UNIVERSITÀ DEGLI STUDI DI MILANO

DIPARTIMENTO
Di SCIENZE VETERINARIE E SANITA' PUBBLICA

SCUOLA DI DOTTORATO IN "SANITÀ E PRODUZIONI ANIMALI: SCIENZA, TECNOLOGIA E BIOTECNOLOGIE"

DOTTORATO DI RICERCA IN IGIENE VETERINARIA E PATOLOGIA ANIMALE

XXV CICLO

TESI DI DOTTORATO DI RICERCA

Epidemiology, genomic characterization and evolutionary analysis of Italian H1 swine influenza viruses

SSD 07/H3 - Malattie infettive e parassitarie degli animali

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Anno Accademico 2011-2012

List of Abbreviations

- Agar gel immunodiffusion (AGID)
- Allantoic fluid (AF)
- Amino-acid (aa)
- Antibodies (Ab)
- Avian influenza virus (AIV)
- 2009 A/H1N1 pandemic strain (H1N1pdm),
- A/Port Chalmers/1/73 (PCh-73)
- Chicken embryonated eggs (CEE)
- complementary RNA (cRNA)
- Culture supernatant (CS)
- Effective sample sizes (ESS)
- European (EU)
- Fixed effects likelihood (FEL)
- General time-reversible (GTR)
- Haemagglutinin (HA)
- Haemagglutination inhibition (HI)
- Influenza A viruses (IAVS)
- Maximum Clade Credibility trees (MCCT)
- Markov chain Monte Carlo (MCMC
- Matrix (M)
- Matrix protein 1 (M1)
- Matrix protein 2 (M2)
- Maximum Likelihood (ML)

- Mean dN/dS ratio (ω)
- Monoclonal antibody (Mab).
- Madin-Darby canine kidney (MDCK)
- National Center for the Biotechnology information (NCBI)
- Neuraminidase (NA).
- Nonstructural protein (NS)
- Nonsynonymous (dN)
- Nuclear export protein (NEP)
- Phylogenetically important regions (PIRs)
- Phosphate buffered saline (PBS)
- Porcine respiratory disease complex (PRDC)
- *Porcine Reproductive and Respiratory Syndrome virus* (PRRSV)
- Protein data bank (PDB)
- Real time RT-PCR (rt RT-PCR)
- Receptor-destroying enzyme (RDE)
- Relaxed uncorrelated lognormal clock (UCLD),
- Reverse-transcription polymerase chain reaction (RT-PCR),
- Ribonucleoprotein (RNP)
- Single likelihood ancestor counting (SLAC)
- Specific pathogen free (SPF)
- Swine influenza (SI)
- Swine influenza virus (SIV)
- Swine influenza viruses (SIVs)
- Synonymous (dS)
- Three dimensional macromolecular structure (3D-MMS)

- Time of the most recent common ancestor (tMRCA)
- *Porcine Circovirus type 2* (PCV-2)
- Viral ribonucleoprotein (vRNPs),
- Viral RNA (vRNA)
- Virus neutralization (VN)

Abstract

Study n.1 - Swine influenza viruses: virological surveillance in Italy during 1998-2011

Three predominant subtypes of swine influenza viruses (SIVs) circulating in pigs throughout the world are H1N1, H3N2 and H1N2. In Europe over the last years, the epidemiology of SIVs has considerable changed. In particular in Italy, swine monitoring programs have been applied since the nineties and are based on genome detection, virus isolation and sequencing of all respiratory forms. This study reported the swine influenza surveillance programme preformed during the period 1998 – 2011. Investigated samples originated from the entire country but the major part of them was from North Italy where more than 75% of the swine industry is located. In this period 580 SIVs were isolated divided in 243 H1N1, 214 H3N2, 111 H1N2, 12 2009 A/ H1N1 pandemic (H1N1pdm) and 1 H3N1. These results revealed continuous circulation of H1N1, H3N2 and H1N2 viruses and isolation of the H1N1 pdm viruses in pigs starting in 2009. The most frequent subtype was the avian-like H1N1 and the second the H3N2. The H1N2 subtype was rarely isolated in the first part of the investigated period but since 2005, its frequency increased becoming the second subtype in the last three years. In the last two years 12 H1N1pdm strains were isolated in 7 different farms also when these viruses did not circulate in the human population. Moreover it is worth noting the isolation of one H3N1 strain, which resulted from a reassortment between swine H1N1 and H3N2 influenza viruses. Phylogenetic analysis evidenced homogeneity among the recent Italian H1N1 SIVs, distinguishing these from the earlier strains. Also for the H1N2 subtype the presence of a homogeneous group, differing from the earlier strains is reported. Interestingly

the neuraminidase (NA) gene of the recent Italian strains showed different characteristics respect to the European H1N2 strains, deriving from the recent human H3N2 viruses. Finally, Italian H3N2 viruses were gradually evolved from A/Port Chalmer/1/73 like human virus, which is considered the most probable ancestor of the swine PCh-73 lineage. These results evidenced reassortment events between swine and human influenza viruses and highlight the need for a stringent surveillance in the pig population with particular attention to the H1N2 subtype and the H1N1 pdm viruses.

