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Analysis of Recombinant H7N9 Wild-Type and Mutant Viruses in Pigs Shows that the Q226L Mutation in HA Is Important for Transmission

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ABSTRACT

The fact that there have been more than 300 human infections with a novel avian H7N9 virus in China indicates that this emerging strain has pandemic potential. Furthermore, many of the H7N9 viruses circulating in animal reservoirs contain putative mammalian signatures in the HA and PB2 genes that are believed to be important in the adaptation of other avian strains to humans. To date, the definitive roles of these mammalian-signature substitutions in transmission and pathogenesis of H7N9 viruses remain unclear. To address this we analyzed the biological characteristics, pathogenicity, and transmissibility of A/Anhui/1/2013 (H7N9) virus and variants *in vitro* and *in vivo* using a synthetically created wild-type virus (rAnhui-WT) and two mutants (rAnhui-HA-226Q and rAnhui-PB2-627E). All three viruses replicated in lungs of intratracheally inoculated pigs, yet nasal shedding was limited. The rAnhui-WT and rAnhui-PB2-627E viruses were transmitted to contact animals. In contrast, the rAnhui-HA-226Q virus was not transmitted to sentinel pigs. Deep sequencing of viruses from the lungs of infected pigs identified substitutions arising in the viral population (e.g., PB2-T271A, PB2-D701N, HA-V195I, and PB2-E627K reversion) that may enhance viral replication in pigs. Collectively, the results demonstrate that critical mutations (i.e., HA-Q226L) enable the H7N9 viruses to be transmitted in a mammalian host and suggest that the myriad H7N9 genotypes circulating in avian species in China and closely related strains (e.g., H7N7) have the potential for further adaptation to human or other mammalian hosts (e.g., pigs), leading to strains capable of sustained human-to-human transmission.

IMPORTANCE

The genomes of the zoonotic avian H7N9 viruses emerging in China have mutations in critical genes (PB2-E627K and HA-Q226L) that may be important in their pandemic potential. This study shows that (i) HA-226L of zoonotic H7N9 strains is critical for binding the α-2,6-linked receptor and enables transmission in pigs; (ii) wild-type A/Anhui/1/2013 (H7N9) shows modest replication, virulence, and transmissibility in pigs, suggesting that it is not well adapted to the mammalian host; and (iii) both wild-type and variant H7N9 viruses rapidly develop additional mammalian-signature mutations in pigs, indicating that they represent an important potential intermediate host. This is the first study analyzing the phenotypic effects of specific mutations within the HA and PB2 genes of the novel H7N9 viruses created by reverse genetics in an important mammalian host model. Finally, this study illustrates that loss-of-function mutations can be used to effectively identify residues critical to zoonosis/transmission.

Annual influenza A virus (IAV) epidemics result in 250 to 500 million human infections, which cause 250,000 to 500,000 fatalities worldwide (1) and tens of thousands of deaths in the United States annually (2). The total burden of IAV can escalate dramatically when new pandemic influenza virus strains emerge. For example, a new influenza virus subtype introduced into the human population in 1918 led to the "Spanish flu" pandemic that killed an estimated 50 to 100 million people (3). IAVs have a negative-sense RNA genome consisting of eight segments that can reassort, or mix, during coinfection within an individual cell or animal. Additionally, genetically diverse IAVs with pandemic potential circulate in animal reservoirs, particularly poultry and swine, and are a continual and unpredictable threat to animal and public health.

The zoonotic H7N9 IAV viruses, which emerged in China in March 2013, have resulted in more than 300 infections and 114 deaths. Because of the lack of an existing immunity against H7 subtype influenza viruses in the human population, the H7N9 virus is of concern as a potential cause of a pandemic (4–6). These H7N9 viruses are reassortants containing avian-lineage genomic RNA segments of viruses that circulated in waterfowl and terrestrial birds (e.g., chicken and brambling) (7–9). Importantly, genomic analysis of human H7N9 isolates indicates that they contain several amino acid changes in multiple gene segments, and some have been hypothesized to be important in the adaptation of

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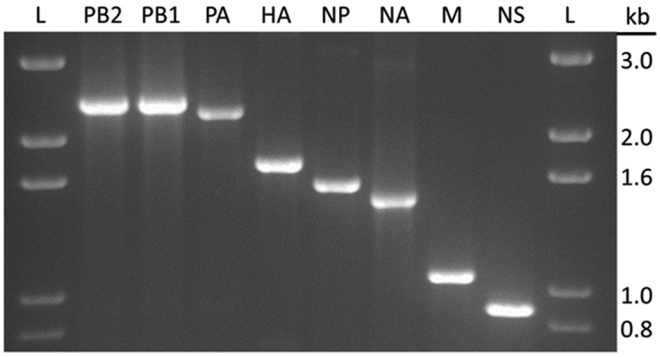

FIG 1 Synthetic generation of the eight full-length genomic segments of the A/Anhui/1-JCVI.1/2013 virus. The products were assembled from oligonucleotides and error corrected. L, 1-kb Plus DNA ladder from Life Technologies.

avian viruses to humans and other mammals ("mammalian-signature" amino acid motifs). Of particular interest are the Q226L (H3 numbering; Q235L, numbering from the initiating methionine of H7) substitution in the sialic acid receptor binding domain of the H7 HA and the E627K substitution in the PB2 protein, which is a subunit of the heterotrimeric viral RNA-dependent RNA polymerase (RDRP). Both mutations have been implicated in the adaptation of different lineage avian IAVs (e.g., H5N1) to humans/mammals (10–14).

It is unclear how this H7N9 lineage will continue to evolve in various avian species, or potentially in swine. Swine have been shown to play a role in the emergence of pandemic influenza A viruses in the past (15–19). Although there is no evidence of H7N9 infection of pigs thus far, hundreds of human infections occurred over a large geographic region in China in a short period of time, suggesting that this H7N9 lineage is widespread in China (20, 21). The close contact between pigs and poultry and humans in China provides continual opportunities for transmission of H7N9 viruses to pigs. Based on the H7N9 genome sequence data from GenBank, more than 99% (82/84) of H7N9 viruses isolated from avian species in China contain PB2-627E, and 66% (67/102) of H7N9 avian strains contain HA-226Q. However, the pathogenesis and transmissibility of the H7N9 viruses containing PB2-627E or HA-226Q in pigs remain unknown. Furthermore, the roles the putative mammalian signatures in the H7N9 virus (i.e., HA-226L and PB2-627K) play in pathogenesis and/or transmissibility remain unclear. In this study, we employed reverse genetics of H7N9 and the pig model to investigate pathogenicity and transmissibility of H7N9 and potentially critical mammalian adaptive mutations in pigs.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells and porcine kidney (PK-15) cells were maintained in Eagle's minimal essential medium (EMEM) with 5% fetal bovine serum (FBS), L-glutamine (Invitrogen, CA), MEM vitamins (Invitrogen, CA), and 1% antibiotic (Invitrogen, CA). Human embryonic kidney (HEK) 293T cells and human lung epithelial (A549) cells were maintained in Opti-modified Eagle's medium (Opti-MEM) supplemented with 10% FBS (HyClone, UT).

Complete genome synthesis and plasmid construction. Nucleotide sequences of the eight gene segments of A/Anhui/1/2013 (H7N9) were retrieved from the EpiFlu database (www.gisaid.org). The synthetic genomics approach used a total of 396 oligonucleotides, 69 to 71 bases in length, which were designed for enzymatic assembly of the eight segments; the noncoding regions inferred by database analysis were also incorporated. The assembly and error correction processes were performed as recently described (22), with minor modifications, such as increased time at all extension steps (from 72°C for 1 min to 72°C for 2 min) for efficient assembly of the polymerase segments. The synthesized segments (Fig. 1) were cloned into the modified bidirectional influenza virus reverse genetics vectors pBZ61A18 (PB2, PB1, and PA) and pBZ61A15 (HA, NP, NA, M, and NS) using the recombination-based method (23) and transformed into Stella-competent *Escherichia coli* cells (Clontech). Colonies were selected and sequenced. The appropriate clones for each segment were propagated for plasmid preparation and verified by sequencing to preclude any mutations or deletions that can emerge in the bacteria if any of the cloned genes are unstable (24). The resulting plasmids are pBZ187A2 (PB2), pBZ188A6 (PB1), pBZ189A26 (PA), pBZ190A3 (HA), pBZ191A1 (NP), pBZ192A3 (NA), pBZ193A2 (M), and pBZ194A4 (NS) (Table 1). The plasmids pWW98A5 (rAnhui-PB2-627E) and pWW101A2 (rAnhui-HA-226Q), with single substitutions, were generated by site-directed mutagenesis using pBZ187A2 and pBZ190A3, respectively, as templates. All constructed plasmids were confirmed by sequencing.

Minigenome assay. The luciferase-mediated minigenome assay was performed as previously described (25). Briefly, human embryonic kidney (293T) cells, human lung epithelial (A549) cells, and porcine kidney (PK-15) cells in 24-well plates were cotransfected with 0.2 μg each of the PB2, PB1, PA, NP, and pPolI-NS-Luc plasmids (pBZ81A36) (25). To control for transfection efficiency, 0.02 μg of the *Renilla* luciferase plasmid pRL-TK (Promega) was also cotransfected. After 18 to 24 h of incubation at 33°C, 37°C, 39°C, and 40°C, luciferase production was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase expression was normalized to *Renilla* luciferase expression (relative activity). For each cell line at each temperature, three independent replicates were conducted, the luciferase activity of the recombinant wild-type H7N9 polymerase was set at 100%, and the activities of the rAnhui-PB2-627E and other viruses were presented relative to that of the wild-type H7N9. GraphPad Prism 5 was used to generate the figures, and one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test was used to determine the significance of the differences ($P < 0.05$) between the wild-type H7N9 polymerase and other polymerases. All results shown are the averages from triplicate experiments, and the standard deviations (SD) are shown. To compare the polymerase activity of the wild-type H7N9 with other influenza viruses, polymerase and NP plasmids from A/PR/8/1934 (H1N1), A/New York/238/2005 (H3N2), A/New York/1682/2009 (pandemic H1N1 [pH1N1]), and A/Hong Kong/213/2003 (H5N1) viruses were employed.

