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Original Article

Emergence of H3N2pM-like and novel reassortant H3N1 swine viruses possessing segments derived from the A (H1N1)pdm09 influenza virus, Korea

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Background Human-to-swine transmission of the pandemic H1N1 2009 [A(H1N1)pdm09] virus in pig populations resulted in reassortment events with endemic swine influenza viruses worldwide.

Objective We investigated whether A(H1N1)pdm09-derived reassortant viruses are present in South Korea and sought to determine the pathogenic potential of the novel swine viruses.

Methods Pig lung tissues were collected from commercially slaughtered pigs. Isolated swine influenza viruses were genetically analyzed and characterized *in vitro* and *in vivo*.

Results We identified reassortant H3N2 (H3N2pM-like) and H3N1 swine viruses containing A(H1N1)pdm09-like segments in Korean pigs that are genetically closely related to strains recently detected in pigs and humans in North America. Although the H3N2pM-like and novel H3N1 reassortants demonstrated efficient replication in mice and ferrets, all the H3N1 strains exhibited growth advantage over the representative H3N2pM-like virus in human airway cells. Interestingly, A/swine/Korea/CY02-07/2012 (H3N1) and A/swine/Korea/CY03-13/2012(H3N1) reassortants were more readily transmitted to respiratory-droplet-contact ferrets compared with the H3N2pM-like (A/swine/Korea/CY02-10/2012) isolate. Furthermore, serologic evaluation showed poor antigenicity to contemporary reference human seasonal H3N2 vaccine strains.

Conclusions We report here for the first time the isolation of H3N2pM-like viruses outside North America and of novel reassortant swine H3N1 viruses with A(H1N1)pdm09-derived genes. Apart from further complicating the genetic diversity of influenza A viruses circulating in domestic pigs, our data also indicate that these strains could potentially pose threat to public health asserting the need for continuous virus monitoring in these ecologically important hosts.

Keywords A(H1N1)pdm09, Korea, reassortment, surveillance, swine influenza virus.

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Introduction

The zoonotic transfer and efficient global spread of a novel reassortant influenza A H1N1 virus in 2009 represent the most extensive human dissemination of a swine-origin virus and perhaps provide the most compelling evidence of the importance of pigs in their genetic evolution and ecology. The pandemic H1N1 2009 [A(H1N1)pdm09] virus, possessing a unique mixture of segments from Eurasian avian-like swine (NA, M) and a North American-like triple-reassortant (human-lineage HA/NA/PB1, avian-lineage PB2/PA, and swine-lineage NP/NS segments) H1 swine viruses,[1,2] have been also detected in various swine herds worldwide shortly after its rapid global spread in humans.[3] With the segmented nature of the viral genome, pigs could promote genetic reassortment of viruses from various hosts and lineages in a co-infected host, re-igniting concerns for the generation of novel strains more virulent than the parental precursors.

By the end of 2009, A(H1N1)pdm09-derived reassortant viruses were already isolated in North American pigs.[4] Since then, various swine A(H1N1)pdm09-like reassortants were additionally identified in Asia,[5–9] Europe[10–12] and the Americas,[13–16] where most of those detected in the United States consistently contained the A(H1N1)pdm09 matrix (pM) gene.[4] More recently though, genetically related novel triple-reassortant H3N2 swine viruses containing the pM gene were predominantly found in North American pigs (termed H3N2pM)[16] and were also alarmingly causing

human infections [termed 'A(H3N2)v'],[17] implying their potential threat to public health.

In the present study, we examined whether the A(H1N1) pdm09 virus remain in circulation among Korean pigs since its first detection here in December 2009[17] and determined its genetic impact on the generation of novel swine viruses. We report here for the first time the emergence of H3N2pM-like viruses outside North America and the characterization of novel reassortant swine H3N1 viruses isolated in lung tissue samples collected from a pig slaughterhouse in Chungcheongbuk-do, Republic of Korea. We provide evidence on the capacity of these reassortant viruses to establish infection and efficient transmission in ferret models, suggesting their potential to cause human infections.

Materials and methods

Virus isolation and growth

Viruses in this study were isolated from Madin-Darby canine kidney (MDCK) cells infected with supernatants of homogenized swine lung tissues collected from clinically healthy, market-aged (≥5 months old) pigs for commercial slaughter from May 2011 through April 2012 (Table S1). All pigs came from different swine farms of Chungcheong Province, Republic of Korea.[18] Viral growth was determined by monitoring cytopathic changes in cells and confirmed by hemagglutinin assay using 0·5% turkey RBC (tRBC). Positive samples were plaque purified and additionally grown in MDCK cells.

Growth characteristics in culture were compared by inoculating MDCK cells with multiplicity of infection (MOI) of 0·001 and lung epithelial (A549) or primary normal human bronchial epithelial (NHBE) cells with MOI of 0·1. Infectious cell culture supernatants were collected at 12, 24, 48, and 72 h post-infection (pi). Fifty-percent tissue culture infective doses ($TCID_{50}$) of stock viruses, nasal washes, tissue homogenates, and supernatants were end-point-titrated in MDCK cells by the Reed and Muench method[19] with results expressed as $\log_{10}TCID_{50}$ per milliliter (ml) or per gram (g) of tissue, respectively. The limit of virus detection was 0·7 $\log_{10}TCID_{50}$ per unit sample tested. Student's *t*-test was used for statistical comparisons; threshold of significance was set at $P < 0{\cdot}05$ (Graph Pad Prism™ ver. 6, San Diego, CA, USA).

