

# Influenza A Subtype H3 Viruses in Feral Swine, United States, 2011–2012

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To determine whether, and to what extent, influenza A subtype H3 viruses were present in feral swine in the United States, we conducted serologic and virologic surveillance during October 2011–September 2012. These animals were periodically exposed to and infected with A(H3N2) viruses, suggesting they may threaten human and animal health.

Swine are proposed as “mixing vessels” to generate novel influenza A viruses (IAVs) by facilitating reassortment among IAVs and providing a potential pathway in which these viruses can move from wild birds to humans (1). Subtype H3N2 is one of the most common subtypes in the US domestic swine population, which possibly resulted from spillover of human seasonal A(H3N2) virus (2,3). Since its introduction in the mid-1990s, A(H3N2) virus has evolved genetically and antigenically in domestic swine. Four genetic groups (so-called clusters I–IV) were identified, and these 4 clusters were also antigenically distinct (4). The viruses in cluster IV formed at least 2 antigenic subclusters, H3N2- $\alpha$  and H3N2- $\beta$  (5). Both subclusters are co-circulating in pigs, and subcluster H3N2- $\beta$  predominated among the isolates obtained from domestic swine at Ohio county fairs during 2010–2011 (5). During July and August 2011, two children were infected with novel

reassortant H3N2 variant (H3N2v), 1 in Indiana and 1 in Pennsylvania (6). This H3N2v virus is antigenically similar to the viruses in subcluster H3N2- $\beta$  and has the matrix gene of influenza A(H1N1)pdm09 virus. It caused illness in  $\approx$ 2,055 persons during August 2011–April 2012 (7).

The role of feral swine in IAV ecology has not been adequately addressed. Feral swine could be a reservoir of IAVs or, possibly, a spatially dynamic mixing vessel, given their free-ranging habits. Such unrestricted movement provides the potential for exchange of IAVs among subpopulations of feral swine and the opportunity for exposure to different IAVs through contact with a variety of habitats and species. Also, feral swine can live up to 8 years, which provides ample opportunities for reinfection with the same subtype IAVs, especially those with antigenically distinct hemagglutinins. The IAVs can be transmitted bidirectionally between feral and domestic swine because contact between them is not unusual (8). Ultimately the IAVs emerging in feral swine potentially could be transmitted to humans.

The United States has  $\approx$ 4–5 million feral swine (9) throughout at least 38 states (10). Feral swine are expanding their range because of a lack of natural predators and intentional introductions for hunting. Our goals in this study were to determine through virologic and serologic surveillance whether, and to what extent, subtype H3 IAVs were present in the US feral swine metapopulation.

## The Study

We collected 1,983 nasal swab samples from swine during October 2011–September 2012 (online Technical Appendix, [wwwnc.cdc.gov/EID/article/20/5/13-1578-Techapp1.pdf](http://wwwnc.cdc.gov/EID/article/20/5/13-1578-Techapp1.pdf)). Matrix gene-based quantitative reverse transcription PCR showed that 9 swabs samples were IAV positive; 1 A(H3N2) feral swine isolate, A/swine/Texas/A01104013/2012(H3N2), was recovered. Phylogenetic analyses showed that all genes of this feral swine isolate are genetically similar to those of A(H3N2v) viruses isolated from humans, and other contemporary subtype H3N2 isolates from swine from county fairs farms. The matrix gene of this feral swine IAV was genetically close to that of A(H1N1)pdm09 and the human A(H3N2v) viruses (online Technical Appendix Figure) (11). Similar to other viruses in the antigenic cluster, H3N2- $\beta$  A/swine/Texas/A01104013/2012(H3N2) had a R189K mutation at the antibody-binding site of the hemagglutinin protein, which caused a recent antigenic drift in subtype H3N2 IAVs (5,12). The 8 genes of A/swine/Texas/A01104013/2012(H3N2) have a minimal 99.59% nt sequence identity to those of the human subtype H3N2v isolate A/Indiana/10/2011(H3N2).

We also collected 1,989 serum samples from swine in 31 states; these samples were tested by using an IAV-specific ELISA (Figure 1). We identified 182 samples as

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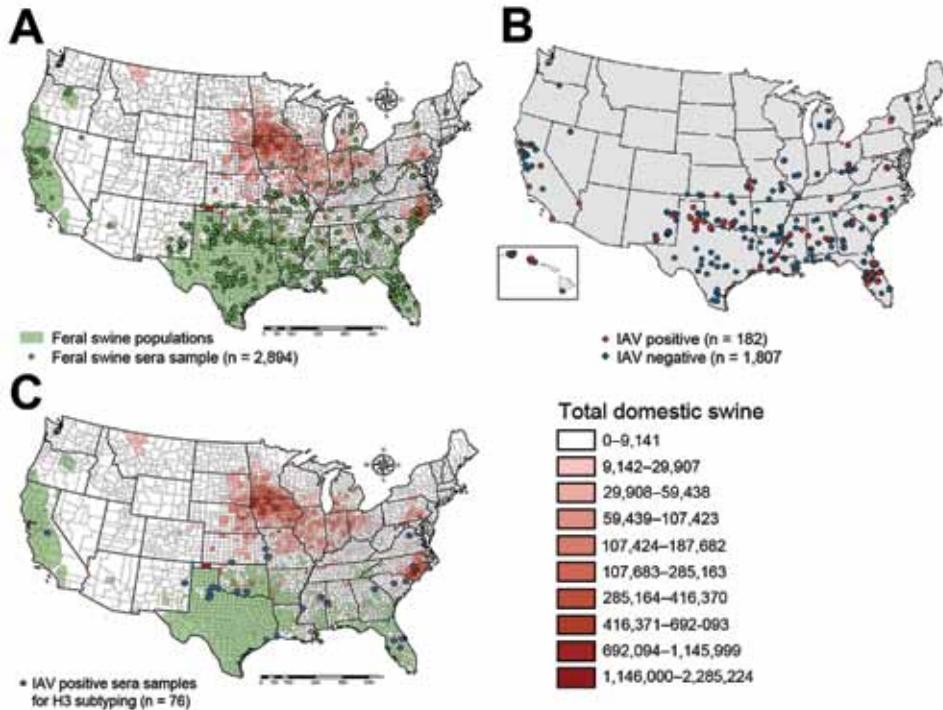


Figure 1. Geographic distributions of serum samples from feral swine, United States, 2011–2012. A) Of 1,989 samples tested by ELISA, 182 were positive (red) and 1,807 were negative (blue). B) The 76 samples (blue) were selected for hemagglutination-inhibition and microneutralization subtyping. C) The distributions of feral swine (green) and domestic swine (orange) were also marked (A and C).