TABLE 1 Plasmids used for rescuing H7N9 viruses

Influenza gene	Plasmid	Description
PB2	pBZ187A2	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) PB2
PB1	pBZ188A6	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) PB1
PA	pBZ189A26	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) PA
HA	pBZ190A3	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) HA
NP	pBZ191A1	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) NP
NA	pBZ192A3	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) NA
M	pBZ193A2	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) M
NS	pBZ194A4	Wild-type A/Anhui/1-JCVI.1/2013 (H7N9) NS
PB2-627E	pWW98A5	A/Anhui/1-JCVI.1/2013 (H7N9) PB2 with a lysine (K)-to-glutamic acid (E) mutation at position 627
HA-235Q	pWW101A2	A/Anhui/1-JCVI.1/2013 (H7N9) HA with a leucine (L)-to-glutamine (Q) mutation at position 235[a] (226[b])

[a] H7 numbering from the first methionine.
[b] H3 structure numbering.

TABLE 2 H7N9 viruses used in the pig infection study

Virus[a]	Details
A/Anhui/1-JCVI.1/2013 (rAnhui-WT)	Wild-type Anhui/1 H7N9 with the mammalian-signature HA (226L[c]) and PB2 (627K) based on sequences from GenBank
rAnhui-HA-226Q[c] (235Q[b])	Avian-like Anhui/1 H7N9 with the avian-signature residue 226Q[c] (235[b]) in the HA
rAnhui-PB2-627E (627E)	Mammalian-like Anhui/1 H7N9 with the avian-signature residue 627E in the PB2

[a] All viruses were reverse genetics derived.
[b] H7 numbering from the first methionine.
[c] H3 structure numbering.

Viruses. Three recombinant H7N9 viruses, including rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E used in this study were generated using synthetic genomics and reverse genetics (Table 2). Briefly, an eight-plasmid (i.e., pBZ187A2, pBZ188A6, pBZ189A26, pBZ190A3, pBZ191A1, pBZ192A3, pBZ193A2, and pBZ194A4) reverse-genetics system was used to generate the rAnhui-WT virus as previously described (26). The pBZ190A3 plasmid was replaced with pWW101A2 to rescue the rAnhui-HA-226Q virus, which contains an L-to-Q substitution at HA residue 226 (H3 numbering system). To generate the rAnhui-PB2-627E virus, which contains a K-to-E substitution at PB2 residue 627, pBZ187A2 was replaced with pWW98A5. All viruses were propagated in MDCK cells and sequence verified prior to the pig infection study. Each challenge virus was diluted in MEM to a final concentration of 10^6 50% tissue culture infective doses ($TCID_{50}$)/ml. A/chicken/Jena/4836/1983 (H2N2) and A/Brisbane/59/2007 (H1N1) were propagated in chicken embryos.

Receptor binding assay. The receptor binding preference of three reverse-genetics-rescued H7N9 viruses was analyzed by hemagglutinating receptor-specific red blood cells (RBCs), including normal turkey red blood cells (containing both α-2,6 and α-2,3 receptor), α-2,3-specific-neuraminidase-treated turkey red blood cells (containing only α-2,6 receptor after treatment), and sheep red blood cells (mainly expressing α-2,3 receptor) (27). Both turkey and sheep red blood cells were washed 3 times before use (Colorado Serum Company, Denver, CO). The α-2,6 receptor-specific red blood cells were prepared by treating turkey red blood cells with α-2,3 specific neuraminidase (NEB, MA). Briefly, 10% RBC in 1,000 μl were incubated at 37°C in the presence of 1,000 IU α-2,3-specific neuraminidase for 1 h. Treated red blood cells were washed three times with PBS before use. The final working solution was 0.5% RBCs in PBS for the hemagglutination assay. To determine the specific receptors on the treated and untreated red blood cells, the avian influenza virus A/chicken/Jena/4836/1983 (H2N2) and human influenza virus A/Brisbane/59/2007(H1N1) were included in the receptor binding assay as controls. Hemagglutination assays were performed in V-bottom microtiter plates by incubating equal volumes (50 μl) of 2-fold serially diluted virus and different red blood cells. The hemagglutination titer was defined as the reciprocal of the highest virus dilution that hemagglutinated red blood cells.

Real-time RT-PCR assay. The real-time reverse transcription-PCR (RT-PCR) assay was performed based on the protocol for detection of novel H7N9 virus developed by the WHO Collaborating Center for Reference and Research on Influenza at the Chinese National Influenza Center (Beijing, China), with modifications (28). Virus RNAs were extracted from 100 mg of tissue using with an RNeasy Plus minikit (Qiagen). cDNA was synthesized by using universal 12 primer (5′-AGCRAAAGCAGG-3′) for influenza virus and SuperScript III reverse transcriptase (Invitrogen, USA). RNA copy numbers of the influenza virus HA gene segment were determined by quantitative real-time PCR in a C1000 Touch thermal cycler (Bio-Rad, USA). Briefly, specific primers and probe targeting the HA gene of the novel H7N9 (forward primer, 5′-AGAAATGAAATGGC TCCTGTCAA-3′; reverse primer, 5′-GGTTTTTTCTTGTATTTTTATA TGACTTAG-3′; and probe, 5′FAM-AGATAATGCTGCATTCCCGCAG ATG-BHQ1–3′) were synthesized by Biosearch Technologies (USA) as described previously (28). The quantitative PCR was conducted in a 25-μl reaction volume, containing 12.5 μl 2× TaqMan real-time PCR master mixes (Invitrogen, USA), 1 μl of each primer (20 μM), 1 μl of probe (10 μM), 4.5 μl RNase-free water, and 5 μl cDNA. The virus RNA copy numbers were determined based on the assay detecting the standard positive plasmid (pBZ190A3-HA) as a molecular reference (detection limit is 10 molecules). The thermocycling conditions were as follows: 95°C for 10 min, then 40 cycles of 95°C for 10 s and 60°C for 45 s. The specimen was considered positive if the cycle threshold (C_T) value was ≤38.0.

Genomic amplification and next-generation sequencing. Viral RNA was extracted from bronchoalveolar lavage fluid (BALF) of inoculated pigs, and the complete genome was amplified using the multisegment RT-PCR strategy as previously described (23). Positive PCR products were subjected to deep sequencing using an Illumina MiSeq personal sequencer and an Ion Torrent PGM sequencer. Sequencing coverage varied from a few hundred reads to many thousands of reads for each nucleotide position, depending on the quantity of multisegment RT-PCR products. Results were analyzed using CLC Genomics Workbench. At each particular nucleotide site, if a variant represented more than 10% of the population (cutoff value = 10), it was counted as a mutation or single-nucleotide variant (SNV).

Pig experiments. The pig study was conducted in biosafety level 3 agriculture (BSL-3Ag) facilities located at the Biosecurity Research Institute at Kansas State University and approved by the Institutional Animal Care and Use Committee at Kansas State University. Three- to 4-week-old pigs (American Yorkshire) that were serum-negative for swine influenza viruses and porcine reproductive and respiratory syndrome were used in this study.

Experimental design. To evaluate the pathogenicity and transmissibility of all three H7N9 viruses (rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E) in pigs, 44 3- to 4-week-old pigs were randomly allocated into 4 groups in separate rooms (12 pigs in each inoculated group and 8 pigs in the control group). Nine pigs from each inoculated group and 8 control pigs were intratracheally inoculated with 10^6 $TCID_{50}$ of each virus (rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E) or virus-free MEM, respectively. Three naive contact pigs were commingled with inoculated animals in each virus group at 3 days postinfection (dpi) to investigate viral transmission (Fig. 2).

Clinical observation. All inoculated and sentinel pigs were observed twice daily for clinical signs (i.e., sneezing, coughing, nasal discharge, respiratory distress) until the end of the experiment (7 dpi). Body temperature was checked and recorded daily after challenge as well as before challenge.

Pathogenesis, virus replication and transmission in pigs. Three pigs from each inoculated group and 2 or 3 control pigs were necropsied at 3, 5, and 7 dpi, and 3 contact pigs were euthanized at 4 days postcontact (dpc). At necropsy the lungs were removed *in toto* from pigs. To investigate virus replication in the lungs, the lungs were flushed with 50 ml fresh MEM to obtain bronchoalveolar fluid (BALF) from each euthanized pig for virus detection and titration as described previously (29). Viral titers in BALF were determined in MDCK cells cultured in 96-well plates, and cytopathic effect (CPE) was observed and recorded daily until 72 h postinfection (hpi). Inoculated MDCK cells were fixed after 72 hpi and immunostained by using NP monoclonal antibody (ATCC clone HB65) to confirm the CPE results. To evaluate nasal viral shedding and transmission in pigs, nasal swabs were collected from inoculated pigs on 0, 3, 5, and 7 dpi and from contact pigs on 0, 2, and 4 dpc. BALF samples were also collected from all contact pigs for virus detection. Virus titers of nasal swabs and BALF samples were determined in MDCK cells as described previously (29). Blood samples were collected from all pigs before challenge and at the time of necropsy.

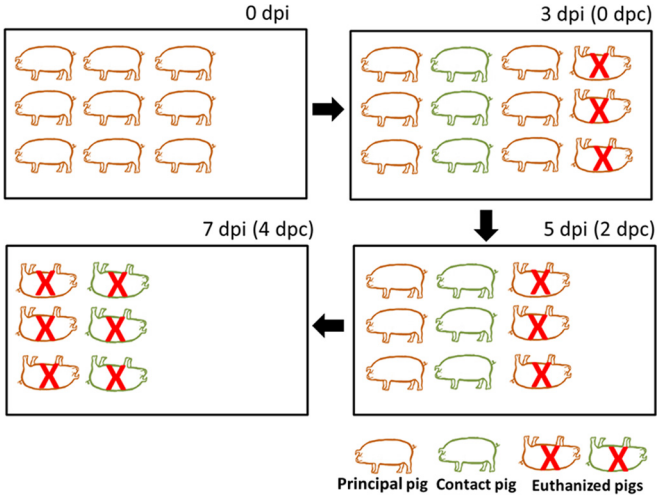

FIG 2 Experimental design for the pig infection study. Forty-four 3- to 4-week-old pigs were randomly assigned to 4 groups housed in separate rooms (12 pigs in each inoculated group and 8 pigs in the mock-inoculated control group). Nine pigs in each group were intratracheally inoculated with 10^6 $TCID_{50}$ of the appropriate H7N9 virus on day 0. Three contact pigs were commingled with each inoculated group at 3 days postinfection (dpi). Three inoculated pigs from each group were necropsied at 3, 5 and 7 dpi, and the 3 contact pigs were sacrificed at 4 days postcontact (dpc).

