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NOVEL SWINE INFLUENZA VIRUS SUBTYPE H3N1 IN ITALY:

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Abstract

To date, three subtypes of swine influenza viruses, H1N1, H1N2, and H3N2 have been isolated in Italy. In 2006, a novel swine influenza virus subtype (H3N1) was isolated from coughing pigs. RT-PCR performed on lung tissues, experimental infection in pigs with the novel isolate, and cloning the virus by plaque assay confirmed this unique H and N combination. The novel isolate was also antigenically and genetically characterized. Genetic and phylogenetic analysis showed that the complete HA gene of the H3N1 strain has the highest nucleotide identity to three Italian H3N2 strains, one isolated in 2001 and two in 2004, whereas the full length NA sequence is closely related to three H1N1 subtype viruses isolated in Italy in 2004. The remaining genes are also closely related to respective genes found in H1N1 and H3N2 SIVs currently circulating in Italy. This suggests that the novel SIV could be a reassortant between the H3N2 and H1N1 SIVs circulating in Italy.

Keywords: Swine, novel subtype H3N1, genetic characterization, Italy

1- Introduction

Swine influenza is an economically important respiratory disease of swine caused by influenza A virus. Currently, three predominant subtypes (H1N1, H1N2, and H3N2) are prevalent in pigs throughout the world. However, subtype H1N7, H4N6, H9N2, and H3N3 viruses have also been isolated sporadically from pigs without becoming established (Karasin et al, 2004; Lipatov et al, 2004; Yu et al, 2008). In Europe over the last years, the epidemiology of swine influenza viruses has considerably changed. The H1N1 viruses now prevalent in European countries are antigenically distinct from the classical H1N1 strains and apparently came from the introduction of an avian virus in toto (Pensaert et al, 1981). These “avian-like” H1N1 viruses that emerged in European mainland pigs in 1979 replaced the previously circulating classical H1N1 strains. The H3N2 viruses present in Europe since 1984 are human-avian reassortants possessing six internal genes from the avian-like H1N1 viruses and HA and NA genes from the earlier isolated human-like H3N2 viruses (Castrucci et al, 1993). The third subtype (H1N2) was first isolated in Great Britain in 1994 (Brown et al, 1995) and subsequently spread to the swine population of continental Europe (Marozin et al, 2002). The H1N2 viruses originally resulted from multiple reassortment events initially involving human H1N1 and H3N2 viruses, followed by reassortment with avian-like swine viruses (Brown et al, 1998). In Italy, three subtypes (H1N1, H1N2, and H3N2) of swine influenza viruses have been reported in the pig population (Castrucci et al, 1993; De Jong et al, 2007; Marozin et al, 2002). Phylogenetic analysis of Italian strains isolated during the period 1998-2007 were recently conducted (Moreno Martin et al. 2008 a, 2008b) and revealed that recent Italian H1N1 SIVs isolated in 2001-2007 were closely related to the A/Sw/IV/1455/99-like H1N1 swine viruses whereas Italian H1N1 strains isolated in the nineties clustered with the A/Sw/Fin/2899/82 SIV. Italian H1N2 strains were genetically similar to H1N2 viruses from the UK but were divided into two distinct clusters, one regarding strains isolated in 1998-2003 closely related to contemporary strains isolated in North Europe and the other related to recent Italian strains. Very interesting was the identification of three reassortant strains. One strain A/Sw/It/2064/99 H1N2 was closely related to H1N1 Italian SIVs (Marozin et al, 2002), the other strain A/Sw/It/5433/01 H1N1 showed 95-97% homology to recent Italian H1N2 SIVs, and the last reassortant A/Sw/It/11271/03 H1N2 showed a high similarity to the Italian H3N2 strains(Moreno Martin et al, 2008a). Regarding the subtype H3N2, De Jong et al (2007) reported that Italian strains isolated in the eighties and nineties displayed antigenic and genetic changes similar to those observed in Northern European viruses in the same period. Phylogenetic analysis showed that the Italian strains were located in the Eurasian virus lineage called A/Port Chalmers/1/73 (PCh lineage) where a gradual evolution of swine viruses starting form A/PCh/1/73-like human influenza virus is observed. This virus is considered the most probable ancestor of the swine PC73 lineage.

