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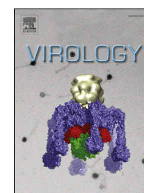

Co-infection of influenza A viruses of swine contributes to effective shuffling of gene segments in a naturally reared pig

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ABSTRACT

Following the 2009 H1N1 pandemic, surveillance activities have been accelerated globally to monitor the emergence of novel reassortant viruses. However, the mechanism by which influenza A viruses of swine (IAV-S) acquire novel gene constellations through reassortment events in natural settings remains poorly understood. To explore the mechanism, we collected 785 nasal swabs from pigs in a farm in Thailand from 2011 to 2014. H3N2, H3N1, H1N1 and H1N2 IAVs-S were isolated from a single co-infected sample by plaque purification and showed a high degree of diversity of the genome. In particular, the H1N1 isolates, possessing a novel gene constellation previously unreported in Thailand, exhibited greater variation in internal genes than H3N2 IAVs-S. A pair of isolates, designated H3N2-B and H1N1-D, was determined to have been initially introduced to the farm. These results demonstrate that numerous IAVs-S with various gene constellations can be created in a single co-infected pig via reassortment.
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Introduction

Influenza A virus of swine (IAV-S) is one of the major respiratory pathogens in pigs, contributing to an increased risk of mortality and economic losses in the swine industry. Previous studies revealed that pigs possess both mammalian-type and avian-type receptors for influenza viruses on their tracheal epithelia, and pigs are now recognized as “mixing vessels,” facilitating reassortment events between human, swine, and avian influenza viruses, which might create strains that are highly transmissible and pathogenic to humans, resulting in the emergence of a pandemic strain (Salomon and Webster, 2009; Scholtissek, 1990–1991). For this reason, the emergence of novel IAV-S strains that may pose a threat to humans has raised concerns, attracting global attention from public health control bodies (Vincent et al., 2013; Webster et al., 1992).

At present, three major subtypes (H1N1, H1N2, and H3N2) of IAVs-S are circulating widely across the globe and accumulating numerous mutations (Vincent et al., 2013). One of the lineages of H1N1 IAVs-S is the classical H1N1 virus, which originated during the human Spanish flu pandemic in 1918. The genetic and antigenic properties of classical swine (CS) H1N1 viruses remained relatively stable in swine in North America and other regions from 1930, when IAV-S was first isolated, to the late 1990s (Vincent et al., 2013). In 1998, the CS H1N1 IAVs-S were reported to have undergone a reassortment event with an avian and a seasonal human H3N2 influenza virus, resulting in emergence of new triple-reassortant H3N2 IAVs-S in the North American swine population (Zhou et al., 1999). The genomic composition of the triple-reassortant IAVs-S is referred to as the triple-reassortant internal gene (TRIG) cassette (Vincent et al., 2008). The TRIG H3N2 IAV-S and its reassortants have been reported not only in North America but also in Asia, in countries including Hong Kong (Vijaykrishna et al., 2011), South Korea (Lee et al., 2008), and Vietnam (Ngo et al., 2012). In addition, an avian-like H1N1 IAV-S was first reported in Belgium in 1979; it has been circulating mainly in the Eurasian swine population and is referred to as the Eurasian avian-like (EA-like) lineage (Scholtissek et al., 1983). H3N2 and H1N2 IAVs-S with human-like surface genes have been isolated in various regions of the world (Vincent et al., 2013). All of these IAVs-S (CS, triple-reassortant, EA-like, and human-like IAVs-S) had been reported in Asia by 2002, indicating rapid and

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wide-ranging intercontinental movement of swine with IAVs-S (Vijaykrishna et al., 2011).

In 2009, a novel H1N1 (H1N1pdm09) virus appeared in the human population in North America, then spread rapidly across the globe, resulting in a pandemic (Garten et al., 2009; Smith et al., 2009). The H1N1pdm09 virus emerged as a reassortant between EA-like and North American TRIG IAVs-S. Following the pandemic, a large number of reassortment events have been reported around the world between endemic IAVs-S and the H1N1pdm09 viruses (Vijaykrishna et al., 2010; Howard et al., 2011; Kitikoon et al., 2011; Lam et al., 2011), resulting in an extraordinary amount of genetic diversity in IAVs-S. Furthermore, a novel reassortant virus, referred to as the H3N2 variant (H3N2v), which possessed seven genes from the North American TRIG H3N2 IAVs-S and the M gene from the H1N1pdm09 virus, has been reported to cause over 300 human cases in the U.S. since the autumn of 2011 (Lindstrom et al., 2012; CDC, http://www.cdc.gov/flu/swineflu/variant-cases-us.htm). The emergence of novel reassortant IAVs-S with the ability to infect humans, such as the H1N1pdm09 and the H3N2v viruses, has drawn global attention to the monitoring and control of newly-generated IAVs-S that have the potential to cause another pandemic. In spite of the increasing availability of data on IAVs-S from worldwide surveillance activities, however, we have little knowledge of the process of reassortment as it occurs in natural conditions, primarily because the number of reports on co-infection of individual pigs with multiple IAVs-S remains limited.

We have previously published data on the genetic and antigenic properties of IAVs-S circulating in pig farms in Thailand (Hiromoto et al., 2012; Takemae et al., 2008, 2011). The hemagglutinin (HA) and neuraminidase (NA) genes of Thai endemic H1N1 IAVs-S originated from the CS and EA-like lineages, respectively, whereas those of Thai H3N2 IAVs-S are derived from seasonal human-like swine virus lineages since 2004 (Takemae et al., 2008, 2011). Similar to other regions of the world, various reassortant IAVs-S between endemic Thai IAVs-S and the H1N1pdm09 virus emerged in Thailand after the H1N1pdm09 virus was introduced into the Thai swine population in 2009 (Charoenvisal et al., 2013; Hiromoto et al., 2012; Kitikoon et al., 2011). In this study, we identified pigs co-infected with H1N1 and H3N2 IAVs-S in a pig farm in Thailand. Our data provide a unique example of IAV-S co-infection in non-experimental conditions. Following independent isolation of the H1N1 and H3N2 IAVs-S, whole-genome sequencing analysis showed that the H1N1 IAV-S was a reassortant previously unreported in Thailand. The H1N1 isolates possessed HA and NA genes from the EA-like lineage and internal genes derived from the H1N1pdm09 virus. The H3N2 IAVs-S had a gene composition similar to H3N2 IAVs-S previously isolated in Thailand (Hiromoto et al., 2012). The IAV-S isolates showed remarkable diversity in their internal genes, and H1N1 IAVs-S possessed much greater genetic variation than the H3N2 isolates. These results provide a better understanding of both the properties of IAVs-S currently circulating in Thailand and the process of reassortment naturally occurring in pig farms.