Macroscopic and microscopic pathology. At necropsy the lungs were removed *in toto* from pigs. The percentages of influenza gross lesions on each lung lobe were scored by a single experienced veterinarian before flushing with 50 ml of MEM to collect BALF samples, and an overall averaged percentage per animal was calculated based on individual lung lobe scores. For histopathologic examination, tissue samples from tracheas, right cardiac lung lobes, and other affected lung lobes were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E). Lung sections were examined by a veterinary pathologist in a blinded fashion, and a score from 0 to 3 was assigned to reflect the severity of bronchial epithelial injury, as described previously (29). Lung sections were scored (on a scale of 0 to 3) for the severity of bronchointerstitial pneumonia (0 = no significant lesions, 1 = mild lesions, 2 = moderate lesions, 3 = severe lesions). The following criteria were used to grade lungs: bronchiolar/alveolar epithelial degeneration and necrosis, neutrophilic inflammation in alveoli/bronchioles, interstitial pneumonia, bronchiolar submucosal lymphocytic inflammation, peribronchiolar lymphocytic inflammation, and bronchiolar/alveolar epithelial regeneration and hyperplasia.

Statistical analysis. Polymerase activity, virus titers, temperature, and macroscopic and microscopic lung scores were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad software Inc., CA); a P value of <0.05 was considered statistically significant. Those response variables were subjected to comparisons for all pairs by using the Tukey-Kramer test. Pairwise mean comparisons between inoculated and control groups were made using the Student t test.

RESULTS

Rapid generation of H7N9 viruses using synthetic genomics technology. To characterize and assess the pandemic potential of the H7N9 viruses, the whole genome derived from the consensus sequence of the original A/Anhui/1/2013 (H7N9) virus isolate was synthesized (designated rA/Anhui/1-JCVI.1/2013 [rAnhui-WT]) from the enzymatic assembly of 396 oligonucleotides (Fig. 1) (22), and cloned into reverse-genetics plasmids (Table 1) (23). We hypothesized that the putative mammalian-signature-to-avian-signature substitutions would be loss-of-function mutations in mammalian hosts and that they could be safely engineered, rescued, and analyzed. Thus, comparing the replication, pathogenesis, and/or transmission of the rAnhui-WT with that of the loss-of-function mutants would demonstrate the role that putative key mammalian-signature substitutions play *in vitro* and in pigs, which are an important reservoir host ("mixing vessel") and experimental model. Consequently, the rAnhui-WT virus and two mutants containing the PB2-K627E (rAnhui-PB2-627E) or HA-L226Q (rAnhui-HA-226Q) substitutions were synthesized and rescued (Table 2) in a biosafety level 3 laboratory using enhanced precautions which are in place at the BSL-3Ag Biosecurity Research Institute (BRI) at Kansas State University (KSU).

Critical roles of the PB2-E627K and HA-Q226L substitutions determined *in vitro*. The putative mammalian-signature substitution PB2-E627K has been studied in other avian IAV lineages, and it typically enhances their replication in mammalian cells at lower temperatures (10, 30–33). However, in some virus strains, such as the 2009 H1N1 pandemic virus (pH1N1), PB2-E627K has little if any impact because of other compensatory mutations (25, 34–37). To compare the RNA polymerase activity of the zoonotic H7N9 with that of contemporary human viral polymerases and analyze the effect of the mammalian-signature PB2-627K substitution at the molecular level, we evaluated the RNA polymerase activity in both human (HEK293T and A549) and porcine (PK-15) cells using a luciferase-mediated minigenome assay. In all of the cells, especially at lower temperatures (e.g., 33°C), the rAnhui-WT (H7N9-WT) RNA polymerase showed significantly higher activity than the polymerases from pH1N1 and zoonotic H5N1 viruses (Fig. 3). In contrast, introducing a single avian-signature substitution, PB2-627E, into the H7N9 RNA polymerase significantly decreased enzymatic activity ($P < 0.05$), although the effect was less dramatic in PK-15 cells than in the human cells, especially at higher temperatures (Fig. 3).

Influenza virus-host cell receptor interactions play a critical role in tissue tropism and species specificity. The receptor binding specificity of the HA on the surface of the rAnhui-WT and recombinant variants was examined to analyze preference for avian receptors (sialic acids attached to galactose via an α-2,3 linkage) or mammalian receptors (sialic acids attached to galactose via an α-2,6 linkage). The receptor binding specificity assay showed that the rAnhui-WT and rAnhui-PB2-627E viruses (all viruses have HA-226L) bound to both α-2,3 and α-2,6 receptors equally well, while the rAnhui-HA-226Q virus bound much better to α-2,3 receptors than to α-2,6 receptors (Table 3). Thus, the HA-Q226L substitution identified in human H7N9 isolates is critical for α-2,6 receptor binding specificity and likely facilitates zoonotic transmission. These *in vitro* results suggest that the PB2-E627K and HA-Q226L substitutions in the novel H7N9 viruses may be critical for transmission, replication, and virulence in humans and may have evolved in infected humans or terrestrial poultry, which express both α-2,3 and α-2,6 receptors in their respiratory tracts.

Clinical symptoms and pathology of the rAnhui-WT and recombinant variants in pigs. Pigs have played a central role in the evolution of many influenza viruses, and we hypothesized that infection of pigs with the rAnhui-WT, rAnhui-HA-226Q, or rAnhui-PB2-627E viruses would delineate the importance of specific amino acid substitutions in the infection of pigs, and by extension their putative role in human infection and transmissibility. To test our hypothesis, forty-four 3- to-4-week-old piglets

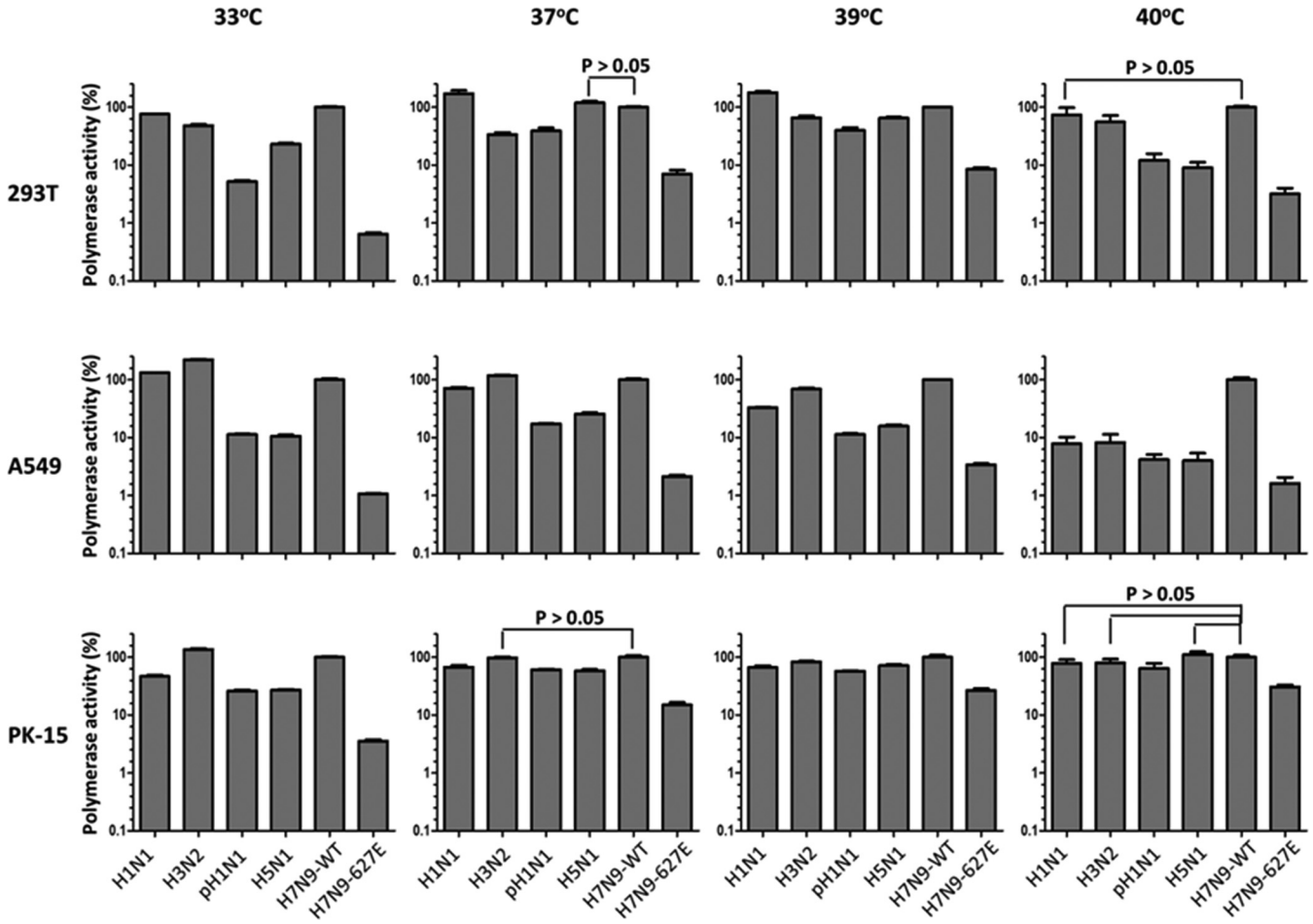

FIG 3 Comparison of RNA polymerase activity of the H7N9 virus with contemporary human or avian viruses and analysis of the effect of the PB2-K627E substitution on the rAnhui-WT (H7N9-WT) polymerase activity. RNA polymerase activities from a range of human and avian viruses were compared: H1N1, A/PR/8/1934; H3N2, A/New York/238/2005; pH1N1, A/New York/1682/2009; H5N1, A/Hong Kong/213/2003; H7N9-WT (rAnhui-WT); H7N9-627E (rAnhui-PB2-627E) polymerase substitution mutant. For each cell line at each temperature, the polymerase activity of H7N9-WT was set at 100%, and all other viruses were compared to H7N9-WT. Each virus was significantly different than the H7N9-WT using a one-way ANOVA with Dunnett's multiple-comparison test ($P < 0.05$), unless noted on the figure as having no significant difference ($P > 0.05$). Bars represent means ± SD for 3 independent replicates.

were randomly assigned to 4 groups housed in separate biocontainment rooms (12 pigs in each inoculated group [principal plus sentinel animals] and 8 pigs in the control group) in the BSL-3Ag BRI facility at KSU. Nine pigs from each group were intratracheally inoculated with 10^6 $TCID_{50}$ of the three H7N9 strains (rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E), and 8 pigs were mock inoculated as a control group. As shown in Fig. 2, three pigs from each group were sacrificed at 3 days postinfection (dpi); subsequently, three naive sentinel animals were commingled with each of the inoculated groups to investigate transmissibility of the recombinant wild-type H7N9 virus and variants in a direct-contact scenario. At 5 and 7 dpi, we necropsied three inoculated pigs from the individual groups, except for 7 dpi, when we sacrificed two mock-inoculated control animals. The sentinel contact animals (three pigs per group except for the mock-inoculated control group) were sacrificed 4 days postcontact (dpc), which was 7 dpi for the principal inoculated animals. No obvious clinical signs (i.e., sneezing, coughing, nasal discharge, or respiratory distress) were observed in any of the animals throughout the observation period. Elevated body temperatures were observed in some pigs (<50%) inoculated with rAnhui-WT (1, 3, and 5 dpi), rAnhui-HA-226Q (1, 3, and 4 dpi), and rAnhui-PB2-627E (1, 3, and 5 dpi) viruses (Fig. 4). Necropsy revealed macroscopic lung lesions typical of influenza (plum-colored, consolidated areas) in

TABLE 3 Virus receptor binding specificity of recombinant H7N9 viruses, avian H2N2 and human H1N1 influenza viruses

Virus	HA-226	Receptor binding specificity[a]		
		α-2,3 + α-2,6	α-2,6	α-2,3
rAnhui-WT	L	256	128	128
rAnhui-PB2-627E	L	256	128	256
rAnhui-HA-226Q	Q	256	4	256
A/chicken/Jena/4836/1983(H2N2)	Q	256	2	128
A/Brisbane/59/2007(H1N1)	—[b]	512	512	2

[a] Values are titers, calculated as the reciprocal of the highest virus dilution that hemagglutinated RBCs bearing various sialic acid receptors.
[b] Not applicable.