Recently the isolation of a novel swine influenza virus H3N1 was reported in Taiwan, USA and Korea (Lekcharoensuk et al, 2006; Ma et al, 2006; Shin et al, 2006) but it has never been isolated in Europe. This paper describes the isolation and characterization of a novel H3N1 swine influenza virus from pigs in Italy. Antigenic typing and genetic characterization suggested that the new isolate was a recombination between the H1N1 and H3N2 viruses circulating in Italy.

2- Materials and methods

2.1- Clinical samples

In March 2006, a severe respiratory disease, characterized by coughing and increased mortality, was observed in a feeder to finisher swine farm with 10000 head, located in the province of Cremona (North Italy). SIV vaccination had not been applied. Two dead pigs were submitted to the laboratory for respiratory disease diagnostic tests. Gross lesions consisted in purple areas of consolidation in the apical and cardiac lobes of the lungs, interlobular edema, mediastinal lymph nodes enlarged and pleuritis.

2.2- Virus isolation and subtype determination

The novel SIV isolate was obtained from a 10% lung homogenate applied onto Madin-Darby canine kidney (MDCK) cells and further inoculated through the allantoic sac route of 9-11 day old SPF chicken embryonated eggs (CEE). Culture supernatant (CS) after observed cytophatic effect and allantoic fluid (AF) were tested with an haemagglutination assay (HA) using chicken erythrocytes

performed as described (OIE Manual, 2005). The presence of influenza A was detected by using a double antibody sandwich ELISA (DAS-ELISA) with an anti-NPA Mab (ATCC n. HB65 H16-L10-4R5) carried out as previously described (Siebinga and de Boer, 1988). The subtype of the isolate 66945/06 was determined from culture supernatant, allantoic fluid and lung tissues by two multiplex RT-PCR assays (Chiapponi et al, 2003).

2.3- Anti-sera

For the antigenic characterization, hyper immune anti-sera collected 21 days after experimental infection of SPF chickens were used. The strains used to produce the anti-sera were the following: Reference strains A/Sw/Fin/2899/82 (2899/82) H1N1, A/Sw/CA/3633/84 (3633/84) H3N2 and swine field strains A/Sw/It/1521/98 (1521/98), A/Sw/It/2064/99 (2064/99) H1N2, A/Sw/It/125746/05 (125746/05) H1N1 and A/Sw/It/79604/06 (79604/06) H3N2 isolated in Italy. The selection of the Italian field strains to produce chicken anti-sera was based on the genomic characteristics and phylogenetic analysis previously conducted, strains 1521/98, 2064/99 (Marozin et al, 2002) and 125746/05 and 79604/06 (Moreno Martin et al, 2008a, 2008b); The partial nucleotide sequence of HA gene of the last two strains is available at GenBank database (http://www.ncbi.nlm.nih.gov/).

2.4- Antigenic characterization

The SIV strain 66945/06 was antigenically characterized by HI, NI and virus neutralization (VN) as previously described (OIE manual, 2005; Van Deusen et al, 1983; Van Reeth et al, 2003) using the anti-sera above described.