Results

Virus isolation from mono-infected and co-infected samples

We collected nasal swabs from pigs at a farrow-to-weaning farm, at which thousands of pigs are systematically reared in a livestock farming-dense area of Thailand, beginning in 2011 (Table 1). Real-time PCR analysis of the 60 swabs collected in February 2012 showed that 12 samples from weaning piglets were positive for the influenza A virus matrix (M) gene (Table 1). Subtype determination, using conventional PCR analysis targeting

Table 1
The surveillance schedule, the number of swabs, and the viruses isolated in this study.

Collection date	Feb 2011		Jul 2011		Oct 2011		Feb 2012		Jun 2012		Feb 2013		Jul 2013		Nov 2013		Mar 2014	
Pigs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs	swab	Isolated SIVs
Sow	20	0	20	0	20	0	20	0	38	0	45	0	45	0	45	0	45	0
Weaning piglets	40	0	40	0	40	0	40	12	56	0	56	0	56	0	56	(1)	56	0

Table 2
Gene constellations of isolated viruses.

Swab No.	Pig			Virus isolation	Subtype	Virus	Gene lineages**								Genotype
	Type	Age	M / F*				HA	NA	PB2	PB1	PA	NP	M	NS	
swab #42	Weaning	4 wks	M	MDCK cells	H3N2	A/swine/Chachoengsao/NIAH105583-042/2012	Human	Human	pdm-2	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-1	H3N2-A
swab #44			M			A/swine/Chachoengsao/NIAH105583-044/2012									
swab #45			M			A/swine/Chachoengsao/NIAH105583-045/2012									
swab #48			M			A/swine/Chachoengsao/NIAH105583-048/2012									
swab #50			M			A/swine/Chachoengsao/NIAH105583-050/2012									
swab #53			F			A/swine/Chachoengsao/NIAH105583-053/2012									
swab #55	Weaning	4 wks	F	MDCK cells	H3N2	A/swine/Chachoengsao/NIAH105583-055/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3b	pdm-1	H3N2-B
swab #58			F			A/swine/Chachoengsao/NIAH105583-058/2012									
swab #52	Weaning	4 wks	F	Plaque purification	H3N2	A/swine/Chachoengsao/NIAH105583-052-06/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3b	pdm-1	H3N2-B
						A/swine/Chachoengsao/NIAH105583-052-11/2012									
						A/swine/Chachoengsao/NIAH105583-052-42/2012									
						A/swine/Chachoengsao/NIAH105583-052-52/2012									
						A/swine/Chachoengsao/NIAH105583-052-64/2012									
						A/swine/Chachoengsao/NIAH105583-052-74/2012									
						A/swine/Chachoengsao/NIAH105583-052-85/2012									
						A/swine/Chachoengsao/NIAH105583-052-01/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3a	pdm-1	H3N2-C
						A/swine/Chachoengsao/NIAH105583-052-02/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3b	pdm-3	H3N2-D
						A/swine/Chachoengsao/NIAH105583-052-46/2012	Human	Human	pdm-1	pdm-1	pdm-3	pdm-1	pdm-3b	pdm-1	H3N2-E
					H3N1	A/swine/Chachoengsao/NIAH105583-062-46/2012	Human	EA-like	pdm-1	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-1	H3N1-A
					H1N1	A/swine/Chachoengsao/NIAH105583-052-05/2012	EA-like	EA-like	pdm-1	pdm-1	pdm-3	pdm-2	pdm-3a	pdm-3	H1N1-A
						A/swine/Chachoengsao/NIAH105583-052-70/2012									
						A/swine/Chachoengsao/NIAH105583-052-07/2012	EA-like	EA-like	pdm-2	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-3	H1N1-B
						A/swine/Chachoengsao/NIAH105583-052-16/2012	EA-like	EA-like	pdm-2	pdm-1	pdm-3	pdm-2	pdm-3a	pdm-3	H1N1-C
						A/swine/Chachoengsao/NIAH105583-052-18/2012									
						A/swine/Chachoengsao/NIAH105583-052-30/2012	EA-like	EA-like	pdm-2	pdm-3	pdm-3	pdm-2	pdm-3a	pdm-3	H1N1-D
						A/swine/Chachoengsao/NIAH105583-052-56/2012	EA-like	EA-like	pdm-2	pdm-1	pdm-3	pdm-1	pdm-3b	pdm-1	H1N1-E
						A/swine/Chachoengsao/NIAH105583-052-82/2012	EA-like	EA-like	pdm-2	pdm-1	pdm-3	pdm-2	pdm-3a	pdm-1	H1N1-F
						A/swine/Chachoengsao/NIAH105583-052-92/2012	EA-like	EA-like	pdm-2	pdm-1	pdm-3	pdm-2	pdm-3b	pdm-3	H1N1-G
					H1N2	A/swine/Chachoengsao/NIAH105583-052-41/2012	EA-like	Human	pdm-1	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-1	H1N2-A
						A/swine/Chachoengsao/NIAH105583-052-48/2012	EA-like	Human	pdm-1	pdm-1	pdm-3	pdm-1	pdm-3b	pdm-1	H1N2-B
						A/swine/Chachoengsao/NIAH105583-052-71/2012	EA-like	Human	pdm-1	pdm-1	pdm-3	pdm-2	pdm-3b	pdm-3	H1N2-C
						A/swine/Chachoengsao/NIAH105583-052-96/2012	EA-like	Human	pdm-1	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-1	H1N2-D
swab #49	Weaning	4 wks	M	Plaque purification*	H3N2	A/swine/Chachoengsao/NIAH105583-049-1/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3b	pdm-1	H3N2-B
						A/swine/Chachoengsao/NIAH105583-049-2/2012									
						A/swine/Chachoengsao/NIAH105583-049-3/2012									
						A/swine/Chachoengsao/NIAH105583-049-4/2012									