IAV positive, from swine that were broadly distributed over 19 states. The average IAV seropositive rate was 9.15% but it varied by month. The highest positive rate (22.9%) was in June 2012 (Figure 2). Although no clear temporal pattern was found in the IAV seropositive rate, the rate was relatively higher in the summer than in other seasons. One explanation could be that noncommercial swine farmers might give their animals more pasture time during the summer, thereby increasing the chance of contact between domestic and feral swine. In addition, our results showed adult feral swine ( $\geq 1$  year of age) had the highest rate of IAV positivity (11.1% [of 1,380 animals]),

followed by subadults (2 months–1 year of age) (5.1% [of 494 animals]), and juveniles (<2 months of age) (3.8% [of 105 animals]) (age was not determined for 10 animals). (Dentition patterns were used to determine the age of feral swine [13].) Female and male pigs were equally as likely to be seropositive (102 [9.6%] of 1,058 vs. 80 [9.7%] of 821).

Of the 182 IAV-positive serum samples, 76 were randomly selected for influenza subtyping. We used hemagglutination-inhibition (HI) and microneutralization (MN) assays for subtyping against A(H1N1)pdm09 virus and 22 H3 IAVs, which represent a wide range of antigenically

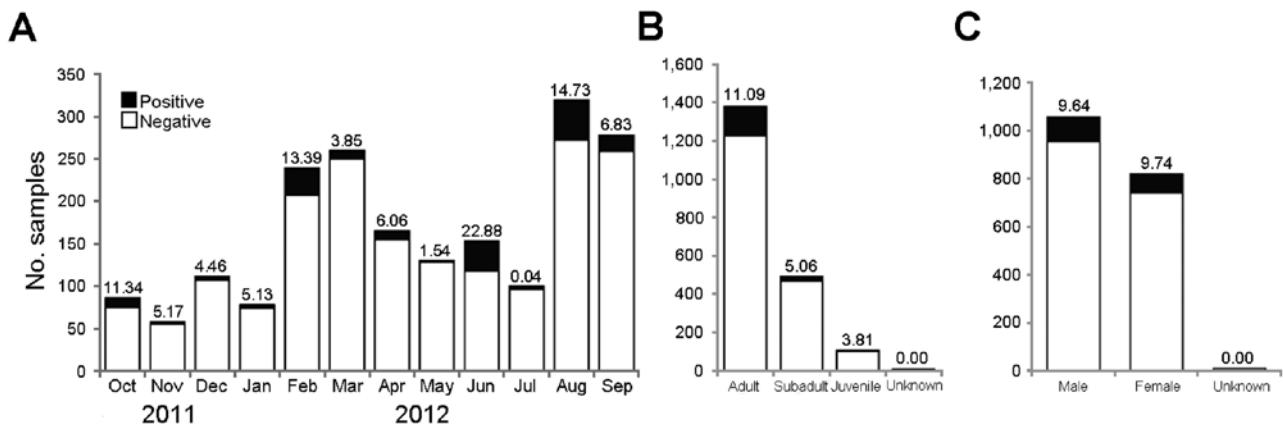


Figure 2. Epidemiologic analyses of feral swine serum samples seropositive for influenza A virus by ELISA, United States, 2011–2012. A) Temporal distribution. B) Distribution of feral pigs, by age. C) Distribution of feral pigs, by sex. The numbers on the bar indicate the influenza A virus–seropositive percentile.

Table. Cross-reactive antibody responses against H3 and influenza A(H1N1)pdm09 virus in 76 influenza-positive serum samples from feral swine, United States, 2011–2012\*

Source, virus	Antigenic cluster†	No. (%) seropositive‡		GMT (95% CI)§		Overall no. (%) seropositive¶
		HI	MN	HI	MN	
Feral swine IAV						
A/swine/Texas/A011040013/2012(H3N2)	H3N2-β	36 (47.4)	36 (47.4)	163 (40–640)	259 (40–1,280)	25 (32.9)
Domestic swine IAV						
A/swine/Ohio/11SW64/2009 (H3N2)	H3N2-α	27 (35.5)	30 (39.5)	65 (40–160)	121 (40–1,280)	17 (22.4)
A/swine/Iowa/12627/2009(H3N2)	H3N2-α	30 (39.5)	31 (40.8)	73 (40–320)	183 (40–1,280)	15 (19.7)
A/swine/Ohio/11SW347/2011(H3N2)	H3N2-β	31 (40.8)	31 (40.8)	122 (40–640)	112 (40–1,280)	17 (22.4)
A/swine/Iowa/6368/2012(H3N2)	H3N2-β	26 (34.2)	36 (47.4)	94 (0–640)	240 (40–1,280)	20 (26.3)
Human H3N2v IAV						
A/IA/07/2011		32 (42.1)	31 (40.8)	109 (40–640)	313 (40–1,280)	21 (27.6)
Human seasonal IAV						
A/Perth/16/2009	H3N2-β					
A/Perth/16/2009	H3N2-β	12 (15.8)	5 (6.6)	76 (40–320]	121 (40–1,280)	1 (1.3)
A/Victoria/361/2011	H3N2-β	11 (14.5)	2 (2.6)	141 (40–640)	226 (40–1,280)	0
A/California/7/2009	H3N2-β	5 (6.6)	3 (3.9)	70 (40–640)	80 (40–320)	1 (1.3)

\*Influenza detected by ELISA. GMT, geometric mean titer; HI, hemagglutination-inhibition; MN, microneutralization; IAV, influenza A virus.

†The antigenic cluster H3N2-α and H3N2-β were defined in (5).

‡Seropositive was defined as the HI or MN titer  $\geq 40$ .

§Only serum with an HI or MN titer  $\geq 40$  are used to calculate the GMT.

¶Overall seroconversion was defined as both HI and MN titer  $\geq 40$ .

distinct H3 IAVs (online Technical Appendix Table). Serum was defined as seropositive if its titer was  $\geq 40$ .

HI results showed that 46 (60.5%) of 76 feral swine samples were positive against at least 1 of the 22 H3 IAVs tested, of which A/swine/Texas/A011040013/2012(H3N2) had the highest seropositive rate (47.4%), followed by 4 human A(H3N2v) isolates (Table). MN results were consistent with those of HI. The geometric mean titers for HI and MN against the feral swine isolate were 163 and 259, respectively. The maximum MN titer among the serum and A/swine/Texas/A011040013/2012(H3N2) was 1,280. The seropositive rates of these serum samples varied from 34.2% to 42.1% against the other viruses from subclusters H3N2-α and H3N2-β (Table), but the viruses in subcluster H3N2-β had significantly higher geometric mean titers than did those in H3N2-α ( $p < 0.001$ ).

