

Influenza A H1N1 virus in Indian pigs & its genetic relatedness with pandemic human influenza A 2009 H1N1

Nagarajan K., G. Saikumar, Rahul Singh Arya, Ashish Gupta, R. Somvanshi & B. Pattnaik*

*Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh & *Project Directorate on FMD, Mukteswar, Nainital, Uttarakhand, India*

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Background & objectives: With the emergence of a new reassortant influenza A H1N1 virus that caused the 2009 pandemic it was felt necessary that pigs should be closely monitored for early detection of any influenza virus infection. Therefore, we investigated disease outbreaks with clinical history suggestive for swine influenza reported to our laboratory by owners of affected pig farms in Uttar Pradesh.

Methods: Detection of swine influenza A virus (SIV) was attempted by isolation in embryonated chicken eggs. Presence of virus was detected by haemagglutination (HA) test and RT-PCR for amplification of different gene segments, cloning and sequencing. BLAST analysis of sequence data, phylogenetic analysis and mutation analysis based on HA, NA and matrix genes was done.

Results: SIV could be isolated from one farm and all eight gene segments amplified by RT-PCR. BLAST analysis of partial nucleotide sequences and phylogenetic analysis using nucleotide sequence of HA (601 nt), NA (671 nt) and M (1031 nt) genes indicated close genetic relationship of the Indian swine isolate (A/Sw/UP-India-IVRI01/2009) with human pandemic 2009 (H1N1). The HA gene showed close relationship with the viruses of "North American Swine" lineage, whereas the NA and M genes clustered with the viruses of "Eurasian Swine" lineage, indicating a novel HA-NA reassortant. The remaining of 5 genes (NP, PA, PB1, PB2 and NS) belonged to "North American Swine" lineage.

Interpretation & conclusions: This is perhaps the first report describing swine influenza among Indian pigs caused by an influenza A H1N1 virus sharing close homology with the human pandemic (H1N1) 2009 virus. Further reassortment with circulating influenza viruses must be closely monitored.

Key words H1N1 - India - phylogeny - reassortant - S-OIV - swine influenza

Swine influenza (SI) is an acute respiratory disease of pigs caused by influenza A virus. SI was first observed in 1918 in the US, Hungary and China^{1,2} and the virus was isolated and identified in 1930³. In India, occurrence of human influenza A virus infections in pigs was first documented in 1978⁴. Later, serological surveys conducted intermittently (1981-

1994) detected antibodies against the prevailing human influenza A viruses (H1N1, H2N2, H3N2 and H0N1) in pigs of Assam⁵, Maharashtra⁶ and West Bengal⁷. The study conducted at Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh, during 1980 documented high prevalence of swine origin H1N1 virus infections (55%) besides human influenza viruses

circulating at that time in pigs of Uttar Pradesh, Andhra Pradesh and West Bengal⁸. Though, serological evidence for the presence of both human and swine influenza virus infections among Indian pigs was reported long ago, clinical disease (SI) in pigs has not been reported and little information is available on the virus subtypes circulating. Since 2006, when highly pathogenic avian influenza (HPAI) H5N1 wreaked havoc in the poultry farms of India⁹, increased threat perception to public health warrants active surveillance of influenza virus infections in humans, birds and pigs. Isolation of influenza virus from pigs preceded that from man and it is an important host acting as a 'mixing vessel' in which further reassortment leading to new subtypes with more pathogenic potential and increased human-to-human transmission can occur¹⁰.

In view of the importance of swine in the epidemiology of influenza and the emergence of a new pandemic virus of swine origin (2009, H1N1), detailed investigation was carried out on disease outbreaks reported in pig farms of Uttar Pradesh suggestive for swine influenza. Phylogenetic analysis of the isolated influenza virus was carried out to ascertain genetic relatedness with other known human and swine influenza viruses.

