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Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States

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Abstract Reassortant H1 swine influenza viruses (SIVs) carrying 2009 pandemic H1N1 virus (pH1N1) genes have been isolated from pigs worldwide. Seven novel reassortant H3N2 SIVs were identified from diseased pigs in the USA from winter 2010 to spring 2011. These novel viruses contain three or five internal genes from pH1N1 and continue to circulate in swine herds. The emergence of novel reassortant H3N2 SIVs demonstrates reassortment between pH1N1 and endemic SIVs in pigs and justifies continuous surveillance. Since the first report on infection of pigs with the 2009 pandemic H1N1 virus (pH1N1) in Canada in 2009 [8], pH1N1 has been isolated from pigs throughout the world, including the USA [21]. Although pH1N1 was generated through reassortment of North American triple reassortant SIVs with the Eurasian avian-like H1N1 SIVs, to date, there is no evidence that the Eurasian avian-like H1N1 SIVs were ever isolated from US pigs. Introduction of pH1N1 into swine has raised concerns that novel reassortant viruses could be generated in pigs, that these reassortants might be more virulent and be transmitted more efficiently among humans than the parental viruses, and that they could cause the next pandemic. Reassortments of SIVs with pH1N1 in swine have been reported worldwide. The first reassortant H1N1 virus was found in Hong Kong, China, in 2009, and contained NA from pH1N1, HA from the Eurasian avian-like H1, and six internal genes from triple reassortant SIVs [22]. Subsequently, a reassortant H1N1 virus consisting of seven genes from pH1N1 and NA from endemic SIVs was isolated in pigs in Germany [18]. In early 2010, three reassortant H1N1 viruses containing NA from endemic H1N1 SIV and the remaining seven genes from pH1N1 were isolated from pigs in Thailand [10]. Recently, two reassortant H1N2 viruses were isolated from pigs in the UK and Italy; one contains six internal genes of pH1N1 and another isolate has HA and six internal genes of pH1N1 and the remaining genes from endemic SIVs [7, 15]. In the USA, nine H1N2 SIVs and one H1N1 reassortant SIV have been detected in swine herds from Indiana, Minnesota and North Carolina. All of these reassortant viruses contain the M gene and additional one to four internal genes from pH1N1 and the remaining genes from endemic triple reassortant SIVs [5, 20].

Here, we report the characterization of seven reassortant H3N2 SIVs containing internal genes from pH1N1. These novel reassortant H3N2 SIVs were isolated between winter of 2010 and spring of 2011 from five swine farms in the Midwestern USA in which outbreaks of respiratory disease had occurred. Genome analysis showed that six viruses contained NP, M and NS genes from pH1N1, and one isolate had PB2, PA, NP, M and NS from pH1N1 and the remaining genes from endemic H3N2 SIVs (Table 1).

In December 2010, an outbreak of respiratory disease occurred in nursery pigs in a commercial swine farm (farm #1) in the Midwestern USA. In mid-January 2011, 50% of the sows that provided piglets to farm #1 (6000 sows in

Table 1 Reassortant patterns of novel H3N2 swine influenza viruses in the USA

Virus name	Subtype	Gene segment							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Kansas/10-91088/2010	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-101926/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-104465/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-104467/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-107824/2011	H3N2	P	T	P	T	P	T	P	P
A/swine/Kansas/11-109700/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-110529/2011	H3N2	T	T	T	T	P	T	P	P

PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein, NA, neuraminidase; M, matrix; NS, nonstructural; T: gene has closest homology with triple reassortant swine influenza virus; P: gene has closest homology with 2009 pandemic H1N1 virus