2.6- Genomic sequencing and phylogenetic analysis

Viral RNA was extracted from lung homogenate, AF and CS by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Viral RNA isolation by QIAamp® ViralRNA Mini Kit (Qiagen, Hilden, Germany) and RT-PCR by OneStep RT-PCR Kit (Qiagen, Hilden, Germany) were performed either directly on clinical specimens (tissue homogenate and nasal swabs collected after the experimental infection) or AF and CS as described previously (Bragstad et al, 2005). Clinical specimens were screened for the presence of influenza A viruses by partial amplification of the M gene according to standard methods (Fouchier et al, 2000). Partial amplification of HA, NA, PB1, PB2, PA, NP, M and NS1 genes of the described strain and other circulating Italian SIVs was carried out under standard conditions using gene-specific primers (sequence available on request). On the basis of this partial characterization, the full length HA and NA genes of the novel strain and some Italian strains were amplified using universal primers as described (Hoffman et al, 2001). For sequencing, amplified products were separated onto agarose gel and then purified with Qiaquick® gel extraction kit (Qiagen, Inc, Valencia, CA, USA). Sequencing reactions were performed by means of BigDye® Terminator Cycle Sequencing kit v1.1 (Applied Byosistems, Foster City, CA, USA). Both strands of the amplicons were sequenced using the same forward and reverse primers used for the amplification. Full length HA and NA amplicons were also sequenced with internal primers (Chiapponi et al, 2003). Sequences were resolved by using an ABI 3130 DNA automatic sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were combined and edited using the Lasergene sequencing analysis software package (DNASTAR, Madison, WI). Multiple sequence alignments were made using ClustalW and maximum parsimony phylogenetic trees were created using MEGA4 (Tamura et al, 2007). Each tree is a consensus of 1,000 bootstrap replicates.

2.7- Plaque assay

In order to confirm the presence of the novel subtype H3N1, the novel SIV isolate was plaque cloned as previously described (Youil et al, 2004) and retested by HI and NI tests and again subtyped by RT-PCR.

2.8- Experimental swine infection

Two 8-week-old SPF pigs were infected intra-tracheally with $10^{7.5}$ EID_{50}/swine of the novel strain prepared in CEE. Nasal swabs were collected 2, 5, 7, 9 and 12 days after infection and analyzed by RT-PCR using gene M specific primers to evaluate virus shedding. RT-PCR positive samples were then inoculated into a monolayer of MDCK cells and SPF CEE. Serum samples were collected 15, 27, and

35 days after infection and were tested for antibodies anti-NPA, and against H1 and H3 subtypes. A Mab-based (Mab HB65) competitive ELISA was performed to detect anti-NPA antibodies as described (De Boer et al, 1990). Antibodies against H1 and H3 were detected by HI test performed as described (OIE Manual, 2005), using the SIVs 66945 H3N1, 79604/06 H3N2, and 125746/05 H1N1, 1513/98 H1N1, 1521/98 H1N2 (Marozin et al, 2002) as antigen.

2.9- Detecting swine respiratory pathogens

The lung homogenate was cultured using different media for the most common swine respiratory bacterial pathogens. The presence of Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circoviruses type 2 (PCV2) and *Mycoplasma hyopneumoniae* was determined by using either RT-PCR, multiplex PCR and PCR assays respectively, as previously described (Calsamiglia et al, 1999; Ouardani et al, 1999; Suárez et al, 1994).

2.10- Nucleotide sequence accession numbers

GenBank accession nos. of the complete HA genes are: **EU037014**, **EU045362**, **EU045365**, **EU982298**, **EU045371**, **EU982299**, **EU982297**, **EU982296**, **EU982295**. GenBank accession nos. of the complete NA genes are: **EU037015**, **EU045388**, **EU045389**, **EU045393**. GenBank accession nos. of partial nucleotide sequences of internal genes are: NP gene: **EU091685**, **EU091690**, **EU091695**. PB1 gene: **EU091687**, **EU091692**, **EU091697**. PB2 gene: **EU091688**, **EU091693**, **EU091698**. PA gene: **EU091686**, **EU091691**, **EU091696**. M gene: **EU091684**, **EU091689**, **EU091694**. NS gene: **EU091704**, **EU091705**, **EU091707**.