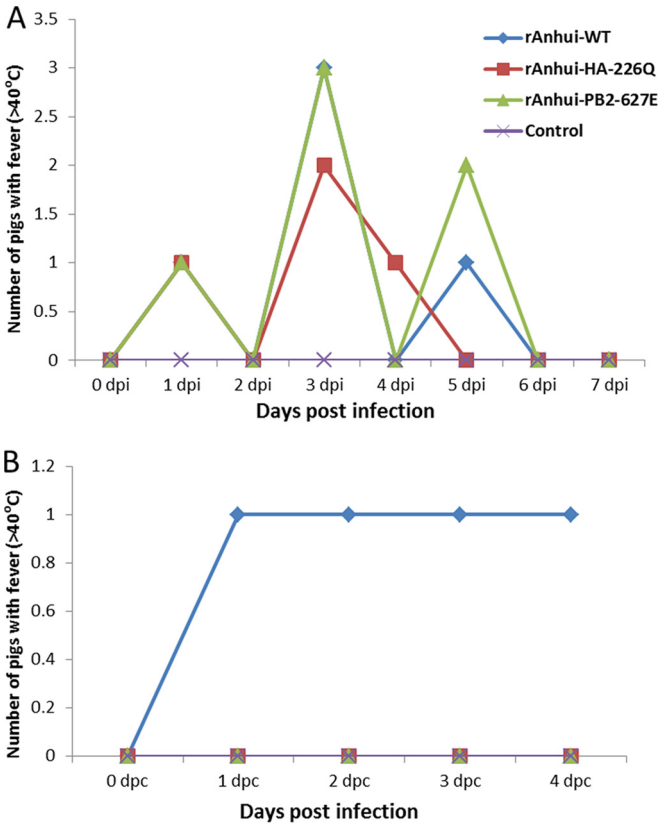

FIG 4 Fever in inoculated and contact pigs. (A) Body temperature changes in pigs that were inoculated with the indicated H7N9 viruses; (B) body temperature changes in contact pigs commingled with pigs that were inoculated with the indicated H7N9 viruses.

virus-inoculated pigs (Fig. 5 and Table 4). Minor, nonspecific lung lesions were infrequently found in control pigs (Table 4), but the lungs from the mock-infected animals were confirmed to be negative for influenza A viruses by virus isolation and influenza virus M-gene-specific real-time RT-PCR assays (including nasal swabs, BALF, and lung tissues) (38, 39). The rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E viruses caused lung lesions in pigs on each necropsy day; however, no significant gross pathological differences were found between the 3 groups (Table 4). Intriguingly, the contact animals in the rAnhui-WT (3/3) and rAnhui-PB2-627E (1/3) groups showed macroscopic lesions typical of influenza (Table 4). In contrast, only very mild gross lung lesions were observed in the rAnhui-HA-226Q contact animals (Table 4), and no virus or viral RNA were detected from any tissue derived from the rAnhui-HA-226Q contact animals (Table 5).

For histopathological examination, tissue samples from the trachea, right cardiac lung lobe, and other affected lung lobes were fixed in 10% buffered formalin, processed, and stained with hematoxylin and eosin (H&E). A histopathological score (0, 1, 2, or 3) was assigned by a board-certified veterinary pathologist in a blinded fashion to reflect the extent of damage to the lung architecture and the severity of bronchial epithelial injury. All three H7N9 variants caused mild to moderate bronchointerstitial pneumonia at 3, 5, or 7 dpi (Table 4) compared to the control group. The three H7N9 viruses preferentially caused lesions in the alveolar region with primarily neutrophilic inflammation in the interstitium and alveoli and very mild bronchiolar epithelial degeneration and necrosis (Fig. 6). No significant differences in the histopathological lung lesions were observed at 3, 5, and 7 dpi between the three groups. The lung sections of the contact animals showed minimal microscopic lung lesions in the rAnhui-WT and rAnhui-PB2-627E groups; none of the three pigs in the rAnhui-HA-226Q virus group showed microscopic lung lesions, suggesting that rAnhui-HA-226Q was not transmitted to contact animals, in contrast to other two H7N9 viruses tested in pigs (Table 4). Some control pigs had an occasional small focus of mild interstitial pneumonia (Table 4), but they were negative for influenza virus infection, as tested by virus isolation and real-time RT-PCR.

H7N9 viruses replicate in pigs, and HA-226L is an important determinant for swine transmission. To investigate viral replication in the tissues of inoculated pigs, infectious virus titers in the bronchoalveolar lavage fluid (BALF) and genome copy numbers in the cardiac lobe of the lung and in the lower trachea were determined. Overall, all three H7N9 viruses (rAnhui-WT, rAnhui-HA-226Q, and rAnhui-PB2-627E) replicated efficiently in pig lungs (Fig. 7). Pigs inoculated with rAnhui-WT had higher titers in the BALF at 5 dpi than those inoculated with rAnhui-HA-226Q or rAnhui-PB2-627E (Fig. 7A). However, this was not consistent at other time points or in other tissues. To evaluate nasal shedding and viral transmission among pigs, nasal swabs were also collected from inoculated pigs at 0, 3, 5, and 7 dpi and from contact pigs at 0, 2, and 4 dpc. Although the H7N9 viruses replicated well in the trachea and lungs, they were not readily identified in nasal swabs after intratracheal inoculation. Only one animal in rAnhui-PB2-627E group was found to have an influenza virus-positive nasal swab at 5 dpi with low viral titers (data not shown). As anticipated, none of the mock-inoculated animals had virus present in any of their tissue or BALF and nasal swab samples (data not shown).

Replication and transmission of the H7N9 variant viruses was also evaluated by analysis of BALF, lung, trachea, and nasal swabs from the various groups of contact animals (Fig. 7). Titers and/or viral RNA were low in the contact animals and were often identified in one or few of the specimens from individual animals (Table 5). Interestingly, despite poor nasal shedding of the H7N9 variants in principal infected pigs, rAnhui-WT and rAnhui-PB2-627E were detected in various tissues of 2/3 and 3/3 contact pigs, respectively (Table 5). In contrast, the rAnhui-HA-226Q was not detected in any tissues of the contact pigs (Table 5). Thus, comparison of the genetically homogeneous recombinant viruses shows that 5/6 of the pigs exposed to viruses that have HA-226L were positive for virus transmission by direct contact, whereas none of the three pigs exposed to the HA-226Q virus was positive (Table 5). Taken together, our data show that the putative mammalian-signature HA-226L is critical in transmissibility of H7N9 in pigs.

Next-generation sequencing and analysis of viral genomes in infected pigs identifies additional residues in the viral genomes. Genomes of the viruses in the BALF samples from inoculated pigs were amplified by multisegment RT-PCR (23) and subjected to next-generation sequencing to identify nucleotide polymorphisms. Two amino acid substitutions (F87L and V195I) in the HA protein were selected in pigs infected with the recombinant Anhui-WT (a clonal population). V195I, located in one end of the 190 helix, is close to 226L, which is in the 220 loop of the receptor binding pocket (Fig. 8), indicating that additional adaptations around the receptor binding pocket occur rapidly in an individual host. The F87L substitution is located underneath the HA head,

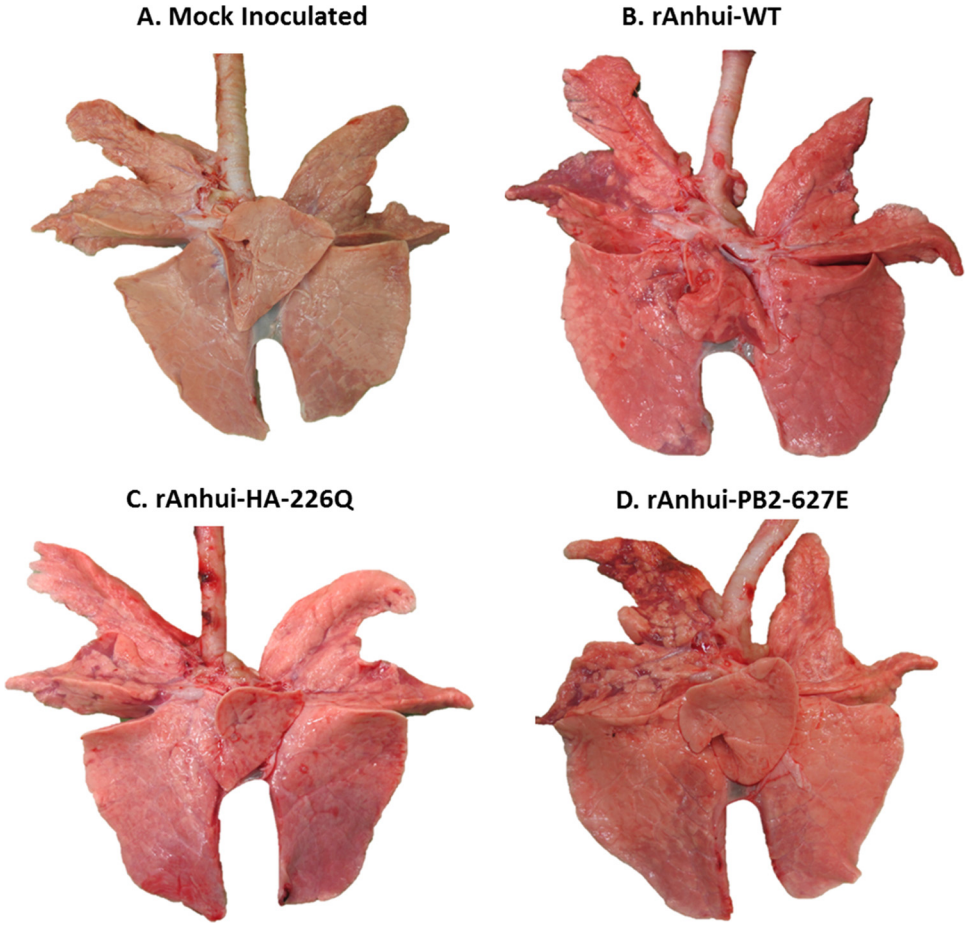

FIG 5 Gross lung lesions from pigs inoculated with the indicated H7N9 viruses at 5 days postinfection (dpi). Lung lesions due to influenza were characterized by multifocal to coalescing, red-plum-colored, consolidated areas. No gross lesions were seen in the mock-inoculated control group (A). Pigs inoculated with the rAnhui-WT virus and variants typically had multifocal, red-plum-colored lesions on the right apical and cardiac lung lobes (B, C, and D).

