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Prevalent Eurasian-avian H1N1 swine influenza virus with 2009 pandemic viral genes facilitating human infection

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Author Contributions

J.L., G.F.G., H.S., Y.X., and S.L. designed research; H.S., Y.X., J.L., F.L., J.Z., C.L., J.S., H.S., Z.J., L.L., X.Z., K.W., D.H. and Q.T. performed research; J.L., H.S., J.L., J.P., C.W., Y.B. and D.W. analyzed data; J.L., G.F.G., H.S., J.P., Y.S. and K.C.C. wrote the paper.

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Abstract

Pigs are considered as important hosts or “mixing vessels” for the generation of pandemic influenza viruses. Systematic surveillance of influenza viruses in pigs is essential for early warning and preparedness for the next potential pandemic. Here, we report on an influenza virus surveillance of pigs from 2011 to 2018 in China, and identify a recently emerged genotype 4 (G4) reassortant Eurasian-avian (EA) H1N1 virus, which bears 2009 pandemic (pdm/09) and triple-reassortant (TR)-derived internal genes and has been predominant in swine populations since 2016. Similar to pdm/09 virus, G4 viruses bind to human-type receptors, produce much higher progeny virus in human airway epithelial cells, and show efficient infectivity and aerosol transmission in ferrets. Moreover, low antigenic cross-reactivity of human influenza vaccine strains with G4 reassortant EA H1N1 virus indicates that pre-existing population immunity does not provide protection against G4 viruses. Further serological surveillance among occupational exposure population showed that 10.4% (35/338) of swine workers were positive for G4 EA H1N1 virus, especially for participants of 18-35 years old who had 20.5% (9/44) seropositive rates, indicating that the predominant G4 EA H1N1 virus has acquired increased human infectivity. Such infectivity greatly enhances the opportunity for virus adaptation in humans, and raises concerns for the possible generation of pandemic viruses.

Significance Statement

Pigs are intermediate hosts for the generation of pandemic influenza virus. Thus, systematic surveillance of influenza viruses in pigs is a key measure for pre-warning the emergence of the next pandemic influenza. Here, we identified a triple reassortant EA H1N1 virus possessing pdm/09 and TR-derived internal genes, termed as G4 genotype, which has become predominant in swine populations since 2016. Similar to pdm/09 virus, G4 viruses have all the essential hallmarks of a candidate pandemic virus. Of concern is that swine workers show elevated seroprevalence for G4 virus. Controlling the prevailing G4 EA H1N1 viruses in pigs and close monitoring in human populations, especially the workers in swine industry, should be urgently implemented.

Main Text

Introduction

Influenza A virus (IAV) is a global pathogen of humans and a wide range of mammalian and avian species. Reassortment of influenza viruses is a major mechanism to generate progeny viruses with novel antigenic and biological characteristics, which can cause catastrophic human epidemics and pandemics. Historically, pandemic influenza A viruses from 1957, 1968 and 2009 are all reassortants derived from human and animal influenza viruses (1, 2). Pigs, being susceptible to avian, swine and human influenza A viruses, are regarded as “mixing vessels” in the generation of influenza viruses with pandemic potential (3-5). The emergence of the pdm/09 H1N1 virus vividly illustrates the importance of pigs in new outbreaks (6-8). Therefore, continuous surveillance of swine influenza viruses (SIVs) in pigs and assessment of their zoonotic potential are essential for the preparedness of human pandemics.

China has arguably the most complex SIVs ecosystem with classical swine (CS) lineage, North American triple-reassortant (TR) lineage, and Eurasian-avian (EA) lineage SIVs co-circulating in pigs (9). EA H1N1 SIVs are found in 2001, and gradually become the dominant lineage in China (9-11). However, after 2009, the 2009 pandemic (pdm/09) H1N1 virus in humans has spread back into pig herds around the world (12, 13). Subsequently, reassortants between the swine EA H1N1 virus and human pdm/09 H1N1 virus have been sporadically detected in pigs in China and other countries (10, 14-20), some of which have caused human infections in China (21-23).

However, the current prevalence and biological properties of these emergent EA reassortants and their infectivity in human population are unknown.
In this study, we performed an extensive SIVs surveillance program in 10 provinces with high density pig populations between 2011 and 2018. We identified a predominant emergent EA reassortant G4 virus in pigs, which has pdm/09 and TR-derived internal genes and shows efficient infectivity and transmissibility in the ferret model. Serological surveillance among swine workers and general population showed that G4 EA H1N1 viruses have acquired increased human infectivity. Thus, the emergent G4 EA H1N1 viruses pose a serious threat to human health.

Results

EA H1N1 Viruses Exhibit Increased Genetic Diversity Since 2013. To investigate the epidemiological status of SIVs, from 2011 to 2018, we performed active surveillance and collected a total of 29,918 nasal swab samples from normal pigs in slaughterhouses in 10 provinces with high density pig populations (SI Appendix, Fig. S1). We isolated 136 influenza viruses from these samples, with an isolation rate of 0.45% (SI Appendix, Table S1). In the same period, 1,016 nasal swabs or lung samples were collected from pigs showing respiratory symptoms in our school's veterinary teaching hospital, of which, 43 were positive for influenza virus at an isolation rate of 4.23% (SI Appendix, Table S1). Based on sequence analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of the combined 179 SIVs, they were identified as EA H1N1 (*n* = 165), pdm/09 H1N1 (*n* = 7), CS H1N1 (*n* = 1), H3N2 (*n* = 4), and H9N2 (*n* = 2) viruses (SI Appendix, Table S1), indicating that EA H1N1 is the predominant subtype virus circulating in pig populations in China. Among them, only EA H1N1 virus was isolated every year, while other SIVs such as CS H1N1 and H3N2 were only found in certain years. Seven pdm/09 H1N1 viruses were only found in 2011, indicating that pdm/09 H1N1 virus was not maintained in pigs even though it was generated from pigs (2). All 43 viruses isolated from diseased pigs were of EA H1N1 subtype. It is noted that the mean virus isolation rates from diseased pigs increased annually from 1.40% in 2011 to 8.21% in 2018, with a sharp increase from 2014 (SI Appendix, Fig. S2), indicating that EA H1N1 viruses are a growing problem in pig farms.
To understand the phylogenetic evolution of the prevailing EA H1N1 viruses, a total of 77 viruses were selected for full genome sequencing on the basis of isolation time and location, with at least one strain sequenced per province (SI Appendix, Table S2). The whole genomes of 77 viruses were analyzed along with all available EA H1N1 sequences from both swine and human viruses in mainland China from 2011 to 2018. Based on the unified swine H1 HA nomenclature system (24), the HA genes of all the EA H1N1 viruses isolated in the study belonged to clade 1C.2.3 (SI Appendix, Fig.S3). Viruses isolated during 2011-2013 had relative short evolutionary branches, However, long branches leading to several lineages were found in viruses isolated after 2013 (Fig. 1A). NA genes had a similar genetic evolution pattern (SI Appendix, Fig. S3). Notably, also after 2013, the six internal genes exhibited distinct diversity with multiple origins from original EA, pdm/09, Avian, and TR lineages (SI Appendix, Fig. S3). These results suggest that the genomes of EA H1N1 SIVs have undergone increased diversity since 2013.
Genotype 4 Reassortant EA H1N1 Viruses Have Been Predominant Since 2016. To show viral evolution, we conducted molecular clock phylogenic analysis and genotype characterization (Fig. 2A and SI Appendix, Fig. S4). Based on lineage classification, six genotypes of G1 ~ G6 were found in EA H1N1 viruses from 2011 to 2018 (Fig. 2B). The virus with all eight genes from the "pure" EA H1N1 lineage was designated as G1. G1 viruses were predominately circulating in both southern and northern China from 2011 to 2013 (SI Appendix, Fig. S5). However, prototypical EA H1N1 viruses had largely disappeared since 2014 (Fig. 2B, and SI Appendix, Fig. S5). G2, G3 and G6 reassortant EA viruses appeared transiently during 2011-2015. In 2013, two triple reassortant G4 and G5 viruses emerged in southern China (SI Appendix, Fig. S5). G5 virus

possess the HA, NA and matrix (M) genes from the original EA H1N1 lineage, the viral ribonucleoprotein (vRNP) genes from the pdm/09 lineage, and the nonstructural (NS) gene from the TR lineage. G5 virus was detected continuously from 2013 to 2017, but it declined since 2015 and was not found in 2018 (Fig. 2B). Similar to the G5, G4 was also a triple reassortant except its M gene was derived from pdm/09 lineage. G4 virus showed a sharp increase since 2016, and is the predominant genotype in circulation in pigs detected across at least 10 provinces (Fig. 2B, and SI Appendix, Fig. S5).

To assess the zoonotic potential of the G4 reassortant EA viruses, four representative G4 viruses (A/swine/Shandong/1207/2016 [SW/SD/1207/16], A/swine/Hebei/0116/2017 [SW/HB/0116/17], A/swine/Henan/SN13/2018 [SW/HN/SN13/18], and A/swine/Jiangsu/J004/2018 [SW/JS/J004/18]) were selected for further biological characterization. Two G1 strains (A/swine/Henan/08/2011 [SW/HN/08/11], and A/swine/Hebei/T37/2013 [SW/HB/T37/13]) and a pdm/09 H1N1 virus A/California/04/09 (CA04) were also selected for comparison.

G4 EA H1N1 Viruses Preferentially Bind Human-like SAα2,6Gal Receptor. The binding preference of HA to host SAα2,6Gal receptor is a critical determinant for cross-species transmission of influenza A viruses to humans (25, 26). We determined the binding affinity of EA H1N1 viruses to SAα2,3Gal and SAα2,6Gal sialylglycopolymers. Like pdm/09 virus CA04, all the four G4 EA H1N1 viruses as well as the two G1 viruses bound SAα2,6Gal receptors with high affinity but bound poorly to SAα2,3Gal receptors (SI Appendix, Fig. S6A). Furthermore, all the EA H1N1 viruses were found to bind to human tracheal epithelial lining to a similar extent as CA04 pdm/09 H1N1 virus, but control avian H5N1 virus showed no binding (SI Appendix, Fig. S6B). Thus, these results demonstrate that G4 EA H1N1 viruses preferentially bind human-like SAα2,6Gal receptor, a key prerequisite for infecting human cells.

G4 EA H1N1 Viruses Replicate Efficiently in Human Airway Epithelial Cells. Next, we assessed the replication of G4 viruses in normal human bronchial epithelial (NHBE) cells and alveolar epithelial (A549) cells, the major target cells in human influenza virus infection. In NHBE cells, G4 and pdm/09 viruses replicated to similar levels at each time point, and both of them produced more viable progeny viruses during 36 ~ 60 h post infection (pi) than did G1 viruses ($P < 0.05$ or $P < 0.01$, ANOVA) (SI Appendix, Fig. S7A).

Infection of human A549 cells with G4 viruses gave similar progeny results. G4 and pdm/09 viruses produced more infectious virus than that of G1 viruses from 24 h to 60 h pi ($P < 0.05$ or $P < 0.01$, ANOVA), reaching highest titers of $10^{7.75}$ and $10^{7.5}$ median tissue culture infective dose ($TCID_{50}$)/ml, respectively. In contrast, G1 viruses showed peak titers of $10^{6.5}$ $TCID_{50}$/ml (SI Appendix, Fig. S7B). Collectively, G4 EA reassortant viruses replicate efficiently in human airway epithelial cells, similar to that of pdm/09 H1N1 virus.

G4 EA H1N1 Viruses Exhibit Efficient Infectivity and Transmissibility in Ferrets. Ferrets have been widely used as an experimental model to study human infection and transmission of influenza virus (27). Here, three ferrets were infected intranasally (i.n.) with each virus at a dose of 10^6 $TCID_{50}$ in a 1.0 ml volume. We found that G1 EA or pdm/09 viruses caused only mild clinical signs (SI Appendix, Table S4). Infection with G4 EA viruses, on the other hand, resulted in more severe clinical symptoms such as pyrexia, sneezing, wheezing, and coughing, with higher mean maximum weight loss ranging from 7.3% to 9.8% (SI Appendix, Table S4). Post-mortem and histopathology revealed that G4 virus-infected lungs had more severe lesions than G1 or pdm/09 virus-infected lungs, with pronounced multifocal areas of consolidation, hemorrhage, and edema and exhibited more severe peribronchiolitis and bronchopneumonia (SI Appendix, Fig. S8A). All four G4 viruses replicated to higher titers in the upper respiratory tract (nasal turbinate and trachea) of ferrets, which were similar to pdm/09 viruses and significantly higher than the two G1 viruses ($P < 0.05$ or $P < 0.01$, ANOVA), while no infectious virus was recovered from extrapulmonary tissues (SI Appendix, Fig. S8B). Overall, current G4 reassortant EA H1N1 viruses showed increased replication and pathogenicity in ferrets, indicating that G4 viruses are likely to cause more severe infection than G1 EA H1N1 viruses in humans.

Efficient human-to-human transmission is a critical feature of pandemic influenza viruses. To assess the transmissibility of G4 viruses, we performed direct-contact (DC) and respiratory-droplet (RD) virus transmission experiments on ferrets. The results showed that CA04 pdm/09

H1N1 virus efficiently transmitted to all ferrets by DC and RD (Fig. 3). All four G4 viruses were transmitted to all DC animals. Importantly, three of four G4 viruses, SW/SD/1207/16, SW/HN/SN13/18 and SW/JS/J004/18, were transmitted to all three RD-ferrets. The remaining G4 virus SW/HB/0116/17 was transmitted to 1 of 3 RD-ferrets (Fig. 3). By contrast, with G1 viruses, neither virus transmission in DC or RD groups (Fig. 3), nor seroconversion at 14 dpi was detected in all recipient ferrets (SI Appendix, Table S4). Thus, there is compelling evidence to show that current predominant G4 reassortant EA H1N1 viruses are highly transmissible by DC and RD among ferrets, suggesting their capacity to readily infect humans.

G4 EA H1N1 Viruses Exhibit Low Antigenic Cross-Reactivity with Human Influenza Vaccine Strains. Pre-existing immunity can protect humans from related influenza viruses, but antigenic drift can decrease such protection in a population. Antigenic change is mainly due to variation of the HA gene. In this study, we found that the HA gene of EA H1N1 viruses isolated after 2013 including G4 viruses formed an independent phylogenetic group. To determine the extent of antigenic drift of G4 viruses, 14 representative EA H1N1 viruses (ten G4 and four G1 viruses) were selected based on their HA phylogenic topology for antigenicity test.

A panel of ferret sera were used for hemagglutination inhibition (HI) assays, including sera against pdm/09 H1N1 virus A/Michigan/45/2015 from the current H1N1 human influenza vaccine lineage, G1 EA H1N1 viruses SW/HN/08/11 and SW/HB/T37/13, and G4 EA H1N1 viruses SW/SD/1207/16 and SW/HN/SN13/18. On the basis of reactivity levels in HI assays, EA viruses could be classified into antigenic group A and B (Fig. 1B and SI Appendix, Table S5). The original G1 EA viruses were in antigenic group A, while G4 viruses belonged to antigenic group B. The cross-reactive titers between the two antigenic groups were 8-64 fold lower than those of homologous reactions. Antisera against pdm/09 H1N1 viruses (A/Michigan/45/2015) cross-reacted with antigenic group A viruses (titers 1:160-320) but reacted poorly with antigenic group B strains (titers < 40) (SI Appendix, Table S5). Further analysis showed that several amino acid differences in the HA antigenic sites among G1 and G4 EA H1N1 viruses, including 135 (H1 numbering) and 222 in Ca2, and 185 in Sb (SI Appendix, Table S6). However, which amino acid contributes to the observed antigenic change need to be determined in future. Thus, predominant G4 reassortant EA H1N1 viruses are antigenically distinct from the earlier G1 EA and pdm/09 H1N1 viruses.

To assess cross-protection of human seasonal influenza vaccine against G4 EA viruses, HI assays were performed with 20 serum samples collected from 4-year-old children vaccinated with trivalent vaccines (pdm/09 H1N1+ H3N2 + B/Victoria). All serum samples were reactive (titers ≥ 1:40) to pdm/09 H1N1 and H3N2 viruses (Fig. 1C). However, none of the serum samples cross-reacted with the G4 or even the G1 EA H1N1 virus (Fig. 1C). Collectively, predominant G4 reassortant EA viruses are antigenically distinct from current human influenza vaccine strains, indicating that pre-existing immunity derived from the present human seasonal influenza vaccines cannot provide protection against G4 viruses.