3- Results

3.1- Virus isolation and subtype determination

Allantoic fluid collected 5 days post incubation of CEE and MDCK CS agglutinated chicken erythrocytes in HA (HA titer of 7 and 6 $\log_2$/25µl respectively). The presence of influenza A antigen was detected by sandwich ELISA using the Mab anti-NPA HB65 in both AF and MDCK CS. A multiplex RT-PCR specific for HA and NA was further used to subtype both AF and CS that unexpectedly resulted to be H3N1. Neither H1 nor N2 specific bands were observed. The presence of H3N1 virus in the lung tissues was also confirmed by RT-PCR and was the only H-N combination detected in the sample. The isolate was named A/Sw/It/66945/06 (66945/06). Respiratory pathogens such as bacteria, *M. hyopneumoniae* or PRRSV that could produce lesions similar to SIV were not observed whereas PCV2 was detected by PCR. The virus resulting from the plaque assay and cloning by limited dilution was identified as subtype H3N1 by RT-PCR and HI and NI tests.

3.2- Antigenic characterization

The HI and VN tests were conducted using a panel of six sera from SPF chickens experimentally infected with two early reference SIV strains (2899/82 H1N1 and 3633/84 H3N2), two well known SIV strains H1N2 (Marozin et al, 2002) isolated in Italy at the end of the nineties (1521/98 closely related to the HA of the European H1N2 viruses and 2064/99 closely related to the HA of the European H1N1 viruses) and, finally, with two recent Italian strains, 125746/05 H1N1 and 79604/06 H3N2, that exhibited different HA genetic characteristics compared to early European isolates (Moreno Martin et al, 2008a, 2008b). The same panel of sera was used to determine the antigenic reactivity of the neuraminidase in NI tests. The HI tests showed that the strain 66945 reacted with post-infection chicken antisera to H3N2 SIVs at high titers (8 $\log_2$ using antiserum CA/3633/84 and 9 $\log_2$ using antiserum It/79604/06) and did not react with chicken antisera towards H1N2. Cross reactions at low titers were observed against H1N1 SIVs 2899/82 (4 $\log_2$) and 125746/05 (5 $\log_2$), probably due to the interference in HI with N1 antibodies. The novel strain H3N1 reacted only with chicken anti-sera to CA/3633/84 H3N2 (SIVs using VN, whereas no reactions were observed with H1N2 and H1N1 anti-sera. In NI assay, N activity of the novel strain was inhibited only by antisera produced towards H1N1 SIVs. Results of the HI and NI tests confirmed the presence of a novel subtype H3N1. Results are shown in table n.1.

3.4- Experimental swine infection

Two SPF pigs were infected intra-tracheally with the isolate H3N1 and were reared in isolation for 35 days. No respiratory distress was reported during the observation period except for an increase in body temperature up to 40,5°C for 2-3 days. Virus shedding was demonstrated testing nasal swabs by RT-PCR only 3 and 5 days PI in the two experimentally infected pigs, and these samples were then inoculated in SPF CEE and into MDCK cells. Infected allantoic fluids and cell culture supernatants were characterized by HI and NI and also by RT-PCR confirming that the subtype H3N1 was the only one to be detected. Serum samples were tested for antibodies against NPA and HA of subtypes 1 and 3. Both antibodies against NPA and H3 were detected in all the serum samples collected 15, 27 and 35 days after infection whereas no specific antibodies towards H1 were evidenced. HI titers, expressed as $\log_2$, were: for pig n.1:7, 6, and 5 $\log_2$ against 66945/06 H3N1 and 7, 6 and 6 $\log_2$ against 79604/06 H3N2 for sera collected 15, 27 and 35 days PI respectively; for pig n.2: 5, 6, and 6 $\log_2$ against 66945/06 H3N1 and 5, 6 and 6 $\log_2$ against 79604/06 H3N2 for sera collected 15, 27 and 35 days PI. HI titer against SIV of subtypes H1N1 and H1N2 resulted to be always <3 $\log_2$.

