Identification and characterization of a highly virulent triple reassortant H1N1 swine influenza virus in the United States

Wenjun Ma · Amy L. Vincent · Kelly M. Lager ·
Bruce H. Janke · Steven C. Henry · Raymond R. R. Rowland ·
Richard A. Hesse · Jürgen A. Richt

Abstract A highly virulent H1N1 influenza A virus, A/Swine/Kansas/77778/2007 (KS07), which caused approximately 10% mortality in finishing pigs, was isolated from herds in the Midwestern United States. Molecular and phylogenic analysis revealed this swine isolate was a triple reassortant virus, similar to an H1N1 virus that infected humans and pigs at an Ohio county fair in August 2007. A pig challenge model was developed to evaluate the pathogenicity and transmission capacity of the KS07 virus. The results confirmed that the KS07 virus is highly virulent in pigs and easily transmitted to sentinel animals. The KS07 virus failed to cross-react with a panel of H1-specific swine sera. Interestingly, the KS07 virus shed for a prolonged period up to 7 days in infected pigs, indicating that this virus can spread efficiently between animals. The highly virulent H1N1 swine influenza virus is further evidence of reassortment among avian, human and swine influenza viruses and justifies the need for continued surveillance of influenza viruses in swine.

W. Ma · A. L. Vincent · K. M. Lager · J. A. Richt
Virus and Prion Diseases of Livestock Research Unit,
National Animal Disease Center, USDA, Agricultural
Research Service, Ames, IA 50010, USA

W. Ma · R. R. R. Rowland · R. A. Hesse · J. A. Richt (✉)
Department of Diagnostic Medicine/Pathobiology,
College of Veterinary Medicine, Kansas State University,
K224B Mosier Hall, Manhattan, KS 66506, USA
e-mail: jricht@vet.k-state.edu

W. Ma · B. H. Janke
Department of Veterinary Diagnostic and Production Animal
Medicine, College of Veterinary Medicine, Iowa State
University, Ames, IA 50011, USA

S. C. Henry
Abilene Animal Hospital PA, Abilene, KS 67410, USA

Introduction

Swine influenza is an acute infectious respiratory disease in pigs caused by influenza A virus within the family *Orthomyxoviridae*. Influenza A viruses are negative, single-strand RNA viruses containing eight segments in their genomes. The segmented nature of the influenza A virus genome allows for antigenic shift or reassortment, leading to generation of novel viruses. Influenza A viruses are divided into different subtypes based on two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are 16 HA and 9 NA subtypes of influenza A viruses that have been found from wild waterfowl and seabirds [1]. Only H1, H3, N1 and N2 subtypes have been consistently isolated from pigs [2–4]. The first swine influenza virus (SIV) isolate in the United States was identified in 1930 belonging to the H1N1 subtype; this SIV subtype still circulates in swine populations worldwide. With the exception of one isolation of human H3N2 virus from pigs in Colorado in 1977 [5], only the classical swine H1N1 (cH1N1) virus was isolated in U.S. swine prior to 1998. In 1998, a triple reassortant H3N2 virus emerged and became endemic in North American swine herds. This virus was composed of HA, NA and polymerase basic 1 (PB1) genes from human influenza viruses; matrix (M), nonstructural (NS) and nucleoprotein (NP) genes from classical swine viruses; and polymerase acidic (PA) and polymerase basic 2 (PB2) genes from avian viruses [6]. Reassortment between triple reassortant H3N2 viruses and cH1N1 viruses has produced a variety of novel reassortants. A consistent finding among all of these viruses is the conservation of the internal genes from the H3N2 triple

reassortant virus. This constellation of genes (PB1 gene of human virus origin; PA and PB2 genes of avian virus origin and the remaining internal genes [M, NS and NP] of swine virus origin [7]) has been referred to as the triple reassortant internal gene cassette (TRIG) [8, 9]. In combination with the TRIG, the reassortant H1N1 viruses contain the HA and NA from the cH1N1 virus; the H1N2 viruses contain the HA from the classical swine virus and the NA from human H3N2 viruses [10, 11]; the H3N1 viruses contain the NA from the classical swine virus, the HA and internal genes from the triple reassortant H3N2 viruses [12, 13]; and in the last few years reassortants between human H1N1 and H1N2 genes with the TRIG cassette have become established in North American swine. The recent emergence of the swine origin pandemic H1N1 influenza A virus in humans demonstrates the flow of genes and viruses between humans and swine is a two-way process [14]. Interestingly, this human virus has a modified TRIG, which has picked up a new matrix gene of a Eurasian swine lineage [14, 15]. Although there have been sporadic reports of swine infected with the human pandemic H1N1 virus following contact with infected humans [16], the significance of the modified TRIG for swine and human infections remains unknown. Therefore, the continuous surveillance of influenza viruses and genes in swine populations is necessary for the protection of swine and humans.