and its potential role in replication in pigs remains unknown. Nucleotide polymorphisms that generate three other amino acid substitutions [R149I (38.1%), A156T (13.8%), and N167D (22.8%), numbering from the first methionine of H7 protein] were detected in the HA of BALF samples from pigs infected by the PB2 mutant rAnhui-PB2-627E (Table 6). These substitutions are located in the head of the HA, and positions 149 and 156 are located underneath the 130 loop, which is important in maintaining conformation structure of the receptor binding pocket (Fig. 8). Intriguingly, N167D is located on top of the receptor binding pocket and is close to the 190 helix. All three substitutions surround the receptor binding pocket and impact the structure of the

TABLE 4 Macroscopic and microscopic lung lesions of principal pigs inoculated with H7N9 virus/variants and their contact pigs

		No. of pigs with lesions/total[a]			
		Inoculated pigs			
Lesion	Virus	3 dpi	5 dpi	7 dpi	Contact pigs (4 dpc)
Macroscopic	rAnhui-WT	3/3 (1.95 ± 1.20)	3/3 (7.52 ± 3.20)	3/3 (0.52 ± 0.23)	3/3 (2.52 ± 1.73)
	rAnhui-PB2-627E	3/3 (10.76 ± 4.89)	2/3 (7.21 ± 4.92)	3/3 (3.66 ± 1.69)	1/3 (1.43)
	rAnhui-HA-226Q	3/3 (7.80 ± 1.90)	3/3 (5.42 ± 3.22)	2/3 (1.28 ± 0.54)	1/3 (0.43)
	Control	1/3 (0.14)	1/3 (0.14)	1/2 (0.28)	NA
Microscopic	rAnhui-WT	2/3 (1.50 ± 0.50)	3/3 (1.50 ± 0.28)	2/3 (0.75 ± 0.25)	3/3 (0.50 ± 0.00)
	rAnhui-PB2-627E	3/3 (1.50 ± 0.28)	3/3 (1.50 ± 0.28)	2/3 (1.00 ± 0.00)	2/3 (0.50 ± 0.00)
	rAnhui-HA-226Q	3/3 (1.16 ± 0.33)	2/3 (1.00 ± 0.00)	3/3 (0.83 ± 0.16)	0/3 (0.00)
	Control	1/3 (0.5)	1/3 (0.5)	1/2 (0.5)	NA

[a] Numbers in parentheses are the mean number of macroscopic or microscopic lung lesions ± SEM. NA, not applicable because there were no contact pigs in the mock-inoculated control group.

TABLE 5 Summary of H7N9 virus and variants transmission characteristics

Virus	Animal	Result for contact animal samples[a]					Cumulative no. positive
		Nasal swab, 2 dpc[b]	Nasal swab, 4 dpc[b]	BALF, 4 dpc[b]	Trachea, 4 dpc[c]	Lung tissue[c]	
rAnhui-WT	A	Neg	Neg	Neg	Neg	Neg	2/3
	B	Neg	**Pos**	Neg	**Pos**	Neg	
	C	Neg	Neg	**Pos**	**Pos**	**Pos**	
rAnhui-PB2-627E	A	Neg	**Pos**	Neg	**Pos**	**Pos**	3/3
	B	**Pos**	Neg	Neg	Neg	Neg	
	C	Neg	**Pos**	Neg	Neg	Neg	
rAnhui-HA-226Q	A	Neg	Neg	Neg	Neg	Neg	0/3
	B	Neg	Neg	Neg	Neg	Neg	
	C	Neg	Neg	Neg	Neg	Neg	

[a] Neg, negative; Pos, positive.
[b] Determined by virus titration in MDCK cells.
[c] Tested by real-time RT-PCR.

pocket. In contrast, only one amino acid substitution (N443K) was observed in the HA protein of viruses from pigs infected with the rAnhui-HA-226Q virus, which is located in the HA stalk domain. Significant nucleotide polymorphisms were not found in the NA gene, except a nonsynonymous substitution that results in W175F in the NA protein from rAnhui-PB2-627E-infected pigs (Table 6).

Nucleotide polymorphisms were also selected in the internal protein genes of three viruses after replication in the pig lungs (Table 6). In a BALF sample harvested from an rAnhui-WT-infected pig, a dominant viral population containing a PB2-T271A substitution (74.0%) was detected, and also, a PB2-M402I mutation (57.0%) emerged (Table 6). In four BALF samples harvested from the rAnhui-PB2-627E-infected pigs, an average 13.0% of the viral population contained a PB2-E627K reversion (Table 6). Intriguingly, 11.0% of the viral population in one pig also had a PB2-D701N substitution, which is known to compensate for PB2-627E in mammalian

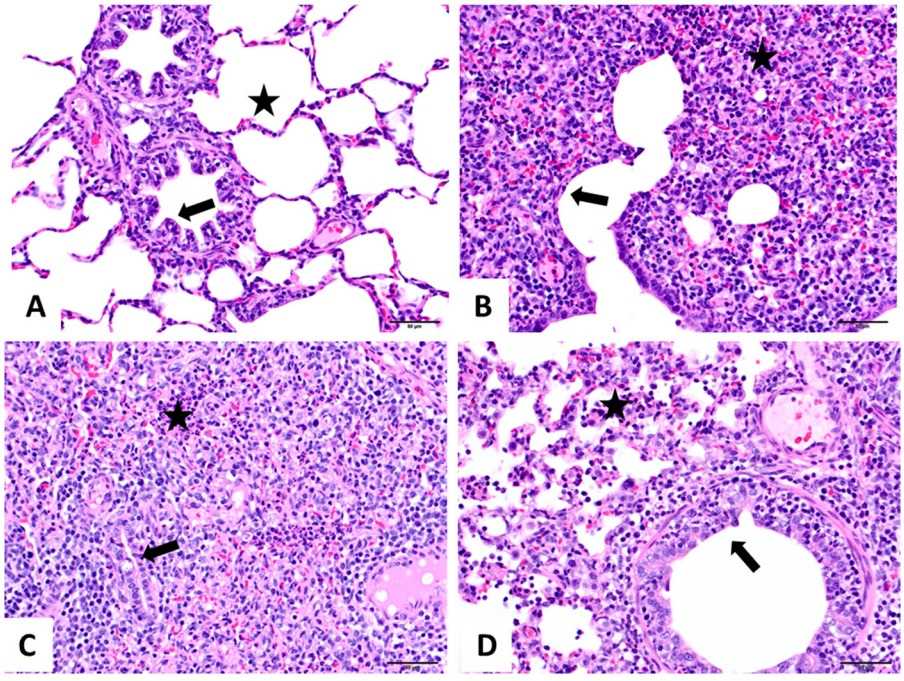

FIG 6 Microscopic lung sections from pigs inoculated with various H7N9 viruses at 5 days postinfection (dpi). (A) The bronchioles are lined by normal cuboidal epithelium (arrow), and the alveoli are clear (asterisk) in the control group; (B) the rAnhui-WT group has damage to bronchioles and mild bronchiolar epithelial hyperplasia (arrow) and moderate numbers of neutrophils in the alveoli (asterisk); (C) the rAnhui-HA-226Q group shows mild degeneration of bronchiolar epithelium (arrow), alveoli infiltrated by moderate numbers of neutrophils (asterisk), and moderate interstitial, peribronchiolar, and perivascular lymphocytic infiltration; (D) the rAnhui-PB2-627E group has minimal to mild bronchiolar epithelial degeneration, as shown by mild attenuation of the bronchiolar wall (arrow), and lesions are alveole-centric and characterized by infiltration of the alveolar lumen with neutrophils and by damage to the alveolar epithelium (asterisk). Bar, 50 μm.

