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

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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-like (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 preexisting 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 18 y to 35 y 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.

swine influenza | Eurasian avian-like H1N1 virus | 2009 pandemic H1N1 virus | reassortant | pandemic potential

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 IAVs 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 IAVs, are regarded as "mixing vessels" in the generation of influenza viruses with pandemic potential (3–5). The emergence of the 2009 pandemic (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-like (EA) lineage SIVs cocirculating in pigs (9). EA H1N1 SIVs are found in 2001, and gradually become the dominant lineage in China (9–11). However, after 2009, the 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 between 2011 and 2018 in 10 provinces with high-density pig populations. We identified a predominant emergent EA reassortant genotype 4 (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.

Significance

Pigs are intermediate hosts for the generation of pandemic influenza virus. Thus, systematic surveillance of influenza viruses in pigs is a key measure for prewarning the emergence of the next pandemic influenza. Here, we identified a 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.

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The authors declare no competing interest.

Data deposition: 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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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 of 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. 1*A*). 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.

G4 Reassortant EA H1N1 Viruses Have Been Predominant since 2016. To show viral evolution, we conducted molecular clock phylogenic analysis and genotype characterization (Fig. 2*A* 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. 2*B*). 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 have largely disappeared since 2014 (Fig. 2*B* and *SI Appendix*, Fig. S5). G2, G3, and G6 reassortant EA viruses appeared transiently during 2011–2015. In 2013, two reassortant G4 and G5 viruses emerged in southern China (*SI Appendix*, Fig. S5). G5 virus possesses 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 has declined since 2015 and was not found in 2018 (Fig. 2*B*). Similar to G5, G4 was also a triple reassortant, except its M gene was derived from pdm/09 lineage. G4 virus has shown a sharp increase since 2016, and is the predominant genotype in circulation in pigs detected across at least 10 provinces (Fig. 2*B* 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 IAVs 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 four of the 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. S6*A*). Furthermore, all of the EA H1N1 viruses were found to bind to human tracheal epithelial lining to an extent similar to CA04 pdm/09 H1N1 virus, but control avian H5N1 virus showed no binding (*SI Appendix*, Fig. S6*B*). 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 h to 60 h postinfection (pi) than did G1 viruses ($P < 0.05$ or $P < 0.01$, ANOVA) (*SI Appendix*, Fig. S7*A*).

Infection of human A549 cells with G4 viruses gave similar progeny results. G4 and pdm/09 viruses produced more infectious virus than did G1 viruses from 24 h to 60 h pi ($P < 0.05$ or $P < 0.01$, ANOVA), reaching highest titers of $10^{7.75}$ median tissue culture infective dose ($TCID_{50}$)/mL and $10^{7.5}$ $TCID_{50}$/mL, respectively. In contrast, G1 viruses showed peak titers of $10^{6.5}$ $TCID_{50}$/mL (*SI Appendix*, Fig. S7*B*). Collectively, G4 EA reassortant viruses replicate efficiently in human airway epithelial cells, similarly to replication 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). Postmortem 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. S8*A*). 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. S8*B*). Overall, current G4 reassortant EA H1N1 viruses showed increased replication and pathogenicity in ferrets,

