Pathogenicity and Transmissibility of Novel Reassortant H3N2 Influenza Viruses with 2009 Pandemic H1N1 Genes in Pigs

Jingjiao Ma,^a Huigang Shen,^{a*} Qinfang Liu,^a Bhupinder Bawa,^a Wenbao Qi,^{a*} Michael Duff,^a Yuekun Lang,^a Jinhwa Lee,^a Hai Yu,^{a,b} Jianfa Bai,^a Guangzhi Tong,^b Richard A. Hesse,^a Jürgen A. Richt,^a Wenjun Ma^a

Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, Kansas, USA^a; Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China^b

ABSTRACT

At least 10 different genotypes of novel reassortant H3N2 influenza viruses with 2009 pandemic H1N1 [A(H1N1)pdm09] gene(s) have been identified in U.S. pigs, including the H3N2 variant with a single A(H1N1)pdm09 M gene, which has infected more than 300 people. To date, only three genotypes of these viruses have been evaluated in animal models, and the pathogenicity and transmissibility of the other seven genotype viruses remain unknown. Here, we show that three H3N2 reassortant viruses that contain 3 (NP, M, and NS) or 5 (PA, PB2, NP, M, and NS) genes from A(H1N1)pdm09 were pathogenic in pigs, similar to the endemic H3N2 swine virus. However, the reassortant H3N2 virus with 3 A(H1N1)pdm09 genes and a recent human influenza virus N2 gene was transmitted most efficiently among pigs, whereas the reassortant H3N2 virus with 5 A(H1N1)pdm09 genes was transmitted less efficiently than the endemic H3N2 virus. Interestingly, the polymerase complex of reassortant H3N2 virus with 5 A(H1N1)pdm09 genes showed significantly higher polymerase activity than those of endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. Further studies showed that an avian-like glycine at position 228 at the hemagglutinin (HA) receptor binding site is responsible for inefficient transmission of the reassortant H3N2 virus with 5 A(H1N1)pdm09 genes. Taken together, our results provide insights into the pathogenicity and transmissibility of novel reassortant H3N2 viruses in pigs and suggest that a mammalian-like serine at position 228 in the HA is critical for the transmissibility of these reassortant H3N2 viruses.

IMPORTANCE

Swine influenza is a highly contagious zoonotic disease that threatens animal and public health. Introduction of 2009 pandemic H1N1 virus [A(H1N1)pdm09] into swine herds has resulted in novel reassortant influenza viruses in swine, including H3N2 and H1N2 variants that have caused human infections in the United States. We showed that reassortant H3N2 influenza viruses with 3 or 5 genes from A(H1N1)pdm09 isolated from diseased pigs are pathogenic and transmissible in pigs, but the reassortant H3N2 virus with 5 A(H1N1)pdm09 genes displayed less efficient transmissibility than the endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. Further studies revealed that an avian-like glycine at the HA 228 receptor binding site of the reassortant H3N2 virus with 5 A(H1N1)pdm09 genes is responsible for less efficient transmissibility in pigs. Our results provide insights into viral pathogenesis and the transmission of novel reassortant H3N2 viruses that are circulating in U.S. swine herds and warrant future surveillance.

Swine influenza is a zoonotic disease that threatens animal and public health. Currently, there are three subtypes of influenza A viruses that predominantly infect pigs worldwide: H1N1, H1N2, and H3N2 (1). Since the emergence of triple-reassortant influenza A viruses containing genes of human, swine, and avian influenza viruses in swine in North America in 1998 (2, 3), triple-reassortant H1N1, H1N2, and H3N2 subtype viruses have been endemic in North American swine herds. In particular, triple-reassortant H3N2 viruses have become a major cause of swine influenza in North America (4, 5) and also sporadically cause human infections (6, 7). The 2009 pandemic was caused by a reassortant H1N1 virus whose genes are from North American triple-reassortant (PB2, PB1, PA, HA, NP, and NS) and Eurasian avian-like (NA and M) H1N1 swine viruses (8). The 2009 pandemic H1N1 [A(H1N1)pdm09] virus circulated in humans and crossed the species barrier to infect other animals, including swine, dogs, cats, and wild mammals (9–13). Importantly, the virus has been isolated from pigs worldwide, including Europe, Asia, South America, and North America (13–16).

Human influenza viruses tend to bind to α -2,6-linked sialic

acids on the host cell surface, which are present in the upper respiratory tract in humans and other mammals (17). In contrast, avian influenza viruses preferentially bind to α -2,3-linked sialic acids. They are reported to be abundant in the avian intestinal tract, as well as in the human lower respiratory tract (18). Swine

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Address correspondence to Wenjun Ma, wma@vet.k-state.edu.

* Present address: Huigang Shen, College of Animal Science and Technology, Zhejiang A&F University, Lin'an, China; Wenbao Qi, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China.

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have been considered “mixing vessels” for avian, human, and swine influenza viruses because the swine respiratory tract has receptors for both avian and mammalian influenza viruses. Thus, if two influenza viruses infect one pig concurrently, they may randomly exchange gene segments, resulting in novel reassortant viruses through an event called reassortment. Since the first reassortant influenza virus containing A(H1N1)pdm09 genes was found in pigs in 2009 in Hong Kong (19), similar reassortant viruses containing genes from influenza viruses endemic in pigs and A(H1N1)pdm09 have been reported from other countries, including novel reassortant H1N2 viruses in the United Kingdom and Italy (20, 21); reassortant H1N1 viruses in Germany and Thailand (15, 22); and reassortant H1N1, H1N2, and H3N2 viruses in the United States (23–25). In addition, reassortant H3N2 viruses were isolated from mink and swine in Canada (26). This has raised concerns that these novel reassortant influenza viruses in swine may pose a threat to humans and gain the ability for human-to-human transmission. Indeed, novel reassortant H3N2 viruses containing the matrix gene from A(H1N1)pdm09 (H3N2 variants [H3N2v]) that emerged in swine have been reported to infect humans in the United States, and most of the infected patients had been either directly or indirectly exposed to pigs (27, 28). Furthermore, limited human-to-human transmission has been found (28).

