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Original Article

Genetic composition of contemporary swine influenza viruses in the West Central region of the United States of America

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Background Because of continuous circulation in different animal species and humans, influenza viruses have host-specific phenotypic and genetic features. Reassortment of the genome segments can significantly change virus phenotype, potentially generating virus with pandemic potential. In 2009, a new pandemic influenza virus emerged.

Objectives In this study, we attempted to find precursor viruses or genes of pandemic H1N1 influenza 2009 among 25 swine influenza viruses, isolated in the West Central region of the United States of America (USA), between 2007 and 2009. The Phylogenetically Similar Triple-Reassortant Internal Genes (PSTRIG) cassette of all the viruses studied here as well as the PSTRIG cassette of pandemic H1N1 viruses have close but equidistant phylogenetic relationships to the early triple-reassortant swine H3N2 influenza A isolated in the USA in 1998.

Methods Samples (nasal swabs and lung tissue lavage) were taken from swine with or without clinical signs of respiratory disease via farmer-funded syndromic surveillance. All studied viruses were isolated in Madin–Darby Canine Kidney cell cultures from the above-mentioned samples according to standard protocols recommended for influenza virus isolation. Sequences were obtained using BigDye Terminator v3.1 Cycle Sequencing kit. Phylogenetic trees were built with MEGA 4.0 software using maximum composite likelihood algorithm and neighbor-joining method for tree topology reconstruction.

Results Among the 25 viruses studied, we have not found any gene segments of Eurasian origin. Our results suggest that pandemic H1N1 viruses diverged and are not directly descended from swine viruses that have been circulating in USA since 1998.

Keywords Influenza, swine, surveillance.

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Introduction

Influenza virus is a great enigma of the past and present. It possesses zoonotic potential in ecological niches and spreads rapidly within populations of its natural hosts; humans, birds, and pigs. American 'classical' swine H1N1 Influenza virus has been circulating in the USA pig populations and was the dominant strains with invariable gene content, only slightly affected by genetic drift from its first isolation in 1930 until 1998.[1] In sharp contrast to the European pig populations, the isolation of human H3N2 influenza virus from pigs in North America prior to 1998 was sporadic, and there was no evidence that these wholly human H3N2 viruses became established in the swine population in North America.[2] Cross-species infection of humans, pigs, and birds is not unique and has occurred in America, Europe, and Asia.[3] However, in 1998, a novel triple-reassortant H3N2 influenza virus was isolated from pigs in the USA. This isolate had PB2 and PA of unknown avian origin, PB1, HA, and NA of human A/Sydney/5/97 (H3N2) – like origin, and NP, M, NS of classical swine A/swine/Iowa/15/1930 (H1N1)-like origin.[1,4] Triple-reassortant swine H3N2 virus underwent further reassortment events in 1999 and 2001 resulting in new H1N2 and H1N1 reassortant subtypes, both of which continue to circulate in pig populations in the USA and have a propensity for reassortment. The most recently described generation of swine triple-reassortants emerged in 2004–2005.[5] H1N2 and H1N1 virus subtypes carrying swine HA (H1) have reassorted with a seasonal human H1N1 A/New

Caledonia/20/99 (H1N1)-like virus. Finally, a virus related to the H1N1 and H1N2 virus subtypes, containing an N1 and M of Eurasian swine lineage had assembled, acquiring epidemiologic features allowing sustained human-to-human transmission and caused the new pandemic (H1N1) in 2009.[6] In this study, we attempted to find precursor viruses or genes of pandemic H1N1 influenza 2009 among 25 swine influenza viruses isolated the West Central USA region between 2007 and 2009.

Methods

Samples (nasal swabs and lung tissue lavage) were taken from pigs with or without clinical signs of respiratory disease via farmer-funded syndromic surveillance. All viruses studied were isolated in Madin–Darby Canine Kidney cell culture from the above-mentioned samples according to standard protocols recommended for influenza virus isolation (http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf). Virus isolation was carried out at the University of Minnesota Veterinary Diagnostic Laboratory. Viral RNA was isolated with Total RNA Isolation kit (Qiagen, Valencia, CA, USA). Segment-specific PCR fragments were obtained with one-step RT-PCR (Qiagen) using influenza A-specific primers, described elsewhere.[7] Two overlapping PCR products for polymerase genes PA, PB1, and PB2 were obtained. PCR products were analyzed in 1% agarose gel. Purified PCR products were sequenced with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), primers available upon request.