G4 EA H1N1 Viruses Showed Increased Infection Rate in Humans Evidenced by Seroprevalence. To determine whether the G4 reassortant EA H1N1 virus can infect across species from swine to humans, serological surveillance was conducted to detect prevalence of virus exposure in swine production workers. From 2016 to 2018, a total of 338 serum samples were collected from swine workers in 15 farms. Serum samples (n = 230) from ordinary households were also collected as a population comparison group. G4 EA virus SW/SD/1207/16, which belonged to antigenic group B, was used as virus antigen in HI assays. To control for interference of H1N1 antibody against pdm/09 and earlier G1 EA viruses, pdm/09 virus A/Michigan/45/2015 and G1 EA virus SW/HN/08/11 were included as viral antigens. Disconcertingly, 10.4% (35/338) of swine workers and 4.4% (10/230) of general population were positive (titers ≥ 1:40) for G4 virus SW/SD/1207/16. In the multivariable analysis, after adjusting for confounders, swine workers had an increased odds ratio [aOR = 2.60, 95% CI (1.24-5.45), P = 0.012] compared with the general population group. After controlling for possible cross-reactivity with pdm/09 virus the odds ratio remained elevated [aOR = 2.25, 95% CI (1.05-4.83), P = 0.038] (Table 1). It is noted that swine workers in 4 of 15 farms were more than 15% seropositive against G4 virus SW/SD/1207/16 (SI Appendix, Table S7). In contrast, 6.5%

(22/338) of swine workers and 2.2% (5/230) of the general population were positive for G1 virus SW/HN/08/11, with no statistically significant difference (P = 0.068) between the two groups after controlling for possible cross-reactivity with pdm/09 virus (Table 1). In addition, swine workers group and general population were 38.8% (131/338) and 31.7% (73/230) seropositive respectively for pdm/09 H1N1 virus A/Michigan/45/2015 (P = 0.082). These results demonstrate that the newly prevalent G4 reassortant EA H1N1 viruses in pigs are more infectious to humans than their predecessors of G1 viruses.

We further investigated the association of sera collection year, age or gender with seroprevalence of G4 reassortant EA H1N1 virus. In the swine workers group, the seropositive rates of G4 EA H1N1 virus were 6.7%, 11.7%, and 11.7% from 2016, 2017 and 2018, respectively (SI Appendix, Table S8). It is noteworthy that participants 18-35 years of age had 20.5% (9/44) seropositive rate against G4 EA H1N1 virus SW/SD/1207/16, which had an increased odds ratio [OR=3.2, 95% CI (1.3-7.7), P < 0.01] compared with other age groups (SI Appendix, Table S8). For gender factor, there were no statistically significant difference in the seroprevalence of any virus tested according to sex were observed (P > 0.05). These results indicate that young adult swine workers carry a higher risk of infection with G4 reassortant EA H1N1 virus.

Discussion

Pigs can independently facilitate the genesis of a human pandemic IAV strain (2, 7). Thus, continual systematic monitoring and assessing potential risks of emerging influenza viruses in pigs are necessary for early warning of future pandemics (28). In this study, based on extensive influenza A virus surveillance in pigs from 2011 to 2018, we identified and characterized a predominant triple reassortant SIVs (G4 genotype) derived from the reassortment of previous EA, pdm/09, and TR viruses. G4 H1N1 viruses are able to bind human-like SAα2,6Gal-linked receptor, replicate well in human airway epithelial cells, and transmit by aerosol among ferrets; they are antigenically distinct from pdm/09 H1N1 viruses. Of concern is that serological surveillance indicates the G4 reassortant EA H1N1 virus exhibits elevated infectivity in humans, especially for swine-exposed younger adults, which increases the opportunity for virus adaptation in humans.

EA H1N1 virus has been circulating in pigs in Europe and Asia for decades (29-31). In 2001, EA virus was found in Hong Kong and gradually became dominant in mainland China (9-11). Here, we also found that the “pure” EA H1N1 viruses of G1 were predominant in swine population from 2011 to 2013. However, since 2014, G4 and G5 reassortant EA H1N1 viruses gradually replaced the prototypical EA H1N1 viruses, and currently G4 viruses are the single predominant genotype circulating in China. Surveillance from farmed pigs with respiratory symptoms had shown that its isolation rate increased sharply after 2014, and increased year by year (SI Appendix, Fig. S2). Others have also reported on the infection of G4-like reassortant EA H1N1 viruses in farmed pigs (16, 18). A typical feature of G4 virus is that vRNP and M genes originate from pdm/09 virus and NS gene is from TR virus, indicating that this gene constellation has a distinct competitive advantage in pigs. All these evidences indicate that G4 EA H1N1 virus is a growing problem in pig farms, and the widespread circulation of G4 viruses in pigs inevitably increases their exposure to humans. So far, a total of 5 human cases of EA-like swine influenza virus infection had been reported in China (21-23, 32, 33). The first three cases were children under 3 years old, but the latest two cases reported in 2016 and 2019 were of a 46 and a 9 year old, respectively. Genetic analysis indicated that the latter two cases were caused by G4-like EA H1N1 virus. Epidemiological survey found that the two patients had neighbors who reared pigs, suggesting that G4 EA virus could transmit from swine to human, and lead to severe infection and even death (22, 23). Thus, it is necessary to strengthen the surveillance effort of G4 EA viruses among swine and human populations.

Pandemic occurs when an IAV with novel HA surface antigen becomes readily able to undertake human to human transmission. G4 genotype of reassortant SIVs, identified in the present study, possess all the essential hallmarks of a candidate pandemic virus. G4 virus has different

antigenicity from current human influenza viruses. Similar to pdm/09 virus, G4 virus preferentially binds human-like SAα2,6Gal receptor and effectively transmits in the ferret model. The G4 virus also shows increased pathogenicity based on the present ferret study and other reports in mice (18, 34, 35). A limited serological investigation found that general population who had little opportunity to contact pigs, lacked antibodies against G4 virus, but swine-exposed adult population showed elevated seroprevalence (10.4%, 35/338), which further supports our hypothesis of G4 virus transmission from pigs to human. It is of concern that human infection of G4 virus will further human adaptation and increase the risk of a human pandemic.
In summary, G4 EA H1N1 viruses possess all the essential hallmarks of highly adapted to infect humans. Controlling the prevailing G4 EA H1N1 viruses in pigs and close monitoring of swine working populations should be promptly implemented.

Materials and Methods

All animal research was approved by the Beijing Association for Science and Technology (approval ID SYXK, Beijing, 2007–0023) and performed in compliance with the Beijing Laboratory Animal Welfare and Ethics guidelines, as issued by the Beijing Administration Committee of Laboratory Animals, and in accordance with the China Agricultural University (CAU) Institutional Animal Care and Use Committee guidelines (ID:SKLAB-B-2010-003) approved by the Animal Welfare Committee of CAU. All experiments with live viruses were performed in biosafety level 2 facilities in CAU.
The detailed methods for this study are provided in SI Appendix.

Data availability

The sequences generated in this study have been deposited in the GenBank database (accession nos. are listed in SI Appendix, Table S3).

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Conflict of Interest

The authors declare no conflict of interest.

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Figures and Tables

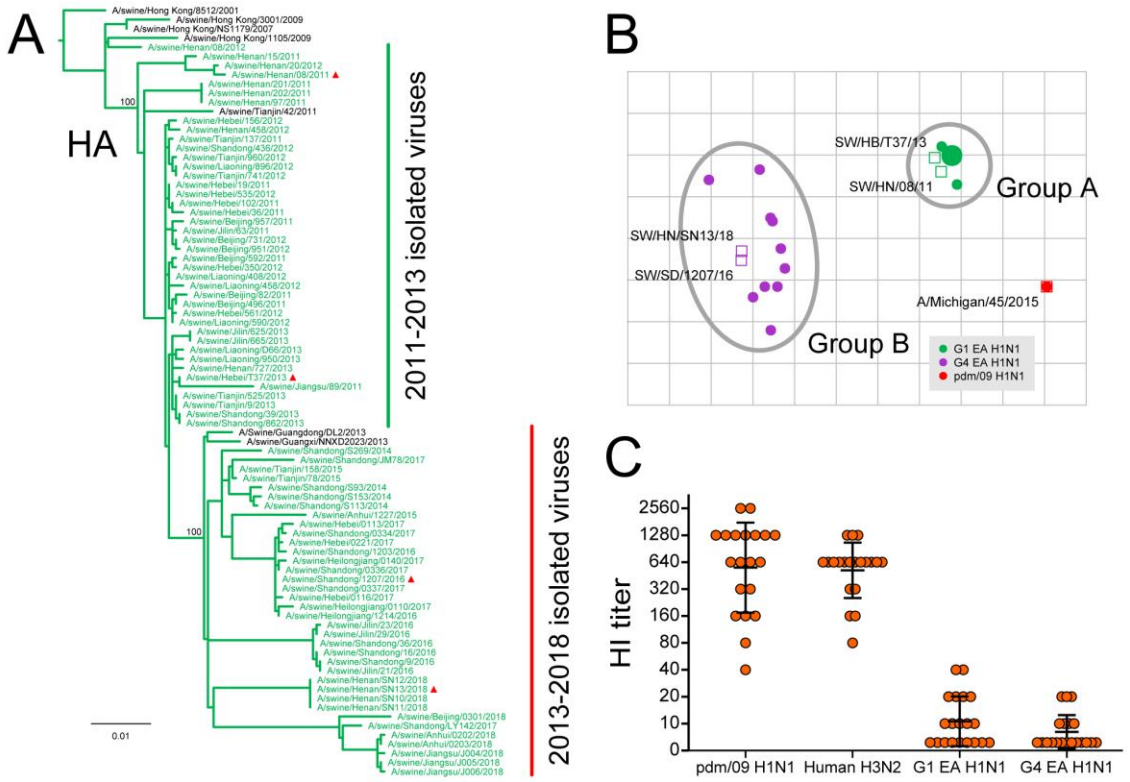

Figure 1. Phylogenetic relationship of HA gene and antigenic characterization of EA H1N1 SIVs in China from 2011 to 2018. (A) Phylogenetic tree of HA gene. Phylogenetic tree was estimated using genetic distances calculated by maximum likelihood under the GTRGAMMA + I model. SIVs isolated in this study were green, sequences of viruses with name in black were downloaded from databases. Scale bar is in units of nucleotide substitutions per site. Node labels represent bootstrap values. Viruses labeled with a red triangle were selected for antiserum generation. A full detailed HA gene tree with the consistent topology is shown in SI Appendix, Fig. S3. (B) Antigenic map based on the HI assay data. Open squares and filled circles represent the positions of antisera and viruses, respectively. Clusters were identified by a *k*-means clustering algorithm. Strains belonging to the same antigenic cluster are encircled in an oval. The vertical and horizontal axes both represent antigenic distance. The spacing between grid lines is 1 unit of antigenic distance, corresponding to a twofold dilution of antiserum in the HI assay. Details of the HI assay data are shown in SI Appendix, Table S5. (C) Antigenic analysis of EA H1N1 and human influenza vaccine strains. Twenty serum samples, collected from 4-year-old children vaccinated with trivalent vaccines [A/Michigan/45/2015 (pdm/09 H1N1) + A/Singapore/INFIMH-16-0019/2016 (H3N2) + B/Colorado/06/2017 (B/Victoria)], were subjected to HI assays. pdm/09 H1N1 virus A/Michigan/45/2015, human H3N2 virus A/Singapore/INFIMH-16-0019/2016, G1 EA H1N1 virus SW/HN/08/11, and G4 EA H1N1 virus SW/SD/1207/16 were used as antigens. HI titers ≥ 40 were considered positive.

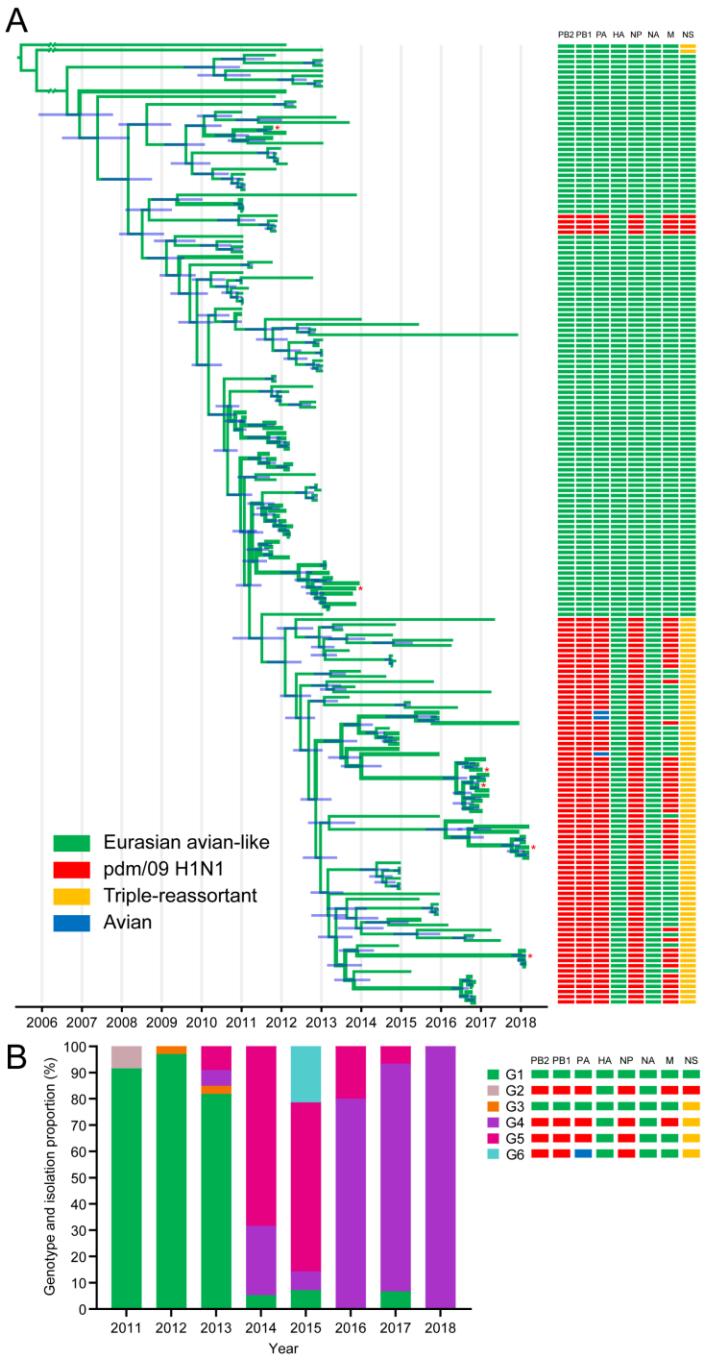

Figure 2. Phylogenetic analysis of EA H1N1 SIVs in China from 2011 to 2018. (A) Phylogeny and divergence time of the HA gene and genotype evolution of EA H1N1 SIVs. The phylogenetic tree of HA gene was generated by Bayesian Markov Chain Monte Carlo framework, using the GTR substitution model with gamma-distributed among site rate heterogeneity and a 'strict molecular clock' model. Colored boxes show the lineage classification of each gene segment of EA H1N1 viruses. Purple node bars represent 95% credible intervals of lineage divergence times. A detailed phylogenetic tree including sequence names is shown in SI Appendix, Fig. S4. (B) Diversity of genotypes of EA viruses isolated from swine in China, 2011–2018.

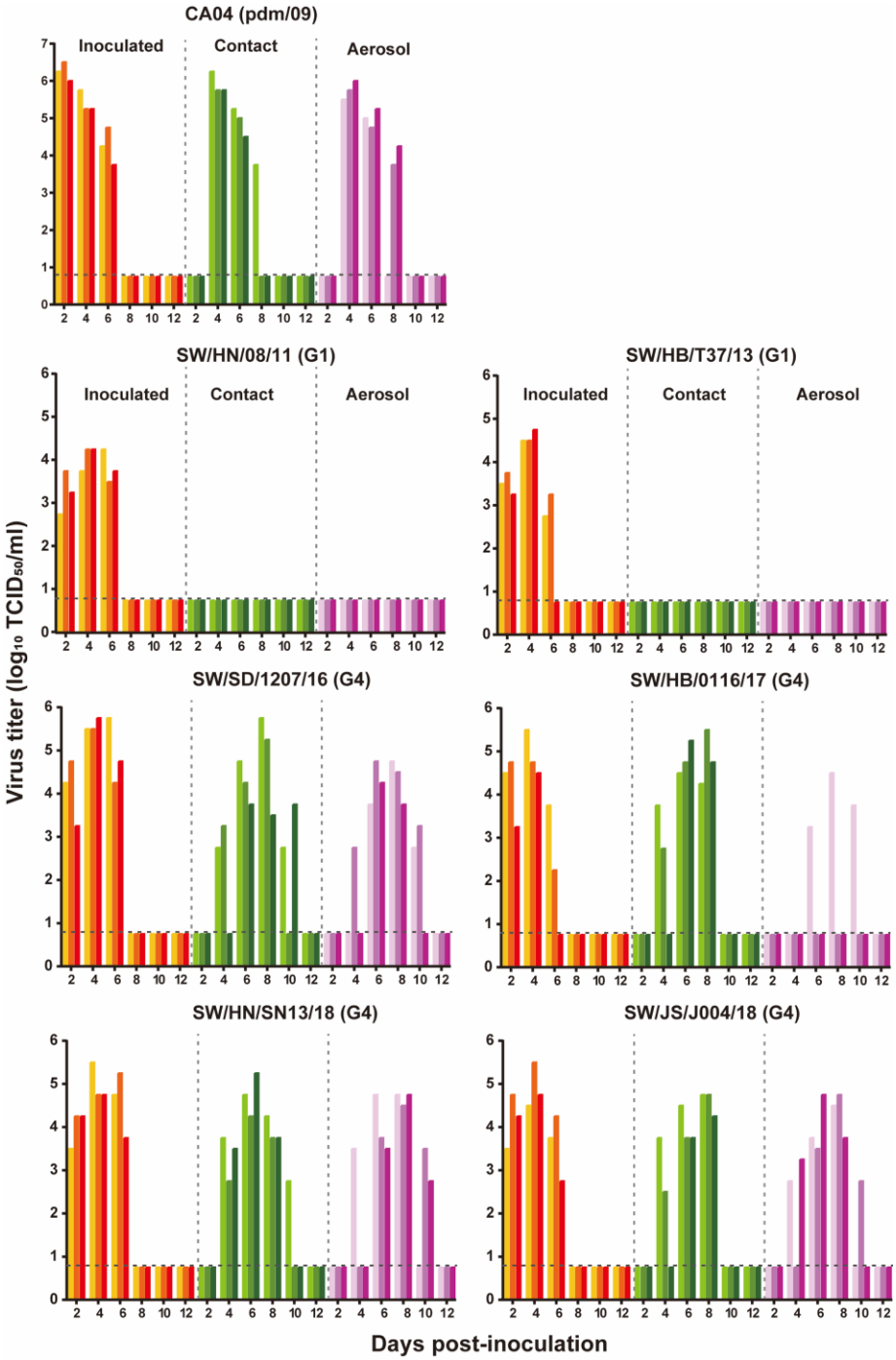

Figure 3. Horizontal transmission of EA H1N1 viruses between ferrets. Groups of three ferrets were inoculated i.n. with 10^6 $TCID_{50}$ of indicated viruses. The next day, infected animals were individually cohoused with an uninfected DC ferret; an uninfected RD contact animal was also housed in a wire frame cage adjacent to the infected ferret. Nasal washes for virus shedding detection were collected every other day from all animals from day 2 of the initial infection. Each color bar represents the virus titer of an individual animal. Dashed lines indicate the lower limit of virus detection.