Previously, we reported the isolation of 7 reassortant H3N2 influenza viruses with 3 or 5 genes derived from A(H1N1)pdm09 from diseased pigs from Midwestern swine farms with outbreaks of respiratory disease (25). To date, the pathogenicity and transmissibility of these novel reassortant H3N2 viruses in pigs remain unknown. Additionally, whether these viruses could be maintained and circulate in swine herds needs to be investigated. To evaluate the pathogenicity and transmissibility of these novel reassortant H3N2 viruses, we selected three novel reassortant viruses carrying either 3 (NP, M, and NS) or 5 (PB2, PA, NP, M, and NS) genes from A(H1N1)pdm09 for the pig study, using a recently isolated endemic triple-reassortant H3N2 influenza virus from diseased pigs as a control.

MATERIALS AND METHODS

Ethics statement. The pig study was conducted at the Large Animal Research Center (a biosafety level 2+ facility) at Kansas State University in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching of the U.S. Department of Agriculture. The protocol was approved by the Institutional Animal Care and Use Committee of Kansas State University (IACUC no. 3146).

Cells. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 1× L-glutamine (Invitrogen, Carlsbad, CA), 1× MEM vitamins (Invitrogen, Carlsbad, CA), and 1% antibiotics (Invitrogen, Carlsbad, CA). Human lung adenocarcinoma epithelial cells (A549) and porcine kidney cells (PK-15) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 1× MEM vitamins, 1× L-glutamine, and 1% antibiotics. The cells were infected with viruses using MEM infecting medium that contained 0.3% bovine albumin (Sigma, St. Louis, MO), 1% antibiotics (Invitrogen, Carlsbad, CA), and 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO).

Viruses. Three novel reassortant H3N2 influenza viruses isolated from swine and containing either 3 or 5 genes from A(H1N1)pdm09 were used in this study: A/swine/Kansas/10-91088/2010 (KS-91088) [NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409388–95], A/swine/Kansas/11-107824/2011 (KS-

107824) [PA, PB2, NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409420–27], and A/swine/Kansas/11-110529/2011 (KS-110529) [NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409436–43]. The KS-110529 virus contains an NA gene derived from recent human influenza viruses that has genetically diverged from the NAs of the above-mentioned 2 reassortant viruses (their NAs are from early human influenza viruses). One endemic triple-reassortant A/swine/Kansas/10-83533/2010 virus (KS-83533N) and its eight-gene segment, GenBank accession number KP270886–93, which was isolated in the same area from diseased pigs and also contains an NA gene from the recent human influenza viruses, was used as a control in this study.

Growth kinetics. To study the growth kinetics of viruses in different cells, including A549 (multiplicity of infection [MOI] = 0.01), MDCK (MOI = 0.1), and PK-15 (MOI = 0.01) cells, confluent cells were infected with each virus at the indicated MOI. The supernatants of the infected cells were collected at 12, 24, 36, and 48 h postinoculation (p.i.). The virus titers of the collected supernatants were determined by inoculating confluent monolayers of MDCK cells in 96-well plates, and the 50% tissue culture infective dose (TCID₅₀)/ml was calculated by the method of Reed and Muench. A plaque assay was conducted to compare the sizes of plaques formed by each virus on MDCK and PK-15 cells.

Plasmid construction and minigenome replication assay. To determine the polymerase activity of each H3N2 virus, a minigenome assay was performed as described previously (29). pPol1-NS-Luciferase carries an influenza A virus reporter minigenome in which the firefly luciferase gene is flanked by the influenza A virus NS gene noncoding regions, a truncated PolII promoter, and the hepatitis delta virus ribozyme. The polymerase (PB1, PB2, and PA) and NP genes of each virus were cloned into the pGEM-T vector (Promega, Madison, WI) and then subcloned into the pCAGGS/MCS vector. All the plasmids were confirmed by sequencing.

Confluent 293T cells were cotransfected with pPol1-NS-Luciferase (100 ng); pSV-Renilla (50 ng) carrying the *Renilla* luciferase gene under simian virus 40 (SV40) RNA polymerase II promoter as a control; and four pCAGGS plasmids expressing viral PB2, PB1, PA, and NP from each strain (the amounts of PB2, PB1, PA, and NP plasmids used were 50 ng, 100 ng, 100 ng, and 500 ng [29]). Twenty-four hours after transfection, cells were collected and lysed using passive lysis buffer, and then the cell lysates were used to conduct a dual-luciferase reporter assay according to the manufacturer's protocol (Promega). The influenza virus polymerase activity derived from the firefly luciferase plasmid (pPol1-NS-Luciferase) was calculated and normalized based on transfection efficiency using the *Renilla* luciferase activity values from pSV-Renilla. Each cotransfection experiment was repeated three times.

Neuraminidase activity assay. The NA activities of endemic and reassortant H3N2 viruses (KS-83533N, KS-91088, KS-107824, and KS-110529) were determined with an NA-XTD Influenza Neuraminidase Assay Kit (Life Technologies). The assay was performed following the manufacturer's instructions. Briefly, all the viruses were diluted in the same titer of 10⁵ TCID₅₀ in 50 μl NA-XTD assay buffer as the virus stock. Serial 1:2 dilutions of virus stocks were made using the NA-XTD assay buffer. The diluted viruses (50 μl) were mixed with 25 μl of NA-XTD chemiluminescent substrate [5 μM; sodium (3-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.1.3,7]decan}-4-yl-phenyl)-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranoside)onate] in a 96-well plate and incubated at 37°C for 30 min. Then, 60 μl of NA-XTD accelerator was added to each well, and the plate was read with Fluostar Omega (BMG Labtech) using a 1-s/well read time as recommended by the manufacturer. Three independent replicate assays were conducted for each virus.