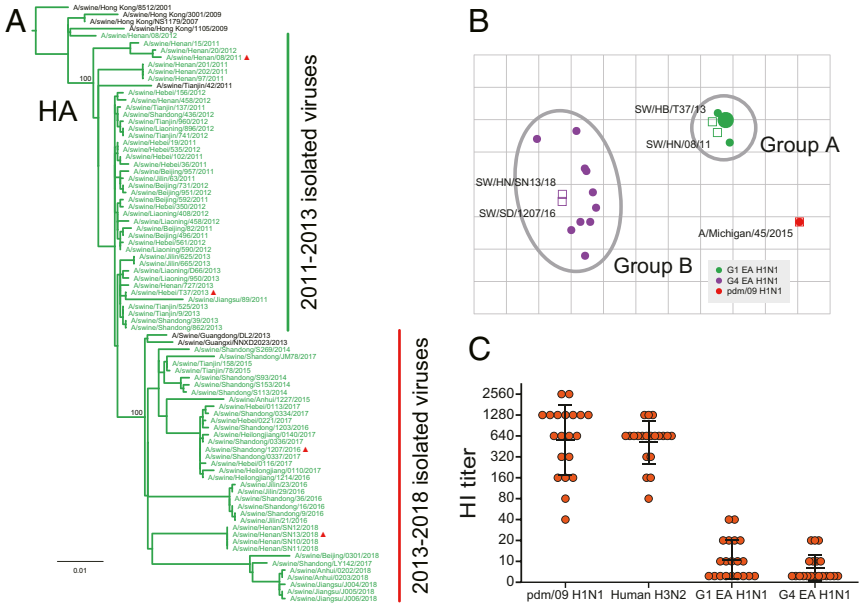

Fig. 1. Phylogenetic relationship of HA gene and antigenic characterization of EA H1N1 SIVs in China from 2011 to 2018. (*A*) Phylogenetic tree of HA gene. Phylogenetic tree was estimated using genetic distances calculated by maximum likelihood under the GTRGAMMA + I model. SIVs isolated in this study are green; sequences of viruses with names in black were downloaded from databases. 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. (Scale bar is in units of nucleotide substitutions per site.) (*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-y-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. The 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.

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 one of three RD ferrets (Fig. 3). By contrast, with G1 viruses, neither virus transmission in DC or RD groups (Fig. 3) nor seroconversion at 14 day pi 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. Preexisting 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 (10 G4 and 4 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 groups A and B (Fig. 1*B* 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- to 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 to 320) but reacted poorly with antigenic group B strains (titers ≤ 40) (*SI Appendix*, Table S5). Further analysis showed 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 needs 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-y-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. 1*C*). However, none of the serum samples cross-reacted with the G4 or even the G1 EA H1N1 virus (Fig. 1*C*). Collectively, predominant G4 reassortant EA viruses are antigenically distinct from current human influenza vaccine strains, indicating that preexisting 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