3.5- Genomic sequence and phylogenetic analysis

Initially the partial nucleotide sequences of the H3N1 isolate genes were compared to those of the SIVs isolated in Italy in the last 10 years. Phylogenetic analysis indicated that the HA gene of strain H3N1 was closely related to three Italian H3N2 strains, one isolated in 2001, A/Sw/It7680/01 (7680/01) and two in 2004, A/Sw/It/297576/04 (297576), A/Sw/It/302219-3/04 (302219-3/04). The NA, NP, PB1, PB2, PA, and M genes showed the highest % of homology to three Italian H1N1 strains isolated in 2004, A/Sw/It/53949/04 (53949/04), A/Sw/It/65296/04 (65296/04), A/Sw/It/247578/04 (247578/04). The remaining NS gene was closely related to other three SIVs currently circulating in Italy (A/Sw/It/1484/02 H3N2, A/Sw/It/4230/02 H1N1, A/Sw/It/207828/02 H1N1).

For a further analysis, BLAST search in the GenBank database demonstrated that the full length HA and NA genes exhibited 96% and 98% nucleotide similarity to those of the A/Sw/Sp/42386/2002 H3N2 and A/Sw/Sp/53207/04 H1N1 respectively. The partial internal genes M, NP, PB1, PB2, and PA showed 98% nucleotide identity with the same Spanish SIVs (53207/04) while the NS gene showed the highest identity (97%) with isolate A/Sw/It/1081/00 H1N2.

The comparison of the full HA and NA genomic sequences of the isolate 66945/06 with some Italian SIVs confirmed the close relationship observed when comparing the partial HA and NA nucleotide sequence between the novel strain and the six Italian SIVs described above (99,0-98,1% at HA and 98,1-98,3 % at NA nucleotide level respectively).

The HA and NA sequences were then aligned to those of other SIVs retrieved from GenBank. Since the highest number of sequences available in GenBank resulted to be mostly partial, the phylogeny reconstruct was limited to only the HA1 region of the H3 SIVs. The resultant maximum parsimony phylogenetic tree indicated that the HA1 region of the HA gene of the novel strain and other recent Italian H3N2 isolates were placed into the antigenic cluster 2 belonging to the PC73-like lineage (De Jong et al, 2007) together with contemporary H3N2 SIVs isolated in South Europe. The H3N1 66945 resulted to belong to a different cluster that the other H3N1 strains isolated in USA and Korea that were closely related (figure n.1). The NA gene of the strain 66945 appeared in the phylogenetic tree (figure n. 2) closely related to Italian and Spanish H1N1 SIVs isolated in the last years but placed in a cluster different from other contemporary H1N1 Italian SIVs related to the Sw/IV/1455/99-like viruses (Marozin et al, 2002).

The comparison of the deduced amino acid sequence of the HA gene of the isolate 66945 with that of other H3N2 and H3N1 SIVs indicates that most of the residues are highly conserved, especially those associated with the sialoside receptor-binding region, Y98, W153, H183, E190, and L194 (Kaverin et al, 2004), and those responsible for host range specificity at positions 226 and 228 (Vines et al, 1998). The strain 66945 H3N1 and the recent Italian isolates H3N2 exhibited the same aa residues, Y98, W153, H183, E190, L194, L226, and S228, which L226 and S228 are mainly responsible for sialyl α2,6-galactose (SAα2,6Gal) specificity. Also the amino acids within the antigenic sites of the HA gene of the Italian H3 SIVs, H3N1 isolate included, resulted to be highly conserved. In addition the novel strain and the related Italian and Spanish H3N2 strains showed the aa residues S137, N145 and N278 characteristic of the antigenic cluster 2.