Genomic sequencing and phylogenetic and analyses

Viral RNA was extracted from cell culture isolates using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RT-PCR and full-genome sequencing were carried out under standard conditions using influenza-specific primers as previously described.[18] DNA sequences were compiled and edited using DNAStar's Lasergene sequence analysis software package version 5.0 (Madison, WI, USA). Multiple sequences were aligned using Clustal_X[20], while rooted phylograms were prepared and plotted with the neighbor-joining (NJ) plot program.[21] Phylogenetic trees were constructed by aligning full-length gene sequences of our swine isolates with those of reference sequences from human and genetically related animal influenza viruses available in GenBank identified through the basic local alignment search tool. Isolates were assigned with GenBank accession numbers KC471342–KC471493.

Animal studies

Groups of 22 5- to 6-week-old female BALB/c mice (weighing ≥18 g) (Samtaco, Seoul, Republic of Korea) were inoculated intranasally with 10^5 $TCID_{50}$/30 µl of designated virus. For each infection group, three mice were sacrificed on 1, 3, 5, and 7 dpi for lung viral titrations; the remaining ten mice were monitored daily up to 14 days for morbidity and mortality. A cutoff value was set to ≥25% for weight losses in which mice will be euthanized.

Groups of two 14- to 16 week-old female outbred ferrets (*Mustela putorius furo*, Wuxi Sangosho Pet Park Co., Ltd., China) that were seronegative by hemagglutination inhibition (HI) and NP ELISA assays for influenza A virus exposure/infection were inoculated intranasally with $10^{5.5}$ $TCID_{50}$/ml of each field virus isolate. At 1 dpi, two contact ferrets were added to the other half of the cage containing one of the inoculated ferrets separated by two stainless steel grids (at 35 mm apart) allowing respiratory-droplet (RD) transmission without direct contact. Signs of morbidity (e.g., weight, temperature, sneezing) and mortality were monitored daily for 14 days. Each test group consisted of two infected and two RD-contacts. Viral growth at the upper respiratory tract was determined by collecting nasal washes in alternate days at 1, 3, 5, 7, and 9 dpi (inoculated) or daily up to 12 dpi (RD-contacts) and. One additional ferret was infected with each virus and humanely euthanized at 3 dpi for virus detection in various tissues (trachea, lung, spleen, intestine, and brain).

Blood samples were collected from mice and ferrets before infection and at 16–18 dpi for HI assays using tRBCs according to standard methods.[22] Animal experiments were performed in a BSL3+ containment facility approved by the Korea Centers for Disease Control and Prevention following general animal care guidelines required by the Institutional Animal Care and Use Committee of Chungbuk National University.

Results

Phylogenetic and genetic characterization of novel reassortant H3 viruses

Of 1,152 tissue samples collected, 19 isolates (1·6%) were recovered and subtyped as H1N1 ($n = 5$), H1N2 ($n = 2$),

H3N1 (n = 4), or H3N2 (n = 8) (Table 1). Phylogenetic analysis of each segment of the five H1N1 and two H1N2 swine viruses revealed that they are descendants of the A (H1N1)pdm09 and pre-existing Korean H1N2 viruses, respectively, without any indication of genetic reassortment (Figures 1, S1). In contrast to the intact H1 viruses, our H3 isolates had a more complex genetic combination. The HA of the four H3N1 isolates grouped together with contemporary cluster IV H3 viruses in North America, which also include the H3N2pM and A(H3N2)v viruses (Figure 1B). The PB1 of A/swine/Korea/CY02-08/2012(H3N1), PB1/NP/NS of A/swine/Korea/CY02-07/2012(H3N1), and PB1/NS of A/swine/Korea/CY03-13/2012(H3N1) appeared to be derived from North American-lineage triple-reassortant H3N2 strains, while the other segments are A(H1N1)pdm09-like. Similarly, all the remaining seven segments of A/swine/Korea/CY03-12/2012(H3N1) were grouped within A(H1N1)pdm09-like viruses (Table 1 and Figure S1).

For the H3N2 isolates, phylogeny of the M genes demonstrated that they were A(H1N1)pdm09-like, whereas all their remaining segments were triple-reassortant swine virus-derived (Figure S1E). Thus, our reassortant H3N2 isolates bear similar genetic constellation as the recent H3N2pM and A(H3N2)v viruses in the United States.[16,17] It is interesting to note that the HA gene of A/swine/KoreaCY02-09/2012(H3N2) was more closely related to the H3N1 strains rather than to the other H3N2pM-like isolates (99·8–100 versus 98·9–99·5% nucleotide sequence homologies) (Figure 1B). Furthermore, the pM segments of the novel H3N1 isolates were also separately clustered. A/swine/Korea/CY02-08/2012(H3N1) and A/swine/Korea/CY03-12/2012(H3N1) grouped together with the Korean H3N2pM-like viruses, while A/swine/Korea/CY02-07/2012(H3N1) and A/swine/Korea/CY03-13/2012(H3N1) appeared to be more closely related to A(H1N1)pdm09-like isolates in this report clustering under a human strain in Korea (A/Daejeon/1840/2009); average sequence identity between these clusters of pM segments is 98·7% (Figure S1E). Overall, phylogenetic analyses demonstrated that the H3 influenza viruses isolated here are reassortant strains between A(H1N1)pdm09-like and triple-reassortant H3N2-like viruses.