Table 2 (*continued*)

Swab No.	Pig			Virus isolation	Subtype	Virus	Gene lineages**								Genotype
						A/swine/Chachoengsao/NIAH105583-049-5/2012 A/swine/Chachoengsao/NIAH105583-049-6/2012 A/swine/Chachoengsao/NIAH105583-049-7/2012 A/swine/Chachoengsao/NIAH105583-049-8/2012 A/swine/Chachoengsao/NIAH105583-049-9/2012 A/swine/Chachoengsao/NIAH105583-049-10/2012									
swab #54	Weaning	4 wks	F	Plaque purification*	H3N2	A/swine/Chachoengsao/NIAH105583-054-1/2012 A/swine/Chachoengsao/NIAH105583-054-2/2012 A/swine/Chachoengsao/NIAH105583-054-3/2012 A/swine/Chachoengsao/NIAH105583-054-4/2012 A/swine/Chachoengsao/NIAH105583-054-5/2012 A/swine/Chachoengsao/NIAH105583-054-6/2012 A/swine/Chachoengsao/NIAH105583-054-7/2012 A/swine/Chachoengsao/NIAH105583-054-8/2012 A/swine/Chachoengsao/NIAH105583-054-9/2012 A/swine/Chachoengsao/NIAH105583-054-10/2012	Human	Human	pdm-1	pdm-1	pdm-1	pdm-1	pdm-3b	pdm-1	H3N2-B
swab #57	Weaning	4 wks	F	Plaque purification*	H3N2	A/swine/Chachoengsao/NIAH105583-057-1/2012 A/swine/Chachoengsao/NIAH105583-057-2/2012 A/swine/Chachoengsao/NIAH105583-057-3/2012 A/swine/Chachoengsao/NIAH105583-057-4/2012 A/swine/Chachoengsao/NIAH105583-057-5/2012 A/swine/Chachoengsao/NIAH105583-057-6/2012 A/swine/Chachoengsao/NIAH105583-057-7/2012 A/swine/Chachoengsao/NIAH105583-057-9/2012 A/swine/Chachoengsao/NIAH105583-057-10/2012 A/swine/Chachoengsao/NIAH105583-057-12/2012 A/swine/Chachoengsao/NIAH105583-057-15/2012 A/swine/Chachoengsao/NIAH105583-057-16/2012 A/swine/Chachoengsao/NIAH105583-057-30/2012 A/swine/Chachoengsao/NIAH105583-057-31/2012 A/swine/Chachoengsao/NIAH105583-057-32/2012 A/swine/Chachoengsao/NIAH105583-057-33/2012 A/swine/Chachoengsao/NIAH105583-057-34/2012	Human	Human	pdm-2	pdm-1	pdm-3	pdm-1	pdm-3a	pdm-1	H3N2-A
					H1N2	A/swine/Chachoengsao/NIAH105583-057-13/2012	EA-like	Human	pdm-2	pdm-1	pdm-3	pdm-2	pdm-3a	pdm-3	H1N2-E

***:Clones were plaque-purified three times as described in the Material and Methods, followed by the direct sequences from the suspension of purified plaques. Partial sequence was determined on each segments in the following regions for genotyping; H1 HA (233-943), H3 HA (371-880), N1 NA (355-803), N2 NA (551-894), PB2 (1068-1598), PB1 (524-1030), PA (553-1403), NP (214-1001), M (130-985) and NS (23-840)

* M means male and F means female.

** Gene lineages are designated as follows: Human, seasonal human-like SIV; EA-like, Eurasian avian-like SIV. pdm-1, pdm-2 and pdm-3 are clusters as shown in Fig. 2.

the H1 and H3 hemagglutinin (HA) genes and the N1 and N2 neuraminidase (NA) genes, showed that 8 swabs were positive for the H3N2 subtype, and 4 were positive for all of the H1, H3, N1, and N2 genes (Table 2). The swabs positive for all of the H1, H3, N1, and N2 genes may have been obtained from pigs co-infected with H1N1 and H3N2 IAVs-S, since H1N1, H1N2, and H3N2 are the predominant IAV-S subtypes in Thailand (Hiromoto et al., 2012; Poonsuk et al., 2013; Takemae et al., 2008, 2011). Eight swabs exclusively positive for the H3N2 subtype were subjected to virus isolation in MDCK cell culture for further genotyping.

We attempted to isolate individual IAVs-S from a swab containing mixed subtypes (swab #49, #52, #54 and #57), using a plaque purification technique to segregate individual strains concurrently replicating in a donor pig. We were able to segregate the expected subtypes, H1N1 and H3N2, from swab #52, and established ten clones of H3N2, one clones of H3N1, nine clones of H1N1 and four clones of H1N2 (Table 2). During plaque purification, we observed that the plaques of the H3N2 isolates were larger than those of the H1N1 isolates (Fig. 3A), suggesting differences in viral replication between subtypes. Contrasting to the variety of subtypes obtained from #52, only H3N2 subtype were identified in #49 and #54, in spite they were determined to be a mixture by PCR subtyping. Seventeen clones of H3N2 and one clone of H1N2 were identified from #57.