Material & Methods

In May 2009, the National CSF Referral Laboratory (NCSFRL), Division of Pathology, IVRI, Izatnagar, Uttar Pradesh, was contacted by a pig farmer who reported a disease outbreak among pigs in village Sakhanu, Badaun, Uttar Pradesh. Ailing animals (20, adult, crossbred) showed fever, lethargy, anorexia, nasal discharge and occasional coughing. During June 2009, another outbreak involving both local non-descript and crossbred pigs (35) with similar clinical signs was reported from a Farm (No. 2) in village Khajuria, Bareilly, Uttar Pradesh. The third suspected outbreak was reported in November 2009, among 45 crossbred pigs in another Farm (No.3) at Lodhipur Rajput, Muradabad, Uttar Pradesh. These farms had procured pigs (Crossbred and local nondescript breed) from nearby farms or market. The outbreaks were sudden in onset with high morbidity but low mortality, with only death of 2 piglets in Farm No. 3. Blood samples collected from 5 ailing pigs from each of the all three Farms were found negative for classical swine fever virus by Real time RT-PCR. Nasal and ocular swabs of 5 pigs from each Farm collected in normal saline solution (NSS) supplemented with antibiotics (Gentamicin 100 µg/ml) and bovine serum albumin (5

mg/ml) were transported over wet ice to the laboratory and stored at -70°C until further processing.

Collected nasal swabs were vortexed for 5 min and centrifuged at 1500-1900 g for 15-30 min at 4°C. The supernatant was collected and treated with antibiotic (penicillin 10,000 units/ml; streptomycin 10,000 units/ml) and fungizone (250 mg/ml) for 30-60 min at 4°C before inoculation into chicken embryos.

As per the Office International des Epizootics (OIE) recommended protocol¹¹, isolation of virus was carried out in 9-11 day old embryonated chicken eggs (ECE). Each sample was inoculated into allantoic cavity of 4 ECEs with 100 to 300 µl of inoculum and incubated at 37°C. Allantoic fluid was harvested after death of embryo or on 3 dpi and tested for the presence of influenza A virus by haemagglutination (HA) test as per the OIE¹¹ using 0.5 per cent chicken red blood cell (RBC) suspension. HA positive samples were further subjected to RT-PCR for determination of identity of the haemagglutinating virus.

Viral RNA was extracted from allantoic fluid by TRIzol (Gibco-BRL, USA) method and reverse transcription was performed with random primers or influenza A specific universal primer¹² and SuperScript III RT-PCR system (Invitrogen, USA). After reverse transcription, PCR was done for initial screening of the samples for the presence of influenza A specific conserved nucleoprotein (NP)¹³ and matrix protein (M) genes. Amplification of 330 nt NP gene fragment was carried out at 95°C for 5 min followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min. Samples showing amplification for NP gene were processed for amplification of all 8 gene segments following protocol described earlier with slight modification¹². The PCR amplicons were analyzed by 0.7 per cent agarose gel electrophoresis.

PCR amplicons from the target region of specific influenza segment were purified using Gel DNA Recovery kit (Zymogen, USA) as per manufacturer's recommended protocol. Gel eluted RT-PCR amplicons were cloned into pTZ57R/T vector using InsT/Aclone™ PCR product cloning kit (Fermentas Life Sciences, USA) as per manufacturer's recommended procedure.

The recombinant plasmids were sequenced using BigDye Terminator v3.1 cycle sequencing kit on an ABI 3130 genetic analyzer (Applied Biosystems, USA). DNA sequences were compiled and edited using the Lasergene sequence analysis software package

(DNASTAR Inc, Madison, USA). BLAST analysis of sequence data was done in the internet influenza virus resource database (<http://www.ncbi.nlm.nih.gov/genomes/FLU/SwineFlu.html>).

Phylogenetic analysis of the available partial nucleotide sequences of HA (601nt), NA (671 nt) and M (1031 nt) genes of the present isolate and those available in the NCBI database (www.ncbi.nlm.nih.gov/sites/nucleotide) was carried out using Molecular Evolutionary Genetic Analysis (MEGA4)¹⁴. Evolutionary history was inferred using the Neighbor-Joining method and the distances were computed using the Maximum Composite Likelihood method available in MEGA and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 2). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The nomenclatures of SIV sequences used in the present study were similar to those at the respective databases. For molecular characterization, the mutations in HA, NA and M genes of Indian swine isolate were compared with A/California/04/2009 (H1N1) virus and all other Indian H1N1pdm viruses¹⁵.