Generation of wild-type KS-107824 and its single-amino-acid-mutated virus (HA G228S) by reverse genetics. Eight-gene segments of the H3N2 KS-107824 virus were amplified using universal primers and cloned into the pHW2000 vector as described previously (30) to establish a reverse-genetic system for KS-107824, resulting in plasmids pHW2000-

PB1, -PB2, -PA, -HA, -NP, -NA, -M, and -NS. All the cloned genes were confirmed by sequencing. A single substitution at position 228 in HA (glycine to serine) was introduced with a site-directed mutagenesis kit (Invitrogen) according to the manufacturer's recommendations, resulting in plasmid pHW2000-HA-G228S, which was confirmed by sequencing. Both the wild-type (rgKS-107824) virus and a mutated virus with a single substitution in HA, G228S (rgKS-107824-G228S), were rescued as described previously (30) by reverse genetics and propagated in MDCK cells for *in vitro* and pig studies. Both wild-type and singly mutated viruses were confirmed by sequencing prior to the animal study.

HA receptor binding preferences of endemic, reassortant, and reverse-genetics-derived H3N2 viruses using hemagglutinating receptor-specific red blood cells. The receptor binding preferences of endemic, reassortant, and reverse-genetics-derived H3N2 viruses were analyzed using hemagglutinating receptor-specific red blood cells (RBCs). For this experiment, normal turkey red blood cells (containing both α -2,6 and α -2,3 receptors), 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) were used (31). To remove α -2,3-linked *N*-acetyl-neuraminic acid residues from oligosaccharides of turkey red blood cells, they were treated with α -2,3 neuraminidase (NEB). Briefly, 10% RBCs in 1 ml of 1X G4 reaction buffer and 1X BSA was incubated at 37°C in the presence of 1,000 IU α -2,3-specific neuraminidase for 1 h. The treated red blood cells were washed three times with phosphate-buffered saline (PBS) before use. The final working solution was 0.5% RBCs in PBS for the hemagglutination assay. The hemagglutination assay was performed with 0.5% different red blood cells with specific hemagglutinating receptors in 96-well V-bottom microtiter plates by incubating equal volumes (50 μ l) of 2-fold serially diluted viruses. The hemagglutination titer was defined as the reciprocal of the highest virus dilution that hemagglutinated red blood cells. To determine the specific receptors on the treated and untreated red blood cells, avian influenza A/chicken/Jena/4836/1983 (H2N2) and human influenza A/Brisbane/59/2007(H1N1) viruses were included in the receptor binding assay as controls.

Solid-phase HA receptor binding assay. Viruses amplified in chicken embryos were collected and centrifuged at 1,200 rpm for 5 min to remove debris. One hundred microliters of sialyl-glycopolymer 3'-sialyl-*N*-acetylactosamine (3'-SLN) and 6'-sialyl-*N*-acetylactosamine (6'-SLN) (V-Lab; 10 μ g/ml or 2.5 μ g/ml in carbonate) was added to each well of the microplates. The microplates were coated at 4°C overnight. After washing the plates with cold PBS five times, the wells were blocked with 100 μ l of PBST (PBS with 0.05% Tween 20) containing 4% lipid-free bovine serum albumin (BSA) at 4°C for 6 h. The plates were washed with 200 μ l of cold PBST five times, and 50 μ l virus supernatant containing 64 HA units was added to each well and incubated at 4°C overnight. The solution was discarded, and the plates were washed with 200 μ l of cold PBST five times. Fifty microliters of solution containing anti-influenza virus NP monoclonal antibody (Thermo Scientific) was added to each well for 2 h at 4°C. After washing, the plates were incubated with 50 μ l of anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Dako-Cytomation, Denmark; 1:1,000) diluted with PBST containing 1% lipid-free BSA for 2 h at 4°C. The plates were washed with 200 μ l of cold PBST five times. The color reaction was developed by incubating with 100 μ l of *o*-phenylenediamine dihydrochloride (OPD) (Sigma) solution (in 100 mM phosphate-citrate buffer, pH 5.0) at 37°C for 15 min. The reactions were stopped by adding 50 μ l of 1 N H₂SO₄ solution (Sigma). The absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Pig study. In the first pig study, 73 5-week-old influenza H1 and H3 subtype virus- and porcine reproductive and respiratory syndrome virus-seronegative crossbred pigs were randomly allocated into 5 groups (4 infected and 1 control groups). Each infected group contained 16 pigs, while the control group had 9 pigs. Twelve pigs from each infected group and the 9 control pigs were intratracheally inoculated with 10⁶ TCID₅₀ of each virus (KS-91088, KS-107824, KS-110529, or KS-83533N) or virus-

free MEM as described previously (32). The remaining 4 naive pigs from each infected group were commingled with inoculated pigs at 2 days p.i. to investigate viral transmission.

Body temperature and clinical symptoms for all experimental pigs were monitored throughout the experiment. Four infected pigs from each inoculated group and 3 control animals were euthanized at 3, 5, and 7 days p.i., and 4 contact pigs were necropsied at 5 days postcontact (p.c.). Blood samples were collected before challenge or contact and on necropsy days. Nasal swabs were collected at 0, 3, 5, and 7 days p.i. for inoculated pigs and at 2, 4, and 5 days p.c. for contact animals. During necropsy, the percentage of gross lesions on each lung lobe was scored by a single experienced veterinarian. Bronchoalveolar lavage fluid (BALF) samples were collected by flushing a lung with 50 ml of MEM. The virus titers of BALF and nasal-swab samples were determined on MDCK cells in 96-well plates. Tissue samples from the nasal turbinate, the trachea, and the right cardiac lung lobe were collected and fixed in 10% buffered formalin for the pathological examination. The lung sections were examined by a veterinary pathologist in a blinded fashion and given a score of 0 to 3 to reflect the severity of bronchial epithelial injury, as described previously (32, 33).