sow farm #1) were sick with acute respiratory signs, and more than 100 sows were suddenly dead within 24 hours after the occurrence of clinical signs. Subsequently, in February 2011, one independent farm (farm #2) located in the same area and farm #1 had an outbreak of respiratory disease in nursery pigs; both received piglets from sow farm #1. In mid-March 2011, an outbreak of respiratory disease occurred in nursery pigs in another independent farm (farm #3) that does not purchase piglets from sow farm #1. At the beginning of April 2011, farm #2 and another independent farm (farm #4) had an outbreak of respiratory disease in nursery pigs, and both farms had purchased piglets from the sow farm #1. During the outbreak, pigs showed respiratory signs, such as coughing, sneezing and nasal discharge. The morbidity was high (≈60%) and the mortality was rather low (≈3%) in the affected herds. The infection persisted in the swine herds throughout the winter in all affected farms. At necropsy, the attending veterinarian observed gross lesions of pneumonia suggestive of influenza. Lung tissues and nasal swab samples from diseased pigs were submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL). SIVs were detected and isolated from samples collected from diseased pigs of all five farms (1 sow farm and 4 nursery farms) by standard real-time RT-PCR and virus isolation in the KSVDL. Lung tissues from these pigs were also found to be positive for porcine circovirus type 2 and *Streptococcus suis*. Porcine reproductive and respiratory syndrome virus was detected in lung tissues of pigs from two affected nursery farms (farm #1 and #2). All seven SIVs were isolated in cell culture using MDCK cells and identified to be of the H3N2 subtype by hemagglutinin inhibition and gel-based RT-PCR assays using standard methods. All isolates were negative by pH1N1 NA-gene specific real-time RT-PCR and positive by pH1N1 M-gene specific real-time RT-PCR [12]. The full genome sequences of all seven isolates were obtained by sequencing all eight gene segments (sequence primers are available upon request). BLAST (<http://blast.ncbi.nlm.nih.gov>) and phylogenetic tree (MegAlign software version 4.1) analyses were conducted to determine the source of the individual genes from the isolates.

Based on sequence analysis, seven H3N2 isolates were identified to be reassortants of pH1N1 and endemic H3N2 SIVs. Phylogenetic analysis revealed that the NP, M and NS genes of all seven novel H3N2 reassortant viruses grouped within the pH1N1 cluster. The PB2 and PA genes of A/swine/Kansas/11-107824/2011 isolated from farm #3 also clustered into the pH1N1 group. The HA genes of these seven viruses belonged to the North American triple reassortant H3N2 virus lineage. The NA gene of A/swine/Kansas/11-110529/2011 isolated from farm #4 grouped within the human-like lineage, whereas the NA genes of the other 6 H3N2 isolates clustered into the North American triple reassortant H3N2 lineage. In addition, all of the other internal genes that are not pH1N1-like were grouped within the triple reassortant SIVs cluster (Fig. 1). Based on the HA phylogenetic tree, the H3N2 triple reassortant viruses in the field are genetically diverse (Fig. 1), because four genetic clusters of H3N2 viruses are circulating presently in US swine herds. These genetically different H3N2 viruses were generated by reassortment events due to at least three introductions of different seasonal human H3N2 viruses into the swine herds in the late 1990s [16, 23, 24].

Molecular analysis showed that the M2 protein of all seven novel reassortant H3N2 viruses had an S31N amantadine-resistance mutation, similar to pH1N1 viruses; the NS1 gene of these seven viruses encodes a truncated 220-amino-acid protein that is identical to pH1N1 NS1. There were no specific mutations for adaptation to mammalian hosts (627E and 701D) [6, 11, 19] in the PB2 of any of the seven novel reassortant H3N2 viruses, but they all had a 271A and SR polymorphism at positions 590/591,

Fig. 1 Phylogenetic trees of the PB2, PB1, PA, HA, NP, NA, M and NS genes of the seven reassortant H3N2 SIVs. The tree was generated by the distance-based neighbor-joining method in the software MEGA 4.1. The reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Horizontal distances are proportional to genetic distance. The viruses isolated in this study are in italic and bold. The viruses (H3N2) that infected two children in Pennsylvania and Indiana are in bold