Virological assessment of H3N2pM-like and novel H3N1 reassortants *in vitro* and in mice

Replication kinetics of the H3N2pM-like (A/swine/Korea/CY02-10/2012) and novel H3N1 (A/swine/Korea/CY02-07/2012, A/swine/Korea/CY02-08/2012, A/swine/Korea/CY03-12/2012, and A/swine/Korea/CY03-13/2012) reassortants were assessed *in vitro*. Titration of infectious viruses released in infected cell culture supernatants showed comparable replication efficiency of all viruses in MDCK cells, although A/swine/Korea/CY02-10/2012(H3N2) demonstrated slightly delayed growth kinetics reaching peak titers at 72 hpi (Figure 2A). In NHBE cells, the novel H3N1 reassortants

Table 1. Genotypes of identified A(H1N1)pdm09-like, H1N2, and reassortant H3 swine influenza viruses.

Isolate	Subtype	Isolation	Genotype								Stock titers ($TCID_{50}$/ml)	MLD_{50}
			HA	NA	PB2	PB1	PA	NP	M	NS		
A/swine/Korea/CY11-01/2011	H1N1	11/21/2011									4·0E+06	ND
A/swine/Korea/CY11-02/2011	H1N1	11/21/2011									2·0E+06	ND
A/swine/Korea/CY12-03/2011	H1N2	12/5/2011									6·3E+06	ND
A/swine/Korea/CY01-04/2012	H1N1	1/2/2012									4·0E+06	ND
A/swine/Korea/CY01-05/2012	H1N1	1/2/2012									1·0E+06	ND
A/swine/Korea/CY01-06/2012	H1N1	1/16/2012									6·3E+06	ND
A/swine/Korea/CY02-07/2012	H3N1	2/7/2012									6·3E+07	> 6·0
A/swine/Korea/CY02-08/2012	H3N1	2/7/2012									6·3E+07	> 6·0
A/swine/Korea/CY02-09/2012	H3N2	2/7/2012									2·0E+06	ND
A/swine/Korea/CY02-10/2012	H3N2	2/20/2012									1·0E+06	> 6·0
A/swine/Korea/CY03-11/2012	H1N2	3/6/2012									2·0E+07	ND
A/swine/Korea/CY03-12/2012	H3N1	3/6/2012									1·0E+08	> 6·0
A/swine/Korea/CY03-13/2012	H3N1	3/6/2012									4·0E+07	> 6·0
A/swine/Korea/CY03-14/2012	H3N2	3/6/2012									4·0E+05	ND
A/swine/Korea/CY03-15/2012	H3N2	3/6/2012									1·0E+06	ND
A/swine/Korea/CY03-16/2012	H3N2	3/27/2012									1·0E+05	ND
A/swine/Korea/CY03-17/2012	H3N2	3/27/2012									2·0E+05	ND
A/swine/Korea/CY03-18/2012	H3N2	3/27/2012									1·0E+06	ND
A/swine/Korea/CY03-19/2012	H3N2	3/27/2012									2·0E+05	ND

Black and gray cells indicate segments of A(H1N1)pdm09 and triple-reassortant virus origin, respectively. The MLD_{50}s of a representative H3N2pM-like and the novel H3N1 reassortants are shown. ND, not determined.