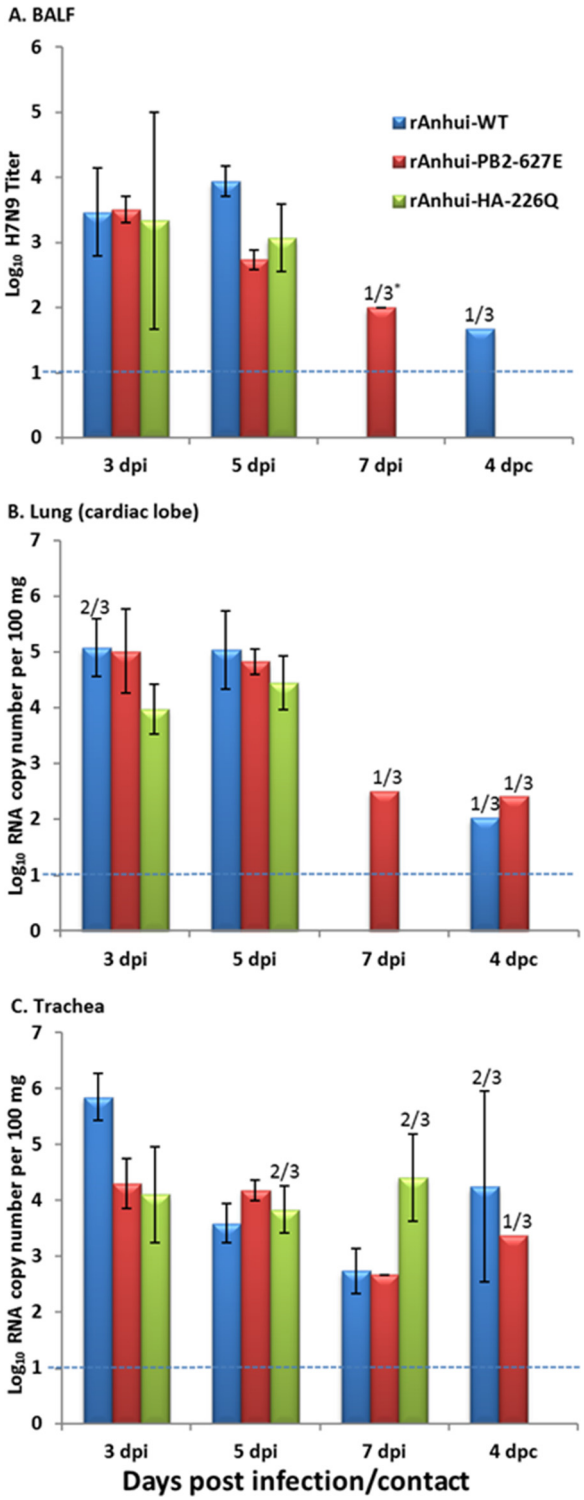

FIG 7 Detection of influenza virus and genomic RNA in inoculated and contact pigs. The bronchoalveolar lavage fluid (A), lungs (B), and tracheas (C) was harvested (3 animals) at 3, 5, or 7 dpi or 4 dpc. Virus titers were determined by $TCID_{50}$ using MDCK cells (A). Tissue homogenates were processed for RNA extraction and quantitative real-time RT-PCR to detect the influenza virus HA gene (B and C). Dotted lines represent the limit of detection. *, All data represent 3/3 positive animals unless otherwise indicated (e.g., 2/3 means that 2 of 3 animals were positive). Error bars represent standard errors of the means (SEM).

hosts. A few nucleotide polymorphisms were found in PB1, PA, and NP proteins, whereas substitutions were not observed in above 10% (cutoff value = 10%) of the population in other proteins (i.e., M1, M2, NS1, and NS2).

DISCUSSION

To date, the roles of putative mammalian-signature substitutions, animal reservoirs, and means of transmission of this novel H7N9 virus among humans and animals remain unclear. During the course of our studies, several articles were published characterizing H7N9 isolates, such as A/Anhui/1/2013, A/Shanghai/1/2013, and A/Shanghai/2/2013, *in vitro* and *in vivo* (6, 21, 40–43). These viruses were isolated in tissue culture or eggs and subsequently propagated most often in eggs, with subtle differences in techniques used by the various laboratories. Although natural isolates contain unique point mutations that can be compared, the results are confounded by other nucleotide and amino acid differences/mutations between the strains and by the fact that IAV evolves so rapidly that culture conditions and propagation/passage methods dramatically impact the viral population. In contrast, this study characterized biological properties, pandemic potential, and molecular mechanisms important in replication and transmissibility in pigs by focusing on one H7N9 strain (A/Anhui/1/2013). We analyzed a synthetically derived wild-type H7N9 virus (rAnhui-WT) and directly investigated the two amino acid residues that may be critical in the adaptation and transmission of these H7N9 viruses to humans (rAnhui-PB2-627E and rAnhui-HA-226Q). Synthetic genomics proved to be a powerful tool that enabled us to rescue and confirm the sequences of all the recombinant viruses even without the wild-type H7N9 isolate.

The reverse-genetics-rescued viruses provided a cleaner background to define the putative loss-of-function mutations in the PB2 and HA viral proteins and generated important data regarding the impact of these substitutions and their role in replication and transmission. The data demonstrate that the rAnhui-WT H7N9 virus, which has putative mammalian-signature amino acids in the PB2 (627K) and HA (226L), and the recombinant viruses (rAnhui-HA-226Q and rAnhui-PB2-627E) replicated in pigs and caused various levels of disease, including fever and pneumonia, which is consistent with findings in another study using an isolate of A/Shanghai/2/2013 virus (21). In contrast, other avian influenza viruses, such as H9N2 (HA-226L and PB2-627-E) viruses, replicate poorly in lungs of pigs despite inoculation of the same amount of viruses by the same route (37). A recent study also showed that human H7N9 viruses replicate efficiently in swine respiratory tissue explants *ex vivo* (44). Collectively, our data suggest that this emerging H7N9 lineage is adapted to mammals and indicate that it could be transmitted to pigs as well as humans.

Comparison of the transmission profiles of the recombinant viruses rAnhui-WT, rAnhui-PB2-627E, and rAnhui-HA-226Q showed that both rAnhui-WT and rAnhui-PB2-627E were transmitted among pigs by direct contact, which demonstrated that HA-226L is required for transmission (Fig. 7 and Table 5). Thus, the HA-226L substitution found in many H7N9 strains circulating appears to play a critical role in transmission. This is likely due to the fact that the HA-226L residue creates a nonpolar area, allowing the Gal-2 C-6 and ring carbons of the α-2,6 human receptors to bind efficiently (45). The mammalian-signature mutation PB2-627K, found in the novel human H7N9 viruses, has been

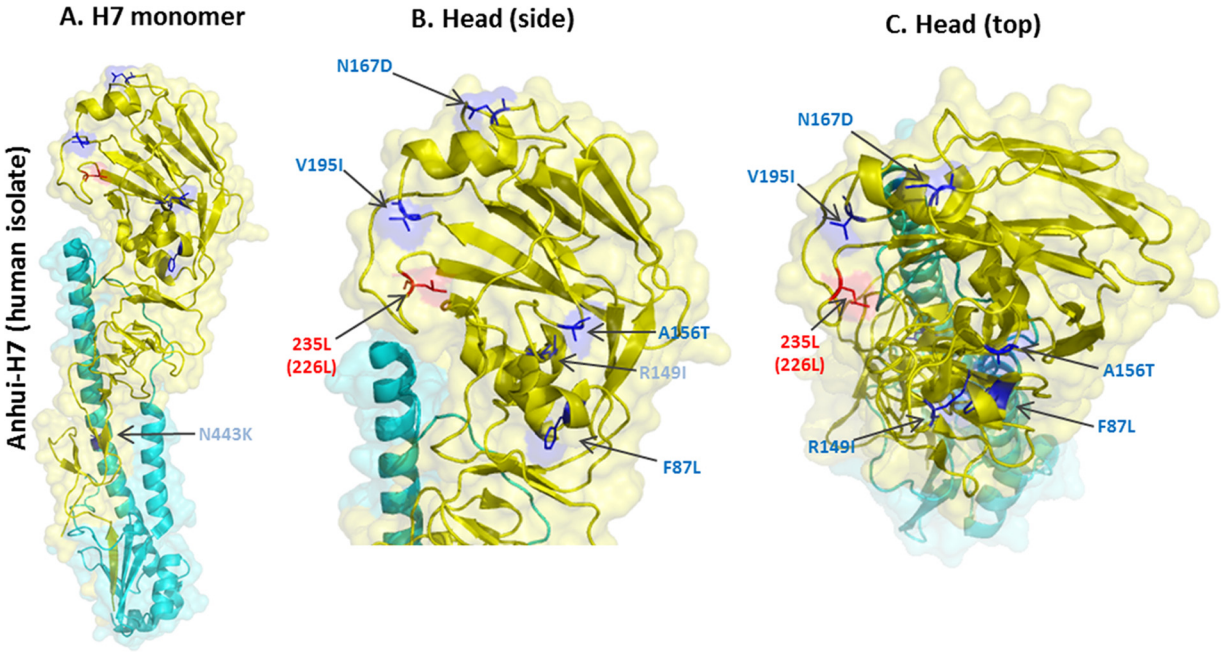

FIG 8 Structure of the H7N9 HA highlighting specific amino acid substitutions. The crystal structure of the hemagglutinin (with Asn-133 glycosylation) from an H7N9 influenza virus isolated from humans (Protein Data Base [PDB] code 4BSA) (45) is shown. The structure was rendered using Pymol, and the surface is shown as transparent. 226L (using H3 numbering; 235L, based on a starting methionine of H7), which was mutated to the glutamine (Q) in rAnhui-HA-226Q, is highlighted in red. The substitutions R149I, A156T, and N167D were found in viruses from the BALF of pigs infected by rAnhui-PB2-627E. F87L and V195I were observed in rAnhui-WT-infected pigs. The single substitution N443K was found in rAnhui-HA-226Q-inoculated pigs. All substitutions found in pig BALF samples are shown in blue. Designations in parentheses indicate the residue based on the starting methionine of the H7 HA.

associated with virulence and transmissibility of zoonotic highly pathogenic H5N1 lineage viruses (26–28). Recent studies using different virus isolates also strongly suggested that the PB2-627K contributes to replication and virulence of H7N9 virus in a mouse model (46, 47). Although the PB2-K627E substitution reduced viral polymerase activity in both human and porcine cells as anticipated (Fig. 3), the rAnhui-PB2-627E mutant was still transmitted to sentinels from the inoculated principal animals at a level comparable to that seen for the rAnhui-WT virus. In contrast, the pigs infected by rAnhui-HA-226Q did not transmit the virus to any of the contact animals (Table 5). This indicates that the mammalian-signature amino acid (226L) in the HA of the H7N9 viruses plays a decisive role in viral transmissibility in pigs. Nevertheless, the reversion of the PB2-627E mutants to 627K and acquisition of PB2-701N suggests that the lysine at position 627 is also important in zoonosis of H7N9 viruses.

TABLE 6 Amino acid substitutions in genomes of H7N9 virus and variants from infected pig BALF samples[a]

	rAnhui-WT		rAnhui-HA-226Q		rAnhui-PB2-627E	
Gene	NT mutation	Amino acid substitution	NT mutation	Amino acid substitution	NT mutation	Amino acid substitution
PB2	A838G T1223C G1233A	T271A (74.0%, 1/3, 3 dpi) I399T (13.5%, 1/3, 5 dpi) M402I (57.0%, 1/3, 3 dpi)	A1604G	K526R (17.3%, 1/3, 5 dpi)	A1906G G2128A	E627K (13.0%, 4/6, 3 and 5 dpi) D701N (11.0%, 1/3, 5 dpi)
PB1	G2173A	A717T (25.1%, 1/3, 3 dpi)	G263A	S80N (12.7%, 1/3, 5 dpi)	—	—
PA	G1700A	R559K (17.1%, 1/3, 5 dpi)	G1051A	A343T (45.8%, 1/3, 5 dpi)	G37A T1418C T1694C	V5 M (11.1%, 1/3, 5 dpi) I465T (34.2%, 1/3, 5 dpi) V557A (12.2%, 1/3, 5 dpi)
HA	T280C G262A	F87L (18.5%, 1/3, 5 dpi) V195I (11.0%, 1/3, 5 dpi)	C1350A	N443K (23.2%, 1/3, 5 dpi)	G467T G487A A502G	R149I (38.1%, 1/3, 5 dpi) A156T (13.8%, 3/3, 5 dpi) N167D (22.8%, 1/3, 3 dpi)
NP	A1535G	D497G(11.5%, 1/3, 5 dpi)	—	—	A1243G	R400G (20.6%, 1/3, 5 dpi)
NA	—	—	—	—	G542T	W175F (13.4%, 1/3, 3 dpi)

[a] NT, Nucleotide. Data in parentheses are the average percentage of the viral population containing the substitution, the number of pigs with the substitution/total at 3 or 5 dpi. —, no mutation.