In the second pig study, 25 3-week-old influenza H1 and H3 subtype virus- and porcine reproductive and respiratory syndrome virus-seronegative crossbred pigs were purchased and randomly allocated into 3 groups (2 infected and 1 control groups). There were 10 pigs in each infection group and 5 pigs in the control group. Since younger pigs were used in this study (in contrast to 5-week-old pigs in the first study), a lower dose of 10⁴ TCID₅₀ of each virus was used for infection. Six pigs from each infected group and the 5 pigs from the control group were intratracheally inoculated with each virus (rgKS-107824 or rgKS-107824-G228S) or virus-free MEM. The remaining 4 naive pigs from each infected group were commingled with inoculated pigs at 2 days p.i. to investigate viral transmission. Three infected pigs and 3 (or 2) control pigs from each group were necropsied at 5 and 7 days p.i., and 4 contact pigs were necropsied at 5 days p.c. The other procedures were conducted in the same manner as in the first pig study.

Statistical analysis. Macroscopic and microscopic lung lesion scores and virus titers were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA); a *P* value of 0.05 or less was considered significant. The response variables shown to have a significant effect by treatment group 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

Novel reassortant H3N2 viruses replicate more efficiently than nonreassortant endemic H3N2 virus in cell cultures. Plaque assays showed that the three novel reassortant H3N2 viruses (KS-91088, KS-107824, and KS-110529) formed plaque sizes similar to those of the nonreassortant endemic control KS-83533N virus in MDCK cells; endemic and reassortant H3N2 viruses with 5 A(H1N1)pdm09 genes formed similar-size plaques in PK-15 cells and in MDCK cells, whereas novel reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes formed smaller plaques in PK-15 cells than in MDCK cells. The novel reassortant viruses grew more efficiently than the control nonreassortant KS-83533N virus in cultured cells, including MDCK, PK-15, and A549 cells. In MDCK cells, the three novel reassortant viruses grew to significantly higher titers than the nonreassortant endemic virus (KS-83533N) at 24, 36, and 48 h p.i. (Fig. 1A). The KS-107824 virus containing 5 (PA, PB2, NP, M, and NS) genes from A(H1N1)pdm09 grew to significantly higher titers than the control nonreassortant endemic KS-83533N virus at all the tested time points on both PK-15 and A549 cells (Fig. 1B and C), whereas the KS-110529 virus with 3 (NP, M, and NS) genes from A(H1N1)pdm09 had significantly

FIG 1 Growth kinetics of reassortant and endemic swine H3N2 influenza viruses in cell cultures. (A) MDCK cells were infected with different viruses at an MOI of 0.1. (B) PK-15 cells were infected with different viruses at an MOI of 0.01. (C) A549 cells were infected with different viruses at an MOI of 0.01. The data points of the curves indicate the means of the results of 3 independent experiments, and the error bars indicate standard errors of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

higher titers only at 24, 36, and 48 h p.i. on these cells (Fig. 1B and C). For the KS-91088 virus, significant differences were observed only at 36 and 48 h p.i. compared to the control endemic KS-83533N virus on both PK-15 and A549 cells (Fig. 1B and C). No significant difference was observed in terms of growth kinetics in canine, human, and swine cells among the three novel reassortant H3N2 viruses.

Novel reassortant H3N2 viruses are pathogenic in pigs. Five-week-old pigs infected with a high dose (10^6 TCID₅₀/pig) of either

of the three novel reassortant H3N2 viruses displayed fever (around 25 to 100% of the pigs from 1 to 7 days p.i.), which was similar to those infected with the endemic control H3N2 virus. No fever was seen in the mock-infected group. All 3 reassortant viruses, as well as the endemic H3N2 virus, caused significantly more macroscopic lung lesions in infected pigs than in the control group (Table 1). However, no significant differences in macroscopic lung lesions were observed among the infected groups.

All 3 novel reassortant H3N2 viruses, as well as the endemic

TABLE 1 Macroscopic and microscopic lung lesion scores of infected and contact pigs

Lesion size	Virus	Lung lesion score ^a			
		Infected pigs			Contact pigs (5 days p.c.)
		3 days p.i.	5 days p.i.	7 days p.i.	
Macroscopic	KS-83533N	13.71 ± 4.95	6.00 ± 1.55	4.71 ± 1.13	3.00 ± 0.61
	KS-91088	10.68 ± 2.75	4.54 ± 1.97	2.43 ± 0.72	4.18 ± 0.87
	KS-110529	11.71 ± 0.61	9.71 ± 0.87	5.86 ± 0.35	7.68 ± 2.73
	KS-107824	15.93 ± 7.66	5.89 ± 1.19	4.11 ± 1.15	1.32 ± 1.96
	Control	0.00	0.00	0.00	NA
Microscopic	KS-83533N	2.50 ± 0.20	2.38 ± 0.24	1.75 ± 0.14	1.50 ± 0.20 ^b
	KS-91088	1.88 ± 0.13	1.88 ± 0.31	1.75 ± 0.32	1.38 ± 0.24 ^c
	KS-110529	2.25 ± 0.25	2.27 ± 0.24	2.50 ± 0.20	2.38 ± 0.24 ^d
	KS-107824	1.75 ± 0.48	2.13 ± 0.38	1.50 ± 0.20	0.75 ± 0.14 ^{b,c,d}
	Control	0.00	0.00	0.00	NA

^a The macroscopic lung lesion scores were determined as percentages of the lung. The microscopic lung lesion scores were determined by the following criteria: 0, no lesion; 1, mild; 2, moderate; 3, severe. The data are means ± standard errors of the mean (SEM). NA, not applicable; there were no control pigs in the contact group.