Study n.2 - First pandemic H1N1 outbreak from a pig farm in Italy

The first outbreak of the 2009 A/H1N1 pandemic virus in a swine breeder farm in Italy in November 2009 was reported. Clinical signs observed in sows included fever, depression, anorexia and agalactia, while in piglets diarrhoea and weight loss. The morbidity in sows was approximately 30% and the accumulated mortality rate was similar with those usually reported in piggeries (<10%). Virus was isolated from piglets (A/Sw/It/290271/09) and the sequencing of the whole genome was then performed. Comparison with all pandemic H1N1 sequences available in GenBank shows in A/Sw/It/290271/09 three unique amino-acid (aa) changes in PB2 (S405T), PB1 (K386R) and PA (K256Q), not yet associated to any well characterized phenotype markers of Influenza viruses. All eight aa at positions representing the so-called species-specific swine-human signatures, found in both swine and in the pandemic H1N1v, are also present. The M2 protein displays the C55F and the PA protein the S409N substitutions, both corresponding to enhanced transmission phenotype markers. Phylogenetic analysis showed that the virus was genetically related to the pandemic H1N1 virus. In addition, serological samples were collected

from 40 sows, of which 20 resulted positive to the pandemic H1N1 virus by HI test proving a virus circulation in the farm.

Study n.3 - Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus

Swine influenza monitoring programs have been in place in Italy since the 1990's and from 2009 testing for the 2009 A/ H1N1 pandemic virus (H1N1pdm) virus was also performed on all the swine samples positive for type A influenza. This paper reports the isolation and genomic characterization of a novel H1N2 swine influenza reassortant strain from pigs in Italy that was derived from the H1N1 pdm virus. In May 2010, mild respiratory symptoms were observed in around 10% of the pigs raised on a fattening farm in Italy. Lung homogenate taken from one pig showing respiratory distress was tested for Influenza type A and H1N1pdm by two real time RT-PCR assays. Virus isolation was achieved by inoculation of lung homogenate into specific pathogen free chicken embryonated eggs (SPF CEE) and applied onto Caco-2 cells and then the complete genome sequencing and phylogenetic analysis was performed from the CEE isolate. The lung homogenate proved to be positive for both influenza type A (gene M) and H1N1pdm real time RT-PCRs. Virus isolation (A/Sw/It/116114/2010) was obtained from both SPF CEE and Caco-2 cells. Phylogenetic analysis showed that all of the genes of A/Sw/It/116114/2010, with the exception of neuraminidase (NA), belonged to the H1N1pdm cluster. The NA was closely related to two H1N2 double reassortant SIVs, previously isolated in Sweden and Italy. NA sequences for these three strains were clustering with H3N2 SIVs. The emergence of a novel reassortant H1N2 strain derived from H1N1pdm in swine in Italy raises further concerns about whether these viruses will become established in

pigs. The new reassortant not only represents a pandemic (zoonotic) threat but also has unknown livestock implications for the European swine industry.

Study n.4. **Genomic characterization and evolutionary analysis of H1N2 swine influenza viruses in Italy**

Three subtypes (H1N1, H1N2, and H3N2) are currently diffused worldwide in pigs. The H1N2 subtype was detected for the first time in Italian pigs in 1998. To investigate the genetic characteristics and the molecular evolution of this subtype in Italy, we conducted a phylogenetic analysis of the HA and NA genes of 53 strains isolated from 1998 to 2012. Moreover the whole genome sequences of 26/53 strains were performed. Phylogenetic analysis of HA and NA genes showed differences between the older (1998-2003) and the more recent strains (2003-2010). The older isolates were closely related to the established European H1N2 lineage, whereas the more recent isolates possessed a different NA deriving from recent human H3N2 viruses. Furthermore, eight Italian H1N2 strains isolated recently in 2008-2012 (A/Sw/It/196875/08, A/Sw/It/310411/09, A/Sw/It/195639/10, A/Sw/It/195399/11, A/Sw/It/274551/11, A/Sw/It/329017/11 A/Sw/It/26654/12 and A/Sw/It/107798/12) showing different features were detected. They were characterised by an HA gene, closely related to the EU H1N1 SIVs. The last two strains exhibited different patterns: A/sw/It/ 58769/10 was an uncommon strain with an HA that is closely related to H1N1 and an NA similar to H3N2 SIVs; A/Sw/116114/10 was a H1N2 reassortant strain derived from 2009 H1N1 pdm viruses. Amino acid analysis revealed interesting features: a deletion of two amino acids (146-147) in the HA gene of the recent isolates and two strains isolated in 1998; the presence of the uncommon aa change (N66S), in the PB1-F2 protein in strains isolated from 2009-2010, which is said to

have contributed to the increased virulence. The evolutionary analysis of the HA and NA genes revealed five different reassortment events between human and swine influenza viruses occurred at different times between 1978 and 2000. These results demonstrate the importance of pigs as mixing vessels for animal and human influenza and show the presence and establishment of reassortant strains involving human viruses in pigs in Italy. These findings also highlighted different genomic characteristics of the NA gene the recent Italian strains compared to circulating European viruses.

The results of the present Thesis have been published or submitted for publication in international scientific peer-reviewed journal:

Moreno A., Di Trani L., Alborali L., Vaccari G., Barbieri I., Falcone E., Sozzi E., Puzzelli S., Ferri G., Cordioli P. 2010. First pandemic H1N1 outbreak from a pig farm in Italy. The Open Virology J, 4: 52-56.