Transmission studies showed that the reverse-genetics-derived wild type (rAnhui-WT) is transmissible in pigs by direct contact. This is in contrast to a study by Zhu et al., who intranasally infected pigs with a different strain derived from the natural isolate A/Shanghai/2/2013 (21). In this study, transmission in direct contact pigs was not identified by virus detection methods, although one of the four contact pigs seroconverted by 14 dpc (21). These results may be attributable to the fact that the A/Shanghai/2/2013 virus was isolated and propagated in embryonated chicken eggs and is likely to have selected a viral population adapting to avian cells, whereas the reverse-genetics-derived virus rAnhui-WT, which represents the consensus sequence of the original human isolate, was propagated by minimal passage in mammalian cells (MDCK) that express both α-2,3 receptors and α-2,6 receptors.

Next-generation sequencing showed that multiple nucleotide polymorphisms rapidly emerge from the molecular clones of the H7N9 viruses during replication in pigs, which demonstrates that this H7N9 lineage rapidly adapts within pigs, and likely humans, suggesting that these viruses have pandemic potential. Five amino acid substitutions (F87L, V195I, R149I, A156T, and N167D) were observed in the HA proteins of rAnhui-WT and rAnhui-PB2-627E viruses after replication in pigs. All of these mutations are close to the receptor binding pocket located in the head of the H7 protein. The functions of these substitutions in receptor binding affinity and transmissibility remain unclear. Nevertheless, the rapid emergence of these substitutions after a single passage in pigs suggests that the H7N9 viruses easily adapted to pigs after initial infection. Mutations also emerged in internal genes of the H7N9 viruses after replication in pig lungs. Notably, in BALF samples harvested from the rAnhui-PB2-627E-infected pigs, a significant proportion of the viral population contained a PB2-E627K reversion. For one of the viruses, 11% of the viral population had a PB2-D701N substitution, which compensates for PB2-627E in mammalian hosts (13, 48). In a sample from one lung of rAnhui-WT-infected pigs, more than half of the viral populations had acquired T271A and M402I substitutions in the PB2 protein; T271A is critical for enhancement of polymerase activity, replication of influenza viruses in mammalian cells, and respiratory droplet transmission of pH1N1 (49, 50). However, the functions of M402I in the PB2 protein and observed mutations in other genes (PA, NP, and NA) remain unclear. Thus, while the PB2-K627E substitution did not have a major impact on transmission, mutations in PB2 appears to be very important for replication of H7N9 in pigs and likely other mammals, such as humans.

In summary, our studies demonstrate that the recombinant human H7N9 virus and variants are infectious for pigs; however, they show different levels of virulence and transmissibility in pigs, and viruses containing HA-226L are more likely to be transmitted between pigs. Recent studies showed that the human A/Anhui/1/2013 isolate showed a limited ability to be transmitted (~33% of contacts) among ferrets by respiratory droplets (40, 42). Collectively, the results of this and other H7N9 studies indicate that the zoonotic H7N9 lineage viruses currently circulating in avian species in China already have the potential for avian-to-human/swine transmission and that additional mutations may be required to enable variants of H7N9 to spread more efficiently among humans. The fact that many mutations (including putative replication/transmission-enhancing mutations, such as PB2-T271A, PB2-E627K, and PB2-D701N) emerged readily during a single passage in pigs is of additional concern; i.e., the H7N9 viruses can rapidly acquire human-signature substitutions. Finally, it is well understood that pigs often act as mixing vessels for human, swine, and avian influenza viruses (51), and our results provide evidence that the H7N9 virus and variants are able to productively infect pigs and suggest that the virus could readily become established in the swine population. If these H7N9 viruses or related viruses become enzootic in pigs, it will create new opportunities for the generation of novel reassortants with other swine influenza viruses circulating in Southeast Asia (e.g., swine, avian, and even human lineage viruses, introduced into pigs by reverse zoonosis), which could increase their pandemic potential (20, 37, 52).

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Q.L., B.Z., R.W., W.M., D.E.W., and J.A.R. conceived and designed the experiments; B.Z. and W.W. carried out the gene synthesis and polymerase assay; Q.L., B.B., J.M., Y. Lang, Y. Lyoo, W.M., and J.A.R. carried out the animal experiment and the related assays; B.Z., R.A.H., X.L., and T.B.S. performed the sequencing and analysis; Q.L., B.Z., W.M., D.E.W. and J.A.R. interpreted the data and wrote the manuscript.

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REFERENCES

1. **WHO.** 2009. Influenza (seasonal) fact sheet 211. http://www.who.int/mediacentre/factsheets/fs211/en/.
2. **Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K.** 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA **289:**179–186. http://dx.doi.org/10.1001/jama.289.2.179.
3. **Johnson NP, Mueller J.** 2002. Updating the accounts: global mortality of the 1918–1920 "Spanish" influenza pandemic. Bull. Hist. Med. **76:**105–115. http://dx.doi.org/10.1353/bhm.2002.0022.
4. **To KK, Chan JF, Chen H, Li L, Yuen KY.** 2013. The emergence of influenza A H7N9 in human beings 16 years after influenza A H5N1: a tale of two cities. Lancet Infect. Dis. **13:**809–821. http://dx.doi.org/10.1016/S1473-3099(13)70167-1.
5. **Zhou J, Wang D, Gao R, Zhao B, Song J, Qi X, Zhang Y, Shi Y, Yang L, Zhu W, Bai T, Qin K, Lan Y, Zou S, Guo J, Dong J, Dong L, Zhang Y, Wei H, Li X, Lu J, Liu L, Zhao X, Li X, Huang W, Wen L, Bo H, Xin L, Chen Y, Xu C, Pei Y, Yang Y, Zhang X, Wang S, Feng Z, Han J, Yang W, Gao GF, Wu G, Li D, Wang Y, Shu Y.** 2013. Biological features of novel avian influenza A (H7N9) virus. Nature **499:**500–503. http://dx.doi.org/10.1038/nature12379.
6. **Richard M, Schrauwen EJ, de Graaf M, Bestebroer TM, Spronken MI, van Boheemen S, de Meulder D, Lexmond P, Linster M, Herfst S, Smith DJ, van den Brand JM, Burke DF, Kuiken T, Rimmelzwaan GF, Osterhaus AD, Fouchier RA.** 2013. Limited airborne transmission of H7N9 influenza A virus between ferrets. Nature **501:**560–563. http://dx.doi.org/10.1038/nature12476.
7. **Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, Bi Y, Wu Y, Li X, Yan J, Liu W, Zhao G, Yang W, Wang Y, Ma J, Shu Y, Lei F, Gao GF.** 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet **381:**1926–1932. http://dx.doi.org/10.1016/S0140-6736(13)60938-1.