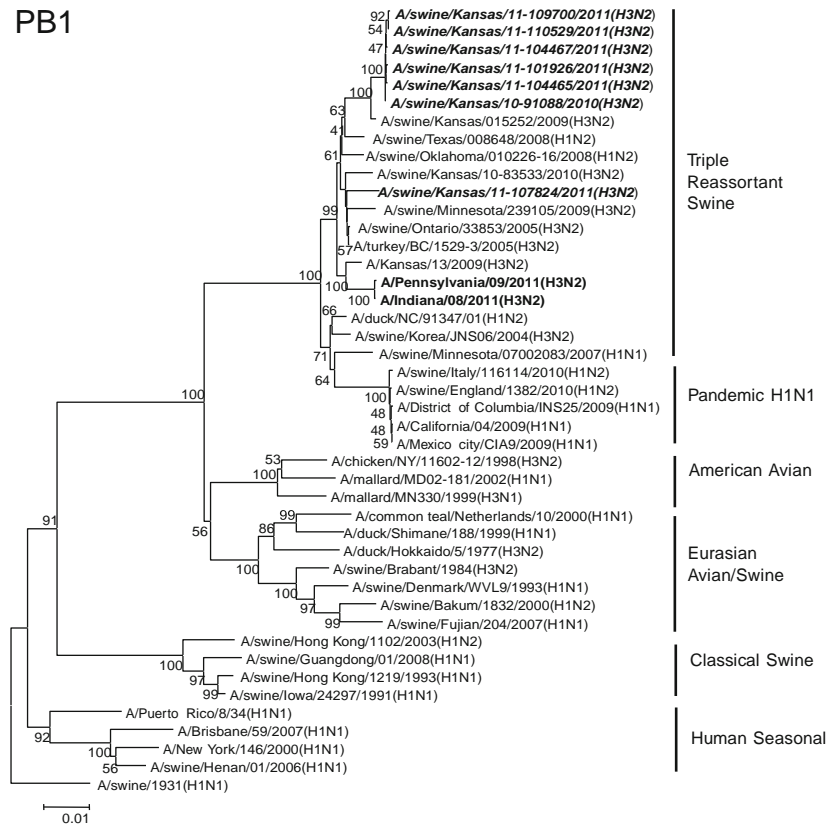
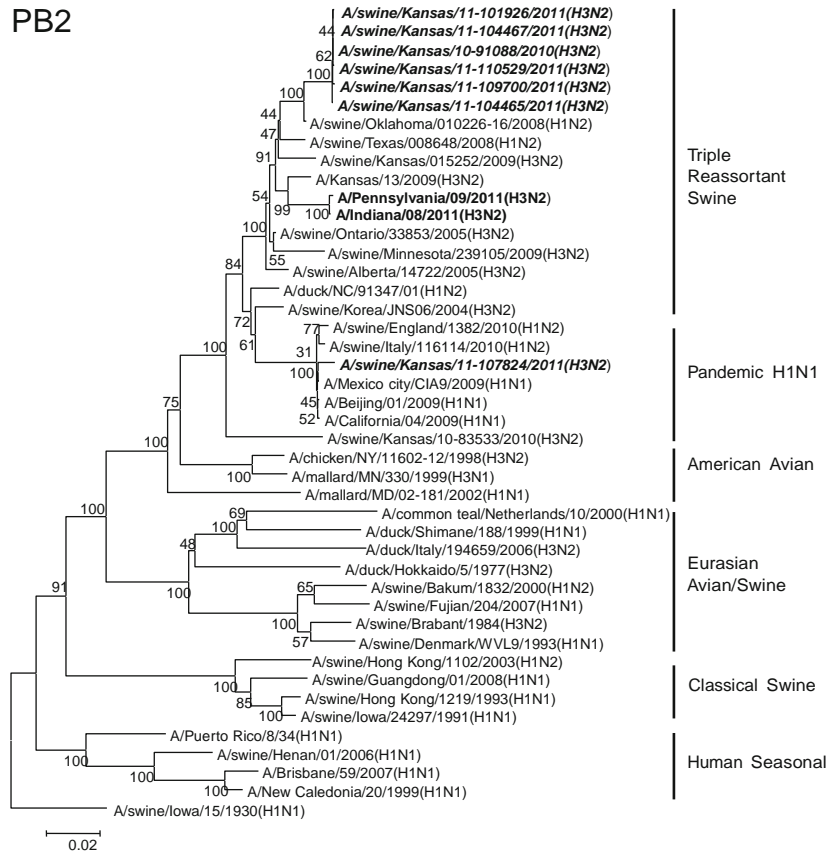