^b Significant differences were observed between the 2 groups ($P < 0.05$).

^c Significant differences were observed between the 2 groups ($P < 0.05$).

^d Significant differences were observed between the 2 groups ($P < 0.001$).

FIG 2 Virus loads in nasal swabs and BALF samples of contact and principal pigs infected with endemic and different reassortant H3N2 viruses. (A) Virus titers in BALF samples of infected and contact pigs. (B) Virus titers in nasal swabs of infected pigs. (C) Virus titers in nasal swabs of contact pigs. All animals were positive for virus isolation at the time points shown unless otherwise indicated (e.g., 2/4 means 2 of 4 animals were positive). The dotted lines represent the limits of detection. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

H3N2 KS-83533N viruses, replicated efficiently in pigs' lungs. Virus was detected in the lungs of pigs infected with either reassortant or endemic H3N2 viruses at 3 and 5 days p.i., with the exception of one pig from the KS-107824 [5 genes from A(H1N1)pdm09] infection group at 5 days p.i. No virus was found in the lungs of pigs infected with the different viruses at 7 days p.i. (Fig. 2A). Although virus titers were variable between the different groups on the indicated necropsy days, no significant differences in virus titers were observed among the infected groups (Fig. 2A). The microscopic pathology score (0 to 3) was between 1.50 and 2.50 at 3, 5, and 7 days p.i. in all 4 inoculated groups compared with a score of 0.00 in control pigs. No significant difference was observed among the infected groups (Table 1). All infected pigs had variable degrees of lung damage, ranging from mild to moderate bronchointerstitial pneumonia, atelectasis, acute to subacute bronchiolitis with epithelial necrosis, and variable lymphocytic cuffing of bronchioles at 3, 5, and 7 days p.i. (Table 1 and Fig. 3).

Novel reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes are transmitted more efficiently than the virus with 5 A(H1N1)pdm09 genes in pigs. All three reassortant viruses and the endemic H3N2 virus were transmitted to sentinel pigs from primary infected animals. Most of pigs infected with the novel reassortant H3N2 virus with 3 genes from A(H1N1)pdm09 (KS-110529 or KS-91088) shed virus via the nasal cavity at 3 (92% to 100%) and 5 (100%) days p.i. with high titers ($10^{3.23}$ to $10^{4.26}$ TCID₅₀/ml), similar to the endemic KS-83533N-infected pigs

(80 to 100%). However, only 42% (5/12) and 25% (2/8) of the pigs infected with the KS-107824 virus with 5 genes from A(H1N1)pdm09 shed virus via the nasal cavity at 3 and 5 days p.i., respectively, with lower titers ($10^{1.60}$ to $10^{2.62}$ TCID₅₀/ml) than the reassortant viruses with 3 A(H1N1)pdm09 genes and the control endemic virus (Fig. 2B).

All contact pigs (100%; 4/4) from groups infected with either endemic KS-83533N or reassortant viruses (KS-91088 or KS-110529) with 3 genes from A(H1N1)pdm09 had fever at 2 and 3 days p.c., and 50% of the contact pigs from these 3 groups still displayed fever at 4 and 5 days p.c. In contrast, only 50% of the contact pigs in the group infected with KS-107824 virus with 5 A(H1N1)pdm09 genes showed fever at 2 and 3 days p.c., and only 25% of contact animals had fever at 4 and 5 days p.c. Only minimal gross lung lesions were observed in the latter contact group of pigs, whereas moderate lung lesions were found in all contact pigs from the other contact groups. Notably, more severe lung lesions were observed in contact pigs of the KS-110529 infection group, in contrast to the other 3 infection groups (Table 1). Virus was detected at 5 days p.c. in the lungs of all contact pigs of each group, with the exception of 2 contact pigs of the KS-107824 [5 A(H1N1)pdm09 genes]-infected group (Fig. 2A). Nasal virus shedding was detected from contact pigs of all infected groups. The reassortant KS-91088 virus with 3 A(H1N1)pdm09 genes displayed kinetics in nasal virus shedding and transmission in contact pigs similar to those of the endemic H3N2 virus. The reassortant KS-107824 virus with 5 A(H1N1)pdm09 genes showed

FIG 3 Microscopic lung sections from pigs infected with various H3N2 viruses at 5 days postinfection. (A) The bronchioles are lined by normal cuboidal epithelium (arrow) and the alveoli are clear (asterisk) in the control group. (B) The bronchiolar and alveolar lumen is filled with large numbers of degenerate and intact neutrophils (asterisks), and moderate interstitial, peribronchiolar, and perivascular lymphocytic infiltration is also seen in the KS-83533N group. (C) Moderate bronchiolar epithelial loss and early regeneration are seen (arrow), and the alveolar and bronchiolar lumen contains small numbers of neutrophils (asterisk) in the KS-91088 group. (D) Moderate to severe bronchiolar epithelial degeneration and necrosis with early regeneration are observed (arrow) in the KS-107824 group. (E) Bronchiolar epithelial necrosis with sloughing of necrotic cells in the lumen was noticed (arrow), and the peribronchiolar and interstitial areas contained moderate numbers of lymphocytes and fewer neutrophils (asterisk) in the KS-110529 group. Scale bars, 50 μ m.

delayed and inefficient transmission and shedding kinetics, since nasal shedding was detected only at 4 days p.c. and not at 2 and 5 days p.c. (Fig. 2C). The reassortant KS-110529 virus with 3 A(H1N1)pdm09 genes was shed most efficiently in contact pigs (Fig. 2C) and also caused significantly more severe microscopic lung lesions in contact pigs than the endemic virus and the other 2 reassortant viruses (Table 1).