Phylogenetic analyses

To identify the genotypes of isolates, we sequenced all gene segments of the isolates and used the Bayesian MCMC method (Drummond et al., 2012) to estimate the phylogenetic relationships among time-stamped sequences of relevant viruses from various hosts and continents. Phylogenetic trees for both the HA and NA genes of the H3N2 isolates revealed that both genes clustered with Thai endemic H3N2 IAVs-S originating from seasonal human-like lineages (Fig. 1A and B, Supplementary Figs. 1 and 2). The H3 and N2 genes, both from individual isolates and from plaque-purified viruses from swab #52, clustered together in the tree, indicating a shared origin. The most closely related strain was the H3N2 IAV-S A/swine/Chonburi/NIAH106952-026/2011 (Sw/Chonburi/2011), isolated in a neighboring province just one year prior (Hiromoto et al., 2012). Several older Thai strains, isolated during 2002–2005, formed clusters with the European IAV-S lineage; however, they were completely replaced with recent Thai strains isolated in and after 2004, and disappeared after 2005 (Fig. 1A and B, Supplementary Figs. 1 and 2). Interestingly, both the H3 and N2 genes from recent Thai IAVs-S formed a cluster separate from the North American IAV-S lineage containing the triple-reassortant internal gene (TRIG) cassette (Zhou et al., 1999) and the European IAV-S lineage (Brown, 2013). In addition, Thai H3N2 isolates were estimated to have diverged from

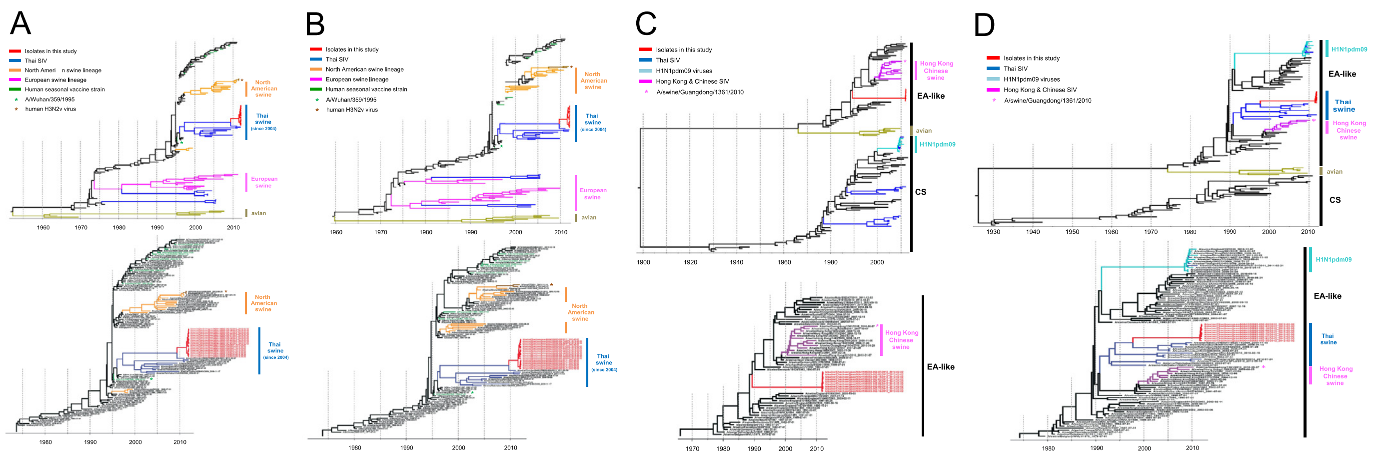

Fig. 1. Phylogenetic relationships of the H3 HA (A), N2 NA (B), H1 HA (C), and N1 NA (D) genes. Both the entire phylogenetic tree (upper) and the enlarged region encompassing the isolates in this study (lower) are shown. In the tree of the H3 HA (A) and N2 NA (B) genes, virus strains are color-coded according to genetic lineage: red, isolates in this study; blue, previously reported Thai swine viruses; black, human strains; orange, North American swine viruses; pink, European swine virus lineages; green, human seasonal vaccine strains; light green, avian viruses. The green and brown asterisks indicate the vaccine strain A/Wuhan/359/1995 and human H3N2v viruses, respectively. In the tree of the H1 HA (C) and N1 NA (D) genes, virus strains are color-coded as follows: red, isolates in this study; blue, Thai swine viruses; black, representative swine virus strains; pink, Hong Kong and Chinese swine virus lineages; light blue, H1N1pdm09 viruses; light green, avian viruses. The pink asterisk indicates a reassortant swine virus strain A/swine/Guangdong/1361/2010. EA-like and CS indicate the Eurasian avian-like and the classical swine virus lineages, respectively. Fully detailed trees that include sequence names are provided in Supplementary Figs. 1–4.

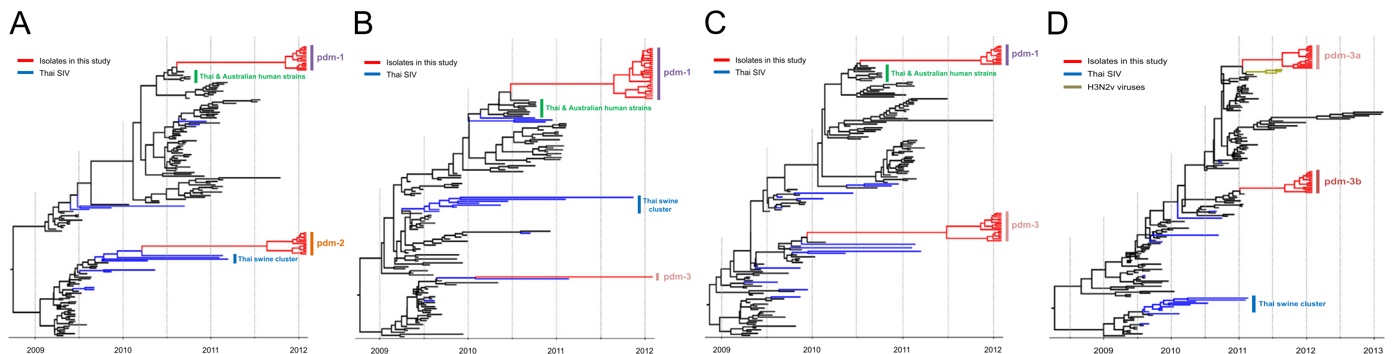

Fig. 2. Phylogenetic relationships of the PB2 (A), PB1 (B), PA (C), and M (D) genes. Virus strains are color-coded as follows: red, isolates in this study; blue, previously reported Thai swine viruses; black, human and swine H1N1pdm09 viruses displaying strong homology to isolates in this study. Isolates formed three clusters designated pdm-1, pdm-2, and pdm-3: pdm-1, the cluster closely related to the Thai and Australian human strains; pdm-2, the cluster linked to the Thai IAV-S cluster; pdm-3, the cluster different from both pdm-1 and pdm-2. Detailed trees of all gene segments that include sequence names are provided in Supplementary Figs. 5–10.