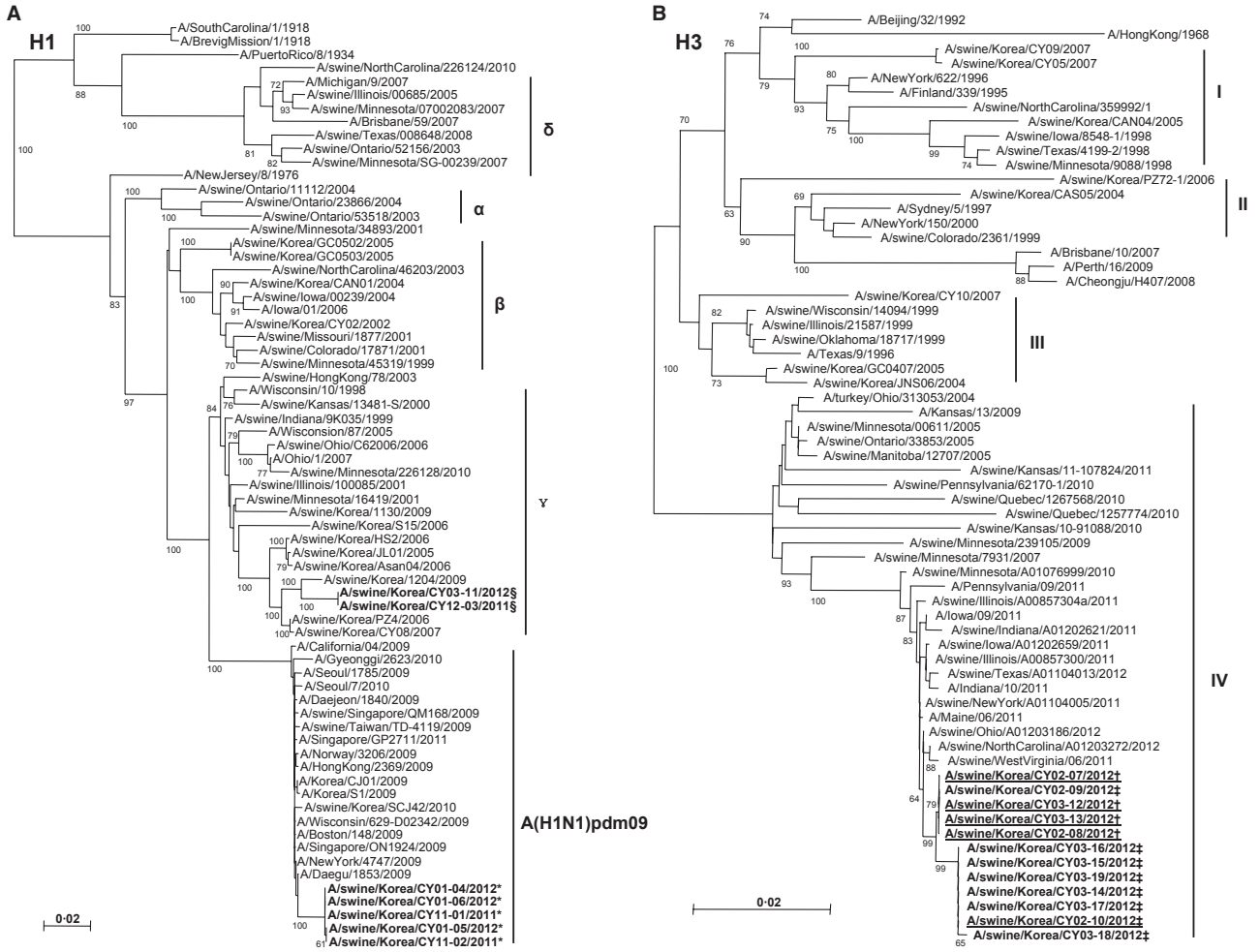

Figure 1. Phylogenetic trees based on the full-length gene sequences of the HA (A, B) and NA (C, D) segments of A(H1N1)pdm09 (*), H1N2 (§), and reassortant H3N1 (†) and H3N2pM-like (‡) viruses in this study. Antigenic clusters of H1 (A) and H3 (B) viruses of the North American swine lineage are indicated. Scale represents the number of substitutions per nucleotide. Branch labels record the stability of the branches over 100 bootstrap replicates. Only bootstrap values >60% are shown. Viruses in boldface are the isolates in this study, whereas underlined strains were used for pathogenesis and transmission studies.

produced at least 100-fold higher peak titers ($P < 0{\cdot}05$) than the H3N2pM-like virus suggesting their more efficient replication in this human airway cell line. No variable growth kinetics was observed in A549 cells where all viruses moderately replicated.

All tested swine H3 isolates proliferated in mice lungs without pre-adaptation. However, only three H3N1 viruses (A/swine/Korea/CY02-07/2012, A/swine/Korea/CY02-08/2012, A/swine/Korea/CY03-13/2012) persisted up to 7 dpi where A/swine/Korea/CY02-07/2012(H3N1) consistently produced the highest lung titers (Figure 2B). Moreover, none of the isolates induced any remarkable signs of morbidity (as indicated by <2% weight losses) nor caused death within 14 days of infection (MLD_{50}s>6·0) (Figure 2C and Table 1).

Pathogenesis and transmission of H3 viruses in ferrets

In general, all ferrets inoculated with the H3N2pM-like and H3N1 isolates exhibited mild-to-moderate signs of influenza disease that were indistinguishable between groups as indicated by mild lethargy, mean maximum weight losses of 6·5–8·5%, and elevated temperatures of up to 2·1°C (Table S2). Sneezing was observed at peak times of viral shedding (1-3 dpi), but was more frequent in groups infected with A/swine/Korea/CY02-07/2012(H3N1), A/swine/Korea/CY03-13/2012(H3N1), and A/swine/Korea/CY02-10/2012(H3N2) viruses. All H3N1 isolates replicated efficiently in the upper respiratory tract producing high peak viral titers (≥5·4 $\log_{10}TCID_{50}$/ml), but only A/swine/Korea/CY02-07/2012 (H3N1) and A/swine/Korea/CY03-13/2012(H3N1) persisted