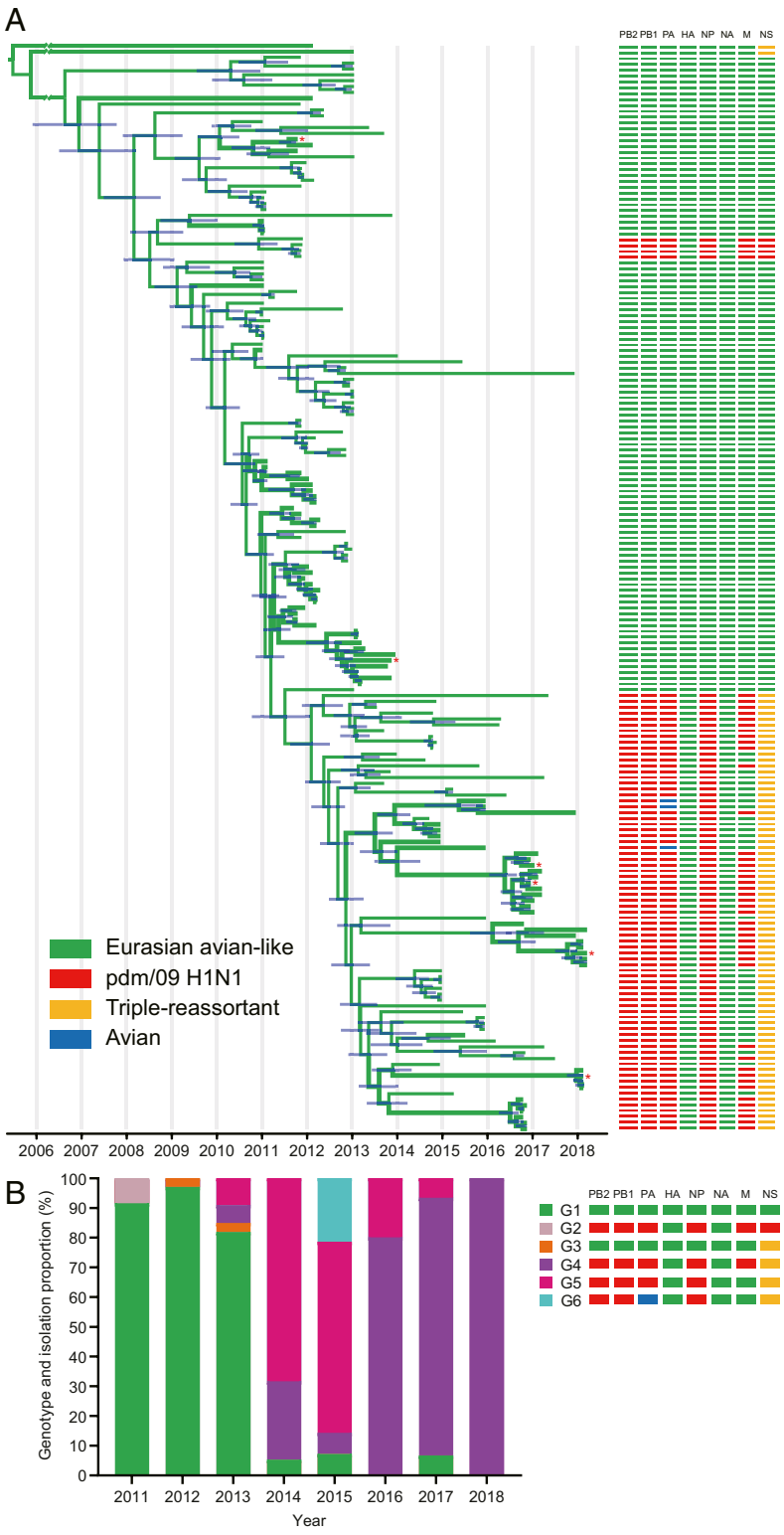

Fig. 2. Phylogenetic analysis of EA H1N1 SIVs in China from 2011 to 2018. (*A*) Phylogeny and divergence time of the HA gene and genotype evolution of EA H1N1 SIVs. The phylogenetic tree of HA gene was generated by Bayesian Markov Chain Monte Carlo framework, using the GTR substitution model with gamma-distributed 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.