Polymerase and neuraminidase activities of the reassortant KS-107824 virus with 5 A(H1N1)pdm09 genes. Compared to endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes, the reassortant KS-107824 virus additionally obtained the polymerase PB2 and PA genes from A(H1N1)pdm09. Therefore, we performed a minigenome assay to investigate the effects of the different polymerase complexes (PB1, PB2, PA, and NP) from each H3N2 virus on polymerase activity. In this assay, the amount of luciferase expression is correlated with the polymerase activity of each virus. The polymerase complex from KS-107824 showed significantly (10-fold) greater polymerase activity than the other 3 combinations from either endemic KS-83533N or reassortant H3N2 (KS-110529 and KS-91088) viruses with 3 A(H1N1)pdm09 genes (Fig. 4A). The polymerase complex from either endemic KS-83533N or reassortant H3N2 (KS-110529 and KS-91088) viruses displayed variable polymerase activities, but no significant difference was observed.

The KS-107824 and KS-83533N viruses, as well as the KS-91088 virus, had an NA from early human influenza viruses, in contrast to the NA of the KS-110529 virus derived from recent seasonal human influenza viruses. To investigate whether the dif-

ferent NA has influence on virus features, we performed a neuraminidase assay. The results showed that the early NA from the endemic KS-83533N virus displayed higher enzyme activity than those from reassortant H3N2 viruses with 3 or 5 A(H1N1)pdm09 genes and from the A(H1N1)pdm09 virus, independent of whether the NA was derived from an early or recent human influenza virus. The NAs from both KS-107824 (an early human influenza virus N2) and KS-110529 (a recent human influenza virus N2), as well as from KS-91088 (an early human influenza virus N2), had enzyme activities similar to that of the A(H1N1)pdm09 virus (Fig. 4B).

HA receptor binding preferences of novel reassortant H3N2 and singly mutated viruses. Our previous studies showed that the KS-107824 HA contains 226V and 228G at the receptor binding sites, whereas the HA proteins of endemic and reassortant H3N2 swine viruses used in this study have 226V and 228S (commonly found in triple-reassortant H3N2 viruses) at their receptor binding sites. The 226V-228G combination in the HA receptor binding site is rarely found, and its role in virus receptor binding, replication, or transmission in pigs remains unknown. Therefore, we first determined its role in receptor binding specificity with a hemagglutination assay using resialylated red blood cells. The three reassortant and endemic H3N2 viruses were able to bind rooster red blood cells that contained both α -2,3 and α -2,6 receptors. The KS-107824 virus bound to both α -2,6 and α -2,3 receptors with a low affinity for α -2,3 receptors. In contrast, the two reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes and the endemic H3N2 virus bound only to α -2,6 receptors. The single substitution

FIG 4 Polymerase and neuraminidase activities of endemic and different reassortant H3N2 viruses in 293T cells at 37°C. (A) Comparison of polymerase activities of endemic and different reassortant H3N2 viruses containing 3 or 5 genes from pH1N1 virus in 293T cells at 37°C. (B) Comparison of neuraminidase activities of different reassortant and endemic H3N2 swine viruses with that of the 2009 pandemic H1N1 virus. For each virus, three independent replicate experiments were conducted. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. TRIG, North American triple-reassortant internal gene; pH1N1, 2009 pandemic H1N1 virus-like gene; CA09, A/California/04/2009.

(G to S) at position 228 in HA resulted in greatly enhanced affinity for α -2,6 receptors and reduced its binding affinity for α -2,3 receptors (Table 2). Although the rescued wild-type rgKS-107824 and the singly mutated rgKS-107824-G228S virus displayed different HA receptor binding preferences, they showed similar growth kinetics in MDCK cells.

To confirm the receptor binding properties of each novel H3N2 virus, we examined the receptor binding affinities of the virus with different glycans using a solid-phase binding assay. As shown in Fig. 5, results similar to those with the hemagglutination assay using resialylated red blood cells were obtained. The endemic virus (KS-83533N) and the reassortant H3N2 virus with 3 A(H1N1)pdm09 genes preferentially bound to α -2,6 sialic acid glycans, whereas the KS-107824 preferentially bound to α -2,3 sialic acid glycans. A single substitution (G to S) at position 228 in HA noticeably switched the receptor binding specificity of the KS-107824 virus from α -2,3 sialic acid to α -2,6 sialic acid glycans.

Glycine at position 228 in HA is responsible for inefficient transmission of the KS-107824 virus. No obvious respiratory

signs were observed in 3-week-old pigs infected with a low dose (10^4 TCID₅₀/pig) of reverse-genetics-derived wild-type rgKS-107824 and singly mutated rgKS-107824-G228S viruses. Fever was observed in one out of six pigs infected with the rgKS-107824-G228S virus at 3 days p.i. and lasted for 3 days. Similarly, only one pig (of six) infected with the rgKS-107824 virus displayed fever at 6 days p.i., which lasted for 1 day. In contrast to the control group, both the rgKS-107824 and rgKS-107824-G228S viruses caused gross lung lesions at both 5 and 7 days p.i. Furthermore, the rgKS-107824-G228S virus induced more severe lung lesions than wild-type rgKS-107824 virus at 5 days p.i. (Table 3). Both viruses were detected in the lungs of all the infected pigs at 5 days p.i., but also in 2 out of 3 pigs at 7 days p.i. Notably, the titers of rgKS-107824-G228S virus were higher than those of wild-type rgKS-107824 at the tested time points, but no significant difference was observed (Fig. 6A).