seasonal human viruses that were closely related to the vaccine reference strain A/Wuhan/359/1995, as shown by antigenic testing in our previous report (Hiromoto et al., 2012) (Fig. 1A and B, Supplementary Figs. 1 and 2). In contrast, the HA and NA genes of the plaque-purified H1N1 isolates clustered with the Eurasian avian-like (EA-like) IAV-S lineage (Fig. 1C and D, Supplementary Figs. 3 and 4). This is the first report of an EA-like H1 gene in Thailand, as the HA gene of previously reported Thai endemic H1N1 IAVs-S belongs to the classical swine (CS) lineage, which includes H1N1pdm09 viruses (Fig. 1C, Supplementary Fig. 3). The EA-like H1 gene of the isolates analyzed in this study was inferred to have diverged from the European lineage ca. 1990; however, no reports on the Thai EA-like H1 gene have been published in the past two decades (Fig. 1C, Supplementary Fig. 3). In contrast to the HA gene, the EA-like NA gene has been reported in Thai swine populations since 2000 (Takemae et al., 2008), and the NA gene of our H1N1 isolates is derived from Thai endemic H1N1 IAVs-S (Fig. 1D, Supplementary Fig. 4). In Asian countries, EA-like H1N1 IAVs-S were first detected in Hong Kong in 2001 (Vijaykrishna et al., 2011) and have been circulating in the swine populations in Hong Kong and China. However, both the H1 and N1 genes of Thai IAVs-S, including the isolates in this study, formed a cluster distinct from those of Hong Kong and Chinese EA-like IAVs-S (Fig. 1C and D, Supplementary Figs. 3 and 4). Additionally, a Chinese H1N1 IAV-S A/swine/Guangdong/1361/2010 (Sw/GD1361) possessing a gene constellation similar to our H1N1 isolates, (i.e., surface genes from EA-like lineages and internal genes from H1N1pdm09 viruses) has been reported (Zhu et al., 2011). Our results revealed that the HA and NA genes of Sw/GD1361 are derived from viruses from Hong Kong and China, as previously reported, and that Sw/GD1361 was unlikely to be relevant to this study (Fig. 1C and D, Supplementary Figs. 3 and 4).

All of the internal genes examined in this study were found to be derived from H1N1pdm09 viruses, indicating that our isolates were reassortants between endemic IAVs-S and H1N1pdm09 viruses. Although all of the internal genes originated from H1N1pdm09 viruses, they formed several distinct clusters (Fig. 2A–D, Table 2, Supplementary Figs. 5–10). We named the clusters as follows: pdm-1, the cluster closely related to the Thai and Australian human strains; pdm-2, the cluster linked to the Thai IAV-S cluster; and pdm-3, the cluster distinct from both pdm-1 and pdm-2. The polymerase basic protein 2 (PB2) and the nucleoprotein (NP) genes of the isolates formed two distinct clusters, pdm-1 and pdm-2 (Fig. 2A, Table 2, Supplementary Figs. 5 and 8). Similarly, the polymerase basic protein 1 (PB1), the polymerase acidic protein (PA), and the nonstructural protein (NS) genes formed two clusters, pdm-1 and pdm-3 (Fig. 2B and C, Table 2, Supplementary Figs. 6, 7, and 10), although the phylogenetic tree for the PA gene did not contain an apparent Thai IAV-S cluster (Fig. 2C, Supplementary Fig. 7). The M genes of our isolates formed two distinct pdm-3 clusters linked to H1N1pdm09 viruses isolated outside of Thailand, while most of the M genes from previously isolated Thai IAVs-S formed a Thai swine cluster (Fig. 2D, Table 2, Supplementary Fig. 9). Notably, no relationship was observed between the subtypes of the isolates and these clusters.

The plaque-purified clones of #52 showed a great variety of combinations of internal genes, in terms of the clusters described above, although the HA and NA genes shared more than 99% homology among isolates of the same subtype (Table 2). In contrast, the clones of #49, #54 and #57 were homogenous in their subtype, H3N2 subtype, and gene constellation, except one H1N2 clone from #57. To identify gene constellations that IAVs-S initially introduced into this farm might have possessed, each gene constellation was examined and classified into one of five genotypes for H3N2 isolates (H3N2-A to H3N2-E) and one of seven for H1N1 viruses (H1N1-A to H1N1-G) (Table 2). Gene constellations of clones of H1N2 were classified into one of five genotypes (H1N2-A to H1N2-E), while that of H3N1 was H3N1-A alone (Table 2). Notably, all constellations could be created via reassortment of two genotypes, H3N2-B and H1N1-D, suggesting that these two IAVs-S are candidates for those initially introduced to the farm and causing co-infections, such as that isolated on swab #52. Interestingly, almost all internal genes of the genotype H3N2-B were derived from Thai and Australian human H1N1pdm09 strains, whereas those of the genotype H1N1-D were closely related to a Thai H1N1pdm09 IAVs-S, A/swine/Chachoengsao/NIAH107037-21/2011 (Sw/Chachoengsao/2011), which was isolated in the province in which this study was performed (Hiromoto et al., 2012). Furthermore, the internal genes of H1N1 isolates showed higher divergence than those of H3N2 IAVs-S, although the number of H3N2 isolates was twice that of H1N1 viruses.

Replication kinetics of each genotype

To test whether a particular gene constellation could be advantageous for viral replication, we examined the replication kinetics of strains with different gene constellations in MDCK cells. Among H3N2 isolates, although all H3N2 genotypes showed identical replication ability at 24 h p.i., the H3N2-A genotype showed decreased replication after 24 h p.i. (Fig. 3B). In contrast, only the H3N2-E genotype could replicate after 48 h p.i. (Fig. 3B). Most of the H1N1 isolates showed similar replication ability, and only the H1N1-D genotype showed a replication ability lower than that of the other H1N1 genotypes (Fig. 3C). Thus, reassortment