Table 1. Seropositive rate of influenza virus in swine workers (SW) and common household population (CHP)

	Univariable analysis					Multivariable regression analysis		
Strain (Genotype)	SW (*n* = 338) No. Pos	% Pos	CHP (*n* = 230) No. Pos	% Pos	*P* value Chi^2	aOR (95% CI) SW versus CHP	*P* value	Model covariates
SW/HN/08/11 (G1 EA H1N1)	22	6.5	5	2.2	**0.017***	3.02 (1.11-8.19)	**0.030**	
Controls for possible cross-reactivity with pdm/09 H1N1						2.60 (0.93-7.23)	0.068	Gender, age group, collection year
SW/SD/1207/16 (G4 EA H1N1)	35	10.4	10	4.4	**0.009**	2.60 (1.24-5.45)	**0.012**	
Controls for possible cross-reactivity with pdm/09 H1N1						2.25 (1.05-4.83)	**0.038**	
A/Michigan/45/2015 (pdm/09 H1N1)	131	38.8	73	31.7	0.087	1.38 (0.96-1.99)	0.082	

*Boldface indicates a statistically significant difference ($P < 0.05$).

Supplementary Information for

Prevalent Eurasian avian-like H1N1 swine influenza virus with 2009 pandemic viral genes facilitating human infection

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This PDF file includes:

Methods

Virus Isolation and Identification. From January 2011 to April 2018, influenza surveillance in pigs was carried out in 10 provinces of China (Anhui, Beijing, Hebei, Heilongjiang, Henan, Jiangsu, Jilin, Liaoning, Shandong, and Tianjin). A total of 29,918 nasal swabs were collected from slaughtered pigs at abattoirs and a total of 1,016 nasal swabs or lung tissues were collected from farmed pigs with signs of respiratory disease. For virus isolation, nasal swabs were taken and placed in 1.0 ml transmission medium [50% (vol/vol) glycerol in PBS] containing antibiotics, as previously described (1). Lungs were homogenized in PBS containing antibiotics. All samples were individually inoculated into both embryonated chicken eggs and MDCK cells for virus isolation. Allantoic fluid and culture supernatants were harvested and tested for HA activity. HI assays were performed using antisera against the H1, H3, H5 and H9 subtypes of influenza viruses. Viral RNA was extracted by using the QIAamp Viral RNA Minikit (Qiagen), and standard RT-PCR was performed with segment-specific primers, using the One Step RT-PCR Kit (Qiagen). The HA and NA lineage of the viruses were determined by direct sequencing of the PCR products. Samples positive for SIVs were stored as stock at −80°C until use.

Genomic Sequencing. Based on HA and NA gene sequences, a total of 77 viruses belonging to EA H1N1 lineage were selected for full genome sequencing on the basis of isolation time and location. At least one strain was sequenced per province. Whole genomic DNA was sequenced by Sanger sequencing on an ABI 3730 genetic analyzer (Applied Bio-systems). All nucleotide sequences of segments of the 77 influenza virus isolates detected in this study have been deposited in GenBank (Table S3).

Phylogenetic Analyses. RAxML was used to construct maximum likelihood phylogenies for each segment (2), via CIPRES Science Gateway (3). 1,000 bootstrap replicates were run, and GTR-GAMMA + I was used as the nucleotide substitution model. Each gene was classified to an evolutionary lineage and/or phylogenetic clade (4, 5). Phylogenetic relationship of HA gene sequenced of SIVs was inferred with BEAST version 1.10.4 (6), using a molecular clock that places a timescale on virus evolution. Phylogeny was estimated within a Bayesian Markov Chain Monte Carlo (MCMC) framework, using the GTR substitution model with gamma-distributed among site rate heterogeneity and a 'strict clock' model. For the analysis employing Bayesian MCMC sampling, a chain length of at least 40 million steps was used with a 10% 'burn-in' removed. At least two independent runs of the chain were performed and compared to ensure adequate sampling.

Receptor Binding Assays. α-2,6 glycans (6'SLN: Neu5Acα2-6Galβ1-4GlcNAcβ-SpNH-LC-LC-biotin) and α-2,3 glycans (3'SLN: Neu5Acα2-3Galβ1-4GlcNAcβ-SpNH-LC-LC-biotin) were kindly provided by the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA). Receptor-binding specificity was determined by a solid-phase direct binding assay as previously described (7). Briefly, serial dilutions (0.16 μg/ml, 0.32 μg/ml, 0.63 μg/ml, 1.25 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml, and 20 μg/ml) of biotinylated glycans 3'SLN and 6'SLN were prepared in PBS, and 100μl was added to the wells of the 96-well microtiter plates (Polystyrene Universal-Bind Microplate; Corning) and allowed to attach overnight at 4°C. The plates were then irradiated with 254 nm ultraviolet light for 2 min. After removal of the glycopolymer solution, the plates were blocked with 0.1 ml of PBS containing 2% bovine serum albumin (BSA) at room temperature for 1 h. After washing with ice-cold PBS containing 0.1% Tween 20 (PBST), the plates were incubated in a solution containing influenza virus (64 HA units in PBST) at 4°C for 12 h. After washing with PBST, chicken antisera against SW/SD/1207/16 (H1N1), CA04(H1N1) or A/Anhui/01/2005 (AH/05-H5N1) virus was added to each well and the plates were incubated at 4°C for 2 h. The wells were then washed with ice-cold PBST and incubated with HRP-linked goat-anti-chicken antibody (Sigma-Aldrich) for 2 h at 4°C. After washing with ice-cold PBST, the plates were incubated with O-phenylenediamine in PBS containing 0.01% H_2O_2 for 10 min at room temperature. The reaction was stopped with 0.05 ml of 1 M H_2SO_4 and the absorbance was determined at 450 nm.

Virus Binding to Human Tracheal Tissues. Paraffin-embedded 5 μm sections of normal human tracheal and lung tissues were deparaffinized in xylene and rehydrated by alcohol. Sections were then blocked with 4% BSA in PBS, followed by virus incubation (at 64 HA units in PBS per section)

at 4°C overnight. After four washes in ice-cold PBS, the sections were incubated with mouse monoclonal antibody specific for influenza nucleoprotein (NP; Abcam, Cambridge, United Kingdom) for 3 h at 4°C. Antibody binding was detected by FITC-labeled goat anti-mouse IgG (Abcam) incubated for 2 h at room temperature. The samples were examined by confocal laser scanning microscopy (Leica Microsystems).

Infection of Human Airway Epithelial Cells. Primary NHBE cells were obtained from ATCC. Cells of passage 2 were grown on membrane supports (12-mm Transwell-Clear Insert, Corning) at the air-liquid interface in serum-free and hormone- and growth factor-supplemented medium as described (8). Fully differentiated 4 to 8-week-old cultures were used for all experiments. The apical surfaces of the cultures were washed 5 times to remove accumulated mucins before inoculation with virus at a multiplicity of infection (MOI) of 0.001. The inoculum was removed after 1 h of incubation at 33°C. No trypsin was added to the cultures because previous studies using similar cultures demonstrated efficient proteolytic activation of influenza viruses by endogenous proteases (9, 10). Supernatants were sampled at 12, 24, 36, 48, 60 and 72 hpi, and titrated by inoculating MDCK cells.

A549 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Multistep replication kinetics were determined by infecting A549 cells at a MOI of 0.001. After 1 h of incubation at 37°C, the cells were washed twice and further incubated in serum-free DMEM containing 0.5 µg/ml TPCK-trypsin. Supernatants were sampled at 12, 24, 36, 48, 60, and 72 hpi, and titrated by inoculating MDCK cells. Each experiment was performed in triplicate.

Ferret Pathogenesis and Transmission Experiments. All 6-month-old male Angora ferrets (Angora LTD), seronegative for currently circulating influenza viruses (H1, H3, H5, H7, and H9). Groups of three ferrets were each anesthetized with ketamine (20 mg/kg) and xylazine (1 mg/kg) and i.n. inoculated with 10^6 $TCID_{50}$ of test virus in a 1-ml volume. The animals were subsequently euthanized on 3 day pi, and nasal turbinate, trachea, lung, spleen, kidney and brain samples were collected for virus titration. Lung tissues were also used for histology.

In the transmission experiment, groups of three animals were anesthetized and inoculated i.n. with 10^6 $TCID_{50}$ of virus. The next day, the three inoculated animals were individually paired by cohousing with a DC ferret; a RD-contact animal was also housed in a wire frame cage adjacent to the infected ferret. The infected and RD-contact ferrets were 5 cm apart. To monitor virus shedding, nasal washes were collected from all animals every other day over 12 days and titrated for virus in MDCK cells. Sera were collected from both inoculated and contacted animals at 14 dpi. Seroconversion was analyzed by HI assay. Clinical signs, temperature and body weight were recorded daily in all ferrets.

Antigenic Analyses. Antigenic characteristics of EA H1N1 viruses were compared using a HI assay with ferret antisera raised against representative viruses. Ferret antisera raised against SW/HN/08/11 (G1), SW/HB/T37/13 (G1), SW/SD/1207/16 (G4) and SW/HN/SN13/18 (G4) viruses were produced in our laboratory, hyperimmune goat serum against pdm/09 H1N1 A/Michigan/45/2015 were provided by Chinese National Influenza Center. HI tests were performed in accordance with WHO guidelines. All HI assays were performed in duplicates. Analysis of antigenic properties was performed using the antigenic cartography methods described previously (11). The antigen cartography of the viruses was constructed using Antigenic Cartography software (http://www.antigenic-cartography.org/). Clusters were identified in the antigenic map by a *k*-means clustering algorithm, using average weighting, and $k = 3$.

HI assays were also used to determine the antigenic cross-reactivity of EA H1N1 viruses with prevailing human influenza vaccine strains. Positive human sera were collected from twenty 4-year-old children at 30 days after two immunizations with influenza trivalent vaccines (A/Michigan/45/2015 + A/Singapore/INFIMH-16-0019/2016 + B/Colorado/06/2017) by Chinese National Influenza Center. HI antigens of A/Michigan/45/2015 (pdm/09 H1N1), A/Singapore/ INFIMH-16-0019/2016 (human seasonal H3N2) were provided by Chinese National Influenza Center. HI tests were performed in accordance with WHO guidelines. All HI assays were performed in duplicates.

Serological Survey of G1, G4 EA and pdm/09 H1N1 Viruses Infection in Humans. From 2016 to 2018, a total of 338 serum samples were collected from swine workers in 15 pig farms. Farms were in two main clusters in Hebei and Shandong province, both regions with high density pig populations. At the same time, 230 blood samples were obtained from ordinary household populations to form the population comparison group. General population samples were frequency-matched to swine workers on age group, geographic region, calendar month of blood sample and gender. HI tests were performed in accordance with WHO guidelines. G1 EA virus SW/HN/08/11, G4 EA virus SW/SD/1207/16, and pdm/09 H1N1 virus A/Michigan/45/2015 were used as antigens. 1% turkey erythrocytes were used in HI assays. Based on the WHO guideline for vaccine evaluation, the HI antibody titers ≥ 40 were considered as positive.

Statistical Methods. Experimental groups were statistically compared by ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

In dichotomous outcome examinations, the Pearson's chi-square test or Fisher's exact test was used to compare differences between groups. In multivariable logistic regression analyses, potential confounding effects of gender, age and collection time were examined. As there was strong evidence that G1 or G4 virus seropositivity in humans were associated with seropositivity to pdm/09 H1N1 ($P < 0.001$), pdm/09 H1N1 was also forced into regression models to account for possible cross-reactivity. The magnitude of association between the variables and seropositivity was expressed as an OR with 95% CI. $P < 0.05$ was considered to indicate a statistically significant difference. Analyses were performed using SPSS software version 19.0 (IBM Corporation, New York, NY, USA).

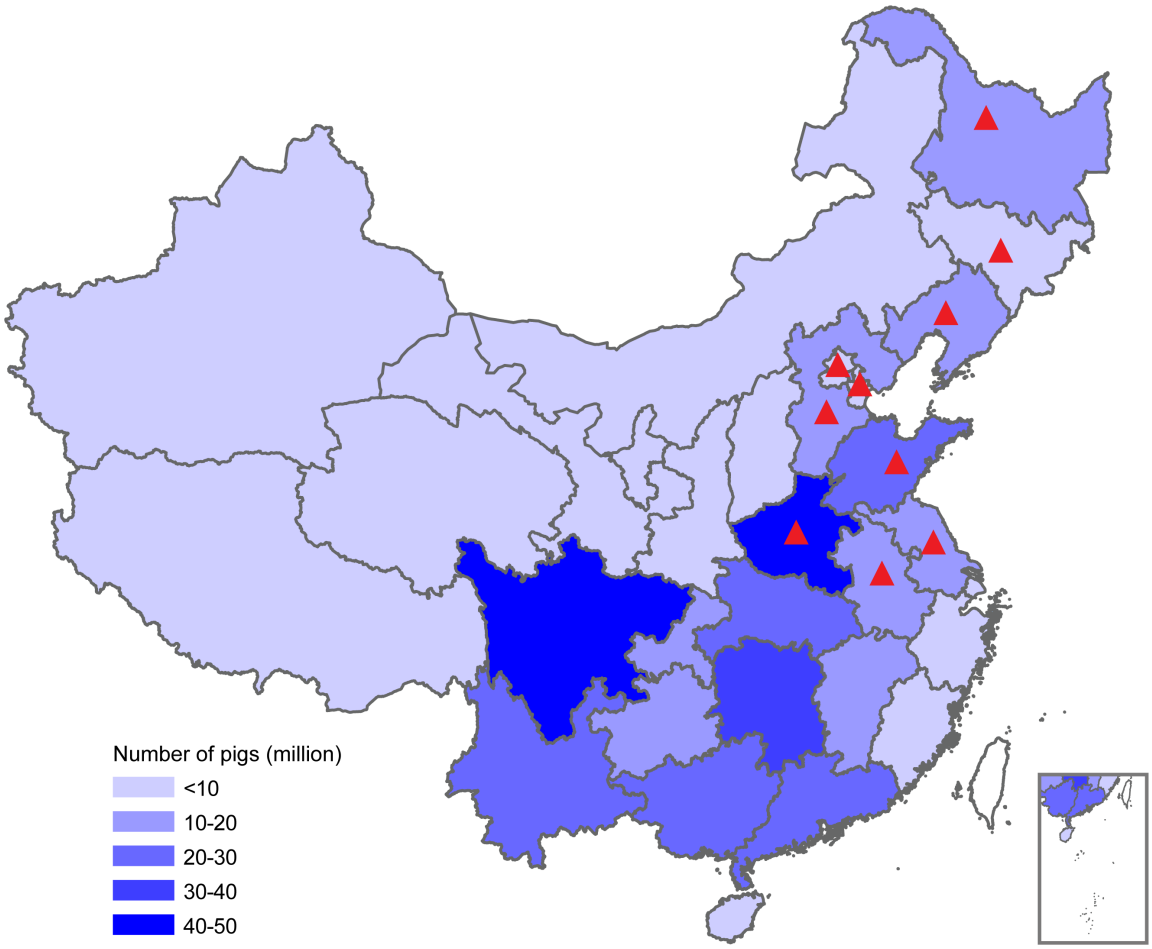


Fig. S1. Map showing pig density in China and geographic location of influenza surveillance in pigs from 2011 to 2018. The number of pigs in each province is the average number from 2014 to 2018; data were from the National Bureau of Statistics of China. Red triangle indicates province where surveillance was conducted.