Results & Discussion

All suspected clinical samples inoculated into ECEs were tested until the third passage for presence of haemagglutinating virus. In all three consecutive passages, the sample from Farm No. 3 showed embryonic mortality before 36 h in all inoculated eggs (4/4) and HA activity was detected (2⁸ and 2¹¹) in the

allantoic fluid. The results confirmed the presence of a haemagglutinating virus which multiplied efficiently in ECEs.

Initial screening for influenza A virus was done by RT-PCR targeting 330 nt fragment of NP gene. None of the nasal /ocular swabs and allantoic fluid samples collected from chicken embryos inoculated with samples from Farm Nos. 1 and 2 were positive. Samples originating from Farm No.3 showed specific amplification of NP gene suggesting the presence of influenza A virus in pigs.

Samples from Farm No.3 yielded positive results in RT-PCR for all eight genes matching their respective sizes. The sequencing results obtained were blasted in the GenBank Database to check their identity and homology with different subtypes of influenza A viruses. Complete sequence of M gene (1031 nt) and partial sequences of HA (654 nt) and NA (671 nt) were found to be very specific for influenza A 2009(H1N1) and are available in the GenBank database (accession Nos. GU 593047, GU593048 and GU593049).

BLAST analysis of partial nt sequence of all 8 gene fragments of the Indian isolate A/Sw/UP-India/2009(H1N1) is shown in the Table. HA and NA gene sequences of the present isolate was almost similar (99%) to the HA and NA genes of 2009 pandemic H1N1 isolates and shared close relationship with other influenza A viruses of North American and Eurasian swine lineages as well. The HA gene of the Indian isolate was of "North American" lineage whereas both NA and M genes belong to Eurasian lineage. This corroborates earlier observations¹⁶.

Table. BLAST analysis of all genes identified in A/Swine/UP-India/IVRI01/2009/H1N1

Gene	Nucleotide length	Strain (NCBI Number)	Lineage	Subtype	Identities (%)
<i>PB2</i>	624 (45-668)	A/Swine/Korea/NS06/2004 (EU301177.2)	North American swine	H3N2	96
<i>PB1</i>	708 (15-722)	A/Wisconsin/10/98 (AF342823.1)	North American swine	H1N1	96
<i>PA</i>	622 (1524-2145)	A/Swine/Korea/CY09/2007 (EU798896.1)	North American swine	H3N2	98
<i>HA</i>	654 (80-733)	A/Swine/Indiana/P12439/00 (AF455600.1)	North American swine	H1N2	95
<i>NP</i>	693 (20-716)	A/Swine/Iowa/553/99 (AF251415.2)	North American swine	H3N2	96
<i>NA</i>	671 (62-732)	A/Swine/England/195852/92 (AF250366.2)	Eurasian swine	H1N1	93
<i>NS</i>	890 (1-845)	A/Swine/Texas/4199-2/98 (AF153261.1)	North American swine	H3N2	96
<i>M</i>	1031 (1-964)	A/Swine/Hong Kong/1197/02 (AY363580.1)	Eurasian swine	H3N2	97

The remaining 5 gene segments (PA, PB1, PB2, NP and NS) belonged to the “North American” lineage. The PA gene of A/Sw/UP-India/2009(H1N1) was closer to 2007 H3N2 subtypes circulating in Korean pigs. The PB2 gene showed maximum homology (99%) with the 2009 pandemic virus (H1N1) and 96 per cent homology with A/Swine/Korea/JNS06/2004 (H3N2) virus. More detailed analysis may reveal whether some swine influenza viruses (H3N2 subtype) in Korean pigs contributed to the evolution of the current pandemic virus.

Previous study on genetic analysis of swine origin H1N1 influenza A viruses of 2009 indicated that the progenitors of the present pandemic virus may have been circulating undetected among swine herds somewhere in the world as only 94-97 per cent maximum homology was found between the 2009 influenza A (H1N1) isolates and viruses circulating during 1992-2004¹⁷. The present Indian swine isolate revealed higher percentage of nucleotide identity with Korean viruses isolated between 2004 and 2007. BLAST analysis of PB1, NP and NS genes revealed 96 per cent homology with A/Wisconsin/10/98(H1N1), A/Swine/Iowa/533/99(H3N2) and A/Swine/Texas/4199-2/98(H3N2), respectively, all North American swine lineage viruses. These observations are similar to an earlier report on the human isolate A/California/04/2009(H1N1)¹⁶.