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

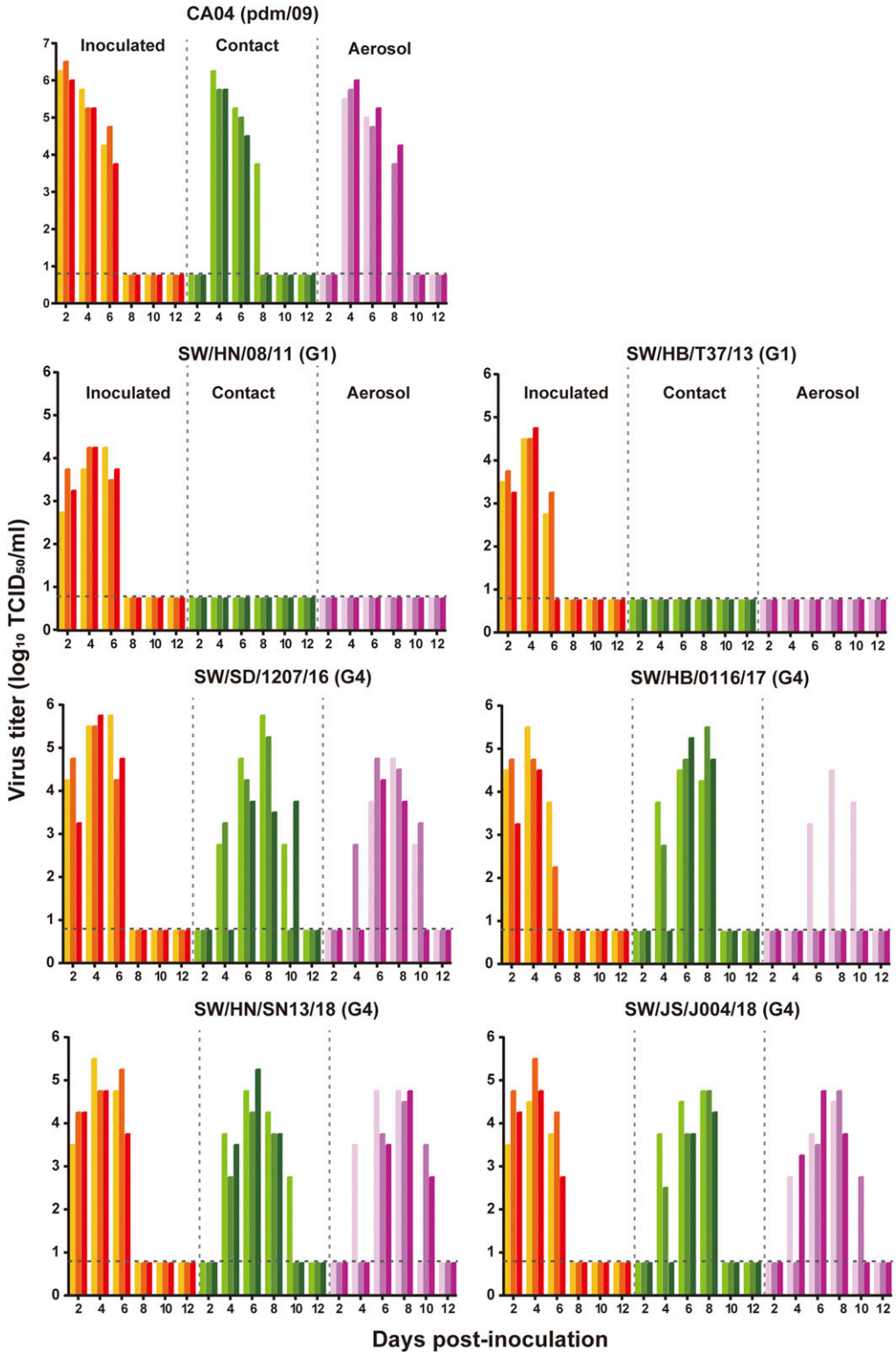

Fig. 3. Horizontal transmission of EA H1N1 viruses between ferrets. Groups of three ferrets were inoculated i.n. with 10^6 $TCID_{50}$ of indicated viruses. The next day, infected animals were individually cohoused with an uninfected DC ferret; an uninfected RD contact animal was also housed in a wire 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.

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 to 5.45], $P = 0.012$) compared with the general population group. After controlling for possible cross-reactivity with

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

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

Boldface indicates a statistically significant difference ($P < 0.05$). Model covariates: gender, age group, and collection year.