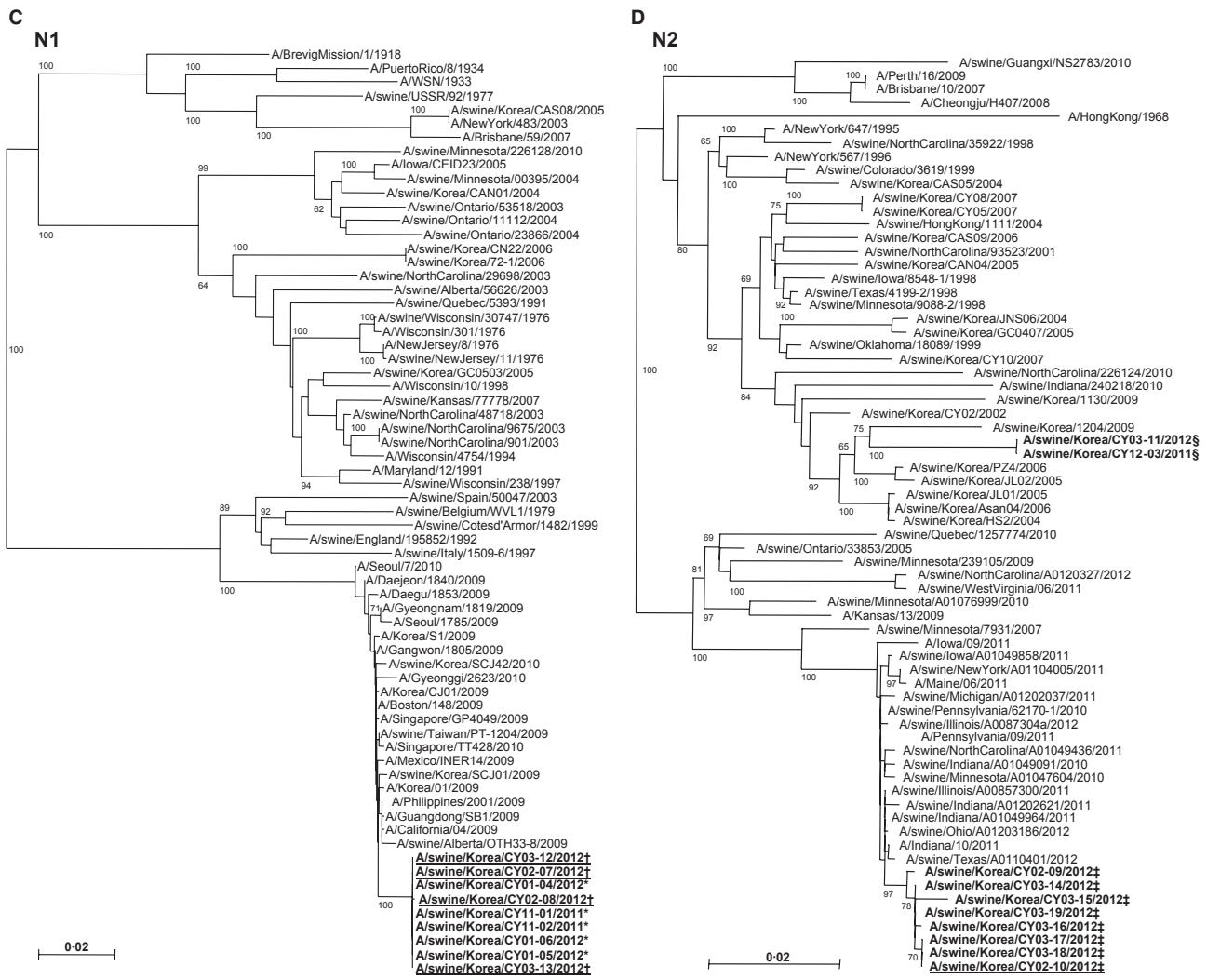

Figure 1b. Continued.

in nasal washes collected up to 5 dpi. On the other hand, A/swine/Korea/CY02-10/2012(H3N2)-infected ferrets had relatively lower titers (4·2 $\log_{10}TCID_{50}$/ml mean peak titer) compared with the H3N1-infected groups although shedding also continued until 5 dpi (Figure 3A–E). Among the H3N1 strains, A/swine/Korea/CY02-07/2012, A/swine/Korea/CY02-08/2012, and A/swine/Korea/CY03-13/2012 were detected in tracheal tissues (at titers 3·3, 2·3, and 4 $\log_{10}TCID_{50}$/g, respectively) collected at 3 dpi, whereas only A/swine/Korea/CY02-07/2012 and A/swine/Korea/CY03-13/2012 were additionally recovered in harvested lung tissue samples (at titers 3·6 and 5·3 $\log_{10}TCID_{50}$/g, respectively) (Figure 3F). In contrast, A/swine/Korea/CY02-10/2012(H3N2) could not produce titers beyond the limit of detection. No infectious virus was recovered in any of the other tissue samples (spleen intestine, brain) tested.

Exposure to A/swine/Korea/CY02-07/2012(H3N1) and A/swine/Korea/CY03-13/2012(H3N1) resulted to positive detection in nasal washes of both RD-contacts as early as 2 days post-exposure (dpe) with 5·5 and 5·3 $\log_{10}TCID_{50}$/ml mean peak titers, respectively (Table S2 and Figure 3A). In contrast, the H3N2pM-like virus exhibited delayed transmission kinetics requiring 4-5 dpe for detection in all RD-contacts (4·8 $\log_{10}TCID_{50}$/ml mean peak titers). Despite negative detection, seroconversion was evident in both RD-contacts of A/swine/Korea/CY02-08/2012(H3N1) at 17 dpe (Table S2), but not in the A/swine/Korea/CY03-12/2012 (H3N1) RD-contact group, suggesting impaired aerosol transmission.

Antigenic cross-reactivity against human seasonal and swine H3N2 viruses

Alignment of the deduced amino acid sequences of individual virus segments did not reveal notable markers for virulence and pathogenicity previously described.[23] Although our H3 isolates, except A/swine/Korea/CY03-12/2012