Moreno A., Di Trani L., Faccini S., Vaccari G., Nigrelli D., Boniotti M.B., Falcone E., Boni A., Chiapponi C., Sozzi E., Cordioli P. 2011. Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet Microbiol 149: 472-477.

Moreno A., Chiapponi C., Boniotti M.B., Sozzi E., Foni E., Barbieri I., Zanoni M.G., Faccini S., Lelli D., Cordioli P. 2012. Genomic characterization of H1N2 swine influenza viruses in Italy. Vet Microbiol. 2012 May 4;156(3-4):265-76.

Moreno A., Sozzi E., Lelli D., Foni E., Chiapponi C., Zehender G., Alborali L., Cordioli P. 2012. Italian H1N2 swine influenza viruses: different evolutionary trends compared to European viruses. 6th annual meeting EPIZONE, 12-14 June 2012 – Brighton, UK, pp 34 (oral presentation). Manuscript in preparation.

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Study n. 4: Genomic characterization and evolutionary analysis of H1N2 swine influenza viruses in Italy

General Introduction

Historical background

Before 1918 influenza viruses had long been described in humans but never in pigs. It was this year that a particularly virulent strain of influenza spread across the world infecting up to one third of the world's human population and killing 40 to 50 million people worldwide (Taubenberger, 2006). Symptoms of this Influenza A strain were seen in the United States, Canada (including the far north), Europe, Asia and even the most remote Pacific islands. Unlike most subsequent influenza virus strains that have developed in Asia, the first wave of the 1918 pandemic probably arose in the United States in the spring of 1918. However, the definitive assignment of a geographic point of origin is difficult considering that influenza appeared almost simultaneously in North America, Europe and Asia. It is though that a mutation or reassortment of the virus occurred in the late summer, resulting in an even more virulent strain of the virus. Consequently, a violent second wave of disease was seen through the word in September – November of 1918. In many places, another severe wave of influenza was held in early 1919. Three extensive outbreaks of influenza within one year are unusual and may point to unique features that could be revealed in its sequence (Taubenberger, 2006).

Figure n.1 - 1918 pandemic H1N1- An Emergency Hospital for Influenza Patients

Figure n. 2 - Influenza victims in 1918 crowd into an emergency hospital near Fort Riley, Kansas

Another unique feature of the 1918 influenza was the simultaneous infection of both humans and swine. Interestingly an epizootic disease characterized by clinical signs and pathological findings similar to influenza in humans was observed in swine

in the United States in the late summer of 1998. While the infection and disease may have existed among swine populations before that time, it is unlikely that a disease with such distinctive characteristics would have gone unnoticed and unreported. Thus, contemporary investigators were convinced that influenza virus had not circulated as an epizootic disease in swine before 1918 and that the virus spread from humans to pigs because of the appearance of illness in pigs after the first wave of the 1918 influenza in humans (Shope, 1936). Thereafter, the disease became widespread among swineherds in the U.S. Midwest. Swine influenza was also first observed in 1918 in Hungary and China (Chun, 1919, Koen, 1919). However, it was not until 1930 that the swine influenza virus (SIV) was isolated and identified by Shope (1931), three years before the isolation of the first human influenza virus A/WS/33 (Smith et al, 1933). Retrospective serological analysis performed later confirmed that the disease in humans and pigs had been caused by closely related influenza A viruses in both cases. The agent was identified as H1N1 influenza A virus. The most relevant points in the history of swine influenza since the first outbreak to nowadays are described in table n. 1.

History of swine influenza	
1918	• Swine influenza H1N1 described in north central USA, Hungary, and China. May have been cause of human pandemic [19], which resulted in 20-40 million human deaths.
1930	• Shope isolated influenza virus from pigs [33]. The prototype classic swine influenza H1N1 strain (A/Swine/Iowa/30) transmitted experimentally to pigs.
1941	• Recognised in Europe and disappeared.
1970	• Transmission of human H3N2 virus to pigs. Avian-like H3N2 in pigs in Asia.
1976	• Classical H1N1 reappears in European pigs.
1979	• Introduction of whole H1N1 virus from birds to pigs. Antigenically distinguishable from classical strains. Still circulating today (2002).
1984	• Reassortment between human H3N2 and avian H1N1 in swine resulting in reassortant H3N2 virus with avian internal gene segments [5]. H3N2 strains first associated with respiratory epizootics. Still circulating today (2002).
1986	• Classical H1N1 reappears in UK, similar to classical H1N1 in continental Europe.
1987	• Reassortant H3N2 associated with respiratory epizootics in UK. Related to A/Port Chalmers/73(H3N2).
1989	• Avian-like swine H1N1 is dominant and widespread in Europe.
1992-1993	• Avian-like H1N1 strains widespread in UK.
1993	• Infection of children with reassortant H3N2 virus from pigs and isolation of avian-like swine H1N1 virus from a pneumonia patient in the Netherlands.
1994	• H1N2 first isolated in pigs in UK, and later also in Belgium. Human-avian reassortant virus [3, 37].
1992-1998	• H3N1 (H3 human, N1 swine) and H1N7 (H1 human, N7 equine) also occurred in swine in the UK but failed to spread.
1998	• H9N2 in pigs and humans in Asia [17]. Apparently an avian virus that has adapted to pigs.
1998	• For the first time, H3N2 viruses cause severe disease in N. America. Viruses are triple (avian-human-classical swine) reassortants, distinct from earlier strains and European strains. H1N2 identical to H3N2, but with H1HA from classical swine H1N1, also isolated.
1999	• Single case of isolation of avian H4N6 from pigs with pneumonia in Canada.
2002	• Current situation in Europe: avian-like H1N1, and reassortant human-like H3N2 and H1N2. In North America: classical swine H1N1, triple reassortant H3N2.