HI results also showed that 12 samples were seropositive against A/Perth/16/2009(H3N2); 11 were seropositive against A/Victoria/361/2011(H3N2), and 5 were seropositive against A/California/7/2009(H1N1). These 3 viruses do not cross-react with the IAVs from subclusters H3N2-α and H3N2-β. Those 5 subtype H1N1-positive samples were also seropositive against subtype H3N2 IAVs, indicating potential previous exposures of these feral swine to both H3 and H1 IAVs.

The HI results demonstrated that only 2 serum samples had a low-level cross-reaction with avian influenza A(H3N2) viruses, and the HI titers for both were 40. This result is consistent with findings in an earlier report (14). Further studies are needed to determine whether additional antigenic clusters of H3 IAVs are present in migratory waterfowl. The discrepancies in the cross-reactivity of these serum samples against the IAVs tested in this study suggested that these feral swine had different exposure histories against antigenically diverse IAVs.

## Conclusions

Our study demonstrated that subtype H3N2 IAVs are periodically infecting feral swine in the United States. Feral swine are a potential source of IAVs with bidirectional transmission to domestic swine or humans. Detection of an H3N2v-like IAV in the feral swine population demonstrates a potential threat to human health. Continued surveillance is recommended to monitor the distribution and the genomic and antigenic diversities of IAVs in feral swine to better assess the risk.

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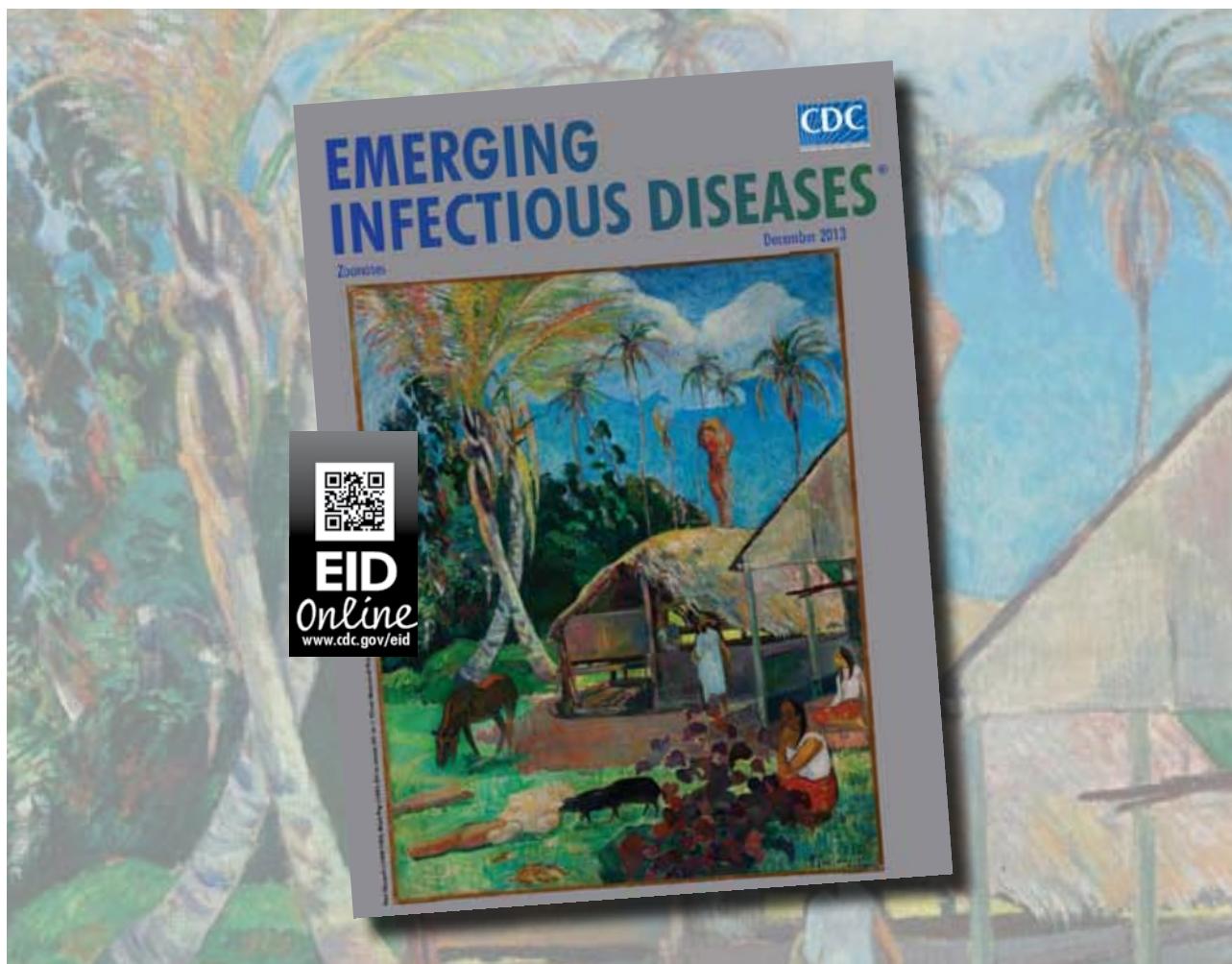
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# Influenza A Subtype H3 Viruses in Feral Swine, United States, 2011–2012

## Technical Appendix

### Material and Methods

#### Sample Collection

Samples were collected opportunistically from feral swine removed for wildlife damage management purposes. Blood samples were collected primarily by cardiac puncture and placed in serum-separating collection tubes. Once the blood was clotted, it was centrifuged, and the serum was transferred into 2-mL cryogenic vials and labeled with a unique barcode. Serum samples were shipped to the US Department of Agriculture (USDA), National Wildlife Disease Program (Fort Collins, CO, USA), where they were stored frozen at  $-80^{\circ}\text{C}$  until testing. Nasal swabs were collected by inserting a sterile Dacron swab in the nasal cavity and gently swabbing the surface of the nasal mucosa. This process was repeated in both nostrils with the same swab, placed in a cryogenic vial with 3 mL of brain-heart infusion broth provided by the USDA National Veterinary Services Laboratories (NVSL), and the vial was labeled with a unique barcode. Vials were kept in a cooler with ice packs until they were shipped to the testing laboratory within 3 days after collection.