Fig. 1 continued

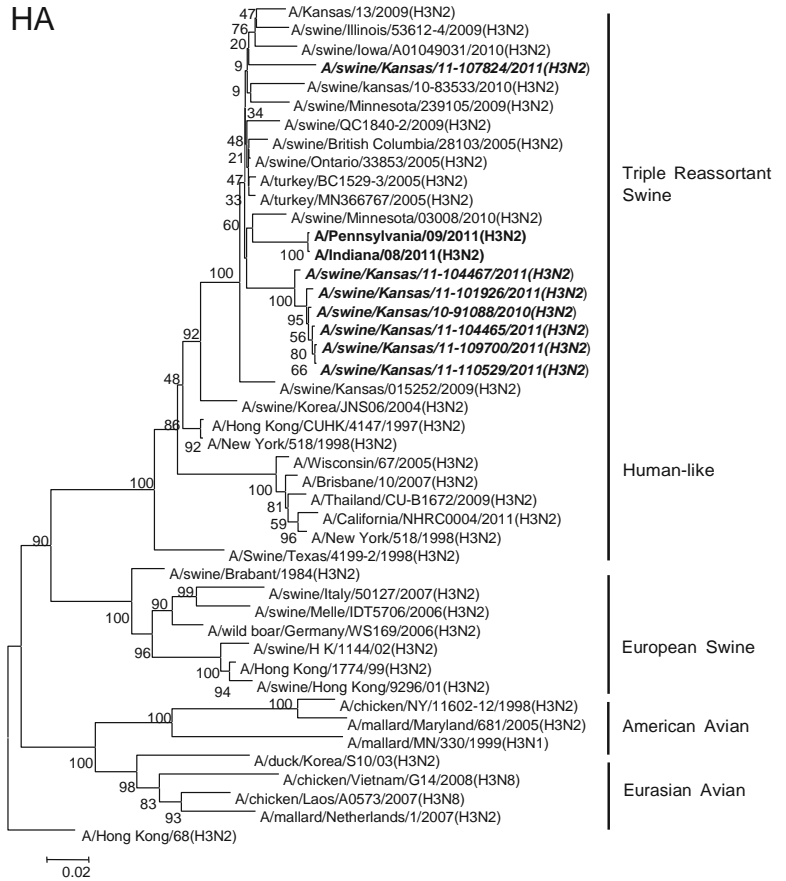
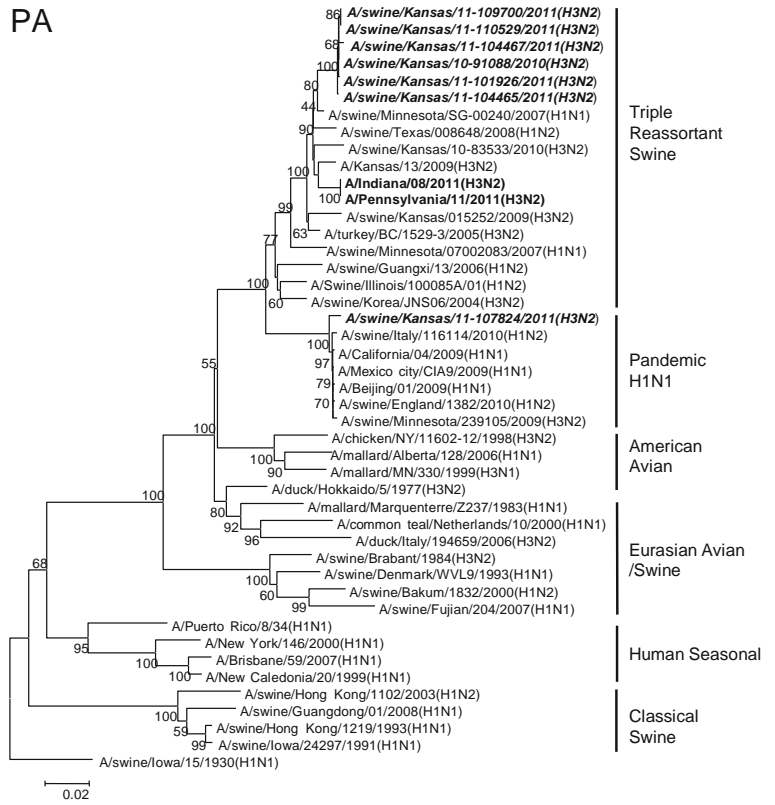
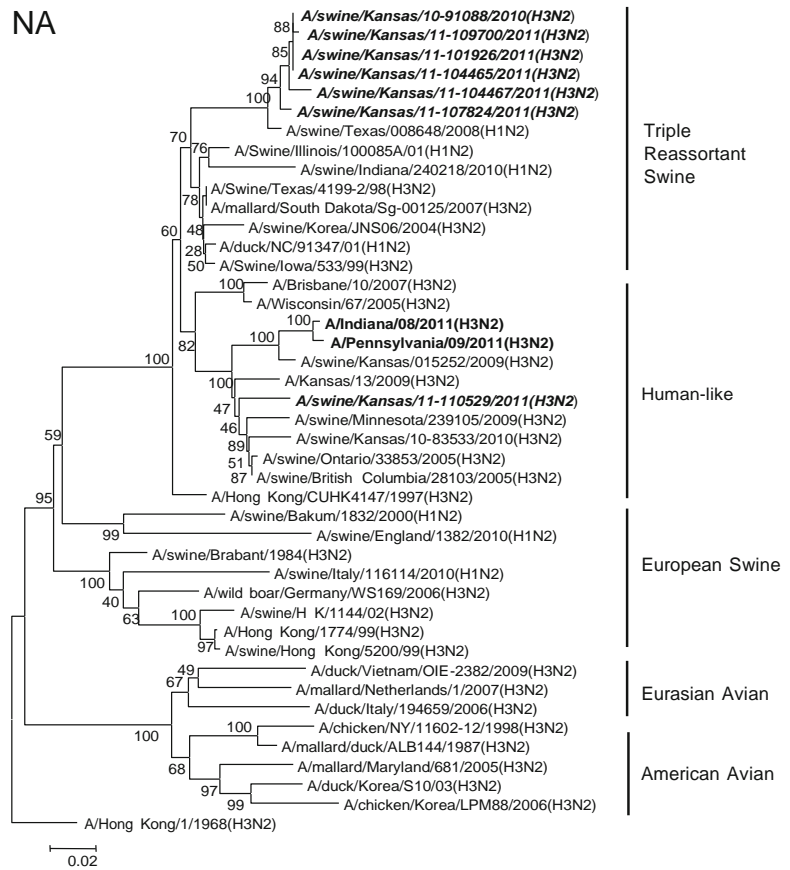