Sequence products were analyzed with a 3730×l DNA Analyzer. Sequence traces were assembled into contigs with Lasergene SeqMan Pro (DNASTAR, Madison, WI, USA). Alignments for phylogenetic studies were created with data provided by The Influenza Virus Resource at the National Center for Biotechnology Information.[8,9] Phylogenetic trees were built with mega 4.0 software (Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA) using maximum composite likelihood algorithm and neighbor-joining method of tree topology reconstruction.[10] The protein coding part of the genes was used. Reliability of rooting was checked using a bootstrap test (500 replications). Values lower than 95% were considered to be unreliable.[11] For the phylogenetics study, we selected the genes that form the PSTRIG cassette (PB2, PB1, PA, NP, and NS). HA and NA genes were analyzed using a BLAST approach.[8,9] We conducted phylogenetic studies of the PSTRIG cassettes, which were similar in all triple-reassortants including pandemic (H1N1) 2009 and viruses that had been included in this study. Influenza viruses H3N2, H1N2 (1999), and H1N1 (2001) with HA of classical swine origin, H1N2 (2004) and H1N1 (2005) HA of human origin and H3N1 subtype viruses representing evolutionary stages of triple-reassortants were selected.[8] For the outgroups, we used gene precursors from viruses isolated prior to triple-reassortant emergence.

Results

We sequenced entire genomes of 25 swine influenza viruses isolated during 2007–2009 from pigs located predominantly in Oklahoma, but also from Texas and Kansas. These states were selected for retrospective surveillance because of their close social and economic relationships, their proximity to the Mexico border, and because of the constant exchange of animals between swine populations in Oklahoma and Kansas and those in Texas.

Among the 25 viruses analyzed, four viruses were H3N2 and 21 were H1N2 subtype. The HA (H3) sequences of the viruses were closely related (97–98% homology) to A/swine/Ontario/33853/2005 (H3N2). The NA (N2) sequence of A/swine/Oklahoma/011506/07 (H3N2) was closely related to A/swine/Oklahoma/18089/99 (H3N2), while all other (Table 1) NA (N2) of H3N2 virus isolates were most closely related to A/swine/British Columbia/28103/2005 (H3N2) (98% homology). This reflects heterogeneity among recently isolated H3N2 influenza viruses circulating in the US pig population. The 21 HA (H1) genes were closely related (97% homology) to A/New York/209/2003 (H1N2). The NA (N2) genes of these viruses were also phylogenetically closely related to A/swine/Ontario/33853/2005 (H3N2) (97–98% homology) (with the exception of A/swine/Texas/008648/08 (H1N2). A/swine/Texas/008648/08 contained a different (N2) that is most closely related to A/swine/Iowa/533/99 (H3N2) (95% homology). These data confirm that viruses isolated recently carry surface proteins from the different eras of triple-reassortant development.

We have not found any gene segments that cluster phylogenetically with those of Eurasian swine or avian origin influenza. To estimate the phylogenetic relationships of the novel pandemic (H1N1) 2009 virus and recently isolated US triple-reassortant swine viruses, we conducted studies on PSTRIG cassettes of H3N2 and H1N2 viruses. The main question was 'is the PSTRIG cassette of the novel pandemic (H1N1) 2009 virus descended from the triple-reassortant swine viruses that emerged in the United States in 1998?. We have found that the PB2, PB1, NS, and NP genes studied here form a phylogenetically indistinguishable and common cluster with triple-reassortants isolated between 1998 to 2009 (Figures 1–4). Surprisingly, the PA genes of pandemic (H1N1) 2009 virus reliably (bootstrap index = 100) segregate from the pool of triple-reassortants isolated in 1998–2009 (Figure 5). These data suggest that the PSTRIG cassette of pandemic (H1N1) 2009 was assem-

Table 1. Triple-reassortant swine influenza viruses isolated from swine in 2007–2009 in United States