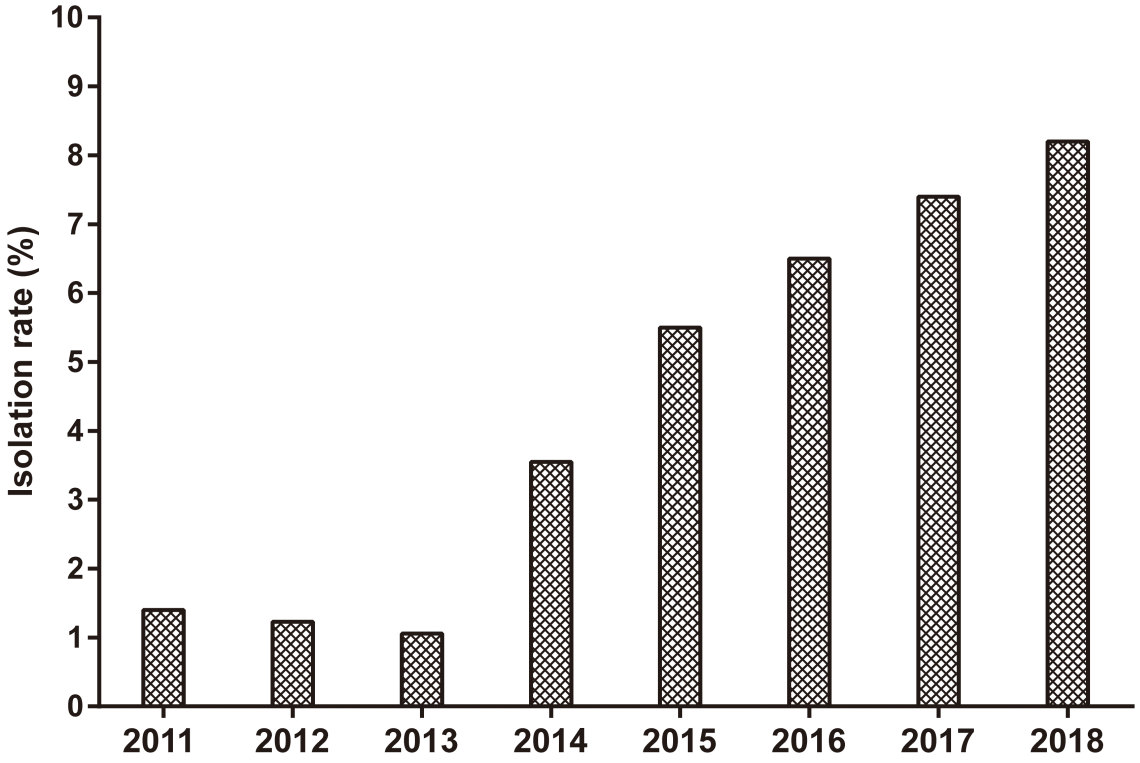
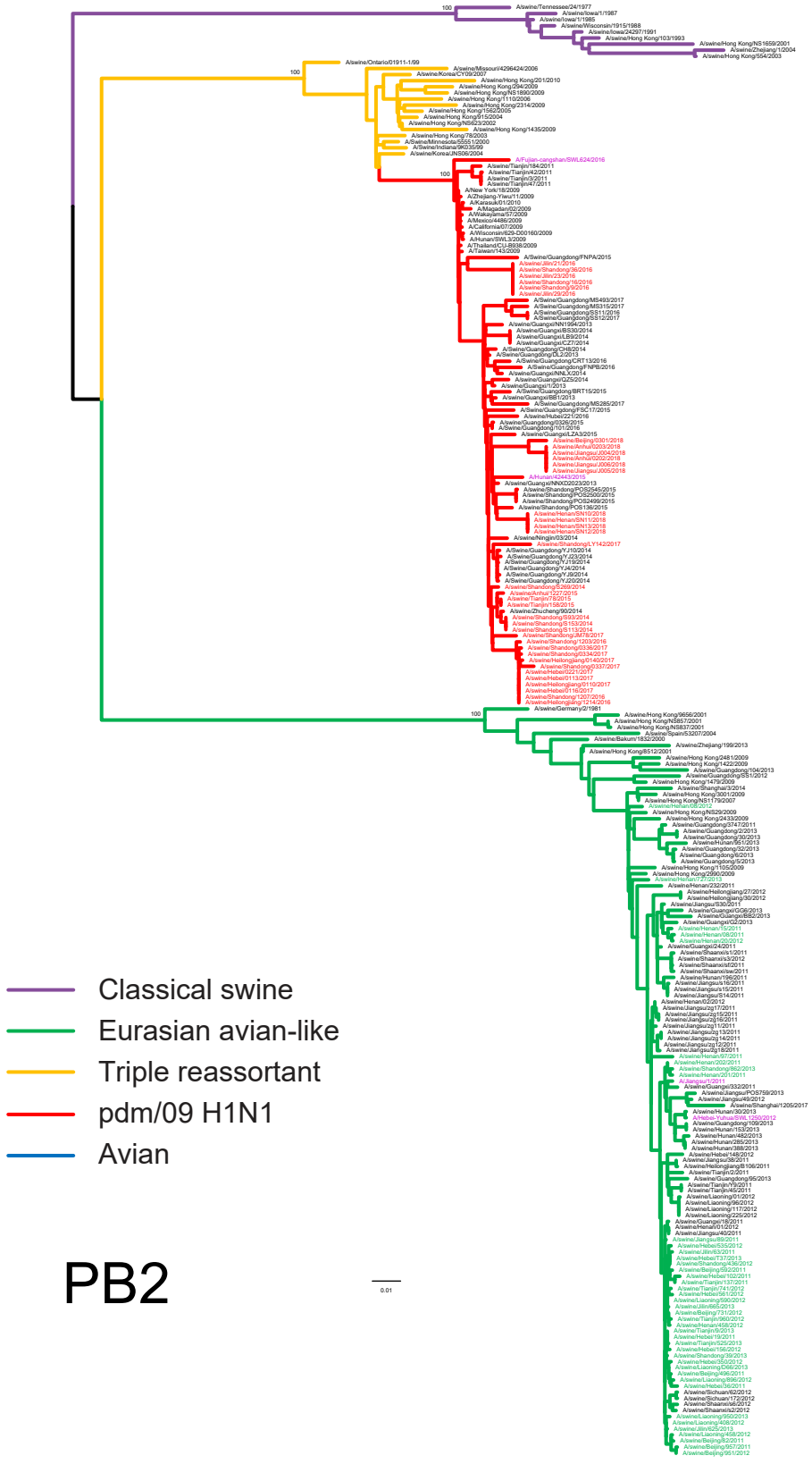


Fig. S2. Isolation rate (%) of SIVs in the period of 2011–2018 from diseased pigs. From 2011 to 2018, number of nasal swabs or lung samples collected from diseased pigs were 143, 162, 94, 141, 128, 93, 121, and 134, respectively.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pdm/09 H1N1
Avian
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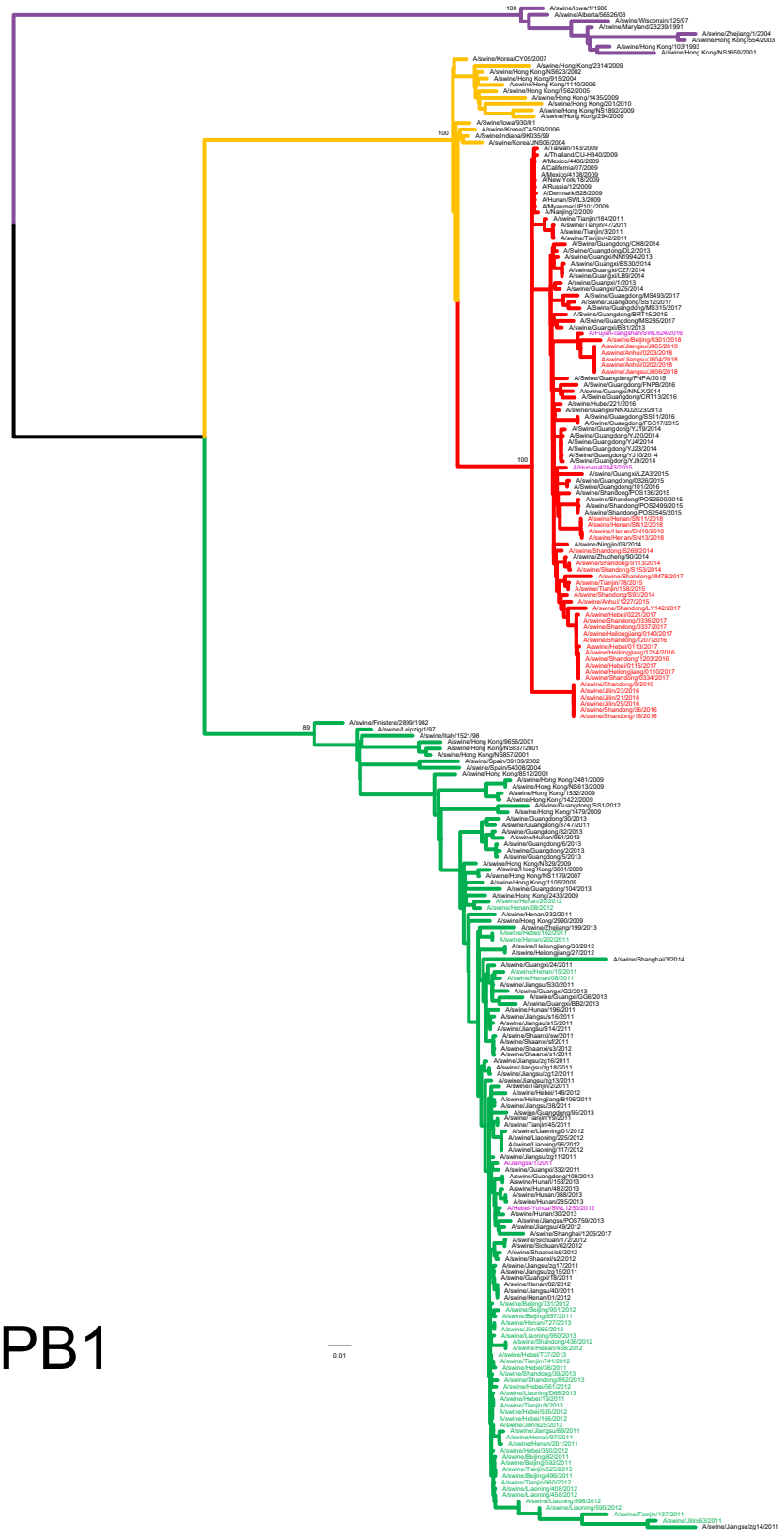
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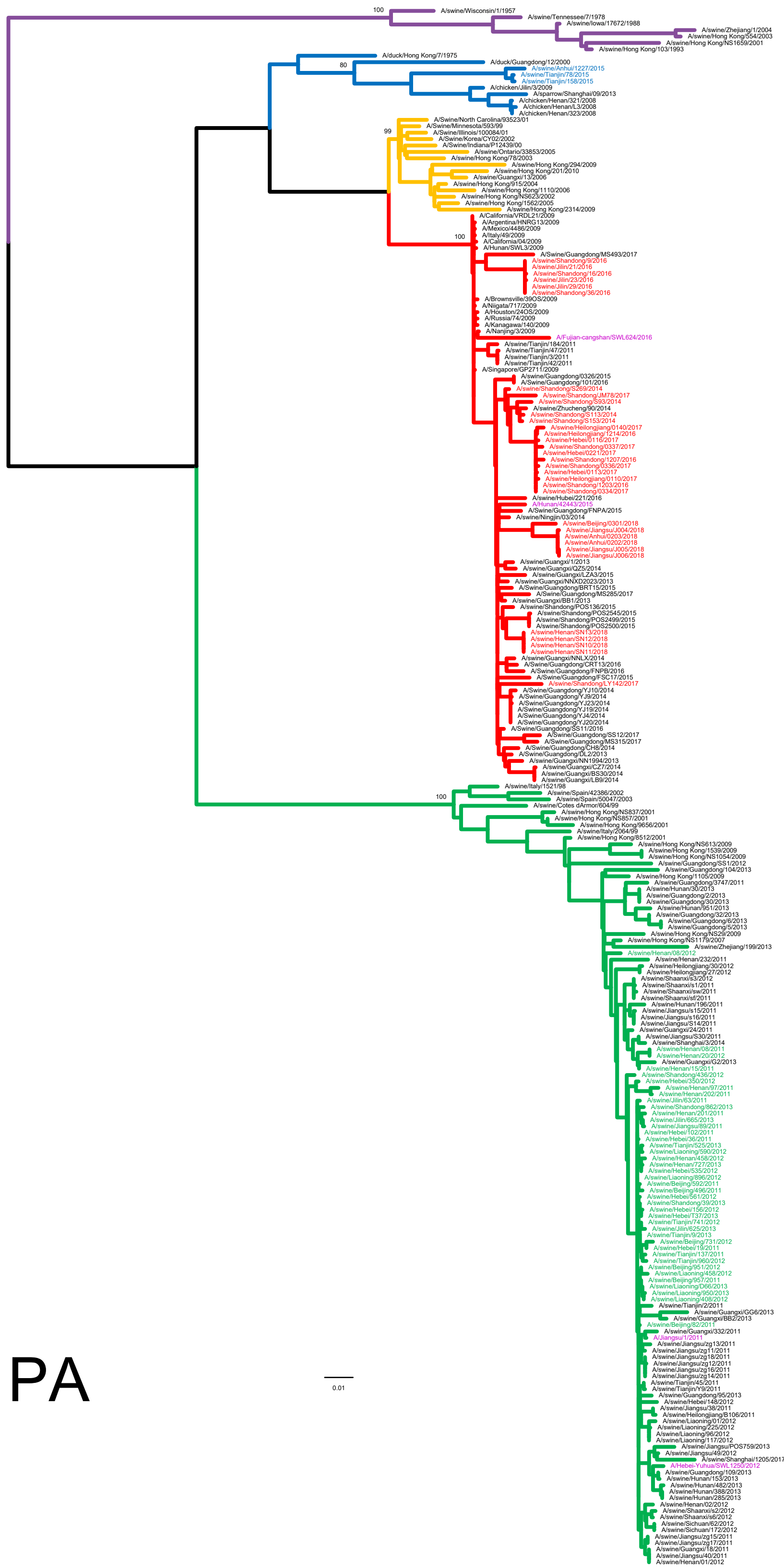