For detailed phylogenetic analysis, available sequence data for, HA (601 nt), NA (671 nt) and M (1031 nt) genes were taken and compared with sequences of influenza A viruses representing different lineages, prevailing in different continents and various species. To ascertain whether the NA gene contained mutations associated with resistance to neuraminidase inhibitors, the NA gene sequence was analyzed.

The phylogenetic trees constructed based on the sequence data for HA, NA and M genes of the Indian isolate are depicted in Fig. a, b, and c, respectively. HA gene of A/Sw/UP-India/IVRI01/2009 (H1N1) showed close homology with other contemporary H1N1 viruses involved in the 2009 pandemic and A/Sw/Guangxi/17/2005 (H1N2), all of which belong to the classical swine North American lineage. This finding suggests the need for more detailed studies on contemporary subtypes of influenza A viruses circulating among avian and mammalian species in Asia for a better understanding of the origin of HA gene of H1N1 2009 pandemic virus (Fig. a). The NA gene was most closely related to influenza A viruses

circulating in swine populations in Eurasia (Fig. b). The close genetic relationship between the NA gene of Indian isolate and other Eurasian swine viruses is in agreement with earlier observations on 2009 (H1N1) pandemic virus¹⁶.

Phylogenetic analysis of the matrix gene showed that the Indian isolate belonged to Eurasian swine lineage with very close relationship to recent 2009 (H1N1) pandemic viruses detected in Mexico, USA, Canada, China and Thailand (Fig. c). Other than recent H1N1 2009 isolates, very close homology was seen with triple reassortant viruses such as A/Swine/HongKong/915/2004 (H1N2) and A/Hong Kong/1774/88 (H3N2) which confirmed earlier suspicion that triple reassortant viruses circulating in the Asian region might have been the progenitors of the 2009 H1N1 pandemic virus¹⁸.

HA of the Indian swine isolate, as in other H1N1 pandemic viruses including Indian human isolates possessed D190, D225, E227, P221 and E216 in the receptor binding site. Other key residues in the receptor binding domain (RBD) predicted to have a role in binding to human receptors such as Y98, T136, W153 and H183 were identical to the earlier reported H1N1pdm viruses¹⁶ including Indian human isolates¹⁵. One significant mutation Q223R present in the Indian swine isolate has also been noticed in A/Blore/NIV236/2009/H1N1¹⁵. The significance of this mutation needs further investigation. The Indian swine isolate possessed residue H274 (position 275 in N1 numbering) a known marker for sensitivity to the neuraminidase inhibitor and the genetic marker S31N in the M2 gene suggesting Amantadine resistance¹⁹.

In conclusion, the results of the present study indicated close relationship between A/Sw/UP-India/IVRI01/2009 (H1N1) and pandemic (H1N1) 2009 influenza virus strains isolated from humans and swine population in India, USA, Canada, Taiwan and Japan. It is not known since when this virus has been circulating among Indian pigs. Phylogenetic analysis based on complete sequences of the genes may shed more light on the origin of the Indian swine isolate. Investigation in Argentina also revealed pandemic (H1N1) 2009 influenza virus among pigs possibly resulting from direct human-to-pig transmission²⁰. In the present investigation, the source of infection in the affected Farm could not be established. H5N1 is already established in the Indian poultry population and is transmissible to pigs. Dual infection in pigs may result in a new