#	Isolate name	Subtype	Sampling date	State	Sample type	Clinical symptoms	GenBank accession numbers
1	A/Swine/OK/008722/07	H3N2	2/21/2007	OK	Lung lavage	Coughing and weight loss	CY045564–CY045571
2	A/Swine/OK/011506/07	H3N2	3/8/2007	OK	Nasal swab	Being carried out for health monitoring	CY045572–CY045579
3	A/Swine/TX/008648/08	H1N2	2/20/2008	TX	Lung lavage	Fever and inactivity	CY045580–CY045587
4	A/Swine/OK/010226-16/08	H1N2	2/29/2008	OK	Lung lavage	Being carried out for health monitoring	CY045588–CY045595
5	A/Swine/OK/010226-17/08	H1N2	2/29/2008	OK	Lung lavage	Being carried out for health monitoring	CY045596–CY045603
6	A/Swine/OK/010710-8/08	H1N2	3/4/2008	OK	Lung lavage	Being carried out for health monitoring	CY045612–CY045619
7	A/Swine/OK/010710-9/08	H1N2	3/4/2008	OK	Lung lavage	Being carried out for health monitoring	CY045604–CY045611
8	A/Swine/OK/011289-8/08	H1N2	3/6/2008	OK	Lung lavage	Polyserositis	CY045636–CY045643
9	A/Swine/OK/011289-9/08	H1N2	3/6/2008	OK	Lung lavage	Polyserositis	CY045620–CY045627
10	A/Swine/OK/011289-10/08	H1N2	3/6/2008	OK	Lung lavage	Polyserositis	CY045628–CY045635
11	A/Swine/OK/011521-4/08	H1N2	3/7/2008	OK	Lung lavage	Parasuis surveillance	CY045644–CY045651
12	A/Swine/OK/011521-5/08	H1N2	3/7/2008	OK	Lung lavage	Parasuis surveillance	CY045652–CY045659
13	A/Swine/OK/016179-8/08	H1N2	4/2/2008	OK	Lung lavage	Pneumonia, open mouth breathing, swollen lymph nodes	CY045668–CY045675
14	A/Swine/OK/016179-9/08	H1N2	4/2/2008	OK	Lung lavage	Pneumonia, open mouth breathing, swollen lymph nodes	CY045660–CY045667
15	A/Swine/OK/020736-1/08	H1N2	4/24/2008	OK	Nasal swab	Health monitoring	CY045692–CY045699
16	A/Swine/OK/0207361-2/08	H1N2	4/24/2008	OK	Nasal swab	Health monitoring	CY045700–CY045707
17	A/Swine/OK/020734-2/08	H1N2	4/24/2008	OK	Lung lavage	Coughing, fever, inappetence, lethargy	CY045684–CY045691
18	A/Swine/OK/020734-3/08	H1N2	4/24/2008	OK	Lung lavage	Coughing, fever, inappetence, lethargy	CY045676–CY045683
19	A/Swine/OK/032726/08	H1N2	6/25/2008	OK	Nasal swab	Disease surveillance	CY045516–CY045523
20	A/Swine/OK/042169/08	H1N2	8/14/2008	OK	Lung lavage	Coughing, pneumonia	CY045708–CY045715
21	A/Swine/TX/050593/08	H1N2	9/26/2008	TX	Lung lavage	Being carried out for health monitoring	CY045524–CY045531
22	A/Swine/TX/050625/08	H1N2	9/26/2008	TX	Lung lavage	Cough and poor growth	CY045532–CY045539
23	A/Swine/OK/053259/08	H1N2	10/10/2008	OK	Lung lavage	Respiratory signs	CY045540–CY045547
24	A/Swine/OK/001142/09	H3N2	1/9/2009	OK	Lung lavage	Poor growth and increased mortality	CY045548–CY045555
25	A/Swine/KS/015252/09	H3N2	3/31/2009	KS	Nasal swab	Being carried out for health monitoring	CY045556–CY045563

bling in the same period as the triple-reassortant viruses but underwent an independent evolution that has been happening in parallel with triple-reassortants circulating in pig populations prior 2009. Comparably long evolution distance between the pandemic (H1N1) 2009 cluster and precursor viruses on all PSTRIG trees reflect this time of undetected circulation.

Discussion

Three influenza virus subtypes, H5N1, H7N7, and H9N2, were proposed as potential pandemic viruses during recent pandemic preparedness planning. Several mechanisms were suggested to explain the direct adaptation of avian viruses to a human host[12–14] but instead, a new pandemic virus most likely emerged according to classical mechanisms by coinfection of the host with two or more subtypes and highlighting swine as a potential 'mixing vessel' for influenza viruses of human, swine, and avian origin.[15,16] Emergence and spreading of the pandemic (H1N1) 2009 influenza virus was fulminant and unpredictable. Preliminary molecular data suggested that this virus was related to the North American triple-reassortant swine viruses.[17] Among the 25 geographically restricted American triple-reassortants, we found that only PB2, PB1, NS, and NP genes were phylogenetically related to pandemic (H1N1)