In December 2007, an outbreak of severe respiratory disease occurred in pigs at a commercial grow-finish swine farm in Kansas and caused approximately 10% mortality in finishing pigs. Influenza A virus was identified from pigs, and the isolates were characterized as swine H1N1 virus genetically similar to the H1N1 virus isolated from an Ohio county fair which infected pigs and humans [17]. Here, we report the genetic and biological characterization of the swine isolate A/Swine/KS/7778/2007 (KS07) in an experimental challenge and transmission study.

Materials and methods

Analysis of clinical samples

In December 2007, an outbreak of respiratory disease occurred in pigs at a commercial grow-finish swine farm in Kansas and caused approximately 10% mortality. At necropsy, the attending veterinarian observed gross lesions of pneumonia. Lung tissues from animals that succumbed to infection and 90 nasal swab samples from affected pigs were submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Bronchial swab samples from the lung tissue were suspended in 2 ml of phosphate-buffered saline and tested for a variety of suspected agents at the KSVDL using standard diagnostic techniques. Presence of *Mycoplasma hyopneumoniae*

(*M. hyopneumoniae*) was tested by PCR [13]. Real-time PCR was used to detect influenza A (matrix), porcine reproductive and respiratory syndrome virus (PRRSV; commercial Tetracore test) and PCR was used to detect porcine circovirus type 2 (PCV2) open reading frame 2.

Lung tissues (~ 5 g) were cultured aerobically for bacteria by inoculation on MacConkey, colistin-nalidixic acid, brilliant green, and blood agar plates with and without nicotinamide adenosine dinucleotide (NAD) factor (*Staphylococcus epidermidis* nurse colonies). In parallel, lung tissues (~ 10 g) were homogenized in Eagle's MEM containing 4% bovine serum albumin, 15 µg/100 ml trypsin, and an antibiotic mixture of ciprofloxacin, penicillin, streptomycin and Amphotericine B and cultivated on Madin-Darby canine kidney (MDCK) cells.

For virus isolation, nasal swab samples were passed through 0.45 µm filters to remove any bacterial contamination and were inoculated on monolayers of MDCK cells in 24-well plates. The MDCK cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 1 µg/ml TCPK-trypsin and 0.3% bovine albumin. The plates were incubated at 37°C in a CO_2 incubator and observed daily. After the appearance of cytopathic effects (CPE), cells were lysed by freezing and thawing and virus was stored for further analysis.

Hemagglutination inhibition (HI) assays

Sera from all experimental pigs before infection were tested by HI assay [18]. For HI assays, sera were heat-inactivated at 56°C, treated with a 20% suspension of kaolin (Sigma Aldrich, St. Louis, MO) to eliminate nonspecific inhibitors, and adsorbed with 0.5% chicken or turkey red blood cells. HI assay was performed to test antibodies against a panel of reference SIV strains including A/Swine/IA/1973 H1N1, A/Swine/Texas/98 H3N2 and A/Sw/NC/2001 variant H1N1 to confirm to be negative for SIVs. HI assays were also conducted for KS07 virus to determine seroconversion of infected and contact pigs and cross-reactivity with other H1 SIV anti-sera. To determine the KS07 virus cross-reactivity with other H1 anti-sera, the fold-decrease between the geometric mean titer of homologous virus/anti-serum and the geometric mean titer of KS07 virus/heterologous anti-serum was calculated with ≤4-fold decrease being cross-reactive; between 4- and 8-fold reduction being moderately cross-reactive; and ≥8-fold reduction being a considerable loss in cross-reactivity.