Fig. 1 continued

NA



NP

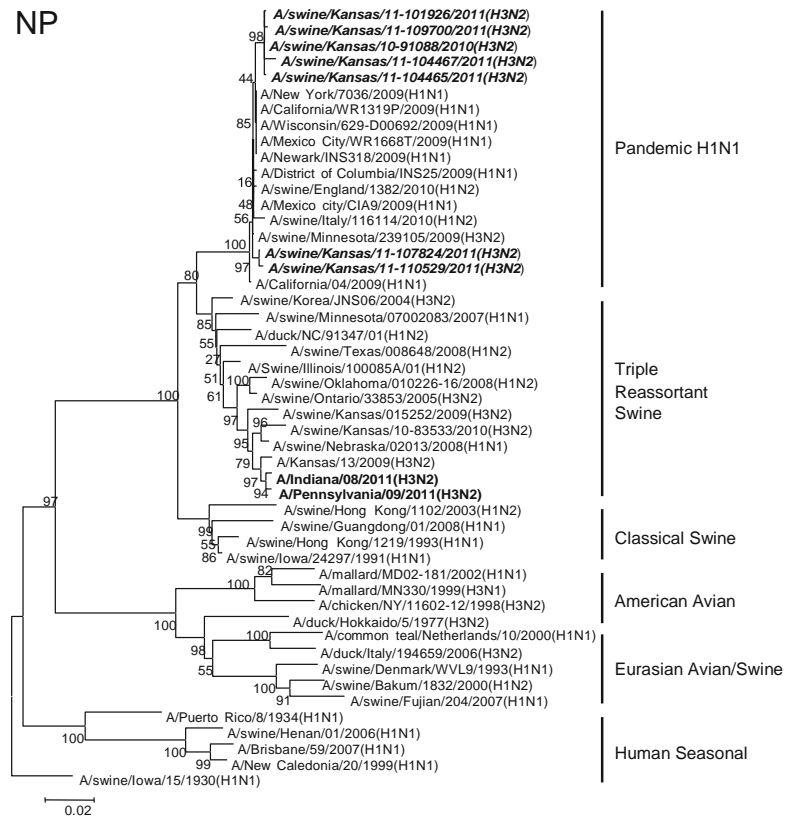
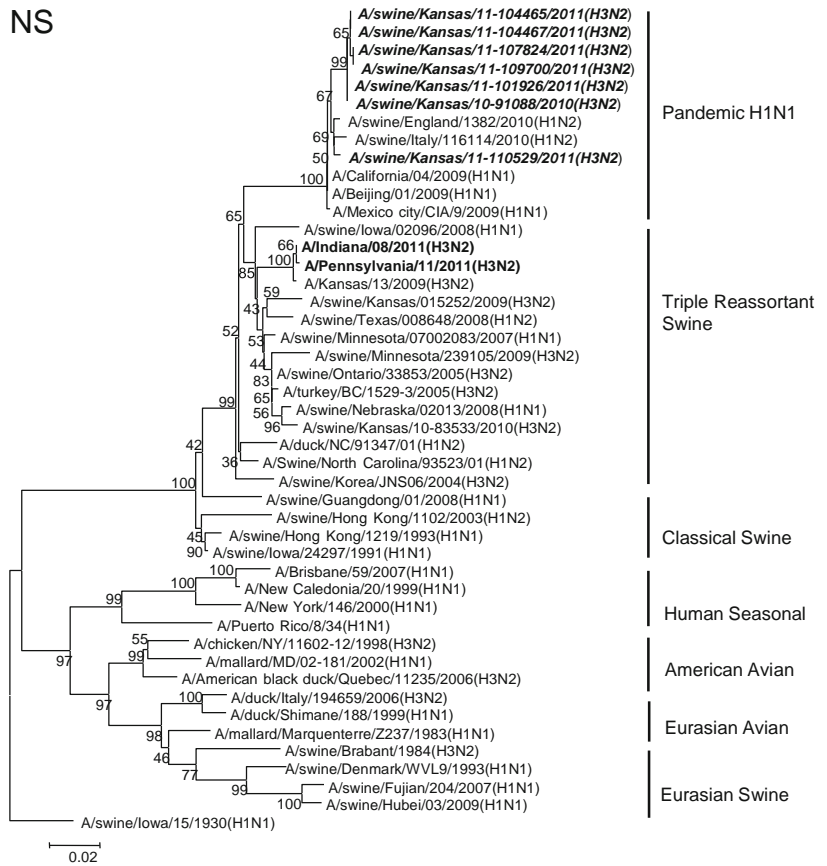
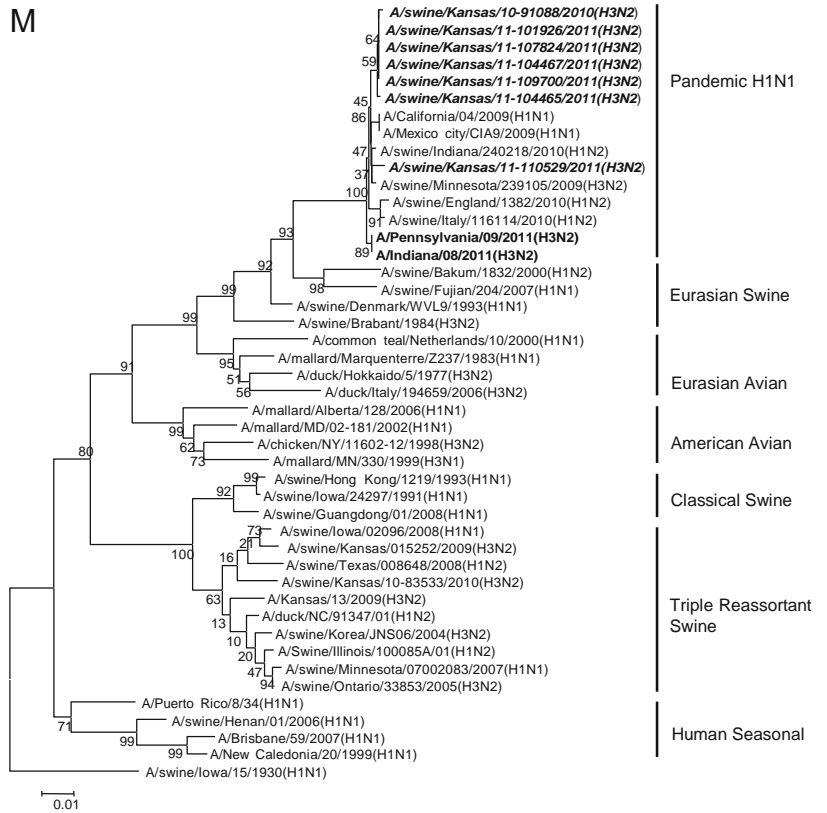


Fig. 1 continued



which is believed to compensate for the lack of 627K [2, 14]. The HA proteins of all seven H3N2 viruses had 226V and 228S at the receptor-binding sites except for the HA of the isolate A/swine/Kansas/11-107824/2011, which contained 226V and 228G. The H3 HA receptor-binding site combination 226V/228S is different from those of most avian (226Q/228G) and human (226L/228S) influenza virus HAs [13], but it is present in the majority of HAs (90%) of North American triple reassortant H3N2 SIVs. The 226V/228G combination in the HA receptor-binding site is rarely found. The receptor specificity of the 226V/228S and 226V/228G combinations remains unknown and needs to be investigated in the future. In addition, the NA protein had 119E, 292R and 274H, suggesting susceptibility to oseltamivir.