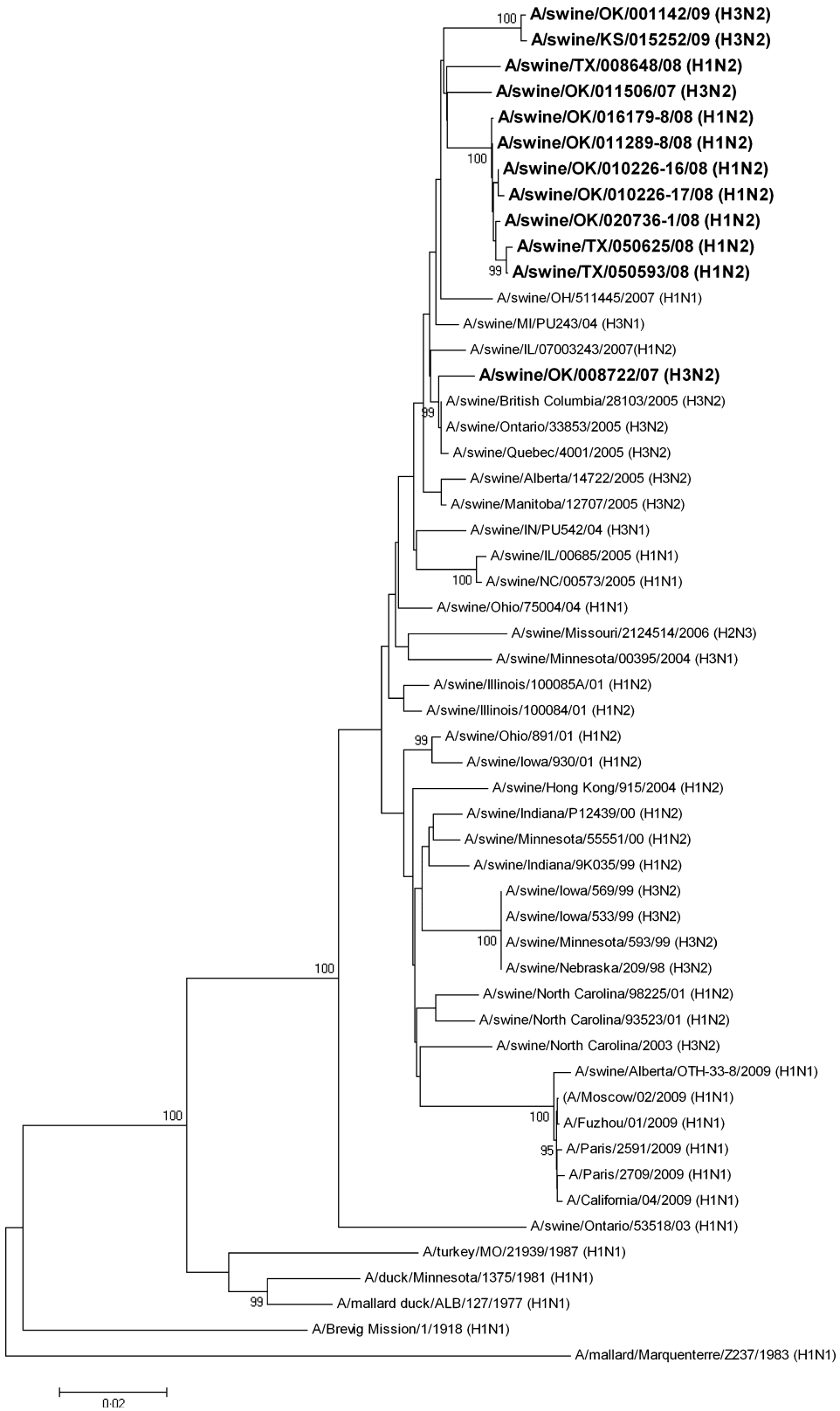

Figure 1. Phylogenetic tree of PB2 genes. Bootstrap indexes >95% are showed. Analogous sequences, among our 25 swine influenza isolates, have been removed for improved presentation. Original sequences type in bold.