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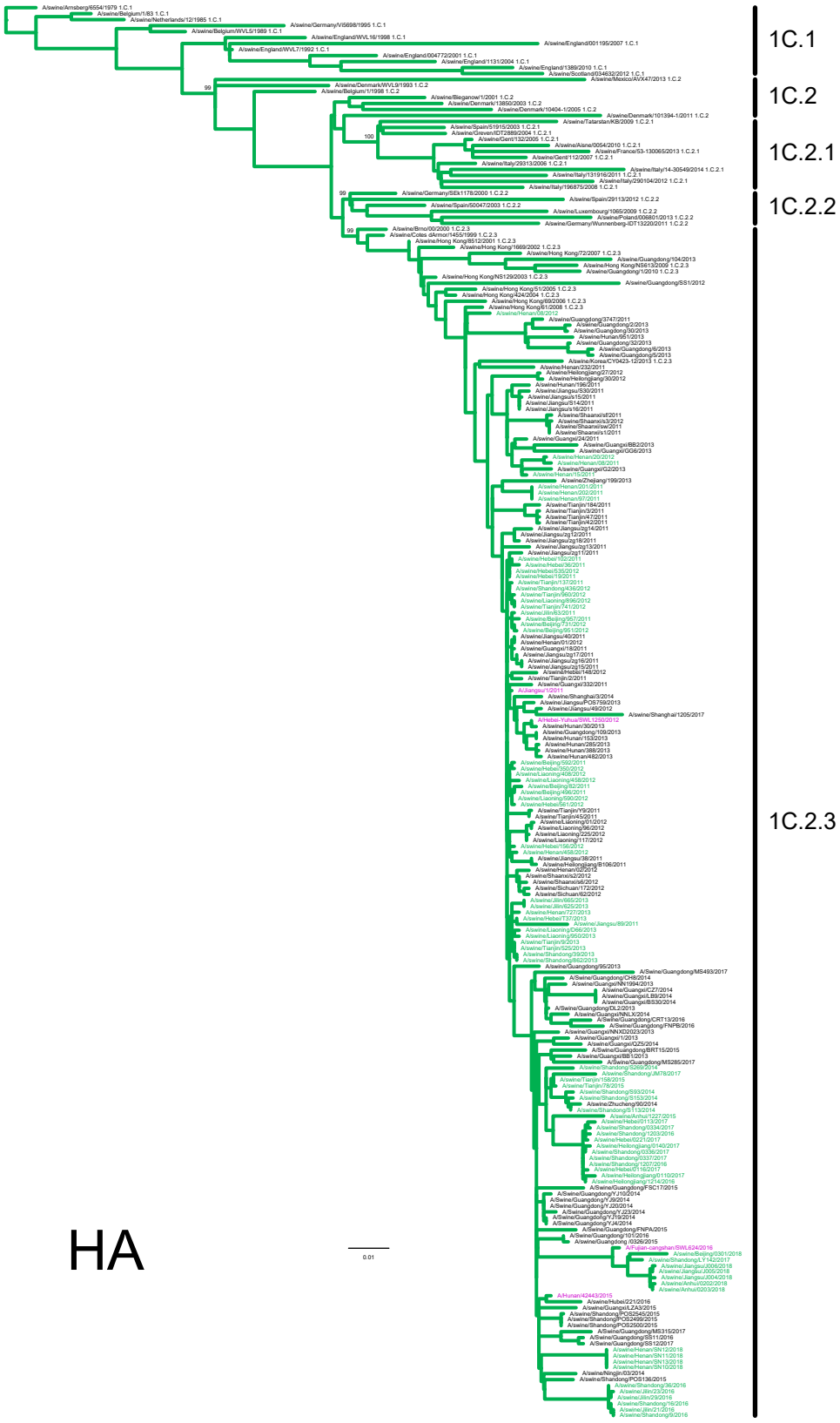
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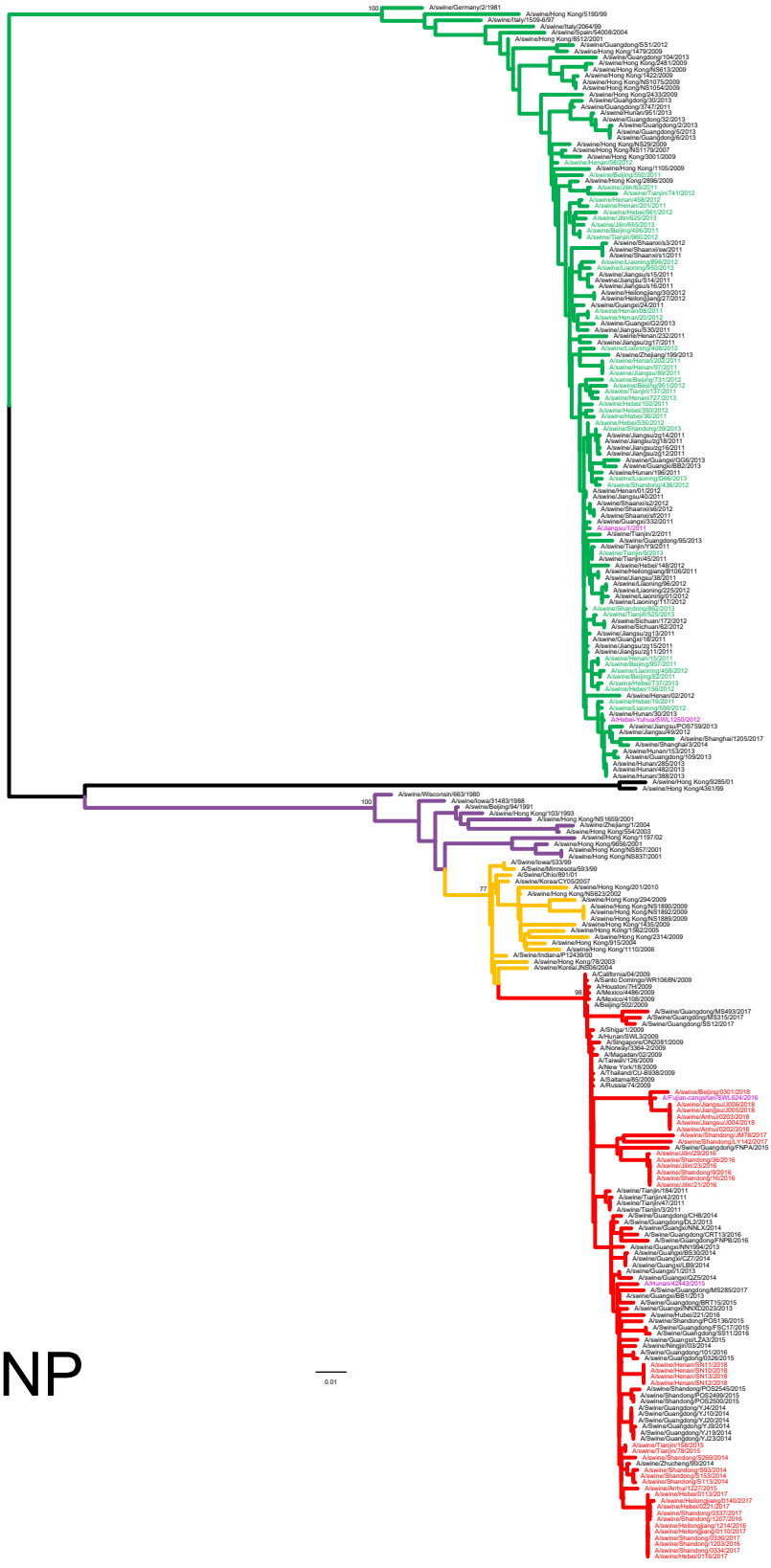
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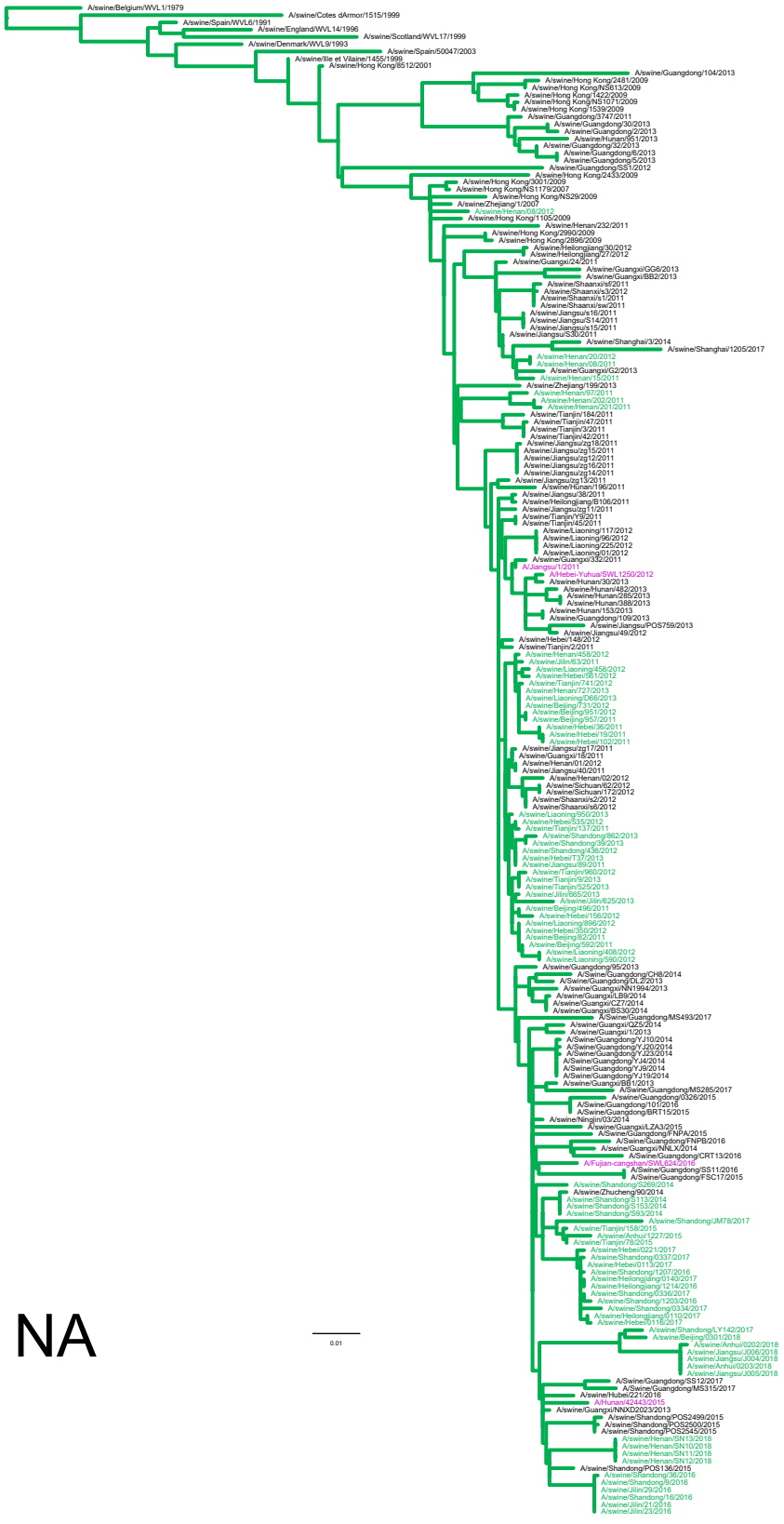
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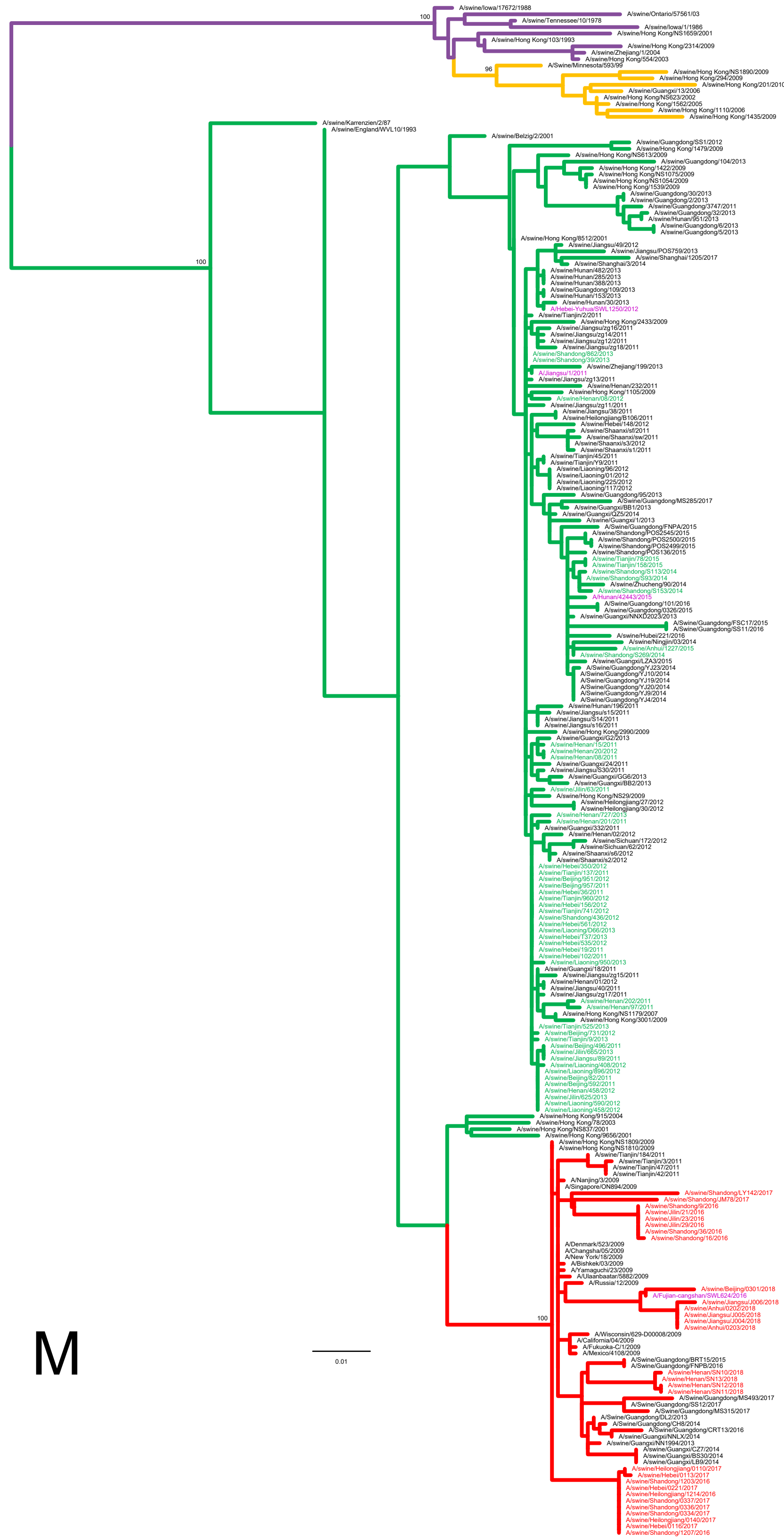
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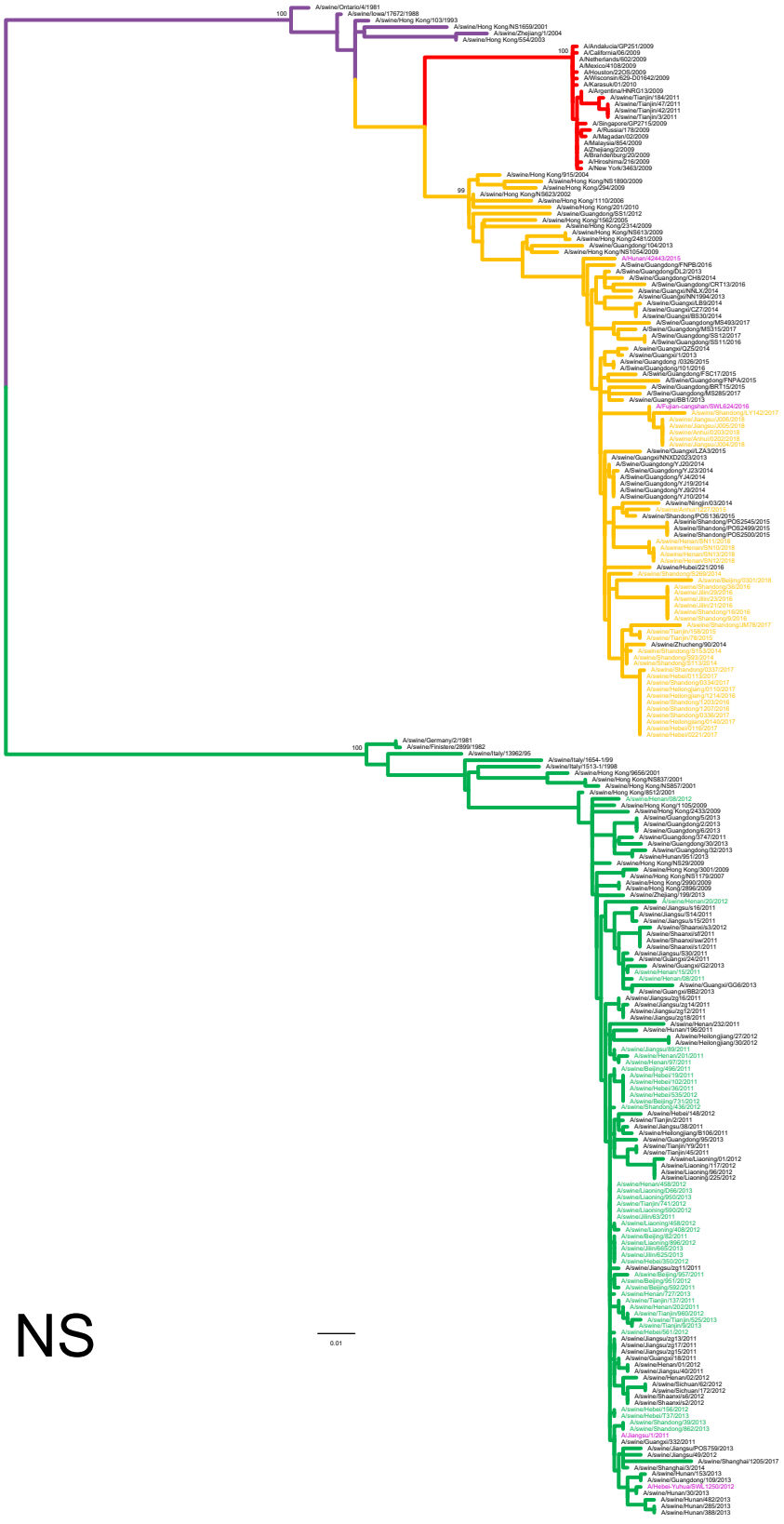
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Fig. S3. Phylogenetic relationships of fully sequenced EA H1N1 SIVs in China in the period of 2011 to 2018. Phylogenetic trees were estimated using genetic distances calculated by maximum likelihood under the GTRGAMMA + I model. SIVs isolated in this study were colored according to the phylogenetic classification of each gene segment. Sequences of viruses with names in black were downloaded from public databases; human EA H1N1 viruses are indicated in purple. Scale bar is in units of nucleotide substitutions per site. Node labels represent bootstrap values.