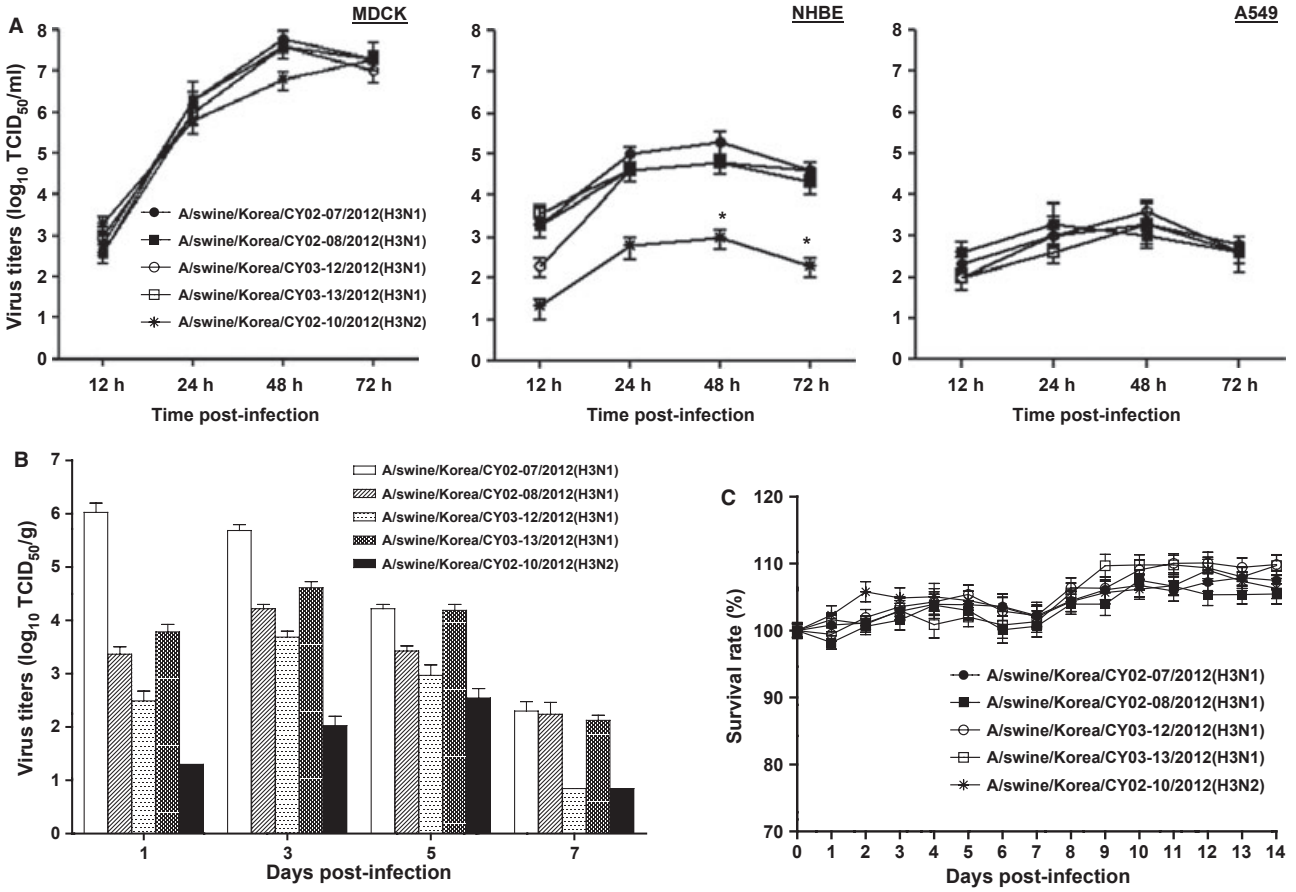

Figure 2. Growth capacity of novel swine viruses in culture (A) and pathogenicity in mice (B-C). (A) Comparative growth curves of the H3N2pM (represented by A/swine/Korea/CY02-10/2012) and novel H3N1 reassortants in MDCK, NHBE, and A549 cells. Virus endpoint titers are expressed as mean $\log_{10}TCID_{50}$/ml from three independent assays (* $P < 0{\cdot}05$). (B) Pathogenicity was assessed in groups of 22 mice inoculated intranasally with the indicated viruses. Three mice from each group were sacrificed on days indicated for lung viral titrations expressed as mean $\log_{10}TCID_{50}$/g ± SD. The remaining ten mice were observed for morbidity and mortality (C).

(H3N1), are predicted to possess a 90-amino acid PB1-F2 protein, none possess the Ser-66 virulence marker. The H3 HA1 region showed consensus residues at sites known for receptor-binding (226-Val and 228-Ser), which have become common among North American H3N2 swine viruses.[16,17] Non-synonymous amino acid differences in each of the predicted antigenic epitopes relative to recent human seasonal and three earlier clusters of triple-reassortant swine H3N2 viruses in North America were noted while a Ser-266-Thr substitution near antigenic site E2 was shared by all our H3 isolates (data not shown).

Using ferret antisera obtained from animals experimentally infected with the H3N2pM-like and H3N1 isolates, antigenic cross-reactivity was not observed against the A/California/04/2009(H1N1) virus when evaluated by HI assays. Antigenicity against homologous viruses was high (at 1280-2560 HI titers) but was slightly lower between the H3N2pM-like and H3N1 isolates (at 640 HI units) perhaps due to the heterosubtypic NAs. Additionally, the A/swine/Korea/CY02-10/2012(H3N2) ferret antisera demonstrated 1280 HI titers against A/swine/Korea/CY02-09/2012 (H3N2), suggesting that the two H3N2pM-like virus clusters (Figure 1B) are not antigenically divergent. However, the three ancestral clusters of triple-reassortant swine H3N2 viruses (represented by A/swine/Korea/CAN04/2005, A/swine//Korea/CAS05/2004, A/swine/Korea/CY07/2007) demonstrated low-to-moderate cross-reactivities (40-160 HI titers), whereas no evident antigenic reaction was observed against A/Brisbane/10/2007 and A/Perth/16/2009 (Table 2) indicating antigenic divergence.