DNA sequencing, phylogenetic analysis and subtype determination

Viral RNA was prepared from 200 µl of virus suspension with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA)

according to the manufacturer's recommendations. Two-step RT-PCR was performed by using universal primers as previously reported [12, 19]. Each gene segment was amplified under standard conditions. PCR products were purified by using a QIAamp Gel extraction kit (Qiagen, Inc., Valencia, CA) and sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). Multiple sequence alignments were made by using SeqMan (DNA-Star, Inc., Madison WI) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [20]. The sequences of influenza A virus published in GenBank were used in the multiple alignments and were identified by their accession numbers. A Megablast search of the Influenza Sequence Database was performed to determine the virus subtype based on the sequence.

Experiments in pigs

SIV and PRRSV-negative pigs were used in this study and all animal experiments were in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center (NADC). The inoculation protocol has been described previously [21]. Briefly, a group of twenty 4-week-old cross-bred pigs were inoculated intratracheally with 1×10^5 TCID$_{50}$/pig of KS07 virus prepared in MDCK cells. Five 4-week-old contact pigs were comingled with inoculated pigs on day 3 post-inoculation (p.i.) to study transmission efficiency. Twenty control pigs were inoculated with noninfectious cell culture supernatant. Nasal swabs were taken on days 0, 3, 5 and 7 p.i., placed in 2 ml MEM and stored at −80°C. Blood was collected from all inoculated, contact and control pigs on days 0, 3, 5, 7, and 14 p.i. Five pigs from the inoculated and control pigs were euthanized on days 3, 5, and 7 p.i., respectively. Bronchioalveolar fluid (BALF) was obtained by the lavage of each lung with 50 ml MEM. Blood was also collected from contact pigs on day 19 post-contact (p.c.), and from the remaining five inoculated and control pigs on day 21 p.i. and tested for HI antibody.

Viral load in BALF was determined in a 96-well plate as previously described [21]. Briefly, 10-fold serial dilutions of each sample were made in serum-free MEM supplemented with TPCK-trypsin and antibiotics. Each dilution (100 µl) was placed on PBS-washed confluent MDCK cells in 96-well plates. Wells were evaluated for CPE after 24 h. At 48 h, plates were fixed with 4% phosphate-buffered formaldehyde and immunocytochemically stained with a monoclonal antibody to influenza A nucleoprotein [22]. The TCID$_{50}$/ml was calculated for each sample by the method of Reed and Muench [23].

Samples from nasal swab were vortexed for 15 s, centrifuged for 10 min at $640 \times g$, and passed the supernatant through 0.45-µm filters to reduce bacterial contamination.

An aliquot of 100 µl was plated on confluent, phosphate buffered saline (PBS)-washed MDCK cells in 48-well plates. After incubation for 1 h at 37°C, 500 µl of serum-free MEM supplemented with 1 µg/ml TPCK trypsin and antibiotics was added. All wells were evaluated for CPE after 48 h. At 72 h, plates were fixed with 4% phosphate-buffered formaldehyde and stained as described above.

BALF was tested for the presence of PRRSV and *M. hyopneumoniae* by diagnostic PCR assays. For PRRSV, the total RNA was isolated from each sample by using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). One microgram of the extracted RNA and a primer pair specific for open reading frame 7 of PRRSV were used in real-time PCR as described previously [13]. DNA was extracted from BALF for detecting *M. hyopneumoniae* as described previously [13].

Examination of lungs of experimental pigs

At necropsy, lungs were removed *in toto*. A single experienced veterinarian recorded the percentage of gross lesions on each lobe. A mean value was determined for the seven pulmonary lobes of each animal [21]. For histopathologic examination, tissue samples from the trachea, the right cardiac pulmonary lobe, and other affected lobes were fixed in 10% buffered formalin, processed and stained with hematoxylin and eosin. 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 [21, 24].

Results

Analysis of clinical samples

The overall mortality in the herd was approximately 10% with the remaining pigs exhibiting severe clinical signs including respiratory distress, lethargy and anorexia. Lungs collected from dead pigs had moderate, subacute to chronic bronchopneumonia and interstitial pneumonia with bronchiolitis and peribronchitis. Pooled lung tissue (from 3 dead pigs) was negative for PCV2 and *M. hyopneumoniae*, but was positive for PRRSV, influenza virus, *Pasteurella multocida*, *Streptococcus suis* and *Streptococcus* sp. Ninety nasal swab samples were positive for influenza virus matrix RNA and 2 of them were also positive for PRRSV RNA. Nasal swabs were negative for PCV2 and *M. hyopneumoniae*.