Table n. 1. Salient points in the history of swine influenza, adapted from Done and Brown, 1999 (Heinen, 2003)

Etiology

Classification and nomenclature

Swine influenza viruses belong to the *Orthomyxoviridae* family that includes RNA viruses with segmented negative-sense single stranded genome. This family include

three genera of influenza viruses named type A, B and C. Swine influenza virusesare classified within the type A. This type affects a wide range of birds and mammals species, including humans. Influenza viruses type B are related with epidemics of mild respiratory in humans whereas influenza viruses type C do not produce epidemics but cause slight respiratory signs. Moreover, influenza C virus has also been isolated from pigs and influenza B has recently been isolated from seals (Osterhaus et al., 2000).

Influenza A viruses (IAVS) are further classified into subtypes based on the antigenic properties of the external glycoproteins haemagglutinin (HA) and neuraminidase (NA). To date seventeen antigenically different HA (H1-H17) and nine NA (N1-N9) have been recognised and their combination designates the subtype of the virus. Recently, a new HA subtype (H17) has been identified in Bats in South America (Tonga et al, 2011).

The current system of nomenclature of influenza viruses were introduced in 1980 and designate the type, host, place, strain number (if any), year of isolation and antigenic subtype of a virus. For example, a swine influenza virus isolated in Wisconsin in 1984 would be designated A/Swine/Wisconsin/1/84(H1N1).

Morphology

Influenza A viruses are 80–120 nm enveloped viruses with segmented, single-stranded, negative-sense RNA genomes (Fig. 1). The eight RNA segments within the viral genome, varying in length between 890 and 2341 nucleotides, encode 10 and in some cases 11 proteins. The virus is enveloped, and the lipid membrane of the virion is derived from the host cell in which the virus replicated. From the surface of the envelope extend the two transmembrane glycoproteins HA and NA, which are commonly called 'spikes'. A third transmembrane protein, matrix protein 2 (M2), also

exists but only 20-60 molecules per virion are present. M2 functions as a pH-activated ion channel that enables acidification of the interior of the virion, leading to un-coating of the virion (Wang et al., 1993). Matrix protein 1 (M1), which is the most abundant protein in the virion, underlies the viral envelope and associates with the ribonucleoprotein (RNP) complex. RNP complex consists of ribonucleic acid (RNA) associated with nucleoprotein (NP) as well as the polymerases PA, PB1 and PB2 that are responsible for RNA replication and transcription. The trimeric RNA polymerase complex (PB1, PB2 and PA) binds to the 50 and 30 ends of the viral RNA forming a noncovalent circular complex (Klumpp et al., 1997). Two non-structural proteins are also associated with the virus (NS1 and NS2). While the NS2 or nuclear export protein (NEP) was originally thought to be a nonstructural protein; it has since been found to be a part of the influenza virion (Richardson and Akkina, 1991). In contrast, although NS1 is abundantly present in infected cells during virus replication, the protein is not incorporated into the progeny virions (Palese et al., 1999). In some virus strains segment 2 (PB1) encodes a second short protein, called PB1-F2, from an additional open reading frame (Conenello and Palese, 2007). The external proteins HA and NA are the main target of the host humoral inmune responses. The HA serves as the viral receptor-binding protein and mediates fusion of the virus envelope with the host cell membrane (Skehel and Wiley, 2000). The HA binds to N-acetylneuraminic acid-2,3-galactose linkage or N-acetylneuraminic acid-2,6-galactose linkage on sialyloligosaccharides for avian and mammalian viruses, respectively (Rogers and Paulson, 1983). The NA is responsible for cleaving terminal sialic acid residues from carbohydrate moieties on the surfaces of the host cell and virus (Gottschalk, 1957), thus assisting in virus cell entry by mucus degradation (Matrosovich et al., 2004) and the release and spread of progeny virions (Palese et

al., 1974). Data about genome segments and encoded proteins of influenza A viruses are reported in table n.2.