4- Discussion

The first isolation and characterization of the novel subtype H3N1 in Europe was described. One strain was isolated in 2006 from a feeder to finisher pig farm located in North Italy where specific respiratory symptoms were observed. The antigenic and genetic characterization confirmed the isolation of a novel H3N1 SIV, never described in Europe previously. The genetic characterization of all genes of the new isolate revealed that it was closely related to the circulating Italian SIV strains. The complete HA gene presented the highest identity to three Italian H3N2 SIVs isolated in 2001 and 2004, whereas the NA gene was instead closely related to three Italian H1N1 SIVs isolated in 2004. The deduced aa sequence of the HA gene showed the aa residues (L226 and S228) responsible for the receptor specificity typical of swine and human influenza viruses. In contrast, the residues involved in the SAα2,3Gal linkage, typical of avian viruses, are Q226 and G228. The partial sequencing of internal genes NP, PB1, PB2, PA and M genes showed the high identity to the same H1N1 Italian strains isolated in 2004. Finally the NS gene exhibited the highest identity to one H3N2 and two H1N1 Italian SIVs isolated in 2002. This suggested that the novel SIV was a reassortant between the H3N2 and H1N1 SIVs circulating in Italy. The experimental infection of two SPF pigs was performed in order to further confirm the presence of the novel subtype. The H3N1 SIV could replicate in the respiratory tract and was shed in nasal secretions although only for a few days. Both virus isolation and typing through serological tests demonstrated the presence of the unique H3N1 subtype.

Monitoring programs performed in the last two years on pig farms with respiratory symptoms brought to the isolation of several strains of the three circulating subtypes but not of subtype H3N1. It is worth notice that the subtype H3N1 has been rarely observed throughout the world whereas the isolation of H1N2 reassortant viruses results frequently. Mitnaul et al (2000) revealed that HA and NA proteins recognized the same molecule (sialic acid) with conflicting activities and a balance of protein activities were essential to ensure an efficient replication of the virus. Drastic changes in this equilibrium could affect viral replication. Even if the reason of the low frequency of H3N1 SIV isolation can not be explained, it could be hypothesized that the activities of H3 and N1 proteins are not optimally balanced compared to the H1 and N2 proteins. This novel subtype does not appear to be so wide spread nor successfully maintained in the Italian swine population. On the contrary, reassortment strains among swine viruses of H3N2, H1N1 and H1N2 were frequently observed in Europe (Marozin et al, 2002; Moreno Martin et al., 2008a, Zell et al, 2008). Swine are also considered as a vessel for reassortment of human and avian viruses because they posses both SAα2,6Gal and SAα2,3Gal receptors characteristic of human and avian viruses respectively (Ito et al, 1998; Lekcharoensuk et al, 2006; Ma et al, 2006). Indeed there is good evidence that pigs are more frequently involved in interspecies transmission of influenza A viruses than are other animals (Pensaert et al 1981; Karasin et al 2004, Yu et al 2008, Zhou et al, 1999). All these findings highlight the need for a stringent surveillance in pig populations in order to better understand the circulation of atypical swine influenza viruses.

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	Antigen	**It/66945/06**		
	subtype	**H3N1**		
	test	VN	HI	NI
Antiserum	Fin/2899/82 H1N1	<10	16	+
	It/125746/05 H1N1	<10	32	+
	It/1521/98 H1N2	<10	<2	-
	It/2064/99 H1N2	<10	<2	-
	CA/3633/84 H3N2	640	256	-
	It/79604/06 H3N2	1280	512	-

289 Table n.1- Results of the antigenic characterization of the novel strain preformed by serological tests. Virus neutralization titres are
290 expressed as ND_{50} reciprocal. Using HI test results as HI titre reciprocal.
291

Figure n. 1. Phylogenetic relationship of the HA1 region of HA gene from the newly identified H3N1 swine influenza virus (A/swine/Italy/66945/2006(H3N1)) with other Italian and database collected H3N2 and H3N1 SIV strains. The tree was created by Maximum Parsimony method and bootstrapped with 1,000 replicate. Only bootstrap values higher than 70% are shown.

Figure n. 2. Phylogenetic relationship of the complete NA gene from the newly identified H3N1 swine influenza virus (A/swine/Italy/66945/2006(H3N1)) to NA gene of other Italian and database collected H1N1 SIV strains. Tree was generated as described in the legend to Fig.n.1.