Subtyping and molecular analysis of the KS07 virus

Nasal swabs with the highest matrix RNA concentration were submitted to the National Animal Disease Center

(NADC) for virus isolation, sequencing and subtyping. The isolate was identified as an H1N1 virus by nucleotide sequence and a BLAST search of the Influenza Sequence Database (www.flu.lanl.gov). The KS07 was repeatedly plaque-cloned, retested, and confirmed by sequencing as belonging to the H1N1 subtype. The 8 gene segments of KS07 (Genbank accession numbers: GQ484355-GQ484362) most closely matched A/Swine/OH/511445/2007 (OH07), an H1N1 virus which infected pigs and humans at an Ohio county fair [17]. Sequence comparison showed that the identity of 8 gene segments between the KS07 and OH07 viruses ranged from 99.7 to 99.9% at the nucleotide level (Table 1). OH07 virus is a triple reassortant virus which carries the surface protein HA and NA genes from swine virus origin and internal genes from human (PB1), avian (PB2, PA) and swine (NP, M, NS) influenza virus origin [17]. Based on the HA genes, the H1 SIV isolates in the U.S. cluster into three distinct genetic groups: H1α (cH1N1), H1β (rH1N1-like) and H1γ (H1N2-like) [11, 25]. Phylogenetic analysis showed that the HA gene of the KS07 isolate can be placed in the H1γ cluster of contemporary H1 SIVs. As expected, the KS07 virus is most closely linked to the OH07 isolate. The same cluster also contains the current pandemic H1N1 viruses (Mexico09 and CA09 H1N1 viruses) (Fig. 1a). The NA gene of the KS07 isolate can be placed in the North American swine N1 cluster. Again the KS07 is closely related to the OH07, but distinct from the NA gene of H1 pandemic viruses, such as Mexico09, which is of Eurasian origin (Fig. 1b).

Molecular comparison of the HA molecules revealed that the HA genes of the KS07 virus had 93.7–99.9%

identity at the nucleotide level and 93.5–100% at the amino acid level to those of other H1 viruses in the same cluster H1γ. The HA of the KS07 H1N1 and other H1 viruses in H1γ cluster depicted in Fig. 1a contain the mammalian receptor binding motif, 190D and 225D (based on H3 numbering). The same motif is found in human H1 viruses. The HA of KS07 and OH07 viruses share putative antigenic determinant sites involving serine at position 71 and asparagine at position 162. These amino acid residues are linked to serological cross-reactivity between OH07 virus with anti-sera raised against viruses in the same genetic cluster [17]. The HA molecule of the current pandemic H1N1 virus (CA09 and Mexico09) contains the same serine at position 71, but a serine not an asparagine at position 162.

Molecular comparison of the NP, PB1 and M1 molecules of the KS07 H1N1 isolate with other H1N1 viruses revealed that they have 100% identity with the OH07 H1N1 virus at the amino acid level. The NS1 showed 99.5% identity between the KS07 and OH07 viruses and NS1 of KS07 differs from that of OH07 by one amino acid substitution (V54I). The NA, PB2 and PA showed 99.6% identity between the KS07 and OH07 viruses at the amino acid level. The PB2 of KS07 virus contained the conserved avian amino acid residue glutamic acid at position 627 as the currently circulating triple reassortant SIVs in North America. Interestingly, KS07, isolated 5 months later than OH07, had two additional substitutions (S35N and G76D) in the NA molecule, three (M344V, M402I and G678D) in the PB2 molecule and three (T32N, D216N and S632T) in the PA molecule when compared to the OH07 virus.

Table 1 Influenza A viruses with the highest and the second highest nucleotide sequence identity to H1N1 swine influenza virus (KS07) as determined by blast search in the Influenza Sequence Database (http://www.flu.lanl.gov/)