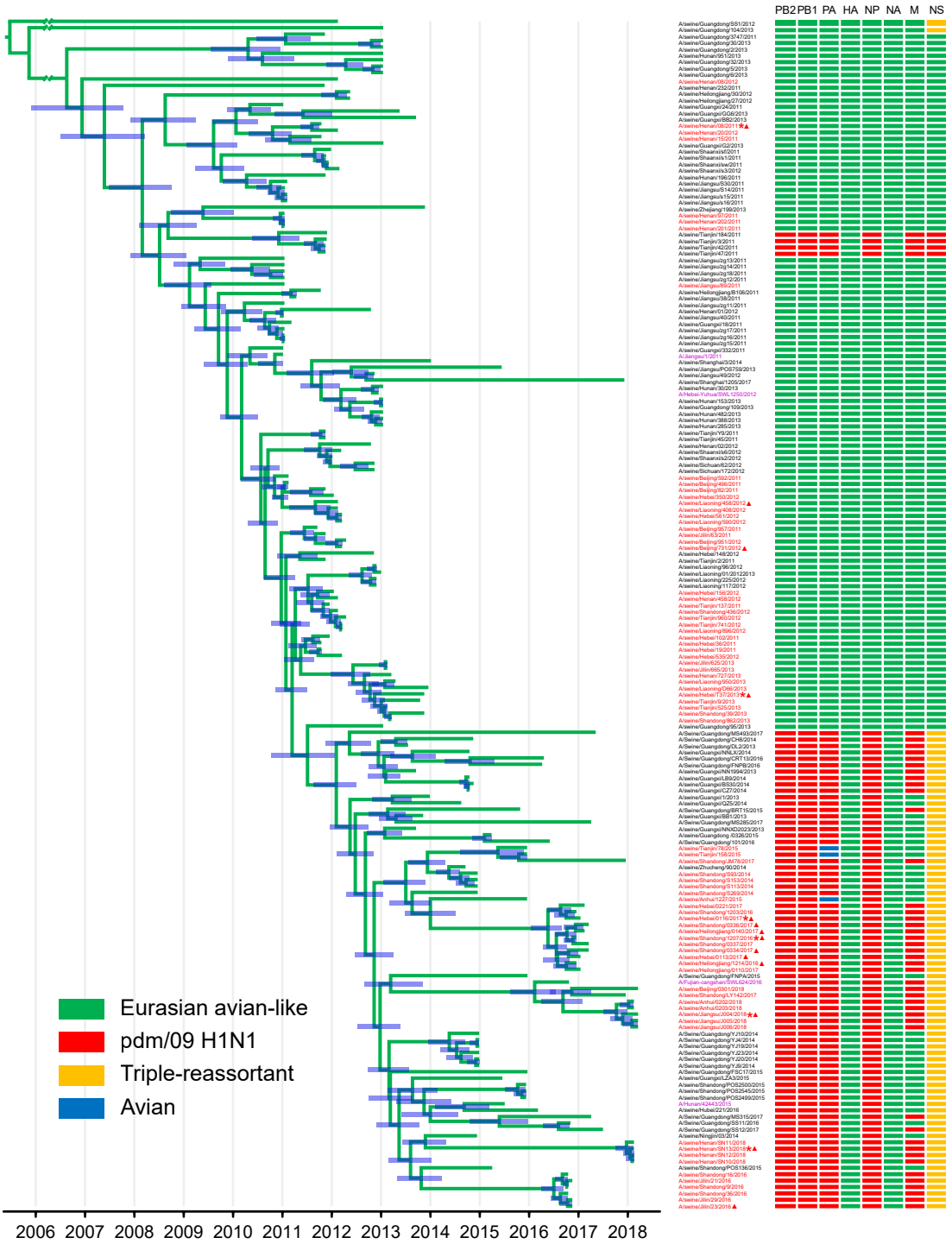

Fig. S4. Phylogeny and divergence time of the HA gene and genotype evolution of EA H1N1 SIVs. The phylogenetic tree of HA gene was generated by Bayesian MCMC framework, using the GTR substitution model with gamma-distributed among site rate heterogeneity and a 'strict molecular clock' model. Colored boxes show the lineage classification of each gene segment of EA H1N1 viruses. Purple node bars represent 95% credible intervals of lineage divergence times. Viruses labeled with a red asterisk were selected for biological characterization. Viruses labeled with a red triangle were selected for antigenic analysis.

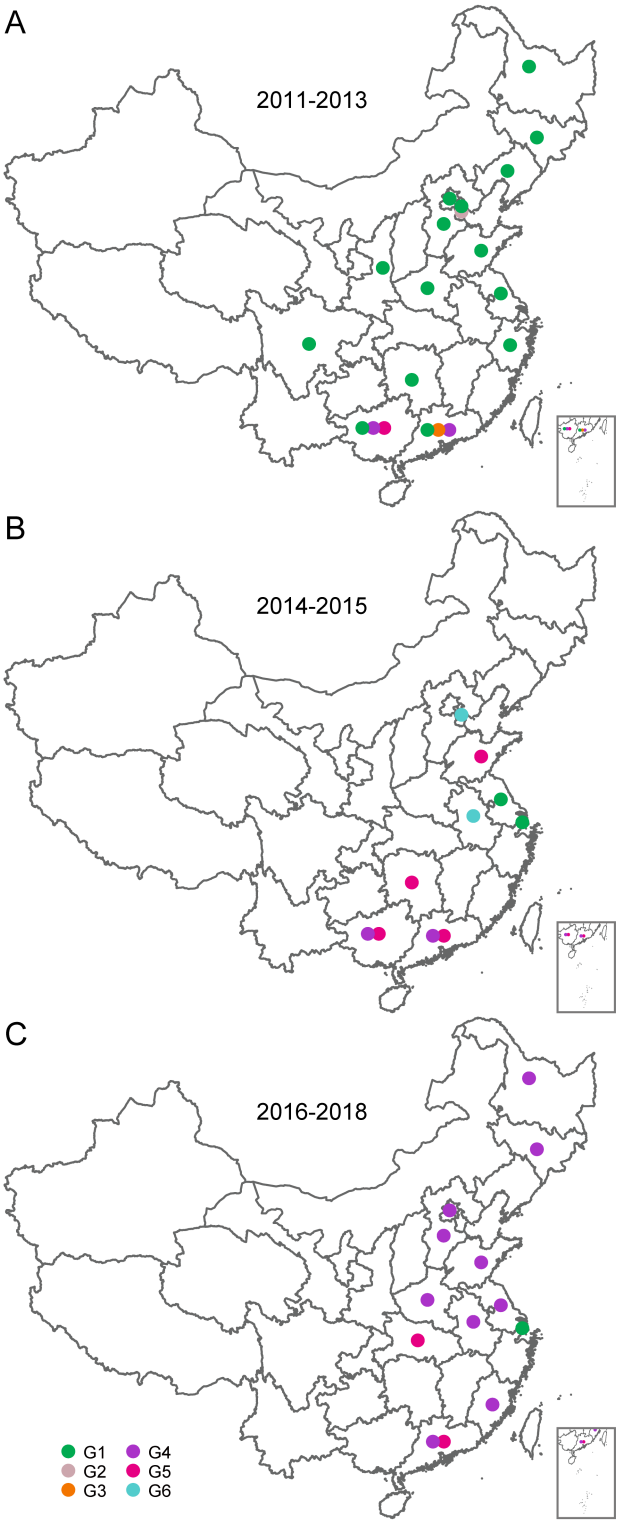

Fig. S5. Geographic distribution of genotypes identified for 187 EA H1N1 viruses isolated in China from 2011 to 2018. The six genotypes of EA H1N1 viruses were mapped chronologically to show the time of genotype isolation in a certain district of China.

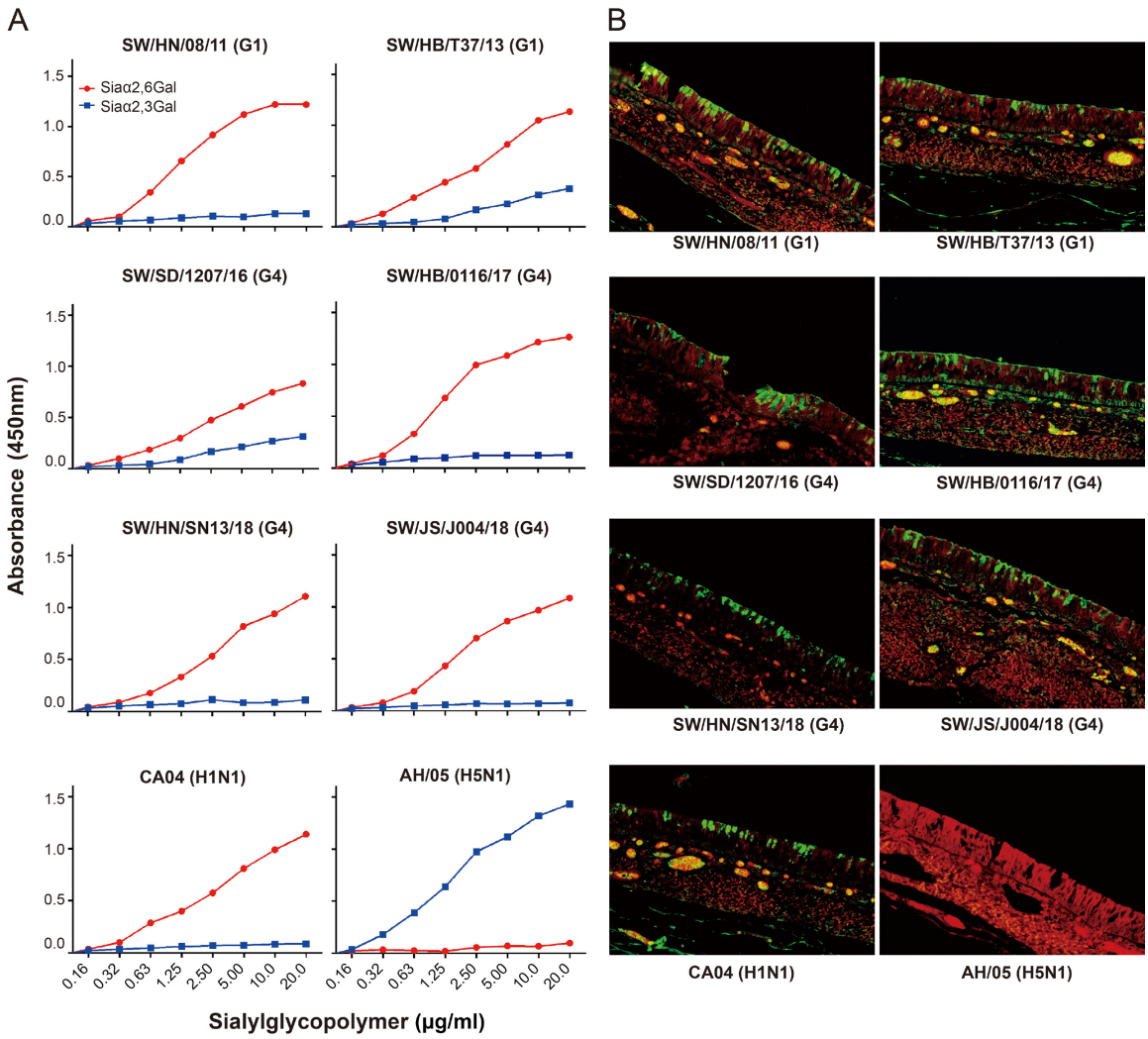

Fig. S6. Host receptor-binding affinity of influenza virus. (A) Strong binding affinity of G1 and G4 viruses to α2,6-linked (red) but not to α2,3-linked (blue) sialic acid polymers. pdm/09 H1N1 virus (CA04) and an avian H5N1 virus (AH/05) that selectively bound SAα2,6Gal and SAα2,3Gal, respectively, were used as controls. (B) Similar binding of G1, G4 and pdm/09 viruses to human respiratory tract. G4 and G1 EA H1N1, control avian H5N1 and human pdm/09 H1N1 viruses were used to incubate human tracheal sections and subsequently immunostained for influenza NP with FITC-labeled goat anti-mouse IgG (green).

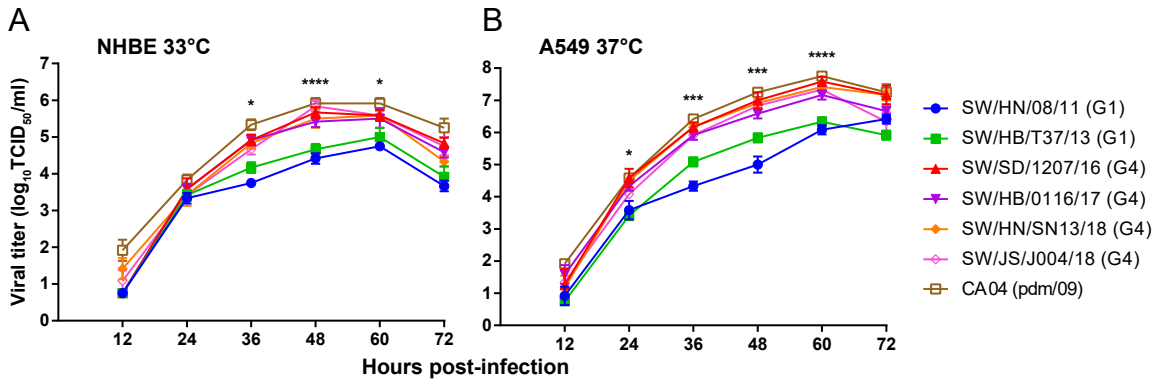

Fig. S7. Viral growth properties in NHBE (A) and A549 (B) cells. Cells were infected with indicated viruses at MOI of 0.001 and incubated at 33°C (A) and 37°C (B), respectively. Supernatants were harvested at the indicated time points and the virus titers were determined in MDCK cells. Values are expressed as means ± standard deviations (SD) (*n* = 3). *, *P* < 0.05, **, *P* < 0.01, between G4 or pdm/09 virus-infected cells and G1 virus-infected cells.

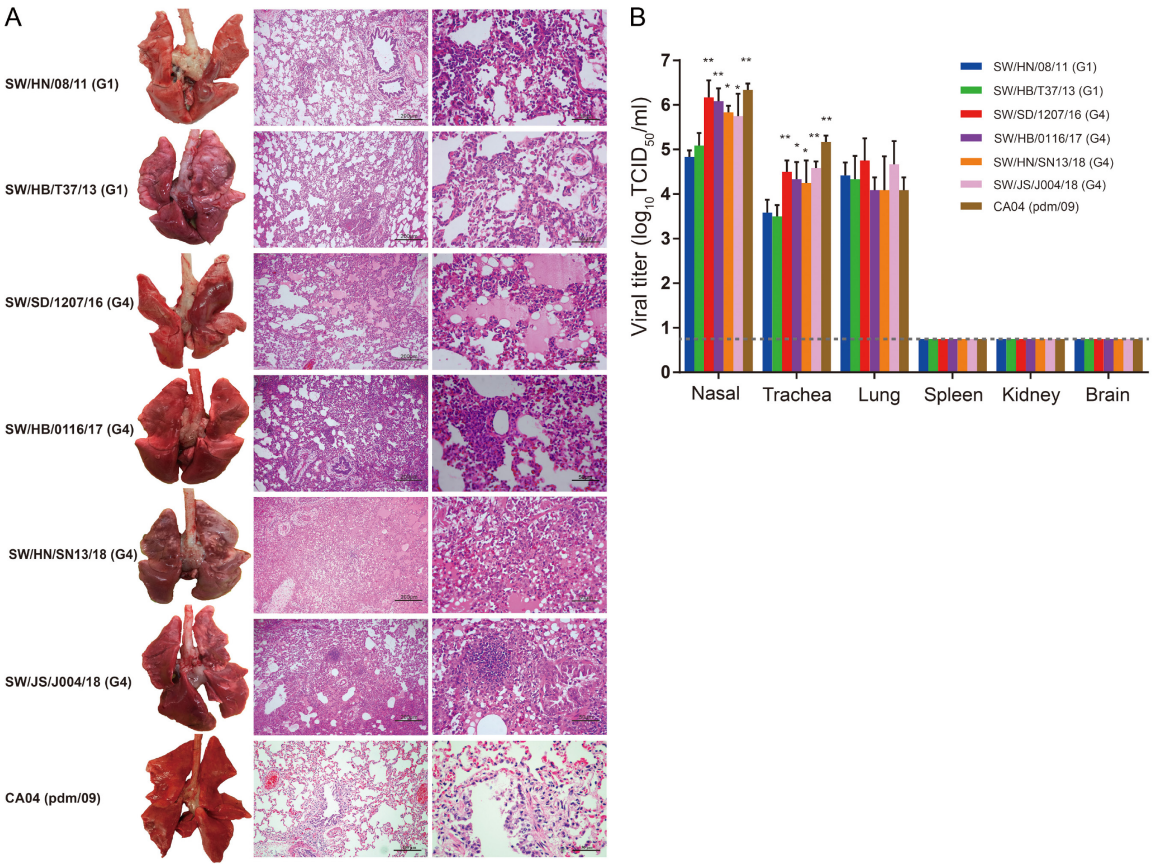

Fig. S8. Pathogenicity and replication of EA H1N1 SIVs in ferrets. (A) Gross and histopathological examination on the respiratory tracts of infected ferrets at 3 dpi. Representative gross (left) and corresponding histological (H&E staining) findings at low (middle) and high (right) magnification are presented. (B) Progeny virus production of influenza viruses in ferrets. Each ferret, in groups of three, was intranasally inoculated with 10^6 $TCID_{50}$ of indicated virus. G4 or pdm/09 progeny virus titers were significantly higher than G1 titers in the upper respiratory tract (nasal and tracheal regions) (*P < 0.05, **P < 0.01, ANOVA). The indicated tissues were collected at 3 dpi for virus titration. All values shown are means ± SD from three independent experiments for each sample. Dashed lines indicate the lower limit of detection.