TABLE 2 HA receptor binding preferences of wild-type and rescued viruses

Virus	Residue at HA 226/228	Titer for receptor(s) ^a :		
		α -2,3 + α -2,6	α -2,6	α -2,3
KS-83533N	V/S	64	64	0
KS-91088	V/S	128	128	0
KS-110529	V/S	128	64	0
KS-107824	V/G	64	32	4
rgKS-107824	V/G	64	32	8
rgKS-107824-G228S	V/S	256	256	0
A/chicken/Jena/4836/1983 (H2N2)	Q/G	256	0	128
A/Brisbane/59/2007 (H1N1)	— ^b	512	512	0

^a The titer shown is the reciprocal of the highest virus dilution that hemagglutinated RBCs bearing various sialic acid receptors.

^b —, not applicable.

FIG 5 Characterization of the receptor binding properties of endemic and different reassortant H3N2 viruses. Binding of the viruses to 2 different glycans (one α -2,3 glycan and one α -2,6 glycan) at 2 different concentrations (10 μ g/ml or 2.5 μ g/ml) was tested. Each bar shows the ratio of the average of 3'-SLN or 6'-SLN absorbance from three independent experiments.

TABLE 3 Macroscopic lung lesions of contact and principal pigs infected with the rgKS-107824 or singly mutated rgKS-107824-G228S virus

Virus	Lung lesion score ^a		
	Infected pigs		Contact pigs
	5 days p.i.	7 days p.i.	(5 days p.c.)
rgKS-107824	15.33 ± 4.84	7.66 ± 3.84	7.25 ± 4.13
rgKS-107824-G228S	35.33 ± 11.92	8.66 ± 2.72	5.50 ± 2.06
Control	0.00	0.00	NA

^a The lung lesion scores were determined as percentages of the lung; the data are means ± SEM. NA, not applicable; there were no control pigs in the contact group.

Although both viruses were transmitted to contact pigs, the efficiencies of nasal shedding of the two viruses were very different. The rgKS-107824-G228S virus could be detected in 3 out of 6 nasal-swab samples collected from infected pigs as early as 3 days p.i., whereas virus was found in nasal-swab samples from only one pig infected with rgKS-107824. At later time points (5 and 7 days p.i.), virus was detected in nasal swabs collected from both infected groups; more infected pigs with higher virus titers were found in the rgKS-107824-G228S-infected group than in the wild-type rgKS-107824-infected group (Fig. 6B). Virus was detected in the lungs of all contact animals (4/4) in each group, but the titers detected in the rgKS-107824-G228S group were higher than those detected in the rgKS-107824 group (Fig. 6A). The wild-type rgKS-107824 virus exhibited delayed and inefficient nasal shedding in sentinel animals, as no contact pigs in the rgKS-107824 group

shed virus at 2 days p.c. and only one and two pigs shed virus at 4 and 5 days p.c. In contrast, all contact pigs (4/4) in the rgKS-107824-G228S group shed virus with significantly higher titers at both 4 and 5 days p.c. (Fig. 6C).

DISCUSSION

At least 10 different genotypes of reassortant H3N2 viruses with 1, 2, 3, 4, 5, or 6 genes from A(H1N1)pdm09 have been detected in U.S. swine (24, 25, 34, 35). Viruses with an A(H1N1)pdm09 gene(s), called H3N2 variants, or H3N2v, have been transmitted to and infected humans, mainly during state fair events (7, 27). From August 2011 to October 2014, 343 cases of human infections with the H3N2 variant have been reported, including 18 hospitalizations and one death (36). To our knowledge, the H3N2 variant with a single M gene from A(H1N1)pdm09 is responsible for most human infections, although other genotypes of reassortant H3N2 viruses containing A(H1N1)pdm09 PA and M genes; A(H1N1)pdm09 NP and M genes; or A(H1N1)pdm09 PA, NP, and M genes have been reported to infect humans. This has raised the question of whether other genotypes of novel reassortant H3N2 viruses not yet detected in humans will be maintained or even become the predominant viruses in swine herds and whether they might cross the species barrier to infect humans.

To date, three genotypes of novel reassortant H3N2 viruses with an A(H1N1)pdm09 gene(s) have been evaluated in ferret and pig models (24, 35, 37). Ducatez et al. showed that an H3N2 reassortant virus with A(H1N1)pdm09 PA, NP, and M genes caused only mild clinical signs and replicated in ferrets to the same extent

FIG 6 Virus titers in nasal swabs and BALF samples of contact and principal pigs infected with the rgKS-107824 and/or singly mutated virus. (A) Virus titers in BALF samples of principal infected and contact pigs. (B) Virus titers in nasal swabs of infected pigs. (C) Virus titers in nasal swabs of contact pigs. The error bars represent SEM. *, *P* < 0.05; **, *P* < 0.01. The dotted line indicates the detection limit of the assay.

as the A(H1N1)pdm09 virus and an early triple-reassortant H3N2 swine virus (24), indicating that no enhancement of virulence occurred in ferrets through reassortment. Another study performed in pigs compared the pathogenicity and transmissibility of a human H3N2 variant with those of a reassortant H3N2 swine isolate with 5 A(H1N1)pdm09 (PA, PB1, NP, M, and NS) genes using an endemic H3N2 swine virus as a control. The study showed that no increased virulence and transmissibility were detected in either the human variant or the swine reassortant H3N2 isolate compared to the endemic H3N2 virus (35). A recent study showed that H3N2 variant human isolates could be transmitted efficiently by direct contact and respiratory droplets to naive ferrets and that they replicated in human Calu-3 cells to significantly higher titers than seasonal H3N2 viruses (37).