pdm/09 virus, the odds ratio remained elevated (aOR = 2.25, 95% CI [1.05 to 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, the 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 y to 35 y of age had a 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 to 7.7], $P < 0.01$) compared with other age groups (*SI Appendix*, Table S8). For gender factor, no statistically significant difference in the seroprevalence of any virus tested according to sex was 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 IAV surveillance in pigs from 2011 to 2018, we identified and characterized a predominant reassortant SIV (G4) 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 has 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 of this evidence indicates 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 five human cases of EA-like SIV infection have been reported in China (21–23, 32, 33). The first three cases were children under 3 y old, but the latest two cases, reported in 2016 and 2019, were of a 46- and a 9-y-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, possesses all of 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 the general population, who had little opportunity to contact pigs, lacked antibodies against G4 virus, but swine-exposed adult populations 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 being 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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1. Y. Kawaoka, S. Krauss, R. G. Webster, Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603–4608 (1989).
2. G. J. Smith *et al*., Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* **459**, 1122–1125 (2009).
3. W. Ma, R. E. Kahn, J. A. Richt, The pig as a mixing vessel for influenza viruses: Human and veterinary implications. *J. Mol. Genet. Med.* **3**, 158–166 (2008).
4. T. Ito *et al*., Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.* **72**, 7367–7373 (1998).
5. Y. Shi, Y. Wu, W. Zhang, J. Qi, G. F. Gao, Enabling the "host jump": Structural determinants of receptor-binding specificity in influenza A viruses. *Nat. Rev. Microbiol.* **12**, 822–831 (2014).
6. F. S. Dawood *et al*.; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med.* **360**, 2605–2615 (2009).
7. R. J. Garten *et al*., Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* **325**, 197–201 (2009).
8. Y. Guan *et al*., The emergence of pandemic influenza viruses. *Protein Cell* **1**, 9–13 (2010).
9. D. Vijaykrishna *et al*., Long-term evolution and transmission dynamics of swine influenza A virus. *Nature* **473**, 519–522 (2011).
10. H. Yang *et al*., Prevalence, genetics, and transmissibility in ferrets of Eurasian avian-like H1N1 swine influenza viruses. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 392–397 (2016).
11. J. Liu *et al*., Emergence of European avian influenza virus-like H1N1 swine influenza A viruses in China. *J. Clin. Microbiol.* **47**, 2643–2646 (2009).
12. H. M. Weingartl *et al*., Genetic and pathobiologic characterization of pandemic H1N1 2009 influenza viruses from a naturally infected swine herd. *J. Virol.* **84**, 2245–2256 (2010).
13. A. Pereda *et al*., Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. *Emerg. Infect. Dis.* **16**, 304–307 (2010).
14. H. Liang *et al*., Expansion of genotypic diversity and establishment of 2009 H1N1 pandemic-origin internal genes in pigs in China. *J. Virol.* **88**, 10864–10874 (2014).
15. H. Zhu *et al*., Novel reassortment of Eurasian avian-like and pandemic/2009 influenza viruses in swine: Infectious potential for humans. *J. Virol.* **85**, 10432–10439 (2011).
16. P. He *et al*., Novel triple-reassortant influenza viruses in pigs, Guangxi, China. *Emerg. Microbes Infect.* **7**, 85 (2018).
17. Y.-F. Sun *et al*., Novel triple-reassortant H1N1 swine influenza viruses in pigs in Tianjin, Northern China. *Vet. Microbiol.* **183**, 85–91 (2016).
18. Z. Cao *et al*., Continuous evolution of influenza A viruses of swine from 2013 to 2015 in Guangdong, China. *PLoS One* **14**, e0217607 (2019).
19. S. J. Watson *et al*.; ESNIP3 Consortium, Molecular epidemiology and evolution of influenza viruses circulating within European swine between 2009 and 2013. *J. Virol.* **89**, 9920–9931 (2015).
20. D. Vijaykrishna *et al*., Reassortment of pandemic H1N1/2009 influenza A virus in swine. *Science* **328**, 1529 (2010).
21. W. Zhu *et al*., Reassortant Eurasian avian-like influenza A(H1N1) virus from a severely ill child, Hunan province, China, 2015. *Emerg. Infect. Dis.* **22**, 1930–1936 (2016).
22. J.-F. Xie *et al*., Emergence of Eurasian avian-like swine influenza A (H1N1) virus from an adult case in Fujian province, China. *Virol. Sin.* **33**, 282–286 (2018).
23. X. Li *et al*., Human infection with a novel reassortant Eurasian-avian lineage swine H1N1 virus in northern China. *Emerg. Microbes Infect.* **8**, 1535–1545 (2019).
24. T. K. Anderson *et al*., A phylogeny-based global nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza A viruses. *MSphere* **1**, e00275-16 (2016).
25. R. J. Connor, Y. Kawaoka, R. G. Webster, J. C. Paulson, Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* **205**, 17–23 (1994).
26. M. Matrosovich *et al*., Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J. Virol.* **74**, 8502–8512 (2000).
27. J. A. Maher, J. DeStefano, The ferret: An animal model to study influenza virus. *Lab Anim. (NY)* **33**, 50–53 (2004).
28. X.-H. Song *et al*., Serological surveillance of influenza A virus infection in swine populations in Fujian province, China: No evidence of naturally occurring H5N1 infection in pigs. *Zoonoses Public Health* **57**, 291–298 (2010).
29. M. Pensaert, K. Ottis, J. Vandeputte, M. M. Kaplan, P. A. Bachmann, Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. *Bull. World Health Organ.* **59**, 75–78 (1981).
30. C. Scholtissek, H. Bürger, P. A. Bachmann, C. Hannoun, Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. *Virology* **129**, 521–523 (1983).
31. A. Krumbholz *et al*., Origin of the European avian-like swine influenza viruses. *J. Gen. Virol.* **95**, 2372–2376 (2014).
32. D.-Y. Wang *et al*., Human infection with Eurasian avian-like influenza A(H1N1) virus, China. *Emerg. Infect. Dis.* **19**, 1709–1711 (2013).
33. X. Qi *et al*., Antigenic and genetic characterization of a European avian-like H1N1 swine influenza virus from a boy in China in 2011. *Arch. Virol.* **158**, 39–53 (2013).
34. G. Wang *et al*., Characterization of swine-origin H1N1 canine influenza viruses. *Emerg. Microbes Infect.* **8**, 1017–1026 (2019).
35. J. A. Pulit-Penaloza, J. A. Belser, T. M. Tumpey, T. R. Maines, Mammalian pathogenicity and transmissibility of a reassortant Eurasian avian-like A(H1N1v) influenza virus associated with human infection in China (2015). *Virology* **537**, 31–35 (2019).