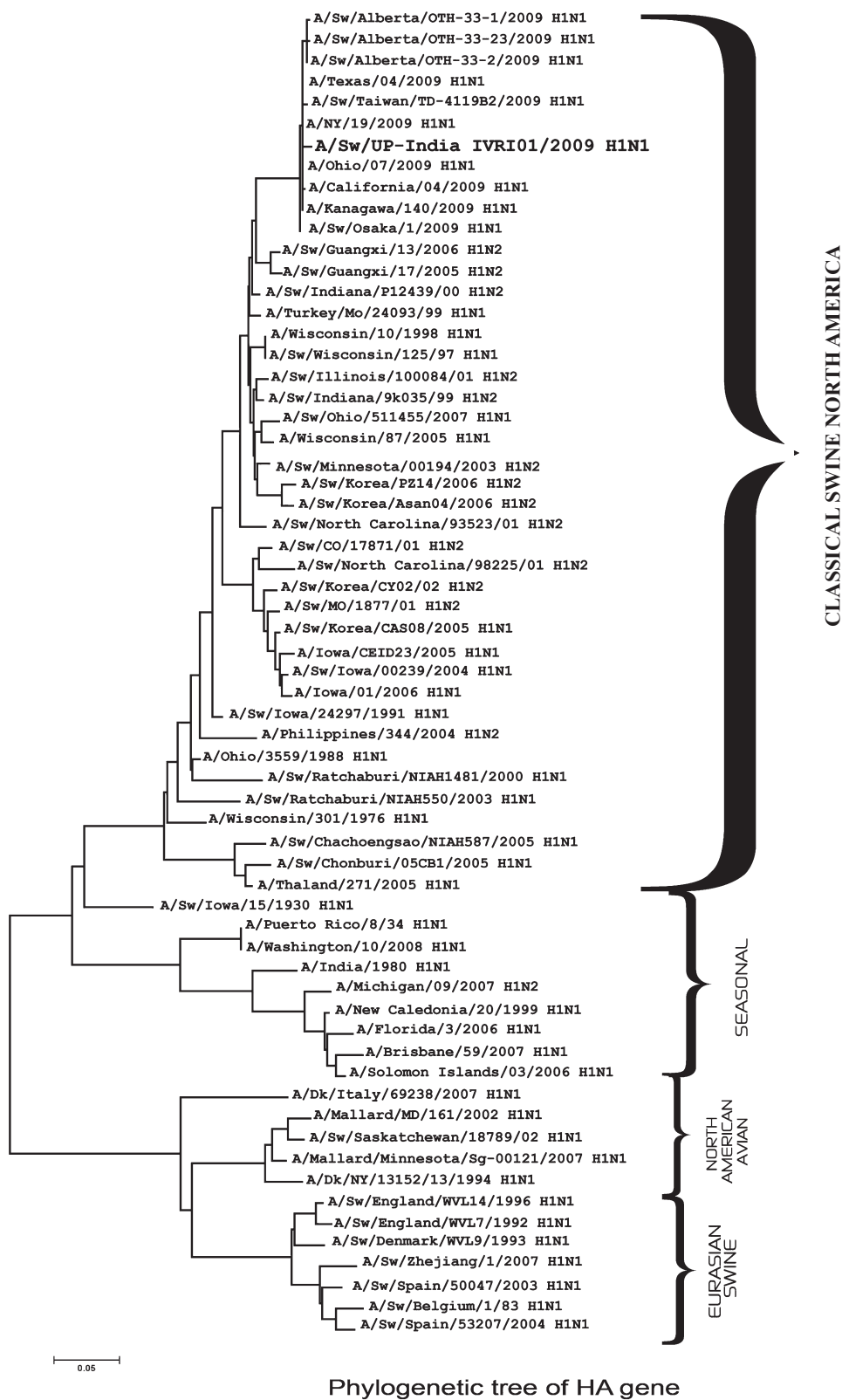


Fig. a. Phylogenetic tree based on nucleotide sequence of haemagglutinin (HA) gene segment of influenza A viruses human, swine and avian origin. A/Sw/UP-India/IVRI01/2009 H1N1 is highlighted with bold letters.