Influenza A virus gene segments and encoded proteins			
RNA segment	Nucleotides (Bp)	Proteins	Amino acids
1	2341	Polymerase PB2	759
2	2341	Polymerase PB1	757
		PB1-F2 protein	< 90
3	2233	Polymerase PA	716
4	1778	Haemagglutinin HA	566
5	1565	Nucleoprotein NP	498
6	1413	Neuraminidase NA	454
7	1027	Matrix protein M1	252
		Matrix protein M1	97
8	890	Non structural protein NS1	230
		Non structural protein NS2	121

Table n.2 . Influenza A virus gene segments and encoded proteins

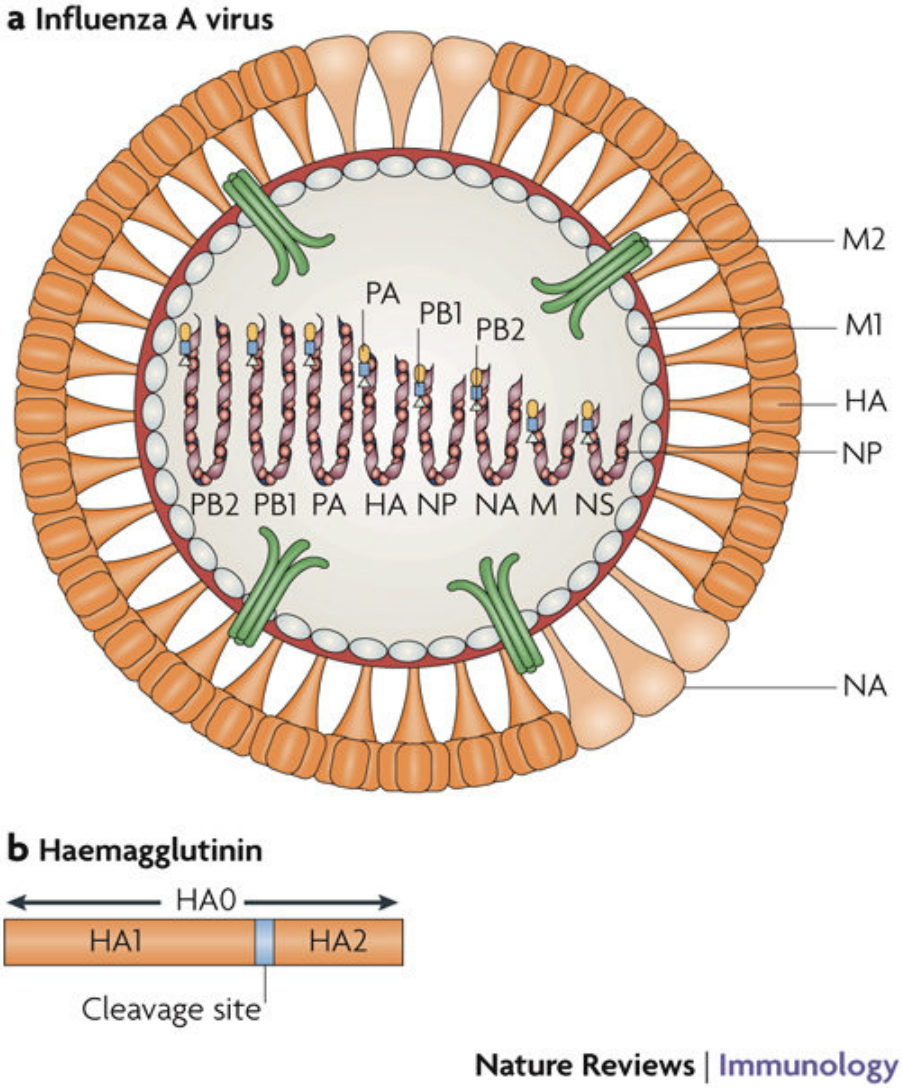


Figure n. 3 - Schematic representation of influenza A virus (Subbarao and Joseph, 2007).

Replication cycle

The replication cycle of influenza virus starts with the cleavage of HA into HA1 and HA2 by enzymes present in the respiratory tract. The HA glycoprotein is synthesized as an HA0 molecule that is post-translationally cleaved into the HA1 and HA2 subunits; this cleavage is essential for virus infectivity. The protease responsible for cleaving HA0 in influenza infections is secreted by cells of the respiratory tract or by co-infecting bacteria or mycoplasma or may be produced in inflammatory responses to infection. Virus grown in cells that lack a cleavage enzyme can be activated by

treatment with trypsin. After the HA cleavage, the receptor-binding site of HA1 can attach to a terminal sialic acid residue of a cell surface receptor. Once attached to the host cell the virus is endocytosed (receptor-mediated endocytosis) and the HA2 subunit mediates the fusion of the virus envelope with the cell membrane. The cleavage of the HA is induced by the low PH of the endosome that is triggered by the activity of the M2 protein (figure 2). After the fusion of the endosomal membrane and the virus envelope, the viral genome, in the form of viral ribonucleoprotein (vRNPs), is released into the cytoplasm and then transported into the nucleus for replication. In the nucleus, the viral RNA (vRNA) is transcribed by the viral RNA-dependent RNA polymerase into two positive-sense RNA species, a messenger RNAs (mRNA) and a complementary RNA (cRNA). The cRNA serves as template from which the polymerase transcribes more copies of negative-sense genomic vRNA that will form the virions. The mRNA is exported out of the nucleus into the cytoplasm for protein synthesis. Some of the newly synthesised proteins are imported into the nucleus to assist in viral RNA replication and vRNP assembly, which also occur in the nucleus. Other newly synthesised viral proteins are processed in the endoplasmic reticulum and the Golgi apparatus where glycosylation occurs (HA, NA, M2). These modified proteins are transported to the cell membrane where they stick in the lipid layer. Virus assembly is a highly inefficient process, where more than 90% of the virus particles are not infectious with packing of too few or excessive viral gene segments. Finally, NA functions as a receptor-destroying enzyme by cleaving terminal sialic acid residues from the receptor. Thus, NA releases progeny virions from the host cell in which they arose and facilitates virus spread. The progeny virions can infect other cells or can be transmitted to another individual. Figure 3 shows a schematic diagram of the influenza viral life cycle.