Gene	Identity (%)	Virus designation	Subtype	Genbank accession no.
HA	99.9	A/Swine/Ohio/511445/2007	H1N1	EU604689
	99.9	A/Swine/Ohio/02/2007	H1N1	FJ986621
NA	99.9	A/Swine/Ohio/511445/2007	H1N1	EU604690
	99.7	A/Swine/Ohio/24366/07	H1N1	EU409949
PB1	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604692
	99.7	A/Swine/Ohio/24366/07	H1N1	EU409945
PB2	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604691
	99.6	A/Swine/Ohio/24366/07	H1N1	EU409946
PA	99.8	A/Swine/Ohio/511445/2007	H1N1	EU604693
	99.1	A/Turkey/Ohio/313053/2004[a]	H3N2	DQ335776
NP	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604694
	97.8	A/Swine/Indiana/PU542/2004	H3N1	DQ150434
M	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604695
	99.8	A/Swine/Quebec/4001/2005	H3N2	EU826546
NS	99.7	A/Swine/Ohio/511445/2007	H1N1	EU604696
	98.2	A/Turkey/Ohio/313053/2004[a]	H3N2	EU735822

[a] Triple reassortant virus

Fig. 1 Phylogenetic trees of HA and NA genes based on the nucleotide sequences of the ORFs from the KS07 and other influenza A virus sequences available from GenBank. **a** HA phylogenetic tree with three clusters of related viruses, H1α (cH1N1), H1β (rH1N1-like) and H1γ (H1N2-like). **b** NA phylogenetic tree with N1 subtype. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA 4. The reference viruses used in the analysis are abbreviated with their state and year of origin, subtype preceded by host abbreviation (*sw* swine, *hu* human, *dk* duck, *ck* chicken, *ge* goose) and GenBank accession number
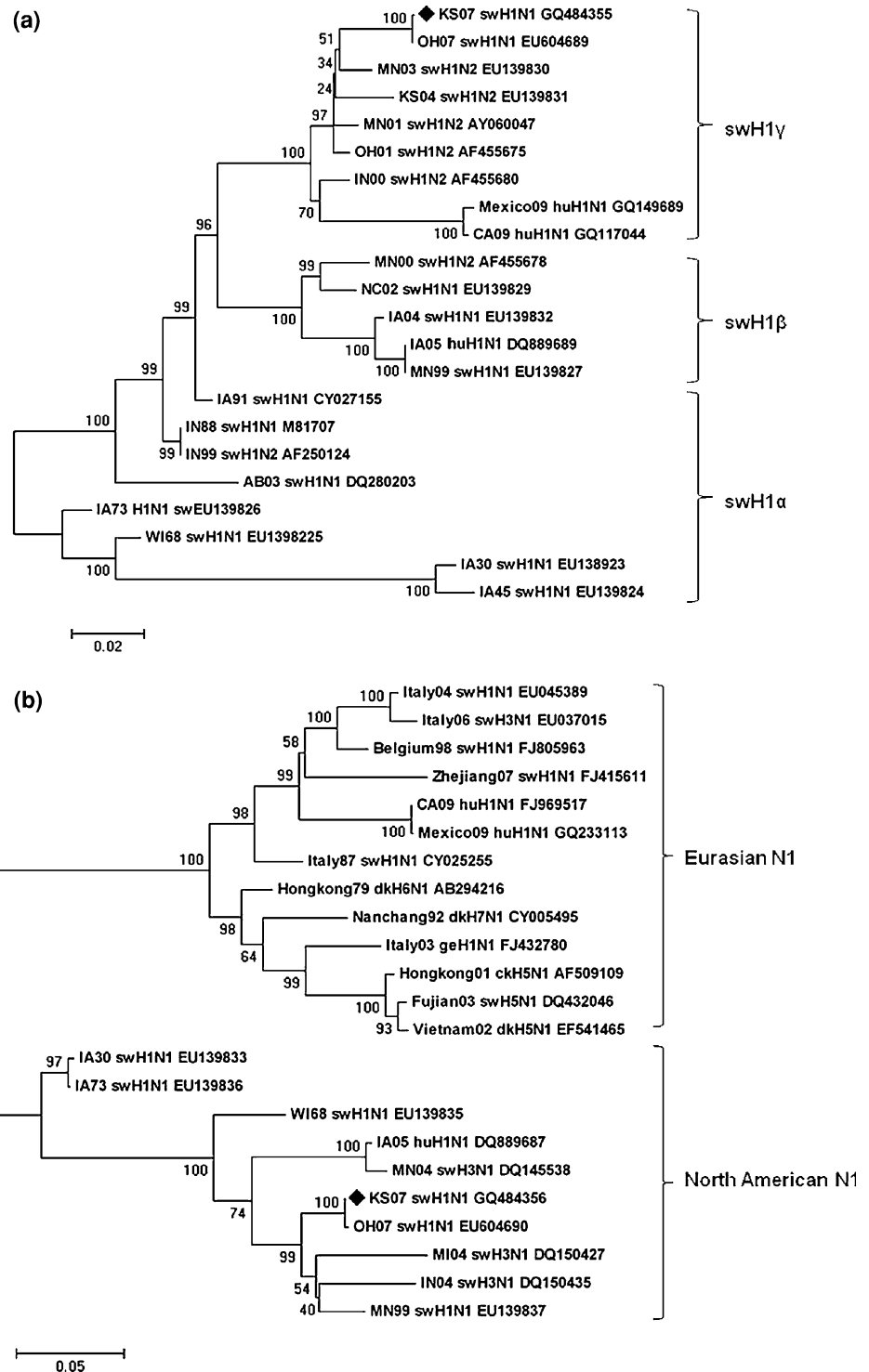
Antigenic cross-reactivity with H1 SIV anti-sera