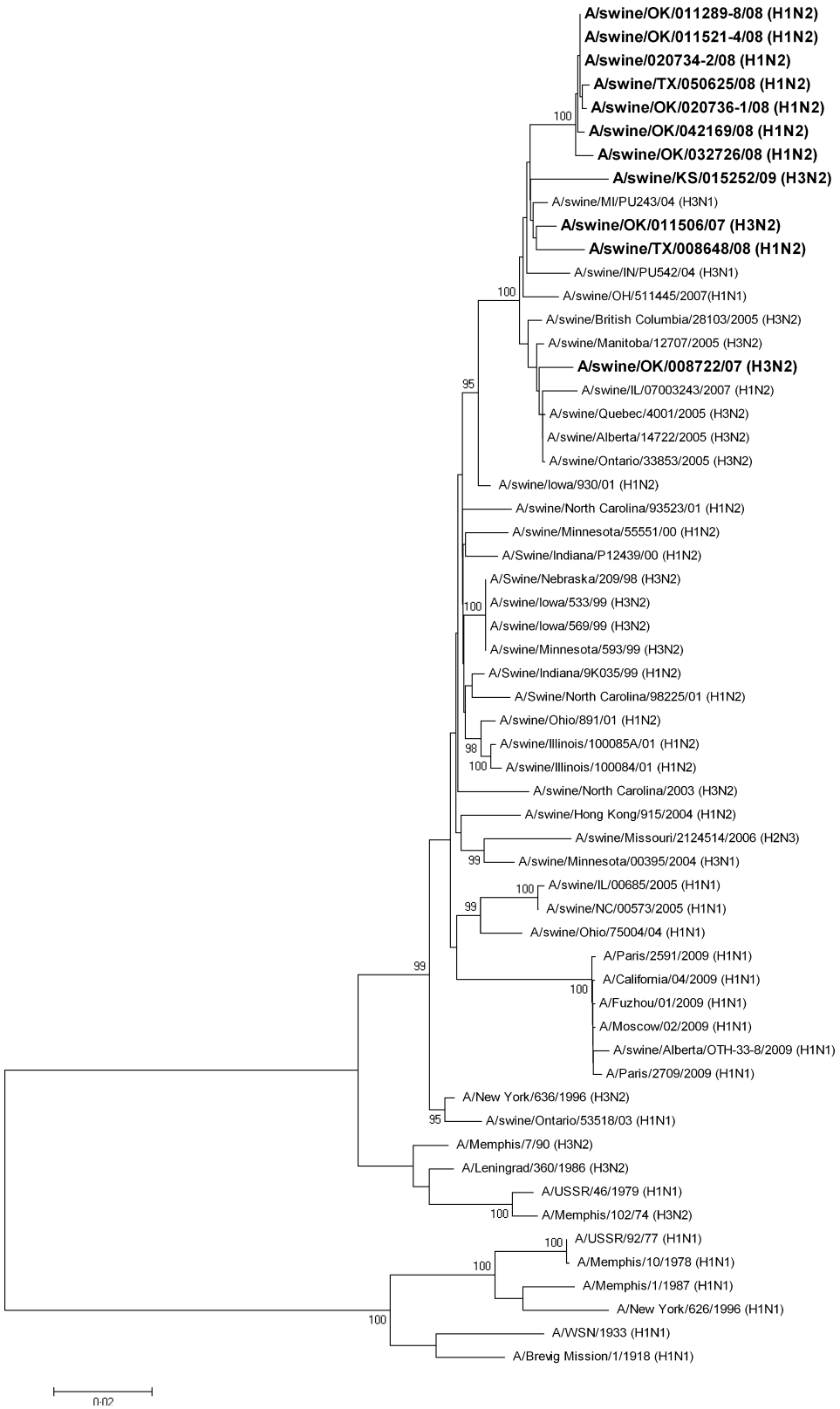

Figure 2. Phylogenetic tree of PB1 genes. Bootstrap indexes >95% are showed. Analogous sequences, among our 25 swine influenza isolates, have been removed for improved presentation. Original sequences type in bold.