H1 subtype (i.e., H1N1 and H1N2) reassortants of pH1N1 and endemic SIVs have been isolated from pigs worldwide [5, 7, 10, 15, 18, 20, 22]. A reassortant H3N2 SIV was only recently isolated from Minnesotan pigs and had PA, NP and M genes from the pH1N1 [5]. In this report, seven novel H3N2 reassortants were isolated from diseased pigs from five different farms. Six of the H3N2 viruses had a similar genetic constellation, i.e., NP, M and NS were derived from pH1N1, and the remaining genes were from endemic H3N2 SIVs; one isolate had PB2, PA, NP, M and NS from pH1N1 and the remaining genes from endemic H3N2 triple reassortant SIVs. Three reassortant H1N2 SIVs containing a similar genetic constellation, carrying the pH1N1 NP, M and NS genes, were recently detected in pigs [5], indicating that this genotype of novel reassortant SIVs seems to be preferred in different subtypes and seem to be stable. The novel reassortant H3N2 viruses having pH1N1 NP, M and NS genes were isolated from diseased sows (sow farm #1) and nursery pigs from three farms (farms #1, #2 and #4) that obtained piglets from sow farm #1, indicating that the piglets may have been infected at the sow farm before transportation to the nursery. Importantly, similar H3N2 reassortant viruses were also isolated from farms #1 and #2 at later time points (two months after the first isolations occurred), suggesting that the virus had been established and continued to circulate within the affected production systems. An H3N2 isolate containing all internal genes from pH1N1 except PB1 was isolated from another farm (farm #3); this farm does not receive piglets from sow farm #1. Whether novel H3N2 reassortant viruses have increased pathogenicity and are transmitted among pigs more efficiently than pH1N1 or parental H3N2 SIVs remains unknown; the role of internal genes from pH1N1 in pathogenesis and transmissibility of these novel viruses need to be investigated.

Concurrent epidemiological surveillance has revealed that these novel reassortant H3N2 SIVs are circulating in Midwest swine herds although triple reassortant H1N1, H1N2 and H3N2 SIVs have also been isolated from other swine farms in the same area. A Kansas boy who was in contact with healthy pigs while attending a county fair in 2009 was reported to have been infected with a triple reassortant H3N2 SIV. This H3N2 virus does not seem to be transmitted efficiently among humans because his three household contacts did not show signs of illness [4]. Recently, two younger children from Indiana and Pennsylvania were infected by reassortant H3N2 SIVs that contained only the M gene from pH1N1 [3]. Two other children in Pennsylvania who were directly exposed to swine at an agricultural fair had confirmed infection with a similar pH1N1 reassortant H3N2 influenza virus [1]. Although no reported human illness due to influenza infection was associated with the Kansas farms where the novel reassortant H3N2 viruses were isolated, it remains unclear whether these novel reassortant H3N2 SIVs can be transmitted to and infect humans. If so, they most likely will pose a threat, especially to children born after 1998, due to the lack of immunity to these viruses. Notably, the isolate A/swine/Kansas/11-110529/2011 has an NA gene that is similar to those of human H3N2 viruses, including the novel reassortant H3N2 viruses that recently infected children in Indiana and Pennsylvania [1, 3]. Although the NA is grouped within the human-like H3N2 influenza lineage, North American triple reassortant SIVs containing a similar NA gene have been circulating in US swine herds for more than 5 years. Nevertheless, continuous circulation of pH1N1 in swine will increase the chance of further reassortment with human, avian or swine influenza viruses [9, 17, 22] and could result in a novel virus with the potential to cause infection and efficient transmission among humans. In conclusion, the emergence of novel reassortant H3N2 SIVs in US swine is further evidence of reassortment between pH1N1 and endemic SIVs. The occurrence of human infection with novel reassortant H3N2 viruses [1, 3] warrants continuous surveillance in swine and humans.

GenBank accession numbers: JN409388-JN409443

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