Table S1. Samples collected for influenza virus isolation from 2011 to 2018

Year	Sampling site	Number of samples collected	Number of virus-positive samples	Isolation rate (%)	Virus subtype				
					CS H1N1	EA H1N1	pdm/09 H1N1	H3N2	H9N2
2011	Slaughter houses	5,540	27	0.49		20	7		
	Veterinary hospital	143	2	1.40		2			
2012	Slaughter houses	4,960	23	0.46		21			2
	Veterinary hospital	162	2	1.23		2			
2013	Slaughter houses	5,310	19	0.36		18		1	
	Veterinary hospital	94	1	1.06		1			
2014	Slaughter houses	2,913	12	0.41	1	11			
	Veterinary hospital	141	5	3.55		5			
2015	Slaughter houses	3,164	17	0.54		16		1	
	Veterinary hospital	128	7	5.47		7			
2016	Slaughter houses	2,156	9	0.42		9			
	Veterinary hospital	93	6	6.45		6			
2017	Slaughter houses	2,755	14	0.51		14			
	Veterinary hospital	121	9	7.44		9			
2018	Slaughter houses	3,120	15	0.48		13		2	
	Veterinary hospital	134	11	8.21		11			
2011-2018	Slaughter houses	29,918	136	0.45	1	122	7	4	2
Eight years	Veterinary hospital	1,016	43	4.23		43			

Table S2. Detailed information of EA H1N1 viruses isolated in this study

Strain name	Collection date	Collection place	PB2	PB1	PA	HA	NP	NA	M	NS	Genotype
A/swine/Henan/201/2011	Jan-2011	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/202/2011	Jan-2011	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/97/2011	Jan-2011	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Jiangsu/89/2011	Jan-2011	Jiangsu	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/496/2011	Feb-2011	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/592/2011	Feb-2011	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/957/2011	Sep-2011	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/19/2011	Oct-2011	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/36/2011	Oct-2011	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/08/2011	Oct-2011	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/15/2011	Oct-2011	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/82/2011	Nov-2011	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Jilin/63/2011	Nov-2011	Jilin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/102/2011	Dec-2011	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Tianjin/137/2011	Dec-2011	Tianjin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/156/2012	Jan-2012	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/350/2012	Jan-2012	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/08/2012	Feb-2012	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/20/2012	Feb-2012	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/458/2012	Feb-2012	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/408/2012	Feb-2012	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/458/2012	Feb-2012	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Shandong/436/2012	Feb-2012	Shandong	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/731/2012	Mar-2012	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/535/2012	Mar-2012	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/561/2012	Mar-2012	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/590/2012	Mar-2012	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/896/2012	Mar-2012	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Tianjin/741/2012	Mar-2012	Tianjin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Beijing/951/2012	Apr-2012	Beijing	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Tianjin/960/2012	Apr-2012	Tianjin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Jilin/625/2013	Feb-2013	Jilin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Jilin/665/2013	Feb-2013	Jilin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Tianjin/525/2013	Feb-2013	Tianjin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Henan/727/2013	Mar-2013	Henan	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Shandong/862/2013	Mar-2013	Shandong	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/950/2013	Apr-2013	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Tianjin/9/2013	Oct-2013	Tianjin	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Hebei/T37/2013	Nov-2013	Hebei	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Shandong/39/2013	Nov-2013	Shandong	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Liaoning/D66/2013	Dec-2013	Liaoning	EA	EA	EA	EA	EA	EA	EA	EA	1
A/swine/Shandong/S113/2014	Nov-2014	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	EA	TRIG	5
A/swine/Shandong/S153/2014	Nov-2014	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	EA	TRIG	5
A/swine/Shandong/S269/2014	Nov-2014	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	EA	TRIG	5
A/swine/Shandong/S93/2014	Nov-2014	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	EA	TRIG	5
A/swine/Anhui/1227/2015	Dec-2015	Anhui	pdm/09	pdm/09	Avian	EA	pdm/09	EA	EA	TRIG	6
A/swine/Tianjin/158/2015	Dec-2015	Tianjin	pdm/09	pdm/09	Avian	EA	pdm/09	EA	EA	TRIG	6
A/swine/Tianjin/78/2015	Dec-2015	Tianjin	pdm/09	pdm/09	Avian	EA	pdm/09	EA	EA	TRIG	6
A/swine/Shandong/16/2016	Oct-2016	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/36/2016	Oct-2016	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/9/2016	Oct-2016	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jilin/21/2016	Nov-2016	Jilin	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jilin/23/2016	Nov-2016	Jilin	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jilin/29/2016	Nov-2016	Jilin	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Heilongjiang/1214/2016	Dec-2016	Heilongjiang	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/1203/2016	Dec-2016	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/1207/2016	Dec-2016	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Hebei/0113/2017	Jan-2017	Hebei	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Hebei/0116/2017	Jan-2017	Hebei	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Heilongjiang/0110/2017	Jan-2017	Heilongjiang	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Hebei/0221/2017	Feb-2017	Hebei	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Heilongjiang/0140/2017	Feb-2017	Heilongjiang	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/0334/2017	Mar-2017	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/0336/2017	Mar-2017	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/0337/2017	Mar-2017	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/JM78/2017	Dec-2017	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Shandong/LY142/2017	Dec-2017	Shandong	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Anhui/0202/2018	Feb-2018	Anhui	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Anhui/0203/2018	Feb-2018	Anhui	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Henan/SN10/2018	Feb-2018	Henan	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Henan/SN11/2018	Feb-2018	Henan	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Henan/SN12/2018	Feb-2018	Henan	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Henan/SN13/2018	Feb-2018	Henan	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4

Table S2. Cont.

Strain name	Collection date	Collection place	PB2	PB1	PA	HA	NP	NA	M	NS	Genotype
A/swine/Beijing/0301/2018	Mar-2018	Beijing	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jiangsu/J004/2018	Mar-2018	Jiangsu	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jiangsu/J005/2018	Mar-2018	Jiangsu	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4
A/swine/Jiangsu/J006/2018	Mar-2018	Jiangsu	pdm/09	pdm/09	pdm/09	EA	pdm/09	EA	pdm/09	TRIG	4

Table S3. Accession numbers for new sequences generated in this study

Strain name	PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Beijing/496/2011(H1N1)	MN416330	MN416417	MN416517	MN416590	MN418734	MN416694	MN418814	MN418660
A/swine/Beijing/592/2011(H1N1)	MN416331	MN416418	MN416518	MN416591	MN418735	MN416695	MN418815	MN418661
A/swine/Beijing/82/2011(H1N1)	MN416333	MN416420	MN416520	MN416593	MN418737	MN416697	MN418817	MN418663
A/swine/Beijing/957/2011(H1N1)	MN416335	MN416422	MN416522	MN416595	MN418739	MN416699	MN418819	MN418665
A/swine/Hebei/102/2011(H1N1)	MN416339	MN416426	MN416526	MN416599	MN418743	MN416703	MN418823	MN418669
A/swine/Hebei/19/2011(H1N1)	MN416341	MN416428	MN416528	MN416601	MN418745	MN416705	MN418825	MN418671
A/swine/Hebei/36/2011(H1N1)	MN416343	MN416430	MN416530	MN416603	MN418747	MN416707	MN418827	MN418673
A/swine/Henan/08/2011(H1N1)	MN416350	MN416437	MN416537	MN416610	MN418754	MN416714	MN418834	MN418680
A/swine/Henan/15/2011(H1N1)	MN416352	MN416439	MN416539	MN416612	MN418756	MN416716	MN418836	MN418682
A/swine/Henan/201/2011(H1N1)	MN416354	MN416441	MN416541	MN416614	MN418758	MN416718	MN418838	MN418684
A/swine/Henan/202/2011(H1N1)	MN416355	MN416442	MN416542	MN416615	MN418759	MN416719	MN418839	MN418685
A/swine/Henan/97/2011(H1N1)	MN416358	MN416445	MN416545	MN416618	MN418762	MN416722	MN418842	MN418688
A/swine/Jiangsu/89/2011(H1N1)	MN416363	MN416450	MN416550	MN416623	MN418767	MN416727	MN418847	MN418693
A/swine/Jilin/63/2011(H1N1)	MN416371	MN416458	MN416558	MN416631	MN418775	MN416735	MN418855	MN418701
A/swine/Tianjin/137/2011(H1N1)	MN416392	MN416479	MN416579	MN416652	MN418796	MN416756	MN418876	MN418722
A/swine/Beijing/731/2012(H1N1)	MN416332	MN416419	MN416519	MN416592	MN418736	MN416696	MN418816	MN418662
A/swine/Beijing/951/2012(H1N1)	MN416334	MN416421	MN416521	MN416594	MN418738	MN416698	MN418818	MN418664
A/swine/Hebei/156/2012(H1N1)	MN416340	MN416427	MN416527	MN416600	MN418744	MN416704	MN418824	MN418670
A/swine/Hebei/350/2012(H1N1)	MN416342	MN416429	MN416529	MN416602	MN418746	MN416706	MN418826	MN418672
A/swine/Hebei/535/2012(H1N1)	MN416344	MN416431	MN416531	MN416604	MN418748	MN416708	MN418828	MN418674
A/swine/Hebei/561/2012(H1N1)	MN416345	MN416432	MN416532	MN416605	MN418749	MN416709	MN418829	MN418675
A/swine/Henan/08/2012(H1N1)	MN416351	MN416438	MN416538	MN416611	MN418755	MN416715	MN418835	MN418681
A/swine/Henan/20/2012(H1N1)	MN416353	MN416440	MN416540	MN416613	MN418757	MN416717	MN418837	MN418683
A/swine/Henan/458/2012(H1N1)	MN416356	MN416443	MN416543	MN416616	MN418760	MN416720	MN418840	MN418686
A/swine/Liaoning/408/2012(H1N1)	MN416373	MN416460	MN416560	MN416633	MN418777	MN416737	MN418857	MN418703
A/swine/Liaoning/458/2012(H1N1)	MN416374	MN416461	MN416561	MN416634	MN418778	MN416738	MN418858	MN418704
A/swine/Liaoning/590/2012(H1N1)	MN416375	MN416462	MN416562	MN416635	MN418779	MN416739	MN418859	MN418705
A/swine/Liaoning/896/2012(H1N1)	MN416376	MN416463	MN416563	MN416636	MN418780	MN416740	MN418860	MN418706
A/swine/Shandong/436/2012(H1N1)	MN416387	MN416474	MN416574	MN416647	MN418791	MN416751	MN418871	MN418717
A/swine/Tianjin/741/2012(H1N1)	MN416395	MN416482	MN416582	MN416655	MN418799	MN416759	MN418879	MN418725
A/swine/Tianjin/960/2012(H1N1)	MN416398	MN416485	MN416585	MN416658	MN418802	MN416762	MN418882	MN418728
A/swine/Hebei/T37/2013(H1N1)	MN416346	MN416433	MN416533	MN416606	MN418750	MN416710	MN418830	MN418676
A/swine/Henan/727/2013(H1N1)	MN416357	MN416444	MN416544	MN416617	MN418761	MN416721	MN418841	MN418687
A/swine/Jilin/625/2013(H1N1)	MN416370	MN416457	MN416557	MN416630	MN418774	MN416734	MN418854	MN418700
A/swine/Jilin/665/2013(H1N1)	MN416372	MN416459	MN416559	MN416632	MN418776	MN416736	MN418856	MN418702
A/swine/Liaoning/950/2013(H1N1)	MN416377	MN416464	MN416564	MN416637	MN418781	MN416741	MN418861	MN418707
A/swine/Liaoning/D66/2013(H1N1)	MN416378	MN416465	MN416565	MN416638	MN418782	MN416742	MN418862	MN418708
A/swine/Shandong/39/2013(H1N1)	MN416386	MN416473	MN416573	MN416646	MN418790	MN416750	MN418870	MN418716
A/swine/Shandong/862/2013(H1N1)	MN416388	MN416475	MN416575	MN416648	MN418792	MN416752	MN418872	MN418718
A/swine/Tianjin/525/2013(H1N1)	MN416394	MN416481	MN416581	MN416654	MN418798	MN416758	MN418878	MN418724
A/swine/Tianjin/9/2013(H1N1)	MN416397	MN416484	MN416584	MN416657	MN418801	MN416761	MN418881	MN418727
A/swine/Shandong/S113/2014(H1N1)	KP735701	KP735705	KP735709	KP735713	KP735717	KP735721	KP735725	KP735729
A/swine/Shandong/S153/2014(H1N1)	KP735702	KP735706	KP735710	KP735714	KP735718	KP735722	KP735726	KP735730
A/swine/Shandong/S269/2014(H1N1)	KP735703	KP735707	KP735711	KP735715	KP735719	KP735723	KP735727	KP735731
A/swine/Shandong/S93/2014(H1N1)	KP735700	KP735704	KP735708	KP735712	KP735716	KP735720	KP735724	KP735728
A/swine/Anhui/1227/2015(H1N1)	MN416328	MN416415	MN416515	MN416588	MN418732	MN416692	MN418812	MN418658
A/swine/Tianjin/158/2015(H1N1)	MN416393	MN416480	MN416580	MN416653	MN418797	MN416757	MN418877	MN418723
A/swine/Tianjin/78/2015(H1N1)	MN416396	MN416483	MN416583	MN416656	MN418800	MN416760	MN418880	MN418726
A/swine/Heilongjiang/1214/2016(H1N1)	MN416349	MN416436	MN416536	MN416609	MN418753	MN416713	MN418833	MN418679
A/swine/Jilin/21/2016(H1N1)	MN416367	MN416454	MN416554	MN416627	MN418771	MN416731	MN418851	MN418697
A/swine/Jilin/23/2016(H1N1)	MN416368	MN416455	MN416555	MN416628	MN418772	MN416732	MN418852	MN418698
A/swine/Jilin/29/2016(H1N1)	MN416369	MN416456	MN416556	MN416629	MN418773	MN416733	MN418853	MN418699
A/swine/Shandong/1203/2016(H1N1)	MN416382	MN416469	MN416569	MN416642	MN418786	MN416746	MN418866	MN418712
A/swine/Shandong/1207/2016(H1N1)	MN416383	MN416470	MN416570	MN416643	MN418787	MN416747	MN418867	MN418713
A/swine/Shandong/16/2016(H1N1)	MN416384	MN416471	MN416571	MN416644	MN418788	MN416748	MN418868	MN418714
A/swine/Shandong/36/2016(H1N1)	MN416385	MN416472	MN416572	MN416645	MN418789	MN416749	MN418869	MN418715
A/swine/Shandong/9/2016(H1N1)	MN416389	MN416476	MN416576	MN416649	MN418793	MN416753	MN418873	MN418719
A/swine/Hebei/0113/2017(H1N1)	MN416336	MN416423	MN416523	MN416596	MN418740	MN416700	MN418820	MN418666
A/swine/Hebei/0116/2017(H1N1)	MN416337	MN416424	MN416524	MN416597	MN418741	MN416701	MN418821	MN418667
A/swine/Hebei/0221/2017(H1N1)	MN416338	MN416425	MN416525	MN416598	MN418742	MN416702	MN418822	MN418668
A/swine/Heilongjiang/0110/2017(H1N1)	MN416347	MN416434	MN416534	MN416607	MN418751	MN416711	MN418831	MN418677
A/swine/Heilongjiang/0140/2017(H1N1)	MN416348	MN416435	MN416535	MN416608	MN418752	MN416712	MN418832	MN418678
A/swine/Shandong/0334/2017(H1N1)	MN416379	MN416466	MN416566	MN416639	MN418783	MN416743	MN418863	MN418709
A/swine/Shandong/0336/2017(H1N1)	MN416380	MN416467	MN416567	MN416640	MN418784	MN416744	MN418864	MN418710
A/swine/Shandong/0337/2017(H1N1)	MN416381	MN416468	MN416568	MN416641	MN418785	MN416745	MN418865	MN418711
A/swine/Shandong/JM78/2017(H1N1)	MN416390	MN416477	MN416577	MN416650	MN418794	MN416754	MN418874	MN418720
A/swine/Shandong/LY142/2017(H1N1)	MN416391	MN416478	MN416578	MN416651	MN418795	MN416755	MN418875	MN418721
A/swine/Anhui/0202/2018(H1N1)	MN416326	MN416413	MN416513	MN416586	MN418730	MN416690	MN418810	MN418656
A/swine/Anhui/0203/2018(H1N1)	MN416327	MN416414	MN416514	MN416587	MN418731	MN416691	MN418811	MN418657
A/swine/Beijing/0301/2018(H1N1)	MN416329	MN416416	MN416516	MN416589	MN418733	MN416693	MN418813	MN418659
A/swine/Henan/SN10/2018(H1N1)	MN416359	MN416446	MN416546	MN416619	MN418763	MN416723	MN418843	MN418689
A/swine/Henan/SN11/2018(H1N1)	MN416360	MN416447	MN416547	MN416620	MN418764	MN416724	MN418844	MN418690
A/swine/Henan/SN12/2018(H1N1)	MN416361	MN416448	MN416548	MN416621	MN418765	MN416725	MN418845	MN418691

Table S3. Cont.