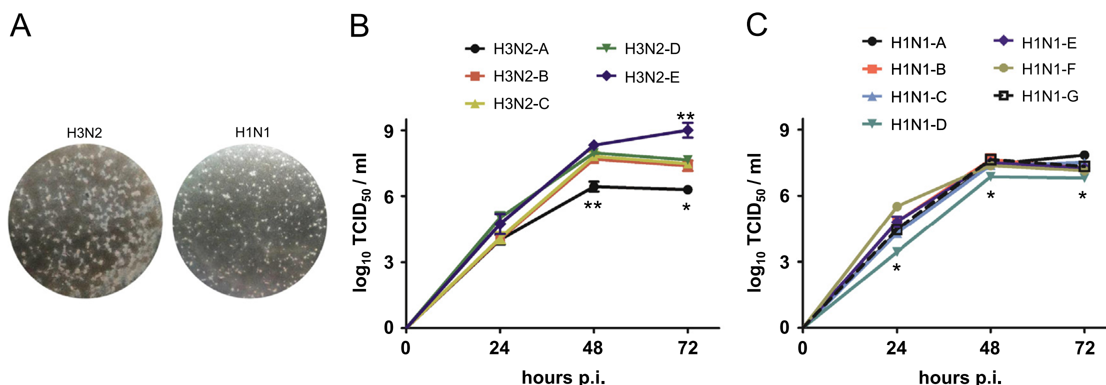

Fig. 3. Comparison of replication ability of each genotype. (A) Images of plaques of the H3N2 and H1N1 isolates. (B) Replication kinetics of each H3N2 genotype in MDCK cells ($N=3$). (C) Replication kinetics of each H1N1 genotype in MDCK cells ($N=3$). In H3N2 isolates, the H3N2-A genotype showed significantly lower replication ability than other genotypes at 48 h and 72 h. In contrast, the H3N2-E genotype retained higher replication ability than other strains at 72 h. In H1N1 viruses, only the H1N1-D genotype displayed significantly lower replication than all other H1N1 genotypes at 24 h, than the H1N1-B and H1N1-G genotypes at 48 h and the H1N1-A, H1N1-C, and H1N1-G genotypes at 72 h. Data are shown as mean ± SEM. $^{*}P<0.05$; $^{**}P<0.01$.

events would significantly contribute to improving the replication ability of IAVs-S, even if only internal genes underwent reassortment, without alteration of surface genes.

Discussion

Pigs are thought of as "mixing vessels" because they are susceptible to both avian and mammalian influenza viruses (Ito et al., 1998; Kida et al., 1994; Scholtissek, 1990-1991). The emergence of triple-reassortant IAVs-S, followed by H1N1pdm09 viruses, in the North American swine population brought global attention to the reassortment events occurring in swine and increased interest in monitoring and controlling emerging pandemic strains (Vincent et al., 2013). In spite of the increase in worldwide surveillance, the details of how reassortment occurs in pigs with multiple IAV-S co-infections under natural conditions remain uncertain.

Although the emergence of H1N1pdm09 viruses has raised serious concerns about human infections with IAVs-S, many cases of swine influenza infection in humans have been reported, aside from the pandemic in 2009 (Freidl et al., 2014; Myers et al., 2007). The hypothesis that pigs are mixing vessels is supported by a number of reports, for example, an experimental demonstration of a reassortment event in a pig between a swine and an avian strain of influenza virus (Kida et al., 1994). In addition, several previous studies have provided evidence not only of infection of pigs with avian viruses in Vietnam (Choi et al., 2005), Canada (Karasin et al., 2004), and other countries, but also of co-circulation and reassortment of human and avian influenza viruses in swine populations (Peiris et al., 2001; Cong et al., 2010). Furthermore, IAVs-S are known, even in mono-infection, to be able to rapidly accumulate mutations during continuous circulation in a swine population (Murcia et al., 2012). These reports have described the virological role of pigs in accumulating influenza viruses from various hosts, mixing their genes by reassortment, accelerating their evolution by acquisition of mutations, and rapidly creating new viruses that can infect humans.

IAV-S reassortment events have mainly been demonstrated under experimental conditions, mostly because it is difficult to obtain samples while the reassortment events are occurring. In the present study, we provide direct evidence of IAV-S co-infection in a natural setting and demonstrate that co-infection with two IAVs-S resulted in creation of at least eighteen different genotypes in a naturally reared pig (Table 2). In humans, rare cases of mixed infection with different types of influenza A viruses have been observed sporadically in the U.S. (Ghedin et al., 2009), New Zealand (Peacey et al., 2010), and China (Liu et al., 2010). Notably, one report showed that a single co-infected human sample could contain a great variety of gene sequences (Ghedin et al., 2009). The researchers concluded that the virus genes originated from two distinct lineages and that intra-host mutations increased their genetic variation, although they did not note how many novel reassortants were generated in the single human sample, because the gene constellations of individual viral isolates were not identified (Ghedin et al., 2009). Our results are concordant with this report showing evidence that a great deal of diversity can be generated within a single co-infected individual. Furthermore, changes in the combination of internal genes, even replacement of one gene with another from a different virus, are known to be able to affect virulence and replication under experimental conditions (Tian et al., 1985; Pappas et al., 2008; Manzoor et al., 2009). We asked which genotype would become established in pigs via acquisition of replication advantages compared to other genotypes. Comparing $TCID_{50}$ measurements, we found that the internal gene combination could affect replication ability, even though all internal genes were derived from H1N1pdm09 viruses (Fig. 3B and C). Although the M gene is known to influence the transmissibility and pathogenicity of H1N1pdm09 viruses (Chou et al., 2011; Lakdawala et al., 2011), our results did not show clear differences in in vitro replication kinetics among viruses possessing M genes from different clusters. However, the H1N1-D genotype, which may be one of the IAV-S strains initially introduced to this farm, exhibited significantly lower replication ability than other H1N1 genotypes, suggesting that H1N1 isolates obtained a more advantageous gene set by reassortment. Our examination of the replication kinetics of each constellation demonstrated that the phenomena observed under experimental conditions also occur under natural conditions (Fig. 3B and C). Further IAV-S surveillance will reveal which constellation identified in the present study would be most likely to establish itself in the Thai swine population in natural settings.