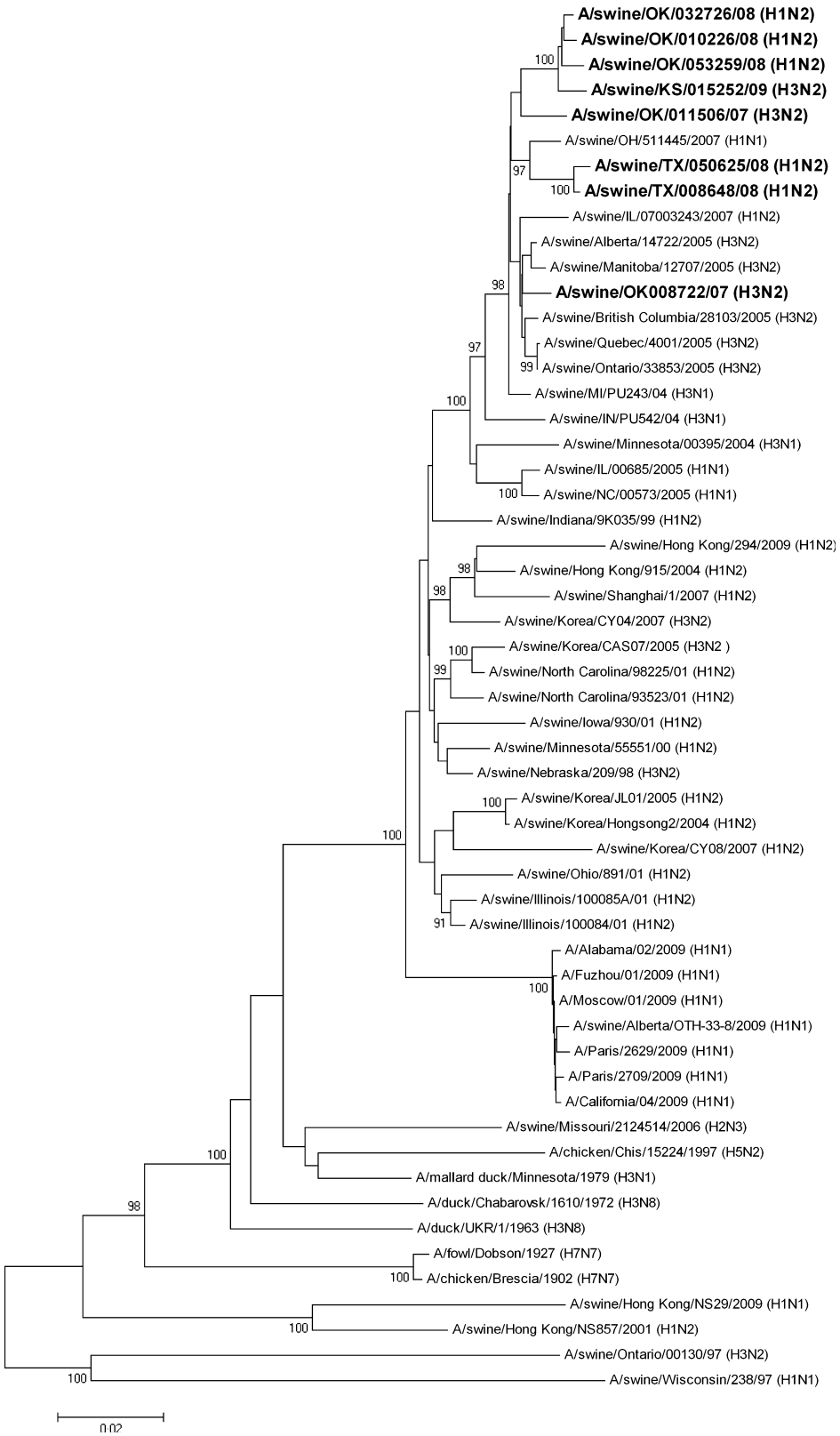

Figure 3. Phylogenetic tree of NP genes. Bootstrap indexes >95% are showed. Analogous sequences, among our 25 swine influenza isolates, have been removed for improved presentation. Original sequences type in bold.

