the HA gene similar to the seasonal human viruses isolated in 1995.<sup>1</sup> Subsequently, genetically similar viruses were isolated from pigs in the United States, Canada, Korea, and China.<sup>2–4</sup> Since 1998, H3N2 triple-reassortant viruses isolated from pigs in 1999, 2001, and 2002 in the United States were identified as three phylogenetic clusters with at least three introductions of the HA genes from viruses circulating in humans in 1995 to 1997.<sup>10</sup> The reassortant we identified in this study could have arisen in Vietnam, following an



**Figure 1.** Phylogenetic tree based on the nucleotide sequences of the ORFs from the H3 HA (A) and N2 (B) genes of selected H3N2 and H1N2 influenza viruses compared with the selected triple-reassortant and human viruses available from GenBank. Internal branching probabilities were determined by bootstrap analysis using 1000 replications. Bootstrap values of more than 90% are shown at the nodes. Scale bars indicate substitution per site. Phylogenetic tree of HA and NA with three clusters of triple-reassortant viruses, cluster 1 (1999–2007 isolates in the United States and Korea), cluster 2 (2004–2007 isolates in the United States, Canada, and Korea), and cluster 3 (2004 isolates in Korea) as indicated by the bar on the right of the trees.

**Table 2.** Antigenic analysis of swine influenza isolated from pigs in Vietnam\*

Viruses	A/Panama/2007/99	A/Wyoming/3/2003	A/New York/55/2004	A/Hiroshima/52/2005
A/Panama/2007/99	<b>640**</b>	640	40	20
A/Wyoming/3/2003	640	<b>2560</b>	2560	160
A/New York/55/2004	20	1280	<b>5120</b>	320
A/Hiroshima/52/2005	80	320	1280	<b>2560</b>
A/swine/Binh Duong/03_08/10	80	320	80	40
A/swine/Binh Duong/03_13/10	160	640	160	40

\*Hemagglutinin inhibition (HI) tests for antigenic analysis of the isolates were performed with 1% guinea pig red blood cells. Post-infection ferret sera to human H3N2 viruses were kindly provided by Dr. Takato Odagiri, of the National Institute of Infectious Diseases, Tokyo, Japan.

\*\*Titers in boldface are the values of the homogeneous reactions.

introduction of the triple-reassortant virus into pig population in Vietnam, because of trade movement of live pigs from North America or other countries. In fact, the pig farm where the novel H3N2 triple-reassortant viruses were isolated in this study had been importing pigs from the

United States until 1997. Also, it is possible that a human seasonal influenza virus entered the pig population in Vietnam as having been observed in other countries.<sup>11</sup> Despite intensive virological surveillance in North America, Korea, and China,<sup>2–4</sup> a virus with the gene constellation found in

**Table 3.** Comparison of deduced amino acid of the HA1 region between the Vietnamese SIVs and human seasonal influenza viruses isolated in 2004 and 2005

Designation of antigenic site*	Amino acid changes position at**																											
	3	10	25	53	62	111	122	124	133	135	138	142	144	145	156	158	159	165	186	188	193	201	209	219	225	253	261	264
A/swine/Binh Duong/03_06/2010	F	M***	M	N	G	I	S***	N	N	K***	S	G	D***	S	H	N	N	K***	G	Y	S	I	N	S	D	A	Q	R
A/swine/Binh Duong/03_08/2010																												P
A/swine/Binh Duong/03_09/2010																												
A/swine/Binh Duong/03_10/2010																												
A/swine/Binh Duong/03_13/2010																												
A/swine/Binh Duong/03_14/2010																												
A/New York/55/2004	L	T	I	D	E	L	N	S						N	N	K	F	N	V	D	R	R	S	Y		R	K	
A/Hiroshima/52/2005	L	T	I	D	E	L	N	S						N	N	K	F	N	D	D	R	R	S		N	R	K	

SIV, swine influenza virus.

\*Designation to the predicted antigenic sites of the H3 HA<sup>8</sup> was shown. —, indicates no designation to the corresponding amino acid.

\*\*Only locations where differences were observed are indicated.

\*\*\*Substitution at the amino acid results in loss of glycosylation on the HA molecules of the Vietnamese isolates. Nucleotide sequences of A/New York/55/2004 (accession number EU501486) and A/Hiroshima/52/2005 (EU121592) were used in this analysis.

**Table 4.** Fifty percent of inhibitory concentration (IC<sub>50</sub>) of the neuraminidase inhibitors against the Vietnamese isolates\*

Viruses	IC <sub>50</sub> (nM ± SD) of NA inhibitors**	
	Oseltamivir	Zanamivir
A/Texas/36/91 Parent (H1N1)***	2.18 ± 0.34	0.89 ± 0.36
A/Texas/36/91 Variant (H1N1) H274Y	695.43 ± 22.96	1.00 ± 0.35
A/Texas/131/02 Parent (H3N2)	0.86 ± 0.17	2.28 ± 0.43
A/Texas/131/02 Variant (H3N2) E119V	41.53 ± 4.06	3.18 ± 0.54
A/swine/Binh Duong/03_06/2010	0.94 ± 0.18	1.74 ± 0.43
A/swine/Binh Duong/03_08/2010	1.67 ± 0.39	2.23 ± 0.14
A/swine/Binh Duong/03_09/2010	1.08 ± 0.11	1.01 ± 0.23
A/swine/Binh Duong/03_10/2010	1.82 ± 0.14	1.11 ± 0.36
A/swine/Binh Duong/03_13/2010	0.81 ± 0.11	0.88 ± 0.39
A/swine/Binh Duong/03_14/2010	0.86 ± 0.21	1.00 ± 0.26

\*NA inhibitors' susceptibility test against the isolates was performed as described previously.<sup>9</sup>\*\*Mean ± SD from a measurement ( $n = 3$ ).