HI assays were conducted to investigate cross-reactivity between KS07 virus with various anti-sera raised against H1 SIV isolates, including viruses from the H1α, H1β and H1γ clusters. As shown in Table 2, the KS07 virus displayed the greatest cross-reactivity with H1 SIV anti-sera from the same genetic cluster. Reactivity was decreased with anti-sera from the H1α and H1β clusters. The least amount of reactivity was obtained using sera against IA/30 virus, the first SIV isolate in the United States. The results showed that the KS07 virus failed to cross-react with most of the other H1 SIV anti-sera presented in our panel (Table 2). As predicted, the highest antigenic cross-reactivity was with anti-serum from OH07

Table 2 Serological cross-reactivity with H1 reference anti-sera

Anti-sera							
H1α cluster			H1β cluster		H1γ cluster		
IA30	IA73	MN99	NC02	IA04	MN01	KS04	OH07
45[a]	8	6	16	32	3	4	1
5120/113[b]	905/113	1810/320	640/40	1280/40	160/56	1280/320	320/226

[a] Fold-decrease as compared to homologous virus/anti-sera geometric mean titer

[b] Geometric mean titers for homologous antigen and antisera/KS07 antigen with heterologous antisera

H1N1 virus due to 100% identity of HA molecules between KS07 and OH07 H1N1 viruses.

Pathogenicity and transmissibility of KS07 H1N1 swine influenza virus in pigs

To investigate pathogenicity of the isolate KS07 virus, we inoculated intratracheally twenty 4-week-old pigs with 1×10^5 TCID$_{50}$/pig of the KS07 virus. Twenty control pigs were mock-inoculated with noninfectious cell culture supernatant. Transmissibility was assessed by the introduction of five age-matched contact pigs with the inoculated pigs, starting on day 3 p.i. All pigs used in this study were seronegative for H1 and H3 SIVs. One inoculated pig died overnight due to complications from the challenge. Five inoculated pigs and five control pigs each were euthanized for necropsy on days 3, 5, and 7 p.i. The contact pigs and four remaining virus-inoculated pigs were serologically tested on day 21 p.i. Although no respiratory

distress, lethargy and anorexia were observed in KS07 inoculated pigs, severe macroscopic lung lesions (plum-colored, consolidated areas) were observed in inoculated pigs at necropsy. The mean lung lesion score was 19.83, 20.91 and 23.14% for inoculated pigs versus 3.97, 1.17 and 0.57% for control animals at days 3, 5 and 7 p.i., respectively (Table 3). Lungs from inoculated pigs exhibited mild to moderate interstitial pneumonia and acute to subacute necrotizing bronchiolitis with slight lymphocytic cuffing of bronchioles and vessels. The histopathologic score (0–3) expressing the extent of damage to lung architecture was >2.5 in inoculated pigs between days 3 and 7 p.i. (Table 3).

Virus was determined in BALF fluid and in nasal swab samples. Virus titers in the BALF averaged $10^{6.20}$ TCID$_{50}$/ml on day 3 p.i., $10^{5.35}$ TCID$_{50}$/ml on day 5 p.i. and were negative on day 7 p.i. in the KS07 challenge group (Table 3). In the KS07 inoculated group, 89.5% (17/19) of pigs shed virus in the nasal swab samples on day 3 p.i. with an average titer of $10^{3.18}$ TCID$_{50}$/ml (Table 4).