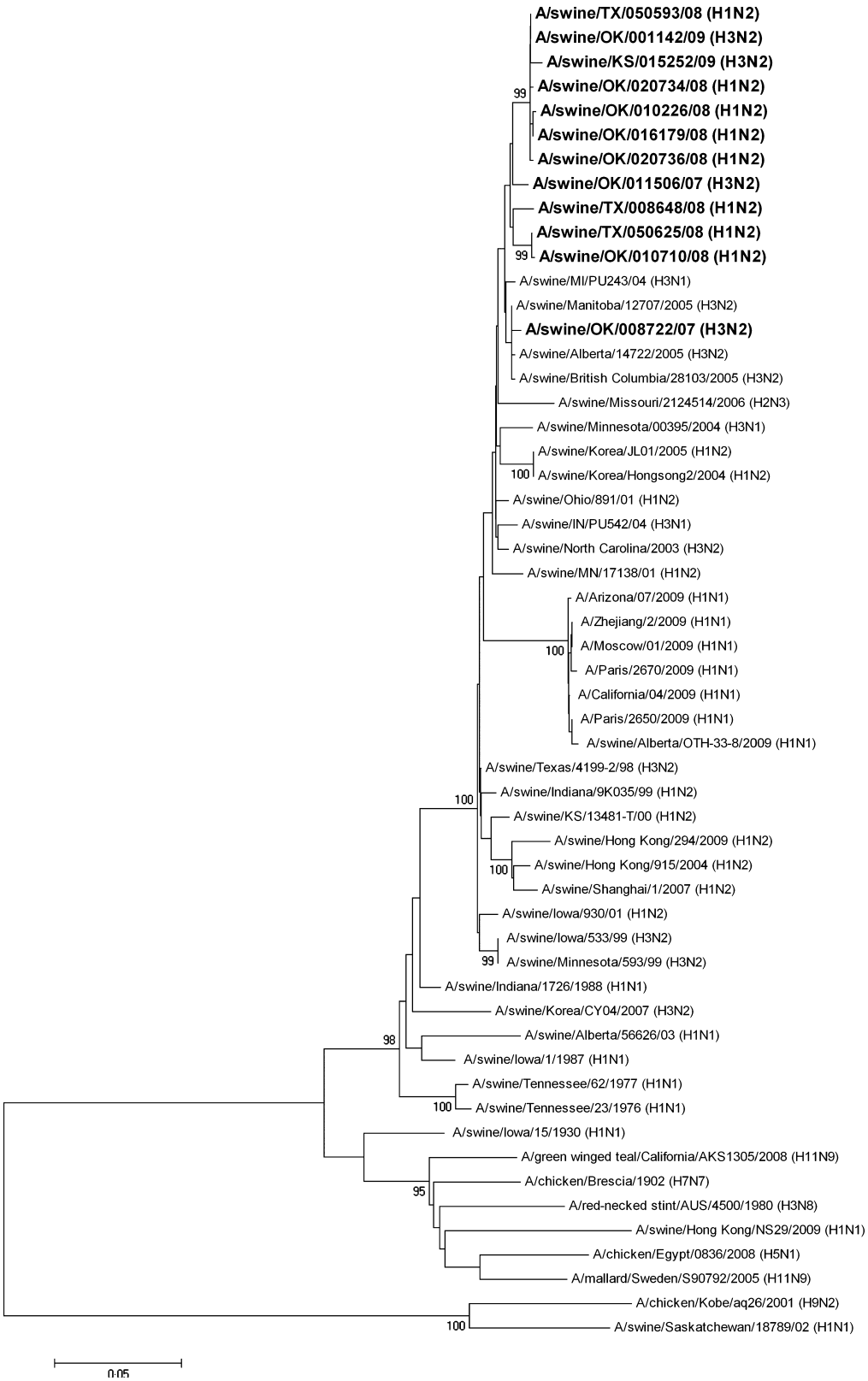

Figure 4. Phylogenetic tree of NS genes. Bootstrap indexes >95% are showed. Analogous sequences, among our 25 swine influenza isolates, have been removed for improved presentation. Original sequences type in bold.