\*\*\*A/Texas/36/1991 (H1N1), A/Texas/131/2002 (H3N2) and the respective Oseltamivir-resistant mutants were kindly provided by Dr. Larisa V. Gubareva.

this study was not reported in those countries. Yet, there is the possibility that the reassortment event had occurred in a country other than Vietnam and that a virus had entered the Vietnamese pig population after the reassortment cannot be ruled out. Owing to the lack of genetic information on the SIVs in Vietnam and extensive international and intra-nation trade movements of live pigs, further surveillance of SIVs in Vietnam is needed to understand the origin of the isolates.

Phylogenetic analysis of the HA genes of all Vietnamese isolates in this study indicated that the virus might linger in the pig population for some time as the branch length derived from human isolates is relatively long (Figure 1). Accumulation of amino acid substitutions from a putative human ancestral strain (Table 3) observed in the HA molecule of the swine isolates contributed to the lengthening of the branch to a certain extent. The HA molecule is one of the factors that determine host-range specificity and tolerated accumulation of amino acid substitutions during the transmission and adaptation to different hosts.<sup>12</sup> Glycosylation in the HA molecule could affect receptor specificity, cell fusion activity, and antigenicity.<sup>13</sup> The Vietnamese isolates lost five potential glycosylation sites at positions 8, 122, 133, 144, and 165 that are conserved among human isolates (Table 3).<sup>13</sup> Among them, glycosylation sites at positions 122 and 133 are located in the vicinity of the receptor-binding site that is conserved in humans but not

in swine isolates.<sup>14</sup> The loss of the glycosylation sites at those positions could affect receptor recognition of the SIVs. Suzuki *et al.*<sup>15</sup> reported that swine tracheal epithelia have Neu5Gc as the terminal sialic acid residue, which had not been detected in human tracheal epithelia along with Neu5Ac, and SIVs preferentially bind Neu5Gc compared with human viruses.

In this study, we identified a novel cluster of viruses with a TRIG cassette. Continuous surveillance of the influenza virus in the pig population in Vietnam could provide vital information for the understanding of the ecology of SIVs in southeastern Asia. Understanding the SIV ecology in that region would be beneficial for preventing zoonotic infections of the SIVs, for establishing improved animal hygiene status in the pig industry, and as an early warning system for an emerging pandemic influenza virus.

## Addendum

Surveillance activity in Vietnam including sampling of pig specimens in this study was arranged by Drs. Nguyen Van Long and Do Huu Dung from the Epidemiology Division, Department of Animal Health, Vietnam; Dr. Binh Xuan Nguyen from the Center for Veterinary Diagnostics, Regional Animal Health Office No.6; and Drs. Tung Nguyen and Do Thi Hoa from the Virology Section, National Centre for Veterinary Diagnostics, Vietnam. Logistical support for the surveillance in Japan was provided by Dr. Yuko Uchida from the Research Team for Zoonotic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Ibaraki, Japan.

The sequences determined in this study are available from GenBank, with accession numbers, AB598480–AB598527.

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## References

- 1 Zhou NN, Senne DA, Landgraf JS *et al.* Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J Virol* 1999; 73:8851–8856.
- 2 Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. Swine influenza viruses a North American perspective. *Adv Virus Res* 2008; 72:127–154.
- 3 Pascua PN, Song MS, Lee JH *et al.* Seroprevalence and genetic evolutions of swine influenza viruses under vaccination pressure in Korean swine herds. *Virus Res* 2008; 138:43–49.
- 4 Vijaykrishna D, Poon LL, Zhu HC *et al.* Reassortment of pandemic H1N1/2009 influenza A virus in swine. *Science* 2010; 328:1529.
- 5 Garten RJ, Davis CT, Russell CA *et al.* Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009; 325:197–201.
- 6 Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *J Virol Methods* 2001; 97:13–22.
- 7 Takemae N, Parchariyanon S, Damrongwatanapokin S *et al.* Genetic diversity of swine influenza viruses isolated from pigs during 2000 to 2005 in Thailand. *Influenza Other Respi Viruses* 2008; 2:181–189.
- 8 Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 1981; 289:373–378.
- 9 Dapat C, Suzuki Y, Saito R *et al.* Rare influenza A (H3N2) variants with reduced sensitivity to antiviral drugs. *Emerg Infect Dis* 2010; 16:493–496.
- 10 Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. Evolution of swine H3N2 influenza viruses in the United States. *J Virol* 2000; 74:8243–8251.
- 11 Brown IH. The epidemiology and evolution of influenza viruses in pigs. *Vet Microbiol* 2000; 74:29–46.
- 12 Bean WJ, Schell M, Katz J *et al.* Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. *J Virol* 1992; 66:1129–1138.
- 13 Abe Y, Takashita E, Sugawara K, Matsuzaki Y, Muraki Y, Hongo S. Effect of the addition of oligosaccharides on the biological activities and antigenicity of influenza A/H3N2 virus hemagglutinin. *J Virol* 2004; 78:9605–9611.
- 14 Karasin AI, Schutten MM, Cooper LA *et al.* Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. *Virus Res* 2000; 68:71–85.
- 15 Suzuki T, Horiike G, Yamazaki Y *et al.* Swine influenza virus strains recognize sialyl sugar chains containing the molecular species of sialic acid predominantly present in the swine tracheal epithelium. *FEBS Lett* 1997; 404:192–196.