Phylogenetic reconstruction using the Bayesian MCMC revealed several interesting features of our isolates. Analysis of the HA and NA genes of H3N2 isolates showed that recently isolated Thai swine viruses, including the isolates in this study, formed a cluster distinct from the major H3N2 IAV-S lineages, which are classified as the North American and European lineages (Fig. 1A and B, Supplementary Figs. 1 and 2). This demonstrates that Thai H3N2 IAVs-S have been independently evolving in Thailand since they were first introduced ca. 1996 from humans. All of our H3N2 isolates were reassortants possessing internal genes derived from H1N1pdm09 viruses (Table 2). Because the HA and NA genes of the present H3N2 isolates were nearly identical to those of the Thai reassortant H3N2 IAV-S strains, such as Sw/Chonburi/2011, which has already been reported in Thailand, they appear to be successfully established in the Thai swine population (Charoenvisal et al., 2013; Hiromoto et al., 2012). Surprisingly, however, the internal genes of the H3N2-B genotype, which is considered one of the strains initially introduced to this farm, showed strong genetic relationships with Thai and Australian human H1N1pdm09 viruses but not with Sw/Chonburi/2011 (Fig. 2A–D, Table 2, Supplementary Figs. 5–10). This indicates that the H3N2-B genotype is a novel reassortant IAV-S between Thai H3N2 IAVs-S and human H1N1pdm09 viruses. In addition, the HA and NA genes of our H3N2 isolates had been circulating for more than 10 years before the emergence of H1N1pdm09 viruses, supporting the hypothesis that the reassortment event between Thai endemic H3N2 IAVs-S and H1N1pdm09 viruses may have occurred in the Thai swine population. Reassortant H3N2 IAVs-S possessing a gene constellation similar to that of the isolates examined here have been isolated in China beginning in November 2010, although they are unlikely to be related to our isolates because the HA and NA genes of Chinese isolates are derived from the Vietnamese TRIG IAV-S, which possesses surface genes from seasonal human-like viruses circulating ca. 2004–2006 (Fan et al., 2012; Ngo et al., 2012).

The H1N1 isolates in this study possessed HA and NA genes derived from the EA-like lineages, demonstrating that they are novel IAVs-S previously unreported in Thailand. Curiously, the EA-like H1 gene has not been reported in Thailand even though this gene branched off from the European lineage some twenty years ago (Fig. 1C, Supplementary Fig. 3). Moreover, the H1N1-D virus, one of the strains initially introduced into this farm, is a reassortant possessing internal genes closely related to the H1N1pdm09 virus Sw/Chachoengsao/2011 isolated in the same province sampled here (Fig. 2A–D, Table 2, Supplementary Figs. 5–10). Previous reports and our results may illuminate the complicated process of the emergence of the H1N1-D genotype. First, the EA-like IAV-S was introduced into Thailand from Europe ca. 1990, and by 2000, these strains had undergone a reassortment event in which they acquired the H1 gene from a strain of CS IAV-S probably circulating in Thailand (Takemae et al., 2008), to yield the ancestral strain of the H1N1-D genotype (ancH1N1-D). Following the 2009 pandemic, the ancH1N1-D underwent secondary reassortment with an H1N1pdm09 IAV-S such as Sw/Chachoengsao/2011, during which it acquired novel internal genes. Finally, the ancH1N1-D underwent reassortment of the H1 gene with EA-like strains, to form the

gene constellation designated H1N1-D (Table 2), although it is unclear whether acquisition of H1N1pdm09 internal genes occurred prior to acquisition of the EA-like H1 gene. In this scenario, how and where the EA-like H1 gene was maintained undetected for over twenty years in Thailand remains to be clarified. We may have overlooked an IAV-S with an EA-like H1 gene circulating in Thailand, due to insufficient surveillance. In Asia, EA-like H1N1 IAVs-S have been maintained in the Chinese swine population since 2001 (Vijaykrishna et al., 2011). Because the surface genes of our H1N1 isolates likely diverged from the European lineage approximately ten years before their entry into China (Fig. 1C and D, Supplementary Figs. 3 and 4), EA-like H1N1 IAVs-S appear to have been independently imported into Thailand and China from Europe. However, experimental inoculation into pigs produced efficient transmission of Sw/GD1361, which possesses a gene constellation similar to that of our H1N1 isolates, through both direct and aerosol contact (Zhu et al., 2011). Among the hundreds of reports of natural human infections with IAVs-S other than H1N1pdm09 viruses, EA-like H1N1 IAVs-S are implicated only in a small number of cases, keeping humans immunologically naïve to these IAVs-S (Freidl et al., 2014). Therefore, the emergence of EA-like IAVs-S that have acquired the ability to cross species boundaries pose a potential threat to public health.

Genetic variation was seen among the clones from #52 and #57 while only clones classified into H3N2-B were obtained from #49 and #54. Reason for this discrepancy is probably due to the numbers of plaques obtained in the first round of plaque-purification because of low virus titers of those swabs. Some twenty plaques were picked from #49 and #54 while hundred or more plaques were able to be picked up from #52 and #57. It might be also possible to consider that a stage of infection affected the genotype variety within a dually infected pig. Later in the infection stage, population of genotype may have skewed to a fewer genotype that obtained growth advantage in the host, although this possibility needs to be explored.

In summary, this study demonstrates that a great deal of genetic variation can be generated during IAV-S co-infection of a single pig under natural conditions. Furthermore, two candidate strains for initial infection of this farm were found to be novel reassortants between Thai IAV-S and H1N1pdm09 viral strains. However, the essential factors for introduction and circulation of IAVs-S in a pig farm remain unclear. Continuous surveillance activities are required to understand, to prevent, and to control IAV-S infections on pig farms.

Materials and methods

Sample collection

We collected nasal swabs from pigs reared on a large-scale pig farm located in Chachoengsao, a neighboring province of Bangkok, in Thailand, beginning in February 2011. The farm raised the following number of pigs: sows (1–3 years old), 1100; suckling piglets (0–3 weeks old), 1800; weaning piglets (4–10 weeks old), 4500; gilts (<1 year old), 100. The farm has implemented strict regulations (for example, prohibition of visitor access to the inside of the farm, disinfection facilities for all vehicles, and long-term employment of workers familiar with daily care of pigs) to maintain an appropriate level of biosecurity. The farm also quarantines newly purchased pigs for three months in a segregated enclosure to avoid introducing pathogens (Supplementary Table 1). From February 2011 to February 2012, twenty swabs were collected from sows, and forty from weaning piglets (4–8 weeks old) at each sampling time. In and after June 2012, we increased the number of swabs collected from both sows and weaning piglets (Table 1). All sampled pigs were clear of influenza-like symptoms. Nasal swabs were placed in preserving medium (minimal essential medium (MEM) containing penicillin (1000 unit/ml), streptomycin (1000 μg/ml), fungizone (25 μg/ml), 0.01 M HEPES, and 0.5% bovine serum albumin) immediately after collection from each pig and stored on ice. The number of samples collected was calculated based on the number required to detect at least one IAV-S-infected pig with a probability of 0.95, if the prevalence rate was $>15\%$ (February 2011–February 2012) or $>5\%$ (June 2012–March 2014) in each sampling population (Thrusfield, 1995).