8. **Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M.** 2013. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. Euro Surveill. **18:**20453. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453.
9. **Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, Bi Y, Wu Y, Li X, Yan J, Liu W, Zhao G, Yang W, Wang Y, Ma J, Shu Y, Lei F, Gao GF.** 2013. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet **381:**1926–1932. http://dx.doi.org/10.1016/S0140-6736(13)60938-1.
10. **Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y.** 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. **3:**1374–1379. http://dx.doi.org/10.1371/journal.ppat.0030133.
11. **Manzoor R, Sakoda Y, Nomura N, Tsuda Y, Ozaki H, Okamatsu M, Kida H.** 2009. PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs. J. Virol. **83:**1572–1578. http://dx.doi.org/10.1128/JVI.01879-08.
12. **Le QM, Sakai-Tagawa Y, Ozawa M, Ito M, Kawaoka Y.** 2009. Selection of H5N1 influenza virus PB2 during replication in humans. J. Virol. **83:**5278–5281. http://dx.doi.org/10.1128/JVI.00063-09.
13. **Steel J, Lowen AC, Mubareka S, Palese P.** 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog. **5:**e1000252. http://dx.doi.org/10.1371/journal.ppat.1000252.
14. **Le QM, Ito M, Muramoto Y, Hoang PV, Vuong CD, Sakai-Tagawa Y, Kiso M, Ozawa M, Takano R, Kawaoka Y.** 2010. Pathogenicity of highly pathogenic avian H5N1 influenza A viruses isolated from humans between 2003 and 2008 in northern Vietnam. J. Gen. Virol. **91:**2485–2490. http://dx.doi.org/10.1099/vir.0.021659-0.
15. **Ma W, Kahn RE, Richt JA.** 2008. The pig as a mixing vessel for influenza viruses: human and veterinary implications. J. Mol. Genet. Med. **3:**158–166.
16. **Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A.** 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature **459:**1122–1125. http://dx.doi.org/10.1038/nature08182.
17. **Webster RG, Shortridge KF, Kawaoka Y.** 1997. Influenza: interspecies transmission and emergence of new pandemics. FEMS Immunol. Med. Microbiol. **18:**275–279. http://dx.doi.org/10.1111/j.1574-695X.1997.tb01056.x.
18. **Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. **72:**7367–7373.
19. **Neumann G, Noda T, Kawaoka Y.** 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459:**931–939. http://dx.doi.org/10.1038/nature08157.
20. **Wong KK, Greenbaum A, Moll ME, Lando J, Moore EL, Ganatra R, Biggerstaff M, Lam E, Smith EE, Storms AD, Miller JR, Dato V, Nalluswami K, Nambiar A, Silvestri SA, Lute JR, Ostroff S, Hancock K, Branch A, Trock SC, Klimov A, Shu B, Brammer L, Epperson S, Finelli L, Jhung MA.** 2012. Outbreak of influenza A (H3N2) variant virus infection among attendees of an agricultural fair, Pennsylvania, USA, 2011. Emerg. Infect. Dis. **18:**1937–1944. http://dx.doi.org/10.3201/eid1812.121097.
21. **Zhu H, Wang D, Kelvin DJ, Li L, Zheng Z, Yoon SW, Wong SS, Farooqui A, Wang J, Banner D, Chen R, Zheng R, Zhou J, Zhang Y, Hong W, Dong W, Cai Q, Roehrl MH, Huang SS, Kelvin AA, Yao T, Zhou B, Chen X, Leung GM, Poon LL, Webster RG, Webby RJ, Peiris JS, Guan Y, Shu Y.** 2013. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science **341:**183–186. http://dx.doi.org/10.1126/science.1239844.
22. **Dormitzer PR, Suphaphiphat P, Gibson DG, Wentworth DE, Stockwell TB, Algire MA, Alperovich N, Barro M, Brown DM, Craig S, Dattilo BM, Denisova EA, De Souza I, Eickmann M, Dugan VG, Ferrari A, Gomila RC, Han L, Judge C, Mane S, Matrosovich M, Merryman C, Palladino G, Palmer GA, Spencer T, Strecker T, Trusheim H, Uhlendorff J, Wen Y, Yee AC, Zaveri J, Zhou B, Becker S, Donabedian A, Mason PW, Glass JI, Rappuoli R, Venter JC.** 2013. Synthetic generation of influenza vaccine viruses for rapid response to pandemics. Sci. Transl. Med. **5:**185ra68. http://dx.doi.org/10.1126/scitranslmed.3006368.
23. **Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, Wentworth DE.** 2009. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. J. Virol. **83:**10309–10313. http://dx.doi.org/10.1128/JVI.01109-09.
24. **Zhou B, Jerzak G, Scholes DT, Donnelly ME, Li Y, Wentworth DE.** 2011. Reverse genetics plasmid for cloning unstable influenza A virus gene segments. J. Virol. Methods **173:**378–383. http://dx.doi.org/10.1016/j.jviromet.2011.01.021.
25. **Zhou B, Li Y, Halpin R, Hine E, Spiro DJ, Wentworth DE.** 2011. PB2 residue 158 is a pathogenic determinant of pandemic H1N1 and H5 influenza a viruses in mice. J. Virol. **85:**357–365. http://dx.doi.org/10.1128/JVI.01694-10.
26. **Ma W, Lager KM, Lekcharoensuk P, Ulery ES, Janke BH, Solorzano A, Webby RJ, Garcia-Sastre A, Richt JA.** 2010. Viral reassortment and transmission after co-infection of pigs with classical H1N1 and triple-reassortant H3N2 swine influenza viruses. J. Gen. Virol. **91:**2314–2321. http://dx.doi.org/10.1099/vir.0.021402-0.
27. **Medeiros R, Escriou N, Naffakh N, Manuguerra JC, van der Werf S.** 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74–85. http://dx.doi.org/10.1006/viro.2001.1121.
28. **WHO.** 2013. Real-time RT-PCR protocol for the detection of avian influenza A(H7N9) virus. http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf.
29. **Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ.** 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. J. Clin. Microbiol. **41:**3198–3205. http://dx.doi.org/10.1128/JCM.41.7.3198-3205.2003.
30. **Subbarao EK, London W, Murphy BR.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. **67:**1761–1764.
31. **Hatta M, Gao P, Halfmann P, Kawaoka Y.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840–1842. http://dx.doi.org/10.1126/science.1062882.
32. **Massin P, van der Werf S, Naffakh N.** 2001. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J. Virol. **75:**5398–5404. http://dx.doi.org/10.1128/JVI.75.11.5398-5404.2001.
33. **Aggarwal S, Dewhurst S, Takimoto T, Kim B.** 2011. Biochemical impact of the host adaptation-associated PB2 E627K mutation on the temperature-dependent RNA synthesis kinetics of influenza A virus polymerase complex. J. Biol. Chem. **286:**34504–34513. http://dx.doi.org/10.1074/jbc.M111.262048.
34. **Herfst S, Chutinimitkul S, Ye J, de Wit E, Munster VJ, Schrauwen EJ, Bestebroer TM, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Osterhaus AD, Perez DR, Fouchier RA.** 2010. Introduction of virulence markers in PB2 of pandemic swine-origin influenza virus does not result in enhanced virulence or transmission. J. Virol. **84:**3752–3758. http://dx.doi.org/10.1128/JVI.02634-09.
35. **Jagger BW, Memoli MJ, Sheng ZM, Qi L, Hrabal RJ, Allen GL, Dugan VG, Wang R, Digard P, Kash JC, Taubenberger JK.** 2010. The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. mBio **1:**e00067–10. http://dx.doi.org/10.1128/mBio.00067-10.
36. **Zhu H, Wang J, Wang P, Song W, Zheng Z, Chen R, Guo K, Zhang T, Peiris JS, Chen H, Guan Y.** 2010. Substitution of lysine at 627 position in PB2 protein does not change virulence of the 2009 pandemic H1N1 virus in mice. Virology **401:**1–5. http://dx.doi.org/10.1016/j.virol.2010.02.024.
37. **Qiao C, Liu Q, Bawa B, Shen H, Qi W, Chen Y, Mok CK, Garcia-Sastre A, Richt JA, Ma W.** 2012. Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus. J. Gen. Virol. **93:**2337–2345. http://dx.doi.org/10.1099/vir.0.044040-0.
38. **Ma W, Oberst R, Li X, Clouser D, Hesse R, Rowland RR, Richt JA.** 2010. Rapid detection of the pandemic 2009 H1N1 virus M gene by real-time and gel-based RT-PCR assays. Influenza Other Respir. Viruses **4:**397–403. http://dx.doi.org/10.1111/j.1750-2659.2010.00180.x.
39. **Richt JA, Lager KM, Clouser DF, Spackman E, Suarez DL, Yoon KJ.** 2004. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. J. Vet. Diagn. Invest. **16:**367–373. http://dx.doi.org/10.1177/104063870401600501.

40. **Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y, Takashita E, McBride R, Noda T, Hatta M, Imai H, Zhao D, Kishida N, Shirakura M, de Vries RP, Shichinohe S, Okamatsu M, Tamura T, Tomita Y, Fujimoto N, Goto K, Katsura H, Kawakami E, Ishikawa I, Watanabe S, Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ, Zhong G, Fan S, Ping J, Maher EA, Hanson A, Uchida Y, Saito T, Ozawa M, Neumann G, Kida H, Odagiri T, Paulson JC, Hasegawa H, Tashiro M, Kawaoka Y.** 2013. Characterization of H7N9 influenza A viruses isolated from humans. Nature **501:**551–555. http://dx.doi.org/10.1038/nature12392.
41. **Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM.** 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature **501:**556–559. http://dx.doi.org/10.1038/nature12391.
42. **Zhang Q, Shi J, Deng G, Guo J, Zeng X, He X, Kong H, Gu C, Li X, Liu J, Wang G, Chen Y, Liu L, Liang L, Li Y, Fan J, Wang J, Li W, Guan L, Li Q, Yang H, Chen P, Jiang L, Guan Y, Xin X, Jiang Y, Tian G, Wang X, Qiao C, Li C, Bu Z, Chen H.** 2013. H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. Science **341:**410–414. http://dx.doi.org/10.1126/science.1240532.
43. **Zhou J, Wang D, Gao R, Zhao B, Song J, Qi X, Zhang Y, Shi Y, Yang L, Zhu W, Bai T, Qin K, Lan Y, Zou S, Guo J, Dong J, Dong L, Wei H, Li X, Lu J, Liu L, Zhao X, Huang W, Wen L, Bo H, Xin L, Chen Y, Xu C, Pei Y, Yang Y, Zhang X, Wang S, Feng Z, Han J, Yang W, Gao GF, Wu G, Li D, Wang Y, Shu Y.** 2013. Biological features of novel avian influenza A (H7N9) virus. Nature **499:**500–503. http://dx.doi.org/10.1038/nature12379.
44. **Jones JC, Baranovich T, Zaraket H, Guan Y, Shu Y, Webby RJ, Webster RG.** 2013. Human H7N9 influenza A viruses replicate in swine respiratory tissue explants. J. Virol. **87:**12496–12498. http://dx.doi.org/10.1128/JVI.02499-13.
45. **Xiong X, Martin SR, Haire LF, Wharton SA, Daniels RS, Bennett MS, McCauley JW, Collins PJ, Walker PA, Skehel JJ, Gamblin SJ.** 2013. Receptor binding by an H7N9 influenza virus from humans. Nature **499:**496–499. http://dx.doi.org/10.1038/nature12372.
46. **Zhang H, Li X, Guo J, Li L, Chang C, Li Y, Bian C, Xu K, Chen H, Sun B.** 2014. The PB2 E627K mutation contributes to the high polymerase activity and enhanced replication of H7N9 influenza virus. J. Gen. Virol. **95:**779–786. http://dx.doi.org/10.1099/vir.0.061721-0.
47. **Mok CK, Lee HH, Lestra M, Nicholls JM, Chan MC, Sia SF, Zhu H, Poon LL, Guan Y, Peiris JS.** 2014. Amino acid substitutions in polymerase basic protein 2 gene contribute to the pathogenicity of the novel A/H7N9 influenza virus in mammalian hosts. J. Virol. **88:**3568–3576. http://dx.doi.org/10.1128/JVI.02740-13.
48. **Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J.** 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. U. S. A. **102:**18590–18595. http://dx.doi.org/10.1073/pnas.0507415102.
49. **Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T.** 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. J. Virol. **84:**4395–4406. http://dx.doi.org/10.1128/JVI.02642-09.
50. **Zhang Y, Zhang Q, Gao Y, He X, Kong H, Jiang Y, Guan Y, Xia X, Shu Y, Kawaoka Y, Bu Z, Chen H.** 2012. Key molecular factors in hemagglutinin and PB2 contribute to efficient transmission of the 2009 H1N1 pandemic influenza virus. J. Virol. **86:**9666–9674. http://dx.doi.org/10.1128/JVI.00958-12.
51. **Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, Richt JA.** 2009. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health **56:**326–337. http://dx.doi.org/10.1111/j.1863-2378.2008.01217.x.
52. **Skowronski DM, Janjua NZ, De Serres G, Purych D, Gilca V, Scheifele DW, Dionne M, Sabaiduc S, Gardy JL, Li G, Bastien N, Petric M, Boivin G, Li Y.** 2012. Cross-reactive and vaccine-induced antibody to an emerging swine-origin variant of influenza A virus subtype H3N2 (H3N2v). J. Infect. Dis. **206:**1852–1861. http://dx.doi.org/10.1093/infdis/jis500.