#### Viral Isolation and Subtyping

The nasal swabs from feral swine were screened by using influenza A virus (IAV) matrix gene based quantitative reverse transcription PCR at 1 of the National Animal Health Laboratory Network facilities across the United States (*1*). Matrix-positive samples were tested with an N1 subtyping PCR. Matrix-positive results were forwarded to NVSL for testing regardless of the N1 subtyping result. Isolates were recovered by using 9- to 11-day-old specific pathogen-free (SPF) eggs according to the protocols described in the Swine Influenza Surveillance Procedure Manual ([www.aphis.usda.gov/animal\\_health/animal\\_dis\\_spec/swine/siv\\_surveillance.shtml](http://www.aphis.usda.gov/animal_health/animal_dis_spec/swine/siv_surveillance.shtml)). The

hemagglutinin (HA) and neuraminidase (NA) subtypes were determined at the NVSL using standard hemagglutination-inhibition (HI) and NA inhibition testing procedures.

### **Viruses**

A total of 22 H3 IAVs were used for comparison in serologic assays. In addition to the feral swine isolate A/swine/Texas/A01104013/2012(H3N2) recovered from this study, 4 human H3N2v isolates, 3 county fair pig H3N2 isolates, 7 domestic pig H3N2 isolates, 2 migratory bird H3N2 isolates, 2 canine H3 isolates, 2 human seasonal H3N2 isolates, and 1 2009 H1N1 isolate were used in the serologic assays (Technical Appendix Table).

To maximize the chances of detecting H3 IAV infection in feral swine samples tested, the 22 isolates described above were selected to represent a wide range of antigenically distinct H3 IAVs (Technical Appendix Table). Ten of them represented 2 of the contemporary H3 antigenic clusters in the swine population: H3N2- $\alpha$  and H3N2- $\beta$  (2). Four human H3N2v isolates were also included, and these isolates antigenically belong to H3N2- $\beta$ .

A/Perth/16/2009(H3N2) and A/Victoria/361/2011(H3N2), 2 seasonal influenza vaccine strains recommended by the World Health Organization (WHO), represent the antigenic variants predominantly circulating in human population. These viruses do not cross-react with the 10 swine IAVs and 4 H3N2v viruses described in the previous paragraph. An avian origin H3N2 canine influenza virus (CIV) (3) and an equine origin H3N8 CIV (4) were also selected, which do not cross-react with swine H3N2, human H3N2v, or H3N2 seasonal influenza viruses. Except for H3N2 CIV, the 2 H3 avian influenza viruses selected do not cross-react with the other isolates listed in the Table. The selected 2009 H1N1 virus also did not react with any H3 IAVs selected for this study (Technical Appendix Table).

All avian influenza viruses were propagated in MDCK cells (ATCC, Manassas, VA, USA) and then stored at  $-70^{\circ}\text{C}$  until analysis.

### **Serologic Assays**

Influenza-specific ELISAs were performed by using IDEXX Influenza A Ab Test (IDEXX, [Westbrook, ME, USA) based on the manufacturer's specifications. HI assays were performed according to the WHO manual on animal influenza diagnosis and surveillances ([http://whqlibdoc.who.int/hq/2002/WHO\\_CDS\\_CSR\\_NCS\\_2002.5.pdf](http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_NCS_2002.5.pdf)). Before HI tests were conducted, feral swine serum was treated with receptor-destroying enzyme (Denka Seiken Co.,

Tokyo, Japan) by 1:3 (v/v) at 37°C for 18 h, and then heat inactivated at 55°C for 30 min. Then, the serum was diluted with phosphate-buffered saline (for a final dilution of 1:10), and 22 influenza A viruses were tested by HI assay with 0.5% turkey red blood cells. The HI for the A/Victoria/369/2011(H3N2) testing was performed using 0.5% guinea pig red blood cells.

Microneutralization (MN) assay was performed in MDCK cells. Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of the viral growth of 100 tissue culture infectious doses of the virus. The MN titers were determined by HA assay using 0.5% turkey red blood cells as described (2).

### **Serologic Data Analyses**

If the titers of a serum against a specific influenza isolate were  $\geq 40$ , the serum was classified as positive to this isolate. Student *t* tests were performed to test the null hypothesis: there was no significant difference among the HI or MN titers between different groups of IAVs.

### **Molecular Characterization and Phylogenetic Analyses**

The multiple sequence alignments were conducted by using the MUSCLE software package (5). The phylogenetic analyses were performed by using maximum-likelihood by GARLI version (6), and bootstrap resampling analyses were conducted with 1,000 runs using PAUP\* 4.0 Beta (7) with a neighbor-joining method, as described (8).

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Technical Appendix Table. Cross-reactive antibody responses among influenza A viruses used in this study by using HI assay against ferret antiserum for H3 influenza A virus and A(H1N1)pdm09 virus\*

Virus†	Antigenic cluster	Ferret antiserum‡									
		Swine influenza virus			Canine influenza virus		Avian influenza virus		Human seasonal influenza virus		
		09SW64	10SW215	11SW347	CIVH3N2	CIVH3N8	99AIVH3N2	11AIVH3N2	Perth09	Victoria11	Pdm09
Isolates from agricultural fairs											
A/swine/Ohio/09SW64/2009(H3N2)	H3N2- $\alpha$	<b>1,600</b>	40	<10	<10	<10	<10	<10	<10	<10	ND
A/swine/Ohio/10SW215/2010(H3N2)	H3N2- $\beta$	80	<b>960</b>	640	<10	<10	<10	<10	<10	<10	ND
A/swine/Ohio/11SW347/2011(H3N2)	H3N2- $\beta$	20	320	<b>576</b>	<10	<10	<10	<10	<10	<10	ND
Isolates from canine											
A/canine/Guangdong/1/2006(H3N2)		<10	<10	<10	<b>1,280</b>	40	320	160	ND	ND	ND
A/canine/Iowa/13628/2005(H3N8)		<10	<10	<10	40	<b>160</b>	<10	<10	ND	ND	ND
Isolates from avian											
A/blue-winged teal/Ohio/99–31/99(H3N2)		<10	<10	<10	ND	<10	<b>70</b>	20	ND	ND	ND
A/blue-winged teal/Ohio/11OS2474/2011(H3N2)		<10	<10	<10	ND	80	20	<b>320</b>	ND	ND	ND
Isolates from vaccine strains											
A/Perth/16/2009(H3N2)		<10	<10	<10	ND	ND	ND	ND	<b>40</b>	80	ND
A/Victoria/361/2011(H3N2)		<10	<10	<10	ND	ND	ND	ND	<10	<b>1,280</b>	ND
A/California/7/2009(H1N1)		<10	<10	<10	ND	ND	ND	ND	<10	<10	ND
Isolates from feral hog											
A/swine/Texas/A01104013/2012(H3N2)		40	>640	320	<10	<10	<10	<10	ND	ND	ND
Isolates from swine farms											
A/swine/Nebraska/9330/2006(H3N2)	H3N2- $\alpha$	320	80	<10	<10	<10	<10	<10	ND	ND	ND
A/swine/North Carolina/1026/2007(H3N2)	H3N2- $\alpha$	640	80	<10	<10	<10	<10	<10	ND	ND	ND
A/swine/Iowa/18469/2008(H3N2)	H3N2- $\alpha$	320	<10	<10	<10	<10	<10	<10	ND	ND	ND
A/swine/Wisconsin/12627/2009(H3N2)	H3N2- $\alpha$	1,280	80	80	<10	<10	<10	<10	ND	ND	ND
A/swine/Michigan/33261/2010(H3N2)	H3N2- $\beta$	160	1,280	1,280	<10	<10	<10	<10	ND	ND	ND
A/swine/Indiana/9622/2011(H3N2)	H3N2- $\beta$	40	1,280	1,280	<10	<10	<10	<10	ND	ND	ND
A/swine/North Carolina/6368/2012(H3N2)	H3N2- $\beta$	320	1280	640	<10	<10	<10	<10	ND	ND	ND
Isolates from patients											
A/Wisconsin/12/2010(H3N2)	H3N2- $\beta$	80	640	320	<10	<10	<10	<10	ND	ND	ND
A/Pennsylvania/14/2010(H3N2)	H3N2- $\beta$	320	640	640	<10	<10	<10	<10	ND	ND	ND
A/Minnesota/11/2010(H3N2)	H3N2- $\beta$	20	320	160	<10	<10	<10	<10	ND	ND	ND
A/Iowa/07/2011(H3N2)	H3N2- $\beta$	80	160	320	<10	<10	<10	<10	ND	ND	ND
Isolates from equine											
A/equine/Miami/1/63(H3N8)		<10	<10	<10	<10	<10	<10	<10	ND	ND	ND