Virus isolation and sequencing analyses

Virus isolation was performed as described previously (Takemae et al., 2011). Viral RNA was extracted directly from swab samples or the supernatant of IAV-S-infected cell cultures using the RNeasy Mini kit (Qiagen). cDNA was synthesized by reverse-transcription with the SuperScript III First-Strand Synthesis System (Life Technologies). All swab samples were subjected to real-time PCR analysis with primers specific to the matrix (M) gene of the influenza A virus to identify IAV-S-positive samples (Ngo et al., 2012). Viruses were isolated from IAV-S-positive swabs by culture with cells of the Madin-Darby canine kidney (MDCK) line in infection medium (MEM containing penicillin (1000 unit/ml), streptomycin (1000 μg/ml), fungizone (2.5 μg/ml), gentamicin (25 μg/ml), MEM vitamin solution, and 0.4% bovine serum albumin) supplemented with 0.5–1 μg/ml trypsin. Genes of isolated viruses were amplified by PCR with primers specific to each gene segment. PCR products were electrophoresed and purified using the QIAquick Gel Extraction kit (Qiagen) and then fully sequenced. Sequencing analysis was performed using the Big Dye Terminator v3.1 Cycle Sequencing Reaction Kit on a 3500xL Genetic Analyzer (Applied Biosystems). All sequences were assembled using the software Sequencher v5.0 (Gene Codes Co.) and consensus sequences were obtained for phylogenetic analysis of each gene segment.

Plaque purification of a co-infected samples

Swab samples, #49, #52, #54 and #57, that contained mixed subtypes was subjected to the standard plaque purification technique with a slight modification (Tobita et al., 1975) to segregate clones of individual viruses. Briefly, serial 10-fold dilutions of a swab sample were prepared with infection medium. After washing three times with PBS, 100 μl aliquots of dilution stocks were inoculated into confluent monolayers of MDCK cells cultured in 6-well plates. MDCK cells were incubated at 37 °C in a CO_2 incubator for 45 min for virus absorption, then covered with 1% agar containing MEM, penicillin (1000 unit/ml), streptomycin (1000 μg/ml), fungizone (2.5 μg/ml), MEM vitamin solution, 0.5% bovine serum albumin, and 1 μg/ml trypsin. After two days of incubation at 37 °C in a CO_2 incubator, 29, 108, 21 and 100 plaques for #49, #52, #54 and #57 were initially picked for further purification and resuspended in 500 μl of infection medium. The subtypes of all resuspended virus stocks were verified using conventional PCR analysis, and virus stocks showing a single subtype were further purified. Following three rounds of purification, viral clones originated from #52 were considered to be established and their genomes were fully sequenced, as described above. Partial sequences of each genome of the purified plaques from #49, #54 and #57 were directly determined from picked plaques after three rounds of purification by RT-PCR and the Big Dye Terminator v3.1 Cycle Sequencing Reaction Kit as described above.

Phylogenetic analyses

Alignments containing the sequences of the isolates in this study and other relevant viruses were created individually for all gene segments, trimmed to include only the coding region, and inspected

for unnecessary gaps using the software BioEdit v7.1.3.0 (Hall, 1999). The relevant sequences to be included in the alignments were determined as follows: for the analyses of H1, H3, N1, and N2 genes, viruses with high scores in BLAST analyses of isolates in this study (less than 10 viruses), IAVs-S previously isolated in Thailand, and several sequences from various lineages and hosts for accurate estimation of phylogenies; for the analyses of internal genes, viruses with high scores in BLAST analyses of isolates in this study (under 100 viruses) and Thai IAVs-S, as well as several H3N2v viruses for analysis of the M gene. The data sets contained 114–171 sequences. Each sequence was tagged with the sample collection date, and those with incomplete information were assigned the middle of the year or the month. We estimated the phylogeny and the time of the most recent common ancestors of the sequence data set using a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST v1.7.5 software package (Drummond et al., 2012). Each data set was analyzed using the SRD06 nucleotide substitution model, the strict clock model, and the constant-coalescent-population model. Multiple Bayesian MCMC runs were conducted independently for 30 million to 500 million generations, with sampling to produce 20,000 trees. Effective sample size values were confirmed to be >300, indicating a sufficient level of sampling, using the software Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Convergence of the runs was also confirmed. Maximum clade credibility trees with time-scaled branches were annotated using the software TreeAnnotator v1.8.0 (Drummond et al., 2012) and visualized using the software FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Replication curves

The replication kinetics of the isolates were investigated by inoculation into MDCK cells at a multiplicity of infection (MOI) of 0.00001 50% tissue culture infectious dose ($TCID_{50}$) per cell; MOIs were adjusted with infection medium. After washing three times, 100 μl viral aliquots were inoculated into confluent monolayers of MDCK cells cultured in 6-well plates. Cells were placed in a CO_2 incubator for 45 min for absorption, and fresh infection medium was added, along with 1 μg/ml trypsin. The supernatants were sampled at 24, 48, and 72 h post-infection (p.i.), and the virus titers of the supernatants were determined by means of endpoint titration in MDCK cells.

Statistical analysis

Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by post-hoc comparisons with Tukey's test. Statistical significance was determined using the software GraphPad PRISM 5.0 (GraphPad Software).

Nucleotide sequence accession numbers

The sequences determined in this study are available from the Global Initiative on Sharing All Influenza Data (GISAID) under the accession numbers shown in Supplementary Table 2.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.06.002.

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