Table 3 Virus titers in BALF, macroscopic lung lesion and microscopic pneumonia in pigs inoculated with KS07 H1N1 virus or mock-inoculated

Group	Virus titer in BALF TCID$_{50}$/ml[a]	Lung lesion score (%)[b]	Histopathologic score (0–3)[b]
KS07 day 3	6.20 ± 0.30	19.83 ± 5.02	2.80 ± 0.34
Control day 3	0.00 ± 0.00	3.97 ± 1.99	0.40 ± 0.29
KS07 day 5	5.35 ± 0.28	20.91 ± 4.69	2.50 ± 0.52
Control day 5	0.00 ± 0.00	1.17 ± 0.36	0.20 ± 0.12
KS07 day 7	0.00 ± 0.00	23.14 ± 4.82	2.80 ± 0.34
Control day 7	0.00 ± 0.00	0.57 ± 0.27	0.00 ± 0.00

[a] Numbers are log$_{10}$ geometric mean BALF TCID$_{50}$/ml ± SEM

[b] Values are mean ± SEM

Table 4 Shedding and transmission of the KS07 virus

	Nasal shedding			Seroconversion
	Day 3	Day 5	Day 7	Day 19/21
Primary	17/19[a] (3.18 ± 0.23)[b]	12/14 (3.40 ± 0.27)	2/9 (1.00 ± 0.00)	4/4 (226)[c]
Contact	5/5 (3.15 ± 0.17)	5/5 (2.60 ± 0.47)	3/5 (1.75 ± 0.75)	5/5 (105)

[a] Number positive out of total number on days 3, 5, 7, 19 or 21 post-infection of post-contact

[b] Numbers in parentheses are log$_{10}$ geometric mean nasal swab TCID$_{50}$/ml ± SEM

[c] Numbers in parentheses are the geometric mean reciprocal HI titer

On day 5 p.i., 85.7% (12/14) of nasal swabs were positive with an average titer of $10^{3.40}$ TCID$_{50}$/ml. The 22.2% (2/9) of the nasal swab samples were positive on day 7 p.i. with an average titer of 10^1 TCID$_{50}$/ml (Table 4). The remaining KS07 inoculated pigs seroconverted by the end of the experiment (day 21 p.i.) with reciprocal HI titers ranging from 80 to 640 and a geometric mean reciprocal HI titer of 226 (Table 4). A few control pigs had an occasional small focus of mild interstitial pneumonia (Table 3), but were negative for SIV infection. In contrast to the inoculated group, 100% (5/5) of pigs in the contact group shed virus in the nasal swab samples on days 3 and 5 p.c. with average titers $10^{3.15}$ and $10^{2.60}$ TCID$_{50}$/ml, respectively (Table 4). On day 7 p.c., 60% (3/5) of the nasal swab samples were positive with an average titer of $10^{1.75}$ TCID$_{50}$/ml (Table 4). The contact pigs were analyzed for seroconversion at day 19 p.c. with reciprocal HI titers ranging from 80 to 160 and a geometric mean reciprocal HI titer of 105 (Table 4). Our results indicate that the KS07 H1N1 virus is virulent in pigs and is transmissible among pigs.

Discussion

The triple reassortant H1N1 influenza virus has been one of the major endemic subtypes in swine populations of North America. In this study, we characterized a highly virulent triple reassortant H1N1 virus, KS07, which caused approximately 10% mortality in finishing pigs and is genetically similar to the OH07 H1N1 virus. The OH07 H1N1 virus was isolated from infected pigs and humans who exhibited signs of influenza clinical illness [17]. The OH07-like H1N1 viruses did not disappear, but continued to be isolated from the U.S. swine herds. The epidemiological data indicates that OH07/KS07-like viruses have spread and are endemic in U.S. swine populations.

When compared to the OH07 H1N1 virus, the KS07 virus had similar pathogenicity and transmissibility among pigs [17]. Although 10% of mortality was reported in original herds where the KS07 virus was isolated, no obvious clinical signs were observed in experimentally infected pigs. There are several possible explanations for the different outcomes. The field pigs were significantly older and exposed to the virus under field conditions, which included several additional stressors. For example, the presence of PRRSV, *P. multocida*, *S. suis* and *Streptococcus* sp, circulating in the herd at the time of infection, may have also enhanced clinical disease signs. However, the KS07 virus induced detectable macroscopic and microscopic lesions in lungs, which were similar to the OH07 isolate [17] and more severe than lesions associated with other contemporary H1N1 SIVs under similar experimental protocols [25, 26]. The KS07 virus failed to cross-react with sera from previous H1N1 isolates similar as shown for the OH07 virus [17], suggesting that current vaccines might not provide efficient protection for swine herds.