In this study, we evaluated three novel reassortant H3N2 influenza viruses with 3 (NP, M, and NS) or 5 (PA, PB2, NP, M, and NS) genes from A(H1N1)pdm09 virus isolated from diseased pigs *in vitro* and *in vivo*. These reassortant H3N2 viruses have different genetic constellations and belong to different genotypes than the reassortant H3N2 viruses used in previously published studies. Our results revealed that introduction of 3 or 5 genes from A(H1N1)pdm09 virus conferred efficient virus replication in canine, swine, and human cells compared to a contemporary endemic H3N2 virus. Both reassortant H3N2 viruses (KS-110529 and KS-91088) with 3 genes from A(H1N1)pdm09 displayed properties similar to those of the endemic H3N2 virus in infected pigs in terms of virus replication and pathogenicity. However, the KS-110529 virus was more transmissible in pigs than the endemic and KS-91088 viruses, as evidenced by the presence of severe microscopic and macroscopic lung lesions in sentinel pigs and the fact that more sentinels shed viruses via the nasal cavity. The KS-110529 virus has a similar recent human influenza N2 gene, as well as 4 other (PB1, PB2, PA, and HA) genes, but differs from the endemic virus in having NP, M, and NS genes derived from the A(H1N1)pdm09 virus (25); this suggests that introduction of 3 internal genes from A(H1N1)pdm09 enhances viral transmission in pigs. KS-110529 and KS-91088 viruses with 3 genes from A(H1N1)pdm09 have similar genetic constellations but differ in the N2 gene (25), suggesting that the recent human influenza virus N2 plays a critical role in enhancing virus transmission in pigs. Taken together, both the 3 A(H1N1)pdm09 virus internal genes (NP, M, and NS genes) and the recent human influenza virus N2 gene are important for efficient viral transmission. The KS-107824 virus with 5 genes from A(H1N1)pdm09 exhibited virus replication and pathogenesis in infected pigs similar to those of the endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. However, it showed delayed and inefficient pig-to-pig transmission compared to the endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. This is supported by the facts that (i) fewer originally infected pigs shed virus and at a lower titer (virus was detected in the lungs of all infected animals), (ii) virus was detected in the lungs of only 50% of the contact pigs, and (iii) delayed nasal shedding was observed in contact pigs.

Multiple viral factors, including PB2-specific amino acid residues (e.g., 627K/701N) and the balance between the activities of HA and NA have been shown to influence influenza virus transmission and pathogenicity in mammals, including humans (38–42). The cause of inefficient transmission of KS-107824 with 5 A(H1N1)pdm09 genes might reside in its different polymerase complex and NA proteins compared to the endemic and the two

reassortant H3N2 viruses. The KS-107824 virus has the avian-origin PB2 containing 627E and 701D residues, as well as SR polymorphism (590S/591R) that was demonstrated to partly compensate for the absence of 627K in its polymerase activity and virus pathogenicity (43, 44). However, the polymerase complex of the KS-107824 virus showed significantly higher polymerase activity than those from the endemic and the two reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. Furthermore, the NA from both KS-107824 and KS-110529 viruses (derived from early and recent human influenza viruses) had enzyme activities similar to that of the 2009 A(H1N1)pdm09 virus. Whether the presence of an early or recent NA (N2) protein is beneficial for the balance with its H3, resulting in different transmission efficacies, needs to be investigated in future. Taken together, these data indicate that the different polymerase and NA genes present in the reassortant H3N2 viruses might not be responsible for inefficient transmission of the KS-107824 virus.

HA receptor specificity is another important factor that has been known to play a major role in influenza virus cross-species transmission (45–47). The swine H3 HA receptor binding site with the combination of 226V–228S is different from the HAs of most avian (226Q–228G) and human (226L–228S) influenza viruses and is present in 90% of North American triple-reassortant H3N2 influenza viruses in swine (48). The combination 226V–228S at the swine H3 HA receptor binding site binds only to α -2,6 receptors, whereas the combination 226V–228G found in the KS-107824 HA binds to both α -2,3 and α -2,6 receptors in our receptor binding assay. The single substitution G228S in the HA of the KS-107824 virus resulted in a receptor binding preference change. Importantly, in subsequent studies, it could be found that the avian-like 228G within the HA of KS-107824 is at least partially responsible for inefficient viral replication and transmission in pigs. This is supported by the facts that the singly mutated virus (rgKS-107824-G228S) replicated more efficiently than wild-type virus in infected and contact pigs and that more animals in this group shed virus at the tested time points with higher titers.

In addition, our results revealed that host factors are also critical for viral virulence and transmissibility. The KS-107824 virus could not be detected in the lungs of 5-week-old pigs at 7 days p.i., although a high infection dose of virus (10^6 TCID₅₀/pig) was used. However, both the wild-type rgKS-107824 virus and the singly mutated rgKS-107824-G228S virus given at a low infection dose (10^4 TCID₅₀/pig) were able to replicate in 3-week-old pigs until 7 days p.i. These data indicate that both virus and host factors, such as age and immune status, might influence viral replication, transmission, and evolution (49). It would be very interesting to compare the immune responses of the animals at different ages upon infection with influenza virus in future studies. In conclusion, we demonstrate that the reassortant H3N2 virus with 3 A(H1N1)pdm09 genes (NP, M, and NS) and a recent human N2 gene replicates and is transmitted in pigs more efficiently than three other H3N2 viruses (2 reassortant and 1 endemic) and that the HA 228S, in contrast to HA 228G, is critical for the transmissibility of these reassortant H3N2 viruses in pigs. These results are in agreement with our concurrent surveillance data in the Kansas area, where more than 50% of H3N2 swine isolates are KS-110529-like viruses. This is also true for the majority of influenza virus N2 sequences in swine isolates that are circulating in North American swine herds based on national surveillance data (34, 50). This kind of reassortant H3N2 virus continually circulates in Midwestern

swine herds and could become the dominant H3N2 virus in swine populations. Our results provide insights into the pathogenesis and transmission of novel reassortant H3N2 viruses that are circulating in U.S. swine herds and warrant future surveillance and mitigation strategies.

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