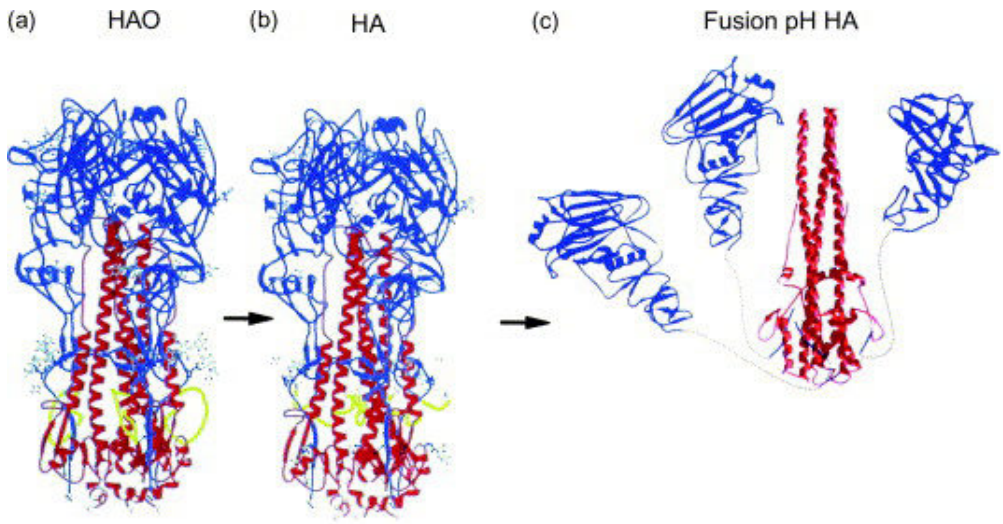


Figure n. 4. Three conformations of the haemagglutinin: (a) uncleaved precursor haemagglutinin, HA0; (b) neutral pH, cleaved haemagglutinin; (c) fusion pH, cleaved haemagglutinin—a composite diagram constructed from data for HA1 and for HA2. In (a) and (b), 19 residues, from 323 of HA1 to 12 of HA2 are coloured yellow. In (a)–(c) HA1 residues are blue, HA2 red. (Skehel and Wiley, 2002).