\*HI, hemagglutination-inhibition; ND, not determined.

†09SW64, A/swine/Ohio/09SW64/2009(H3N2); 10SW215, A/swine/Ohio/10SW215/2010(H3N2); 11SW347, A/swine/Ohio/11SW347/2011(H3N2); CIVH3N2, A/canine/Guangdong/1/2006(H3N2); CIVH3N8, A/canine/Iowa/13628/2005(H3N8); 99AIVH3N2, A/blue-winged teal/Ohio/99–31/99(H3N2); 11AIVH3N2, A/blue-winged teal/Ohio/11OS2474/2011(H3N2); Perth09, A/Perth/16/2009(H3N2); Victoria11, A/Victoria/361/2011(H3N2); pdm09, A/California/7/2009(H1N1)

‡Values in bold are HI titers with homologous influenza isolates that were used to generate ferret antiserum. Each HI value in this table is an average number from 2 experiments.

Figure 1

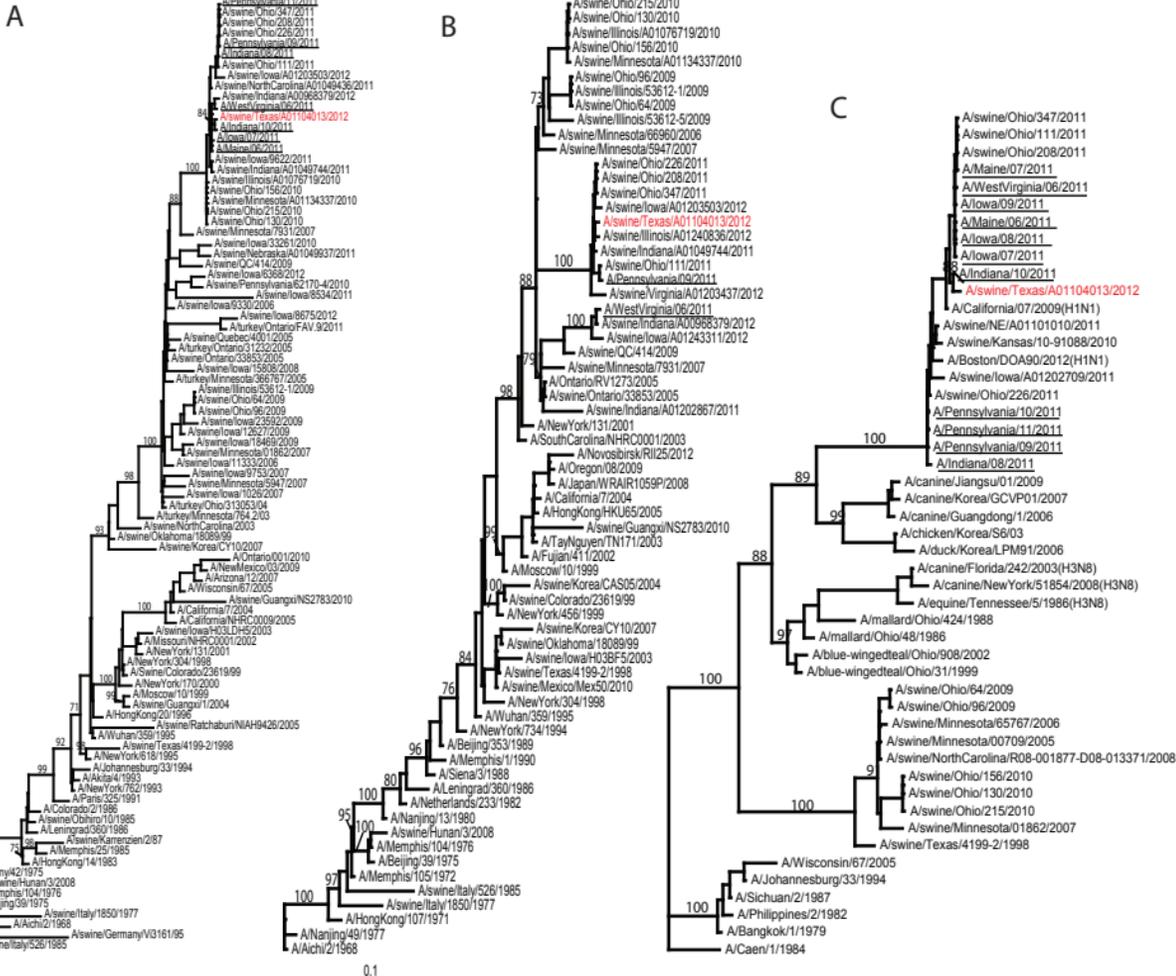




Figure S1D PB2

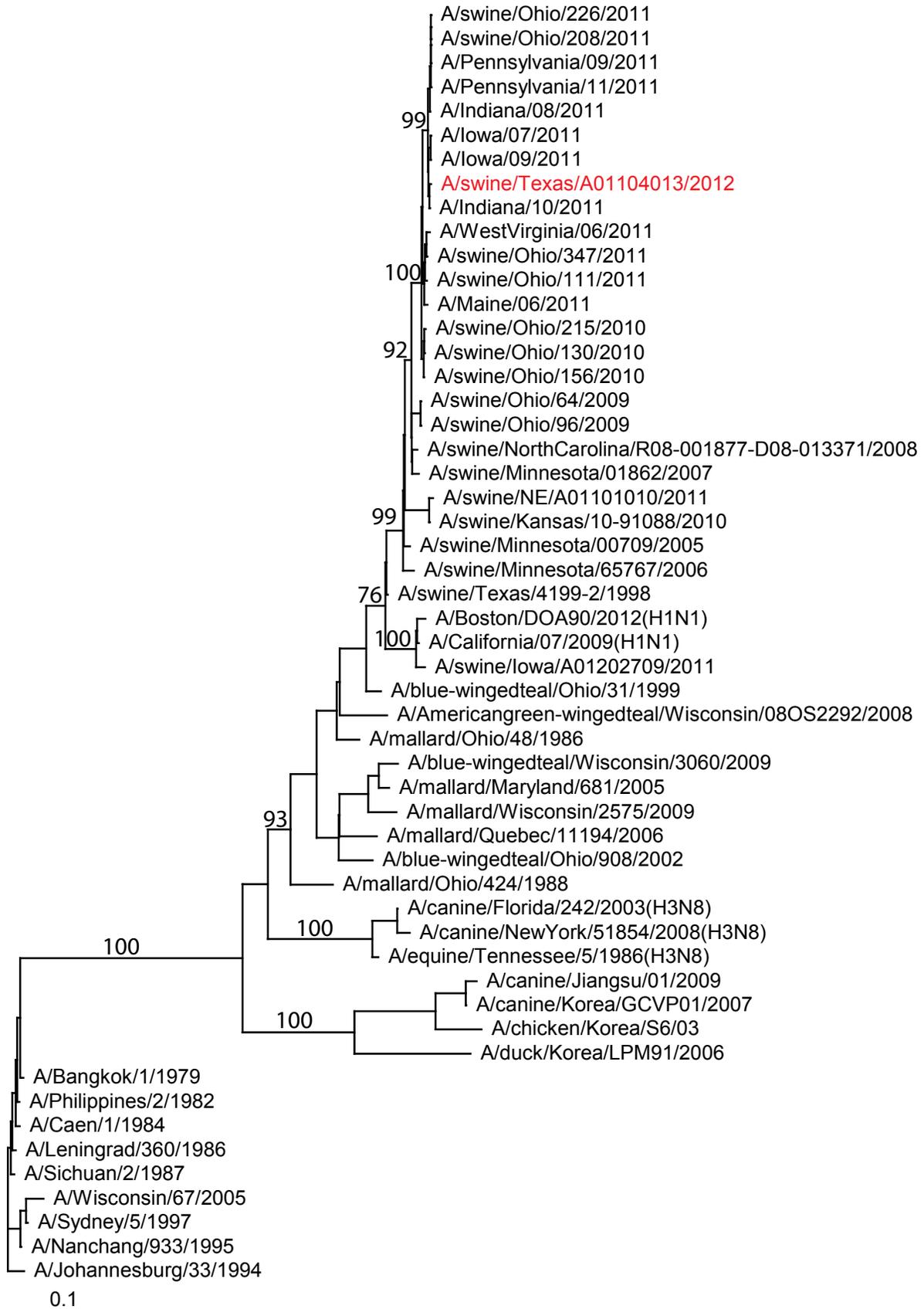


Figure S1E PB1

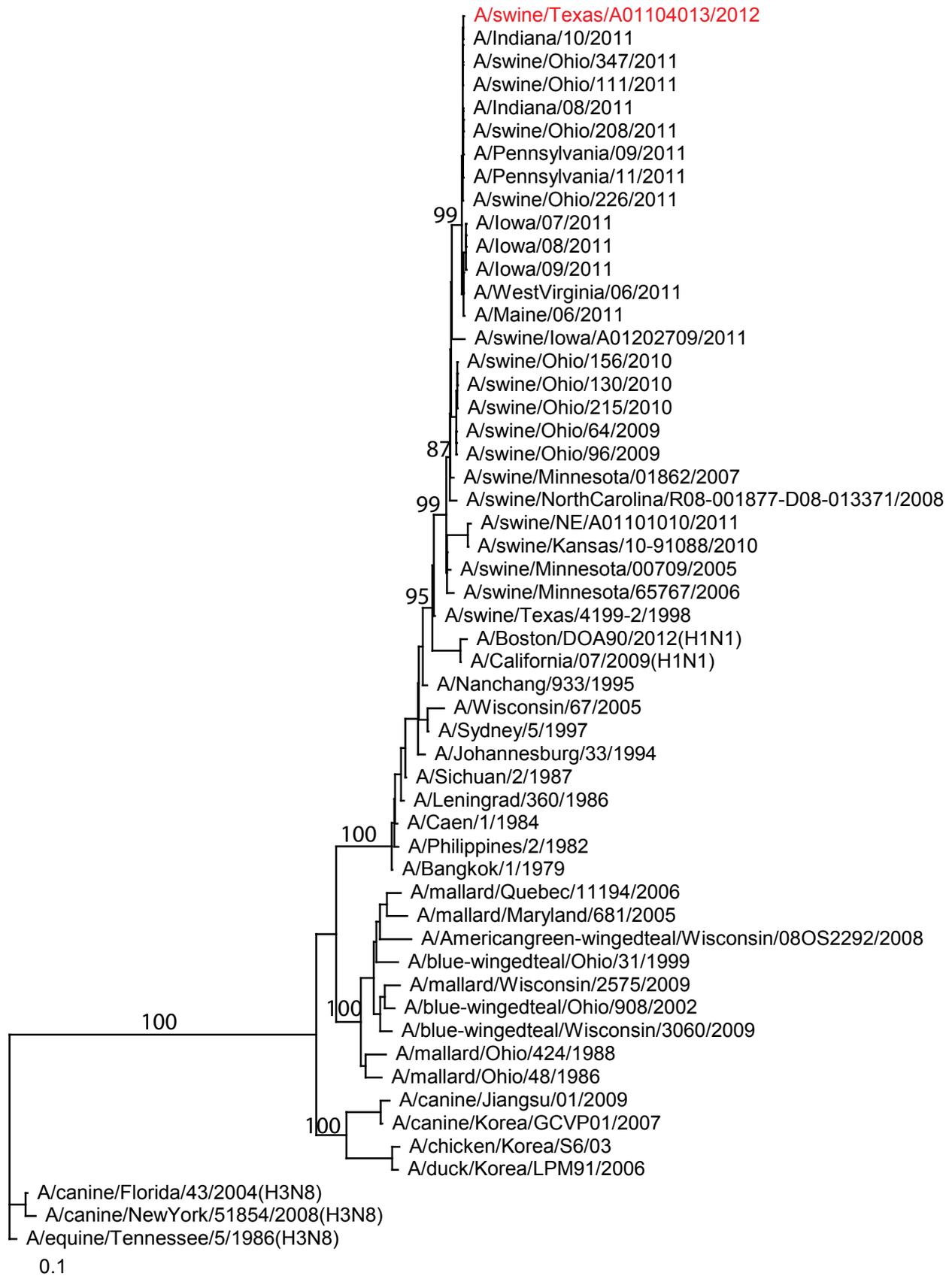


Figure S1F PA