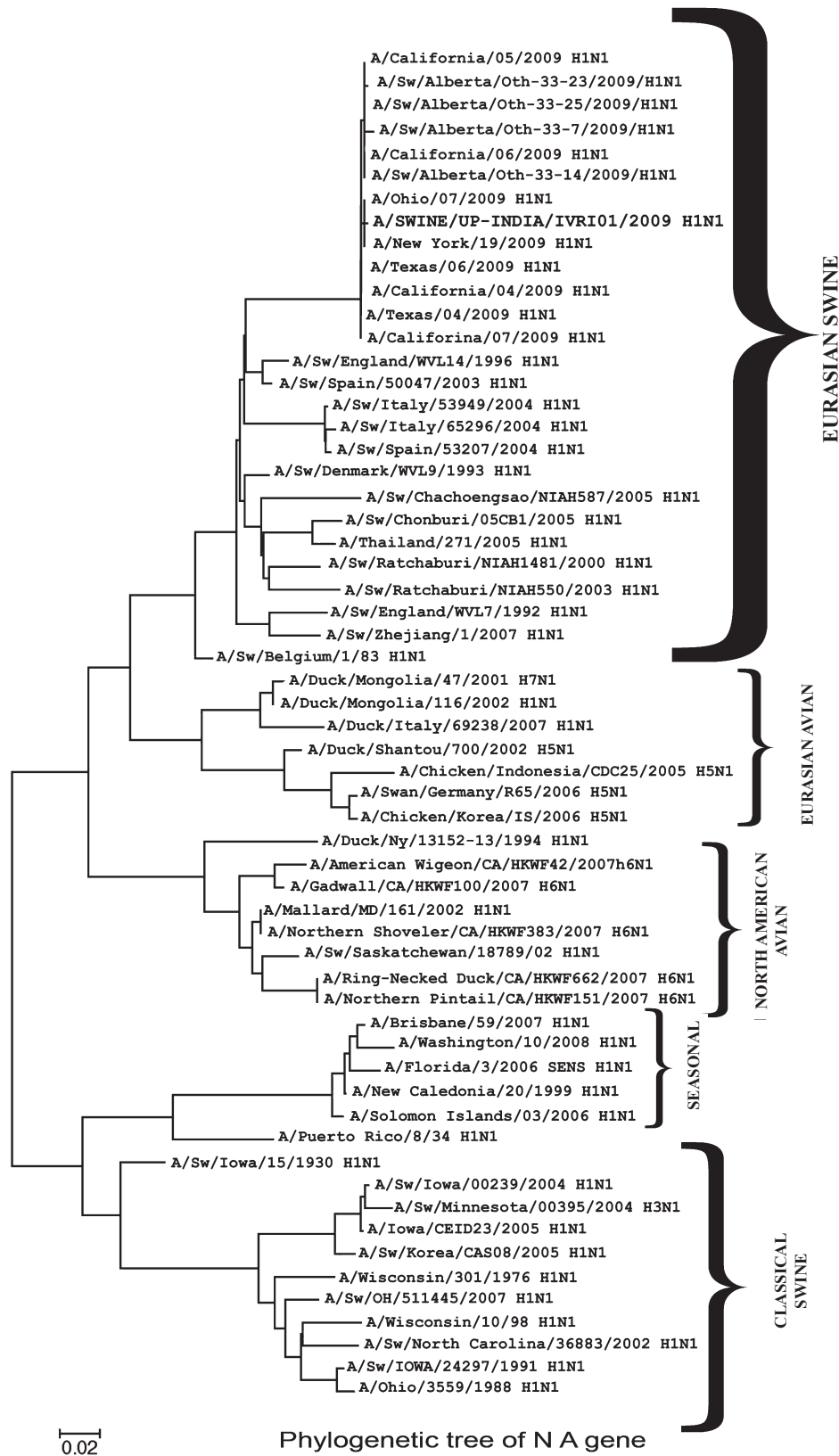


Fig. b. Phylogentic tree based on nucleotide sequence of neuraminidase (NA) gene segment of influenza A viruses human, swine and avian origin. A/Sw/UP-India/IVRI01/2009 H1N1 is highlighted with bold letters.