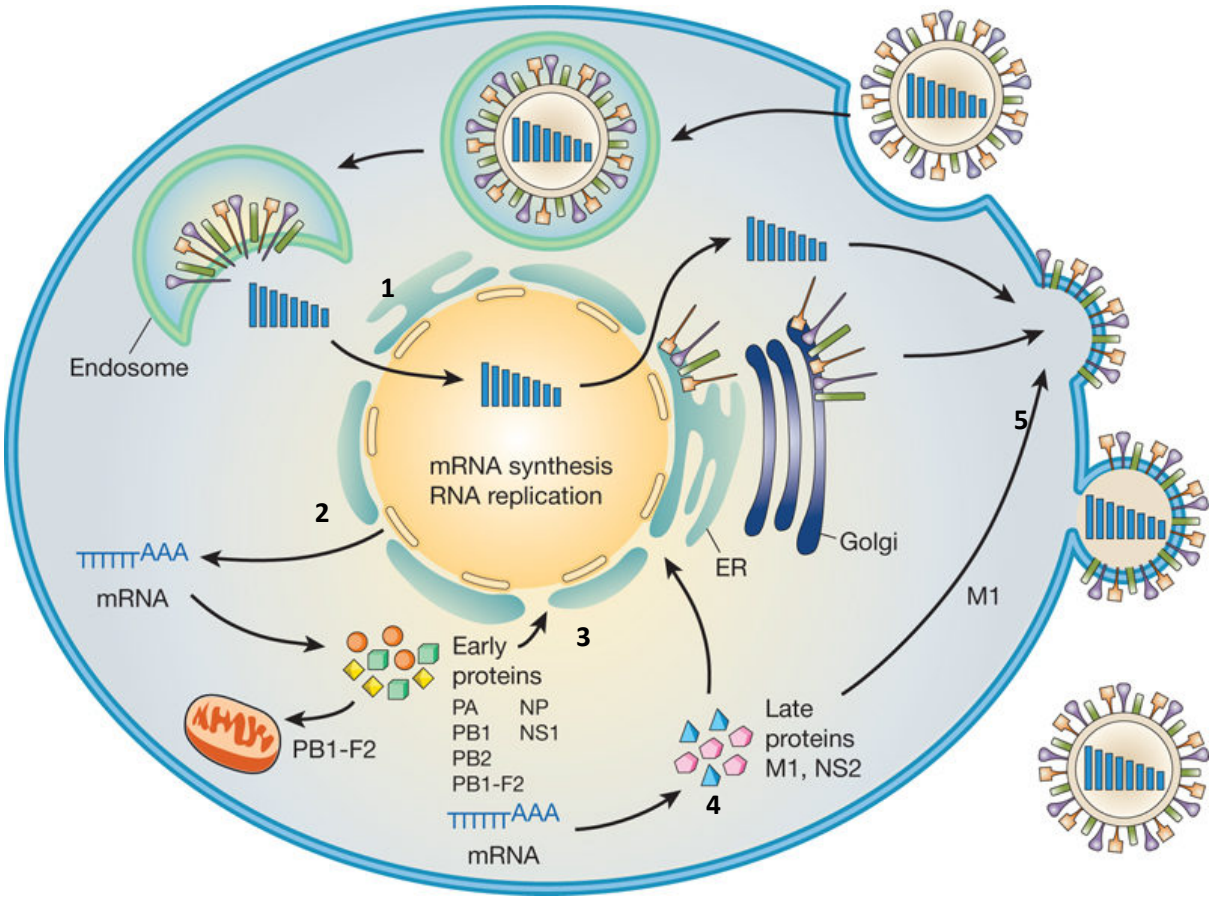


Figure n. 5 - Schematic diagram of the influenza viral life cycle – 1- after receptor-mediated endocytosis, the vRNP complexes are released into the cytoplasm and subsequently transported to the nucleus, where replication and transcription take place. 2- messenger RNAs are exported to the cytoplasm for translation. 3- early viral proteins, that is, those required for replication and transcription, are transported back to the nucleus. 4- late in the infection cycle, the M1 and NS2 proteins facilitate the nuclear export of newly synthesized vRNPs. PB1-F2 associates with mitochondria. 5- the assembly and budding of progeny virions occurs at the plasma membrane (Neumann *et al. Nature* 459, 931-939 (2009) doi:10.1038/nature08157).

Antigenic diversity

Influenza A viruses are characterised by high viral diversity due to the segmented genome and the lack of a proofreading mechanism of replication. Two mechanisms can provide diversity to the viral population: antigenic drift and genetic reassortment.

Antigenic drift is a gradual change of the genome acquired by point mutations. The two surface glycoproteins of the influenza virus, HA and NA, are the most important antigens for inducing protective immunity in the host and show the greatest variation. The HA molecule is the most frequently affected with the antigenic drift, especially the antigenic sites which are exposed to the immunological pressure. The antigenic mutations in the gene codifying by the HA protein cause changes in the structure of the glycoprotein resulting in virus strains that can no longer be neutralized by previous host antibodies. This could result in viruses able to replicate more efficiently and mutants that could be transmitted more easily. This phenomenon is observed frequently human influenza viruses as a result of the selective pressure imposed by the use of vaccines. Other example, evidenced in the avian influenza viruses belonging to the H5 and H7 subtypes, is the mutation from low to high pathogenic strains, as observed in Italy in the H7N1 epidemic in 1999-2001 (Capua and Alexander, 2008).

The antigenic shift can occur through one of these three mechanisms: 1) genetic reassortment when two different influenza virus strains infect a single cell and results in a virus with new antigenic proteins. The genome of the influenza viruses is segmented and therefore gene segments can be exchanged in mixed infections with different strains of the viruses. When two viruses infect the same cell, progeny viruses may inherit sets of segments made up of combinations of segments of the parent viruses; 2) direct transfer of whole virus from another species. This is probably

what occurred in 1918 when the H1N1 'Spanish flu' virus entered the human population; 3) Re-emergence of a virus that may have caused an epidemic many years earlier. The H1N1 'Russian flu' virus, for example, re-emerged in 1977 after having been in circulation in man prior to 1950. It is believed the virus might have escaped from a laboratory.

Epidemiology

Influenza A viruses of subtypes H1N1, H3N2 and H1N2 have been reported in the pig population all over the world. Unlike human influenza, the origin and nature of swine influenza viruses differ on continents. Indeed two lineages of SIVs characterised by different genomic evolutions are recognized: one Eurasian lineage circulating in Europe and Asia and one American lineage predominant in America but also present in Asia. A schematic diagram of distribution of different lineages worldwide is reported in figure n. 4.

Figure n. 6 – Schematic diagram of distribution of two lineages in the world

In Europe SIVs belonging to the three subtypes are endemic in pigs and are responsible for one of the most prevalent respiratory diseases in pigs. These viruses differ in their origin (i.e. transmission from birds or from humans, wholly or after single or multiple reassortment events…) from those found in other continents and various lineages can be distinguished within each subtype. There is periodic exchange of virus or virus genes between different hosts through interspecies transmission and/or genetic reassortment. Analysis of all the stable lineages of influenza viruses in pigs has revealed that they originated from an avian or human source, acquired the capability to infect and transmit between individual pigs, thereby persisting within swine populations. Figure n. 5 gives an overview of the three different subtypes currently circulating in Europe.