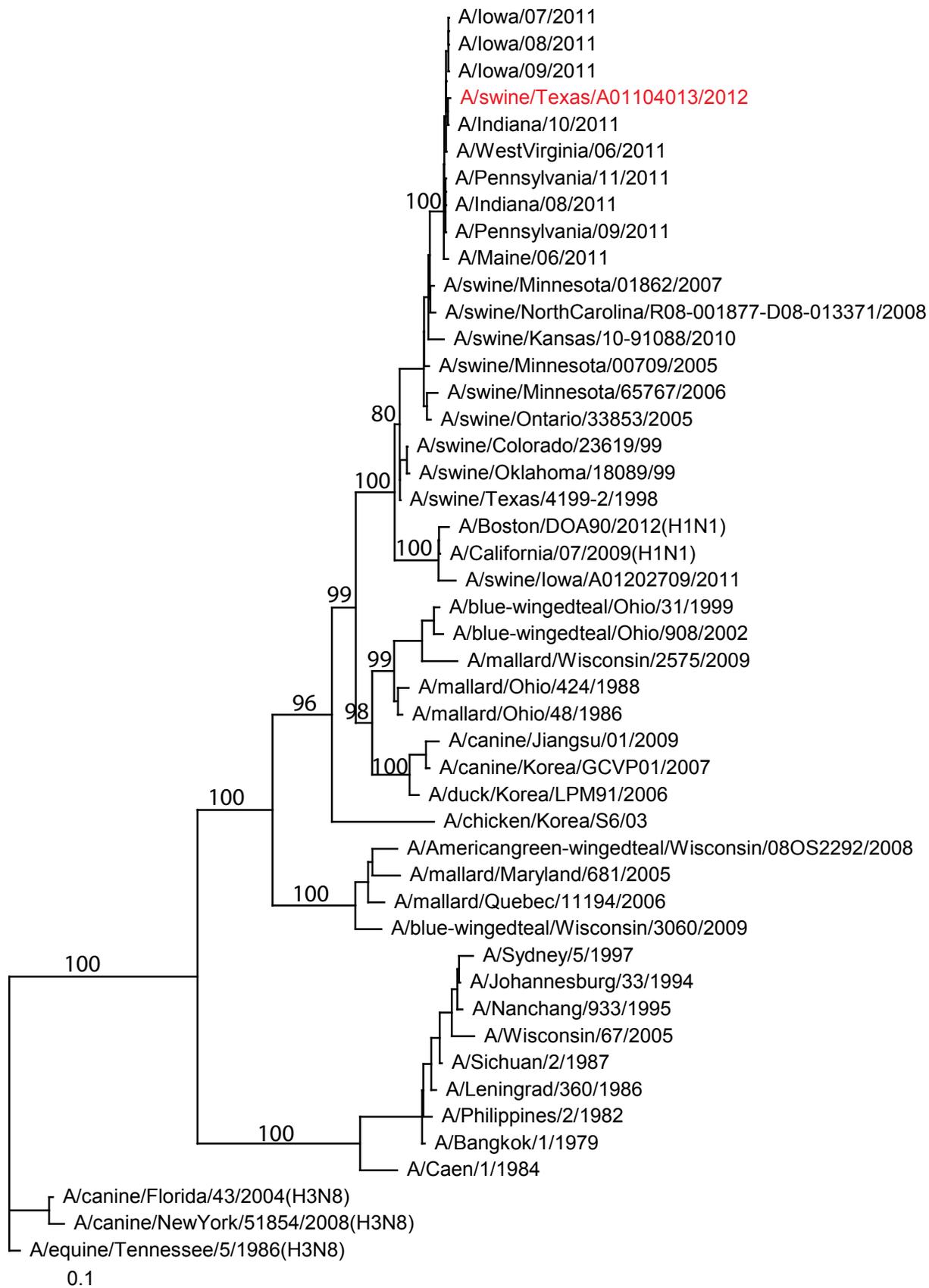


Figure S1G NP

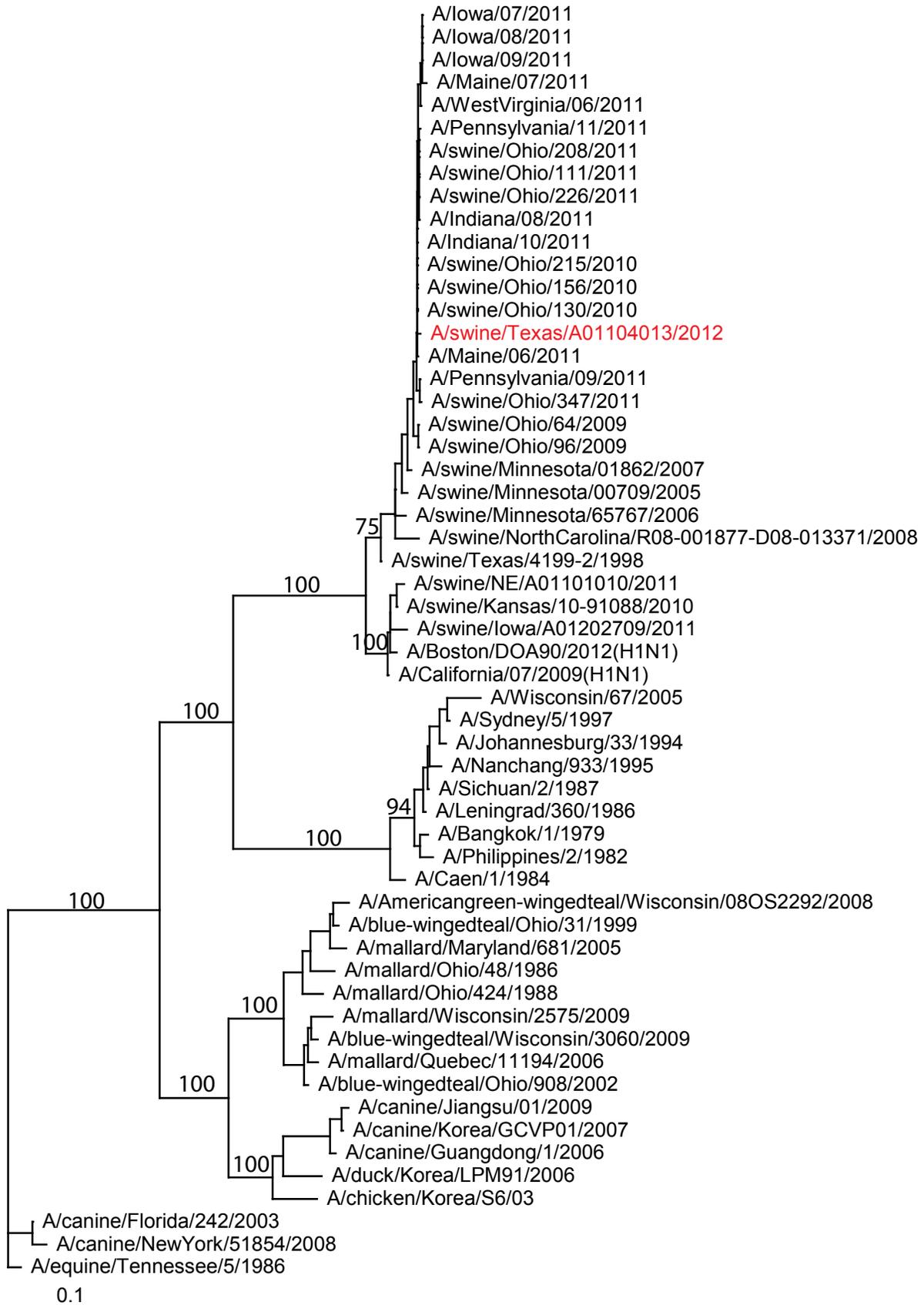
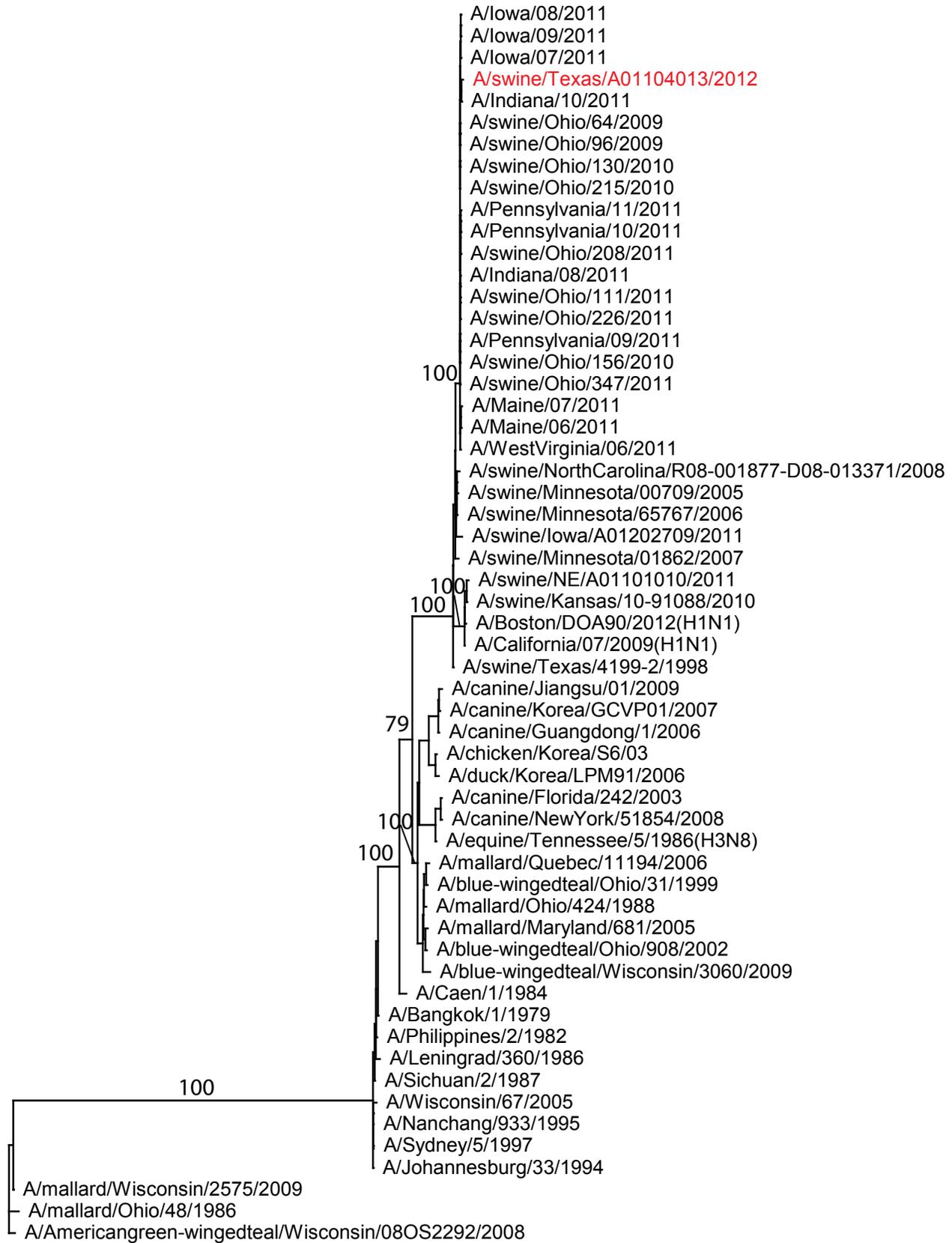


Figure S1H NS



Technical Appendix Figure. Phylogenetic analysis of HA (A), NA (B), PB2 (C), PB1 (D), PA (E), NP (F), MP (G), and NS (H) gene segments of H3N2 influenza A virus recovered from feral swine. The H3N2 feral swine virus is marked in red, and human H3N2 variant (H3N2v) influenza virus isolates are underlined. The phylogenetic analyses were performed by using maximum-likelihood method as described (8). Sequence analyses showed that the HA, NA, PB2, PB1, PA, NP, MP, and NS genes of A/swine/Texas/A01104013/2012(H3N2) have 99.82%, 99.85%, 99.89%, 99.82%, 99.81%, 99.73%, 99.59%, and 99.40% nt sequence identity similar to those of A/Indiana/10/2011(H3N2), isolated from county fairs, respectively.