An important difference between KS07 and OH07 viruses is that the OH07 virus infected humans. There have been no reports of clinically ill humans exposed to herds acutely infected with the KS07 virus. To date, it remains unknown whether the KS07 virus is able to infect humans; one likely possibility is the limited interaction of swine herds with humans due to increased biosecurity. As reported recently, the sequences of the OH07 swine and human isolates are identical [17]. The genome sequence of the KS07 virus was shown to be almost identical to the OH07 virus. Importantly, the amino acid sequence of the KS07 and OH07 HA are 100% identical. Two amino acid residues (E190D and G225D, H3 numbering) in the receptor binding sites have been described to have a fundamental role in avian and human receptor binding specificity of H1 viruses [27], and these two residues are conserved between human and swine H1 isolates. As predicted, these positions are mammalian-like D190 and D225 in the HA of both OH07 and KS07 H1N1 viruses. The PB2 gene has been shown to be crucial for interspecies transmission barriers for avian influenza viruses to infect mammals, especially the amino acid residue E627K [28]. Since the PB2 in the contemporary SIVs is of avian viral origin, it is of interest to evaluate the PB2 at this amino acid position. Interestingly, the avian type (627E) is present in both KS07 and OH07 PB2 genes and also in currently circulating triple reassortant SIVs' PB2 genes, indicating this site is not critical for SIVs. The KS07-like H1N1 viruses cause severe respiratory disease outbreaks in young to mature pigs and have continued to be identified in swine populations. Therefore, it is necessary to continue surveillance due to its high virulence in pigs and potential threat for humans.

Pigs have been considered to be a "mixing vessel" for human and avian influenza viruses because the epithelial cells in pigs have receptors for both influenza viruses [29]. Transmission of SIVs to humans has been documented. However, most transmission events are isolated events and do not lead to outbreaks in human populations [30, 31]. As mentioned above, pigs can be infected with wholly avian and/or human viruses, allowing swine viruses to acquire avian and/or human virus gene segments to generate novel SIVs [7, 32–36]. Further supporting the occurrence of such events is our recent identification of a swine and avian reassortant H2N3 virus [24], an HA subtype not seen in the human population since the 1957–1968 H2N2 pandemic. The TRIG cassette from the contemporary swine triple reassortant viruses seems to have offered currently circulating SIVs the ability to acquire new HA and NA genes as well as an increased rate of antigenic drift [9, 37]. This was

not previously observed with classical swine H1N1 viruses. The appearance of the new swine-origin pandemic influenza A H1N1 virus has demonstrated the promiscuity and cross-species adaptability of these SIVs. As of 6 August 2009, it has infected more than 177,400 people and caused the deaths of 1,462 persons in over 170 countries and territories worldwide [38]. The WHO raised the alert level for the pandemic H1N1 to the highest possible level 6. This entirely novel H1N1 virus has not circulated previously in humans. The genome of the pandemic H1N1 virus contains 6 genes (PB1, PB2, PA, HA, NP and NS) from currently circulating SIVs in North America and 2 genes (NA and M) from Eurasian SIVs [14, 15]; this indicates that pigs may have played a central role in the generation of this virus. So far the pandemic H1N1 virus has been found in several swine farms in Canada [39], Argentina [40] and Australia [41], indicating the potential for the infection of pigs. How and when this virus evolved is still unknown, but genetic analysis indicated the reassortment between North American swine triple reassortant viruses and Eurasia SIVs might have occurred years before emergence in humans and have been circulating undetected among swine herds somewhere in the world [14, 15] Notably, OH07/KS07-like viruses as well as the current pandemic H1N1 virus belong to the same HA cluster and share similar putative antigenic determinant sites compared to other H1 viruses in the same cluster. The OH07 virus was transmitted to humans, but seemed to not transmit among them. Therefore, it is critical to study transmission capacity of swine H1N1 viruses to elucidate the molecular mechanism by which the pandemic H1N1 virus can successfully transmit from human-to-human in order to prevent and control future pandemics.

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