Strain name	PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Henan/SN13/2018(H1N1)	MN416362	MN416449	MN416549	MN416622	MN418766	MN416726	MN418846	MN418692
A/swine/Jiangsu/J004/2018(H1N1)	MN416364	MN416451	MN416551	MN416624	MN418768	MN416728	MN418848	MN418694
A/swine/Jiangsu/J005/2018(H1N1)	MN416365	MN416452	MN416552	MN416625	MN418769	MN416729	MN418849	MN418695
A/swine/Jiangsu/J006/2018(H1N1)	MN416366	MN416453	MN416553	MN416626	MN418770	MN416730	MN418850	MN418696

Table S4. Clinical symptoms of ferrets infected with influenza viruses

Virus (genotype)	No. with fever (mean maximum temperature increase, °C)	No. with wheezing/ coughing	No. with lung lesion (mean pulmonary consolidation area, %)	No. with weight loss (mean maximum weight loss, %)	No. with virus detection	No. with seroconversion (HI titer)
SW/HN/08/11 (G1 EA)						
Inoculated	3/3 (0.8)	3/3	3/3 (8.5)	3/3 (4.2)	3/3	3/3 (160, 320, 320)
DC contact	0/3	0/3	0/3	0/3	0/3	0/3
RD contact	0/3	0/3	0/3	0/3	0/3	0/3
SW/HB/T37/13 (G1 EA)						
Inoculated	3/3 (1.1)	3/3	3/3 (9.3)	3/3 (5.6)	3/3	3/3 (320, 320, 320)
DC contact	0/3	0/3	0/3	0/3	0/3	0/3
RD contact	0/3	0/3	0/3	0/3	0/3	0/3
SW/SD/1207/16 (G4 EA)						
Inoculated	3/3 (1.6)	3/3	3/3 (25.5)	3/3 (8.6)	3/3	3/3 (320, 320, 320)
DC contact	3/3 (1.3)	3/3	3/3 (23.4)	3/3 (8.4)	3/3	3/3 (160, 160, 80)
RD contact	3/3 (1.3)	3/3	3/3 (22.6)	3/3 (7.1)	3/3	3/3 (160, 80, 80)
SW/HB/0116/17 (G4 EA)						
Inoculated	3/3 (1.4)	3/3	3/3 (19.4)	3/3 (7.3)	3/3	3/3 (160, 320, 320)
DC contact	3/3 (1.2)	3/3	3/3 (17.5)	3/3 (6.6)	3/3	3/3 (160, 80, 80)
RD contact	3/3 (1.3)	1/3	1/3 (16.7)	3/3 (6.2)	1/3	1/3 (160)
SW/HN/SN13/18 (G4 EA)						
Inoculated	3/3 (1.8)	3/3	3/3 (37.1)	3/3 (9.8)	3/3	3/3 (160, 320, 160)
DC contact	3/3 (1.6)	3/3	3/3 (24.5)	3/3 (8.7)	3/3	3/3 (160, 80, 80)
RD contact	3/3 (1.7)	3/3	3/3 (22.3)	3/3 (7.4)	3/3	3/3 (80, 160, 160)
SW/JS/J004/18 (G4 EA)						
Inoculated	3/3 (1.6)	3/3	3/3 (21.8)	3/3 (9.1)	3/3	3/3 (320, 160, 160)
DC contact	3/3 (1.5)	3/3	3/3 (23.4)	3/3 (8.6)	3/3	3/3 (160, 160, 80)
RD contact	3/3 (1.4)	3/3	3/3 (19.5)	3/3 (8.2)	3/3	3/3 (160, 160, 160)
CA04 (pdm/09 H1N1)						
Inoculated	3/3 (0.9)	3/3	3/3 (5.2)	3/3 (4.4)	3/3	3/3 (320, 160, 320)
DC contact	3/3 (0.8)	3/3	3/3 (4.5)	3/3 (4.0)	3/3	3/3 (160, 160, 160)
RD contact	3/3 (0.8)	3/3	3/3 (4.0)	3/3 (3.8)	3/3	3/3 (160, 80, 80)

HI, hemagglutination inhibition.
DC, direct-contact.
RD, respiratory-droplet.

Table S5. Antigenic classification of EA H1N1 SIVs by HI assays*

	Antisera				
Test viruses (genotype)	A SW/HN/08/11	A SW/HB/T37/13	B SW/SD/1207/16	B SW/HN/SN13/18	pdm/09 A/Michigan/45/2015
A/Michigan/45/2015 (pdm/09 H1N1)	-	-	-	-	2560†
A					
SW/HN/08/11 (G1 EA H1N1)	2560†	1280	160	80	320
SW/HB/T37/13 (G1 EA H1N1)	2560	2560†	80	80	160
SW/BJ/731/12 (G1 EA H1N1)	1280	2560	80	160	160
SW/LN/458/12 (G1 EA H1N1)	2560	2560	80	80	160
B					
SW/SD/1207/16 (G4 EA H1N1)	80	80	2560†	2560	20
SW/HN/SN13/18 (G4 EA H1N1)	80	160	2560	2560†	40
SW/JL/23/16 (G4 EA H1N1)	80	320	5120	2560	40
SW/HLJ/1214/16 (G4 EA H1N1)	160	160	1280	1280	<
SW/SD/0334/17 (G4 EA H1N1)	80	40	1280	1280	<
SW/HLJ/0140/17 (G4 EA H1N1)	160	160	1280	5120	20
SW/HB/0113/17 (G4 EA H1N1)	80	80	2560	2560	40
SW/HB/0116/17 (G4 EA H1N1)	160	160	2560	2560	20
SW/SD/0336/17 (G4 EA H1N1)	80	40	1280	1280	20
SW/JS/J004/18 (G4 EA H1N1)	80	40	2560	2560	20

*Virus names are abbreviated as isolation location, strain name and collection year. Data represent HI titers. High HI titers (≥ 640) are shaded black, moderate HI titers (160, 320) are gray and low HI titers (40, 80) are not shaded. Titers < 20 are indicated with the sign <. -, not done.
†Homologous titer.

Table S6. Amino acid differences in the antigenic sites of the HA molecule of the H1N1 viruses (H1 numbering)

		Amino acid in the antigenic site															
		Site Sa									Site Sb						
Virus	Genotype	124	125	155	157	159	160	162	163	164	153	156	185	189	190	193	195
A/swine/Henan/08/2011	G1 EA	P	N	G	S	P	K	S	K	S	K	N	Y	Q	T	Q	N
A/swine/Hebei/T37/2013	G1 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/swine/Beijing/731/2012	G1 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/swine/Liaoning/458/2012	G1 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/swine/Shandong/1207/2016	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Henan/SN13/2018	G4 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/swine/Jilin/23/2016	G4 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/swine/Heilongjiang/1214/2016	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Shandong/0334/2017	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Heilongjiang/0140/2017	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Hebei/0113/2017	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Hebei/0116/2017	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Shandong/0336/2017	G4 EA	P	N	G	S	P	K	S	K	S	K	N	E	Q	T	Q	N
A/swine/Jiangsu/J004/2018	G4 EA	P	N	G	S	P	K	S	K	S	K	N	D	Q	T	Q	N
A/Michigan/45/2015	pdm/09	P	N	G	S	P	K	N	Q	S	K	N	T	Q	S	Q	A

		Amino acid in the antigenic site															
		Site Ca1				Site Ca2						Site Cb					
Virus	Genotype	166	170	204	237	135	137	140	142	221	222	70	71	73	74	75	115
A/swine/Henan/08/2011	G1 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Hebei/T37/2013	G1 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Beijing/731/2012	G1 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Liaoning/458/2012	G1 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Shandong/1207/2016	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Henan/SN13/2018	G4 EA	T	G	T	G	S	S	G	N	R	E	L	L	A	N	S	E
A/swine/Jilin/23/2016	G4 EA	T	G	S	G	S	S	G	N	R	G	L	L	A	N	S	E
A/swine/Heilongjiang/1214/2016	G4 EA	T	G	S	G	S	S	G	N	R	E	L	L	A	N	S	E
A/swine/Shandong/0334/2017	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Heilongjiang/0140/2017	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Hebei/0113/2017	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Hebei/0116/2017	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Shandong/0336/2017	G4 EA	T	G	S	G	A	S	G	N	R	E	L	L	A	N	S	E
A/swine/Jiangsu/J004/2018	G4 EA	T	G	S	G	A	S	G	N	R	K	L	L	A	N	S	E
A/Michigan/45/2015	pdm/09	I	G	S	G	A	P	G	K	R	D	L	S	A	S	S	E

Table S7. Summary of the serological survey of the workers from 15 swine farms by HI assay during 2016–2018

			% Positive (No. Positive)*		
	Farm	No. Total	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)
2016	A1	23	4.3 (1)	8.7 (2)	52.2 (12)
	A2	20	5.0 (1)	5.0 (1)	30.0 (6)
	A3	17	0 (0)	5.9 (1)	29.4 (5)
	A4	20	10.0 (2)	10.0 (2)	50.0 (10)
	A5	10	0 (0)	0 (0)	40.0 (4)
Total		90	4.4 (4)	6.7 (6)	41.1 (37)
2017	B1	29	10.3 (3)	17.2 (5)	44.8 (13)
	B2	21	19.0 (4)	19.0 (4)	33.3 (7)
	B3	30	10.0 (3)	13.3 (4)	43.3 (13)
	B4	26	3.8 (1)	3.8 (1)	19.2 (5)
	B5	14	0 (0)	0 (0)	14.3 (2)
Total		120	9.2 (11)	11.7 (14)	33.3 (40)
2018	C1	25	8.0 (2)	8.0 (2)	48.0 (12)
	C2	29	3.4 (1)	6.9 (2)	34.5 (10)
	C3	26	7.7 (2)	15.4 (4)	42.3 (11)
	C4	29	6.9 (2)	17.2 (5)	44.8 (13)
	C5	19	0 (0)	10.5 (2)	42.1 (8)
Total		128	5.5 (7)	11.7 (15)	42.2 (54)
All 3 years	15	338	6.5 (22)	10.4 (35)	38.8 (131)

*HI titer ≥ 40 is considered as positive sample.

Table S8. Characteristics of swine workers upon enrolment and odds ratios for seroprevalence of reference strains

	% Pos (No. positive) | OR (95% CI)[‡]		
Group (No. total)	SW/HN/08/11 (G1)	SW/SD/1207/16 (G4)	A/Michigan/45/2015 (pdm/09)
Gender			
Male (202)	6.9 (14) | 1.2 (0.5-2.9)	10.9 (22) | 1.2 (0.6-2.4)	41.1 (83) | 1.3 (0.8-2.0)
Female[#] (136)	5.9 (8) | Ref	9.6 (13) | Ref	35.3 (48) | Ref
Age (years)			
18–35 (44)	9.1 (4) | 1.6 (0.5-5.2)	**20.5**[*] (9) | 3.2 (1.3-7.7)	**54.5** (24) | 2.2 (1.1-4.1)
36–45 (51)	7.8 (4) | 1.4 (0.4-4.4)	15.7 (8) | 2.3 (1.0-5.7)	39.2 (20) | 1.2 (0.6-2.2)
> 45[#] (243)	5.8 (14) | Ref	7.4 (18) | Ref	35.8 (87) | Ref
Collection year			
2016[#] (90)	4.4 (4) | Ref	6.7 (6) | Ref	41.1 (37) | Ref
2017 (120)	9.2 (11) | 2.2 (0.7-7.1)	11.7 (14) | 1.8 (0.7-5.0)	33.3 (40) | 0.7 (0.4-1.3)
2018 (128)	5.5 (7) | 1.2 (0.4-4.4)	11.7 (15) | 1.9 (0.7-5.0)	42.2 (54) | 1.0 (0.6-1.8)

[‡]Odds ratio and 95% confidence interval compared to the control group.
[*]Boldface indicates a statistically significant difference compared with the control group ($P < 0.05$).
[#]Control group.

Table S9. Detailed data of positive serum samples of swine workers

		HI antibody titer against reference strain*					HI antibody titer against reference strain*		
Year	Farm	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)	Year	Farm	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)
2016	1	<	<	80	2017	6	<	<	40
2016	1	<	<	40	2017	6	<	40	<
2016	1	<	40	80	2017	6	<	<	40
2016	1	<	<	80	2017	6	<	<	40
2016	1	<	<	80	2017	6	<	40	40
2016	1	<	<	80	2017	6	<	<	40
2016	1	<	<	40	2017	6	<	<	160
2016	1	<	<	80	2017	6	<	<	80
2016	1	<	<	40	2017	6	<	<	40
2016	1	<	<	40	2017	6	<	40	80
2016	1	40	40	160	2017	7	<	<	40
2016	1	<	<	80	2017	7	<	<	80
2016	2	<	<	160	2017	7	<	<	40
2016	2	<	<	40	2017	7	160	40	160
2016	2	<	<	40	2017	7	40	40	<
2016	2	160	80	160	2017	7	40	40	160
2016	2	<	<	40	2017	7	40	40	<
2016	2	<	<	80	2017	7	<	<	40
2016	3	<	<	40	2017	7	<	<	40
2016	3	<	<	40	2017	8	<	<	40
2016	3	<	<	40	2017	8	<	<	80
2016	3	<	<	40	2017	8	<	<	80
2016	3	<	160	80	2017	8	160	160	320
2016	4	<	<	80	2017	8	40	40	80
2016	4	<	<	40	2017	8	<	<	40
2016	4	<	<	80	2017	8	<	<	40
2016	4	<	<	40	2017	8	<	<	40
2016	4	<	<	40	2017	8	<	<	80
2016	4	<	<	80	2017	8	<	<	80
2016	4	40	40	160	2017	8	<	40	<
2016	4	<	<	40	2017	8	<	<	40
2016	4	40	80	640	2017	8	40	<	40
2016	4	<	<	160	2017	8	<	40	160
2016	5	<	<	80	2017	9	40	40	40
2016	5	<	<	80	2017	9	<	<	40
2016	5	<	<	40	2017	9	<	<	40
2016	5	<	<	80	2017	9	<	<	40
2017	6	<	<	80	2017	9	<	<	40
2017	6	40	<	<	2017	10	<	<	40
2017	6	80	80	160	2017	10	<	<	40
2017	6	40	80	160	2018	11	<	<	40
2017	6	<	<	80	2018	11	<	<	40

Table S9. Cont.

		HI antibody titer against reference strain*		
Year	Farm	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)
2018	11	<	<	160
2018	11	<	<	80
2018	11	<	<	40
2018	11	<	<	320
2018	11	<	<	80
2018	11	<	<	40
2018	11	40	40	80
2018	11	80	80	640
2018	11	<	<	80
2018	11	<	<	40
2018	12	<	<	160
2018	12	<	<	40
2018	12	<	160	<
2018	12	<	<	160
2018	12	<	<	40
2018	12	<	<	40
2018	12	<	<	40
2018	12	<	<	80
2018	12	160	160	640
2018	12	<	<	40
2018	12	<	<	40
2018	13	<	<	40
2018	13	<	<	80
2018	13	<	<	40
2018	13	<	40	320
2018	13	40	40	160
2018	13	<	<	80
2018	13	<	<	40
2018	13	<	<	40
2018	13	640	1280	1280
2018	13	<	<	160
2018	13	<	80	160
2018	14	<	<	80
2018	14	<	<	40
2018	14	<	<	320
2018	14	<	<	40
2018	14	<	40	320
2018	14	<	<	80
2018	14	40	80	80
2018	14	<	40	<
2018	14	<	<	40
2018	14	<	80	<
2018	14	<	<	40
2018	14	<	<	40
2018	14	<	<	40
2018	14	80	80	80
2018	14	<	<	80
2018	15	<	<	40
2018	15	<	<	80
2018	15	<	40	160
2018	15	<	<	80
2018	15	<	40	160
2018	15	<	<	160
2018	15	<	<	40
2018	15	<	<	40

*Titers < 40 are indicated with the sign <.

Table S10. Detailed data of positive serum samples of common household population

Year	HI antibody titer against reference strain*		
	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)
2016	<	<	40
2016	<	<	80
2016	<	<	40
2016	<	<	40
2016	<	<	40
2016	<	<	40
2016	<	<	40
2016	<	<	320
2016	<	40	320
2016	<	40	320
2016	<	<	80
2016	<	<	80
2016	<	<	40
2016	<	40	<
2016	<	<	80
2016	<	<	40
2016	<	<	40
2016	40	40	320
2016	<	<	160
2016	<	<	320
2016	<	<	160
2016	<	<	320
2016	<	<	320
2016	<	<	160
2016	40	40	<
2016	<	<	40
2016	<	<	40
2016	<	<	80
2016	<	<	320
2016	<	<	80
2016	<	<	320
2017	<	<	80
2017	<	<	40
2017	<	<	320
2017	<	<	80
2017	<	<	80
2017	<	<	40
2017	<	<	40

Year	HI antibody titer against reference strain*		
	SW/HN/08/11 (G1 EA)	SW/SD/1207/16 (G4 EA)	A/Michigan/45/2015 (pdm/09)
2017	<	<	80
2017	<	40	160
2017	<	<	40
2017	<	<	40
2017	<	<	40
2017	<	<	40
2017	<	<	40
2017	<	<	40
2017	<	<	40
2017	40	40	160
2017	<	<	40
2017	<	<	80
2017	<	<	40
2018	40	80	160
2018	160	320	320
2018	<	<	320
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	80
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	80
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	160
2018	<	<	40
2018	<	<	40
2018	<	<	40
2018	<	<	80
2018	<	40	80
2018	<	<	40

*Titers < 40 are indicated with the sign <.

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