Discussion

In this study, we show that A(H1N1)pdm 09-like viruses remain in circulation among swine herds of Korea, providing opportunities to undergo reassortment with currently

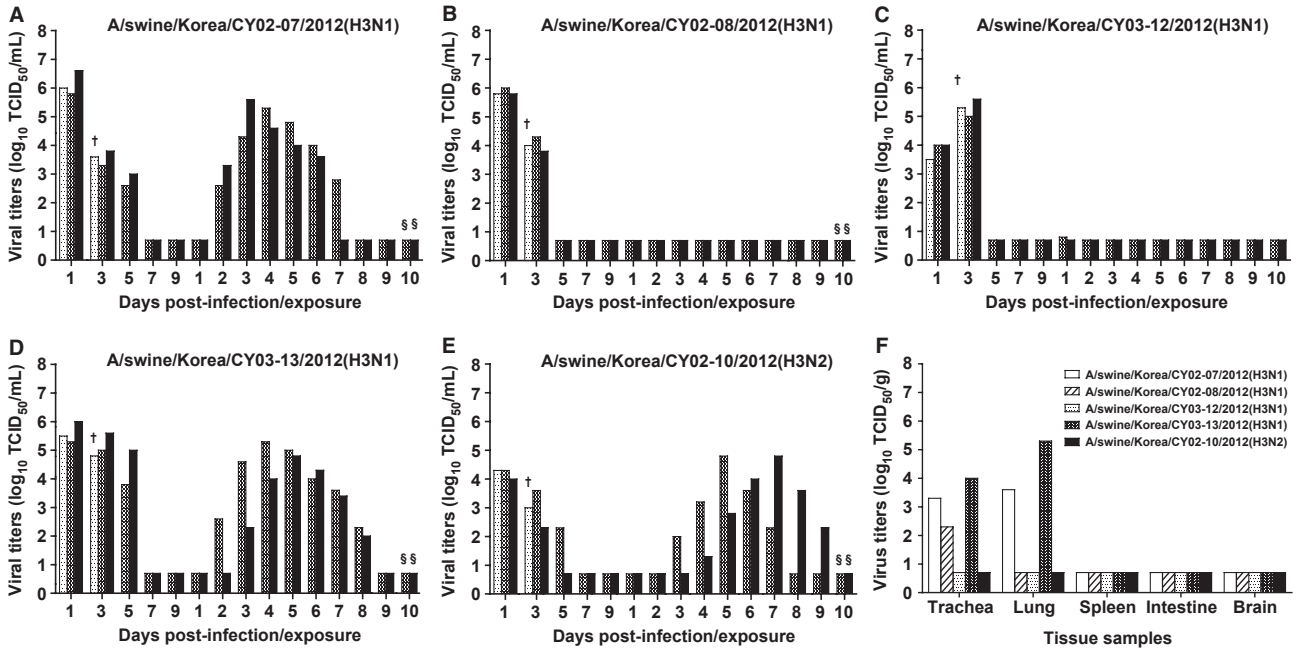

Figure 3. Replication and respiratory-droplet transmission in ferrets. Groups of two ferrets inoculated with the H3N2pM-like and H3N1 swine isolates were paired individually adjacent to RD-contact ferrets at 1 dpi (A–E). Nasal wash titers are shown for individual ferrets (§, seroconversion of RD-contacts at 17 dpe by HI assays). One additional ferret for each group was inoculated and humanely euthanized at 3 dpi (†) for virus titration in various tissues (F). Virus titers in nasal washes and homogenized tissues are expressed as $\log_{10}TCID_{50}$/ml or g tissue collected with the limit of virus detection set at 0·7 $\log_{10}TCID_{50}$/ml or g.

Table 2. Immunologic cross-reactivities of ferret-raised H3 antisera against panel of influenza A viruses

		Ferret antisera					
Viruses	**Subtype**	**A/swine/ Korea/ CY02-07/12**	**A/swine/ Korea/ CY02-08/12**	**A/swine/ Korea/ CY03-12/2012**	**A/swine/ Korea/ CY03-13/12**	**A/swine/ Korea/ CY02-10/12**	**A/Perth/ 16/2009**
A/California/04/2009	H1N1	<20	<20	<20	<20	<20	<20
A/swine/Korea/CY02-07/2012	H3N1	**2560**	2560	2560	2560	640	<20
A/swine/Korea/CY02-08/2012	H3N1	2560	**2560**	2560	2560	640	<20
A/swine/Korea/CY03-12/2012	H3N1	2560	2560	**2560**	2560	640	<20
A/swine/Korea/CY03-13/2012	H3N1	2560	2560	2560	**2560**	640	<20
A/swine/Korea/CY02-09/2012	H3N2	640	640	640	640	1280	<20
A/swine/Korea/CY02-10/2012	H3N2	640	640	640	640	**1280**	<20
A/Brisbane/10/2007*	H3N2	<20	<20	<20	<20	<20	40
A/Perth/16/2009*	H3N2	<20	<20	<20	<20	<20	**1280**
A/swine/Korea/CAN04/2005**	H3N2	40	80	40	40	20	<20
A/swine//Korea/CAS05/2004***	H3N2	160	160	80	80	160	<20
A/swine/Korea/CY07/2007†	H3N2	80	80	40	40	40	<20