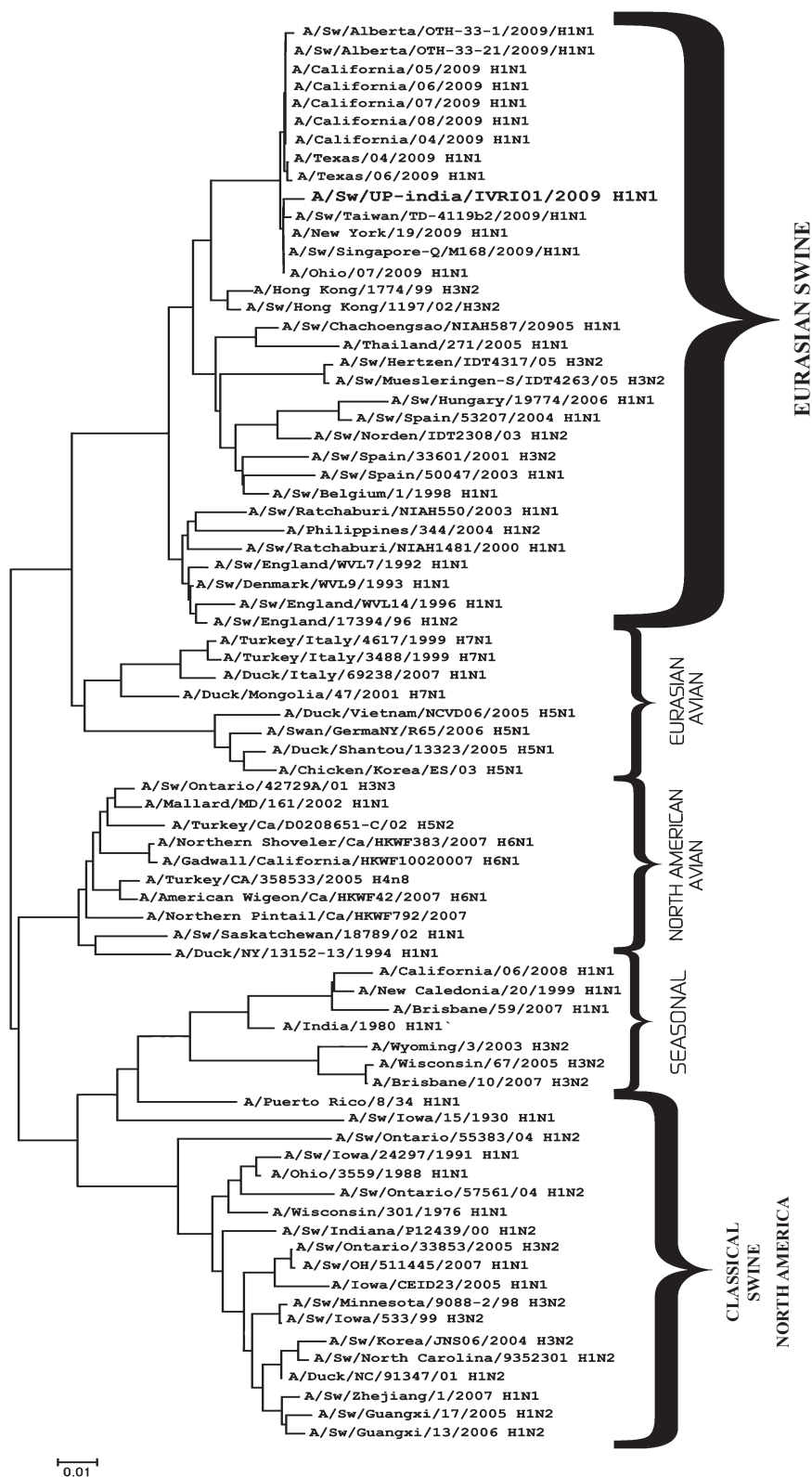


Fig. c. Phylogenetic tree based on nucleotide sequence of M gene segment of influenza A viruses human, swine and avian origin. A/Sw/UP-India/IVRI01/2009 H1N1 is highlighted with bold letters.

reassortant with high transmissibility and case fatality in human beings. To avert such a situation, intensive surveillance of humans, pigs and poultry for influenza virus infections is needed.

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Reprint requests: Dr G. Saikumar, Senior Scientist & I/C National CSF Ref. Lab, Division of Pathology, Indian Veterinary Research Institute, Izatnagar 243 122, UP, India
e-mail: saikumarivri@gmail.com