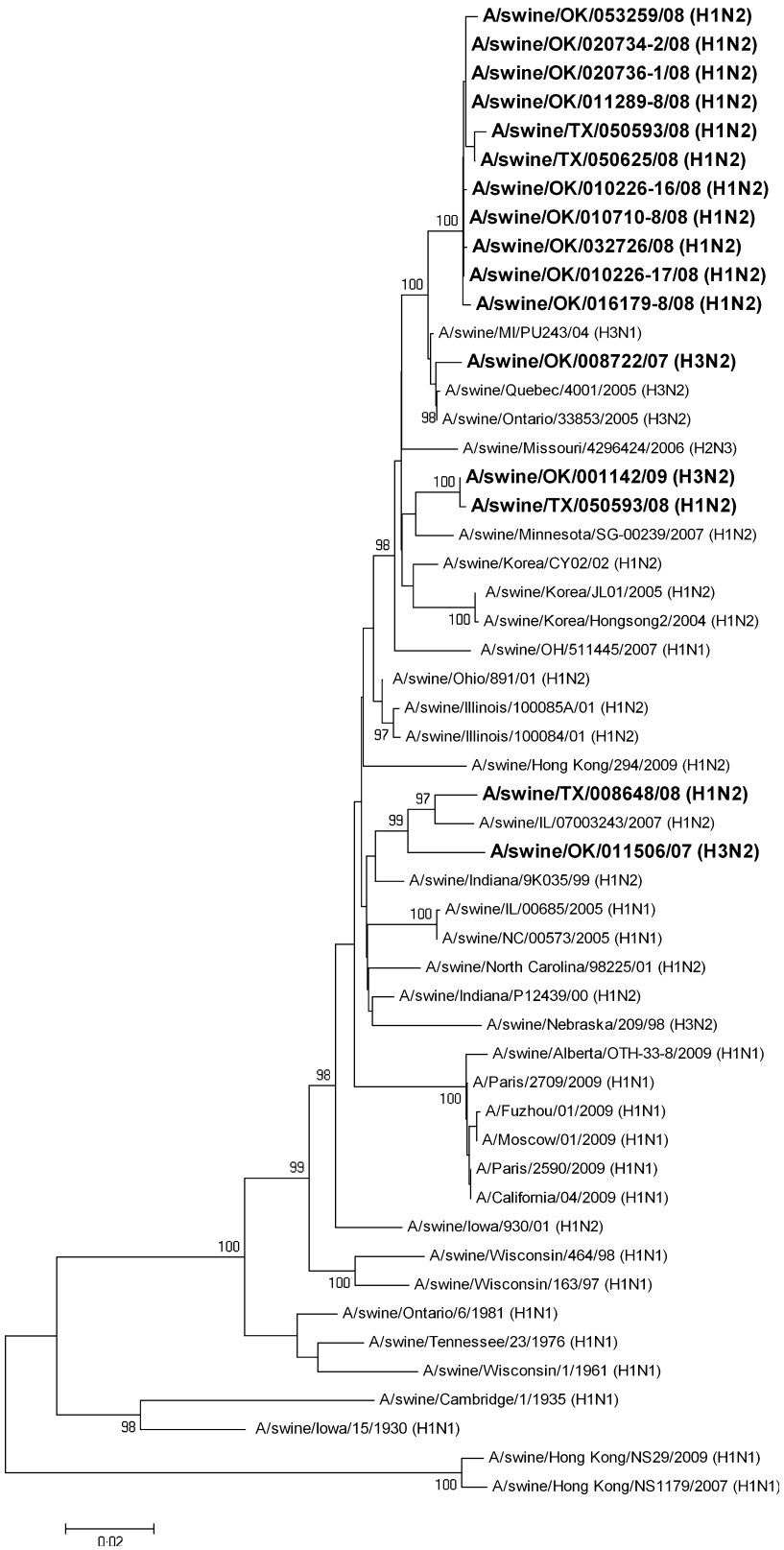

Figure 5. Phylogenetic tree of PA genes. Bootstrap indexes >95% are showed. Analogous sequences, among our 25 swine influenza isolates, have been removed for improved presentation. Original sequences type in bold.

2009. The PA genes forms separate phylogenetic clusters. This suggests that H3N2 triple-reassortants became established in the North American swine population in 1998 after multiple reassorment events and had no single common precursor. Among isolated viruses, we have not found H1N1 or H1N2 triple-reassortants carrying swine HA (H1), viruses originating in 1999. In our study, we have shown that all H1N2 isolates have H1, derived from A/New Caledonia/20/99 (H1N1) – like human influenza. Viruses of this genotype have become dominant in West Central US region since 2003[1] and remain dominant between 2007–2009.

In addition to differences in the PSTRIG cassette, pandemic (H1N1) 2009 influenza contains M and NA genes of Eurasian swine origin. No Eurasian swine lineage genes genetically related to A/swine/Belgium/WVL1/1979 (H1N1) or more recent Eurasian swine influenza reassortants carrying genome segments of A/Port Chalmers/1/1973 (H3N2) – like were found in the swine population in the West Central USA region. In contrast, the circulation of swine influenza viruses of American origin in Asia is widespread,[4,18] most likely due to monopolarity of swine export/import relations (http://www.aphis.usda.gov/publications/animal_health/content/printable_version/AHR_Web_PDF_07/H_Chapter_7.pdfs). Because of the small sample size, the presence of Eurasian swine lineage genes in North American pigs cannot be completely excluded. Our studies agree with surveillance results published earlier[1,2,5,6,19,20] (http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf), and it is possible that there is no significant circulation of Eurasian swine influenza virus in West Central USA pig population.

Previously detected swine influenza cases in humans in the USA did not result in new pandemic viruses. At least 11 sporadic cases of triple-reassortant swine influenza A (H1) in humans were reported in the period 2005–2009 in the USA.[21] Detection of these cases among the myriad seasonal influenza cases almost excludes the possibility of hidden circulation of a novel H1N1 influenza virus during its evolution into a potentially pandemic virus. This circumstantial evidence, along with our studies that show significant phylogenetic distance between pandemic (H1N1) 2009 and triple-reassortant swine viruses based on the analysis of the PSTRIG cassette, and the absence of Eurasian-origin viruses and genes do not support a hypothesis of reassortment and emergence of pandemic (H1N1) 2009 in the USA. However, where these events actually took place remains unclear, but isolation in Hong Kong and characterization of the strain A/swine/HK/915/04 (H1N2), which has seven of eight gene segments, phylogenetically closely related to pandemic (H1N1) 2009 influenza virus,[18] suggests that the possibility of emergence in South-East Asia before 2009 also could not be excluded.

Triple-reassortant swine influenza viruses have demonstrated ability to circulate in swine populations, exchange genome segments, and become endemic in the swine populations of North America and Asia. Even though the pandemic (H1N1) 2009 precursor viruses have not been found in the swine or human populations yet, considerable effort should be made to isolate these precursors to better understand the key factors determining the ability of influenza viruses to cause pandemics.

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