*Human seasonal H3N2 virus; prototype cluster I (**), II (***), III (†) H3 swine viruses are represented. Titers in boldface represent homologous virus reactivity.

circulating animal influenza viruses. Indeed, A(H1N1) pdm09-derived H1N2 reassortants were isolated in 2010 bearing N2 segments from pre-existing Korean H1N2 swine strains.[6] Meanwhile, H1N2 isolates in the present study appear to be intact Korean swine viruses without any indications of genetic reassortment. Some gilts in Korea are imported from North America[24] prompting speculations that North American triple-reassortant swine viruses were

introduced into Korean pigs through importation of infected but asymptomatic pigs.[25] We now report the isolation of H3N2pM-like and novel H3N1 reassortants carrying various combination of A(H1N1)pdm09- and triple-reassortant H3N2-derived segments that are more closely related to North American isolates rather than from swine viruses previously isolated in Korea.[26]

Swine H3N1 viruses with the triple-reassortant gene cascade have been isolated in the United States[27,28] and the Republic of Korea.[29] However, the identification of A(H1N1)pdm09-derived H3N1 viruses with these particular gene constellations has never been reported elsewhere, including the United States. At present, it is unknown when and where these H3 reassortants were generated and whether genetically similar or other reassortant strains are present in different regions of the Korean Peninsula. It is possible that co-infection of A(H1N1)pdm09 and previously undetected triple-reassortant H3N2 viruses in pigs facilitated reassortment. Considering that the M genes of A/swine/Korea/CY02-07/2012(H3N1) and A/swine/Korea/CY03-13/2012(H3N1) demonstrated closer relationship to local A(H1N1)pdm09 strains, it is tempting to speculate that reassortment events may have occurred in domestic pigs of Korea. Furthermore, some viral segments of our H3 isolates, such as HA, do not phylogenetically form a single lineage possibly suggesting independent introductions or short-term evolution following reassortment of viral ancestors.

Infection of reassortant H3 viruses did not induce severe clinical disease in mice and ferrets. Three H3N1 isolates (A/swine/Korea/CY02-07/2012, A/swine/Korea/CY02-08/2012, and A/swine/Korea/CY03-13/2012) persisted longer in mice lungs, whereas A/swine/Korea/CY02-07/2012(H3N1) and A/swine/Korea/CY03-13/2012(H3N1) exhibited tropism for lung and tracheal tissues epitomizing the A(H1N1)pdm09 virus.[30] We also observed that all tested viruses could grow well in cultured NHBE cells. Such results correlate to their efficient replication in the upper respiratory tract of ferrets, suggesting their ability to infect cells of the human upper respiratory airway. Consistently though, the H3N1 reassortants produced higher viral titers in such cells and in ferret nasal washes than the H3N2pM-like virus.

It has been proposed that the pM segment is a determinant of the aerosol transmission and zoonotic potential of H3N2pM viruses.[16] Our results show that the H3N2pM-like isolate was transmitted to RD-contact ferrets with kinetics commensurate to genetically related 2011 A(H3N2)v human viruses.[31] Of the four novel H3N1 reassortants, only A/swine/Korea/CY02-07/2012(H3N1) and A/swine/Korea/CY03-13/2012(H3N1) transmitted more readily in naïve RD-contacts despite evident growth in ferrets lungs. The balance between HA-NA activities have been shown to confer RD transmission of the A(H1N1)pdm09 virus in ferrets.[32] Therefore, acquisition of the H3-HA would consequently perturb stability and alter transmission kinetics as exemplified by A/swine/Korea/CY03-12/2012(H3N1). However, results generated here indicated that the presence of H3N2-derived PB1, NP, and NS segments do not essentially impair aerosol dissemination. Given their importance in viral replication and virulence, it is probable that these segments played significant roles in the observed RD transmission. More importantly, our serologic results and of others[33,34] clearly showed that our reassortant H3 isolates are antigenically divergent from recent human seasonal H3N2 strains. Therefore, previous exposure to or immunization with trivalent influenza vaccines may not elicit protective immunity against infection from these novel H3 viruses.

We have shown here that the H3N2pM-like and novel reassortant H3N1 isolates could potentially establish infection and transmission in ferrets, a recognized model for human influenza infection and transmissibility, presenting concerns for human health risks. Although H3N2pM-like viruses appear to be more commonly isolated in this report, such predominance over the H3N1 reassortants remains to be seen. All these strains could still be displaced by more dominant viruses in the field. Due to continuous movement of pigs, it may not also be too surprising to find H3N2 swine viruses outside North America bearing gene constellation similar to the H3N2pM and 2011 A(H3N2)v strains. However, their increased prevalence and dissemination across continents would be notably important given their potential threat to public health. Furthermore, their establishment would certainly complicate further the genetic diversity of swine influenza viruses in Korea. Thus, our report asserts the need for continuous vigilance on the evolution of influenza viruses in this ecologically significant reservoir for the detection of emergent novel strains with potential threat to animal and human populations.

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Conflicts of interest

All authors declare no competing interest.

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Addendum

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic trees based on the full-length gene sequences of the internal gene segments of A(H1N1)pdm09 (*), H1N2 (§), and reassortant H3N1 (†) and H3N2pM-like (‡) viruses in this study (A-F).

Table S1. Monthly distribution of virus isolation from swine lungs.

Table S2. Evaluation of clinical disease in experimentally inoculated ferrets.