Figure n.1

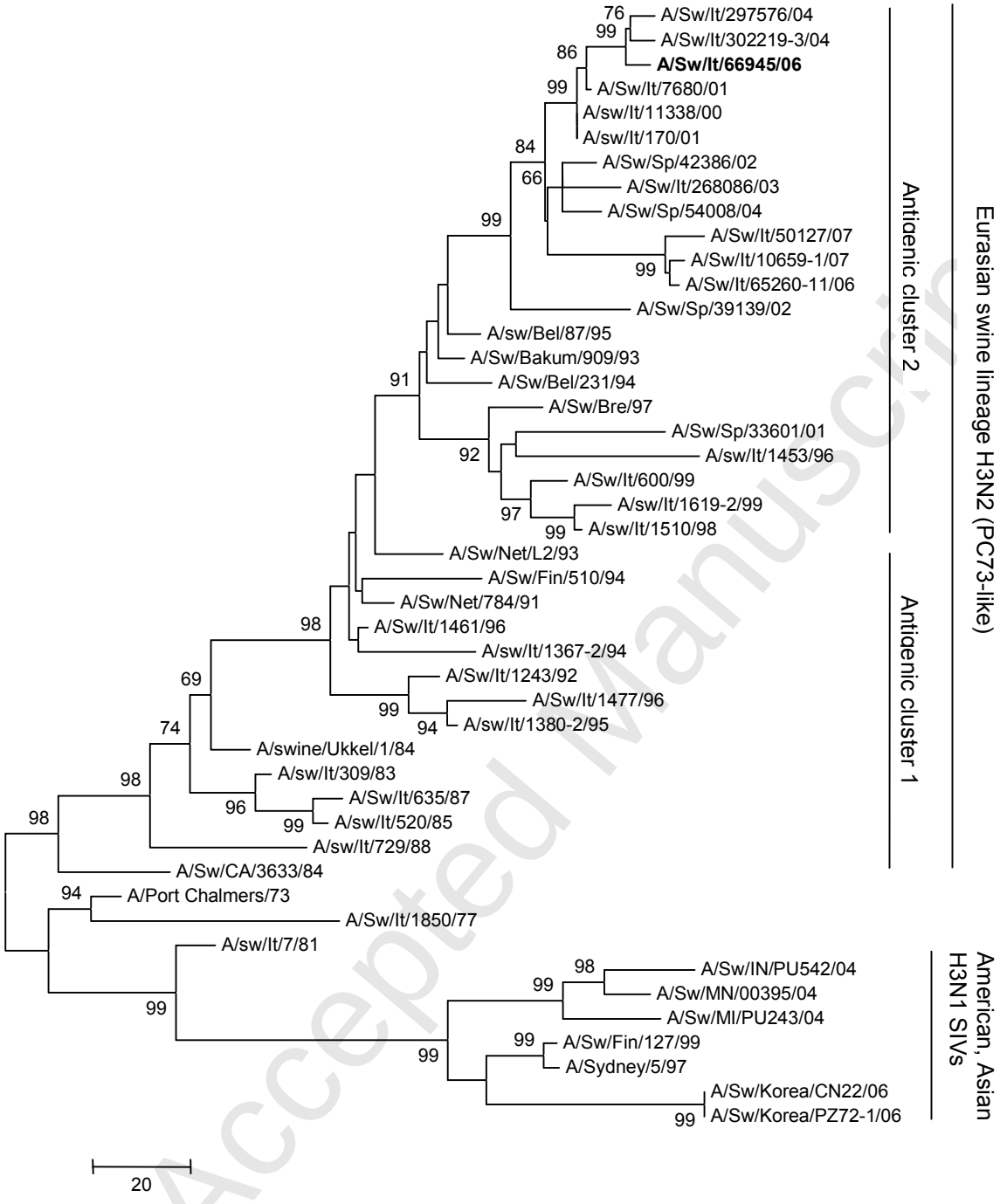

Figure 2