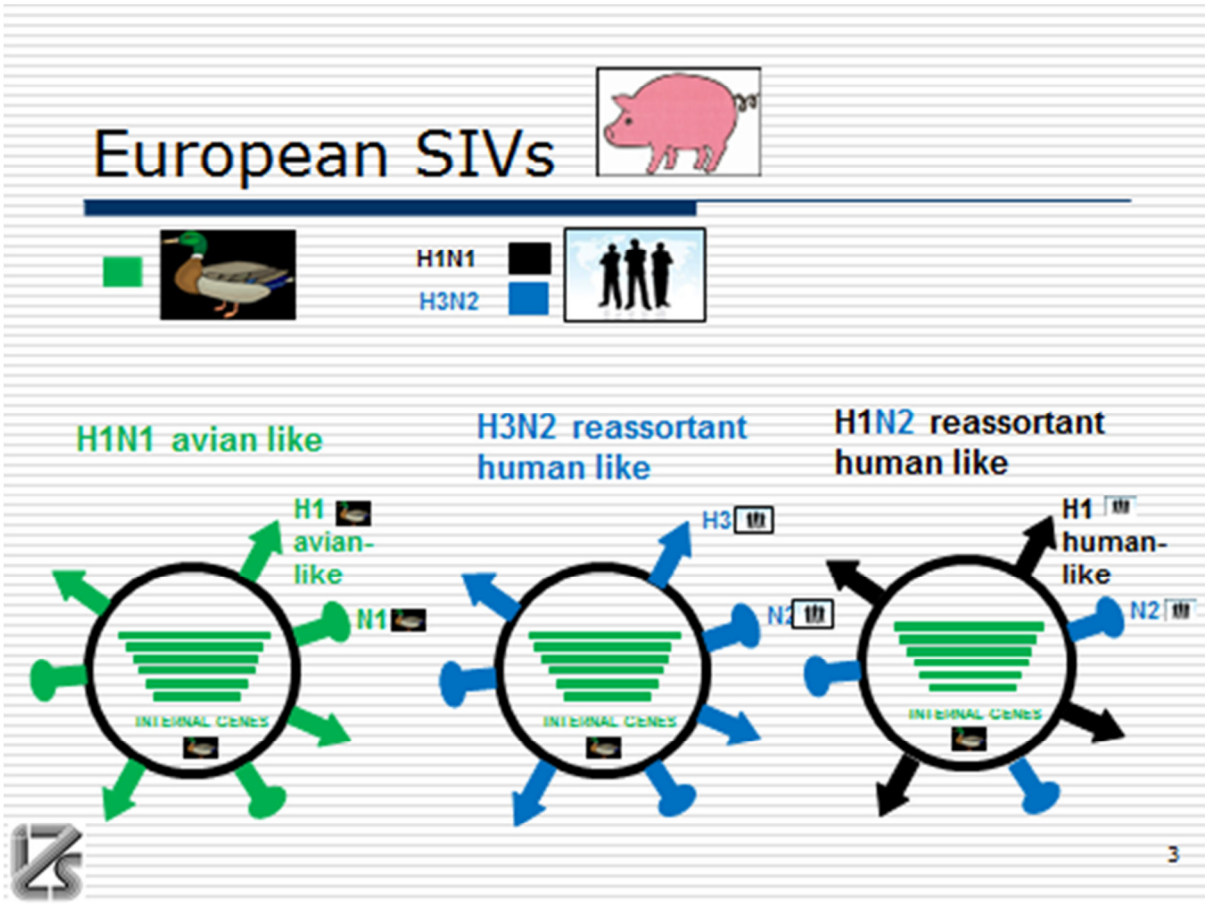


Figure n. 7 - Overview of the three different subtypes currently circulating in Europe

H1N1 subtype

Classical H1N1 SIVs

Influenza viruses of the H1N1 subtype were first detected in Europe in UK in 1938-1940 (Blakemore and Gledhill, 1941). Studies of genomic characterisation performed later showed that the complete genome of these strains was more closely related to the early human strain A/Puerto Rico/8/34 than to the classical H1N1 SIV A/sw/Iowa/1930 (Neumenier et al., 1994).

Classical H1N1 SIVs were clearly demonstrated since the late 70's in different countries. In 1976 they were isolated in Italy from pigs imported from USA (Nardelli et al., 1978) and after, in the 80's, from other outbreaks in the Netherlands (Masurel et al., 1983), Denmark (Sorensen et al., 1981) and Sweden (Abusugura et al., 1987). Thus the virus became endemic in pigs throughout Europe with a seroprevalence of 20-25% (Zang et al., 1989; Brown et al., 1995b). During this period, these viruses remained relatively stable antigenically with no evidence of involvement in virus reassortment (Brown et al., 1997) but disappeared following the emergence of the avian-like H1N1 SIVs (Brown 2000).

Avian-like H1N1 SIVs

In 1979 inter-species transmission of influenza H1N1 viruses from birds to pigs was demonstrated; indeed, the etiological agent that caused swine influenza outbreaks in Belgium and Germany was found to be an avian influenza H1N1 virus wholly transmitted to pigs (Pensaert et al., 1981). This introduction led to the establishment of a stable lineage in swine, the new avian-like H1N1 SIVs, which are antigenically distinguishable from classical SIVs. These avian-like viruses appear to have a selective advantage over classical viruses, since in Europe they have replaced the

classical SIVs (Schultz et al., 1991; Campitelli et al, 1997). They were associated with severe clinical signs in France, Denmark, Italy and the Netherlands. These viruses remain the dominant strain in pigs in many European countries.

H3N2 subtype

Following the 1968 Hong Kong pandemic influenza, the isolation of this human H3N2 virus from pigs in Taiwan in 1970 (Kundin, 1970) provided the first evidence of potential transmission of human strains to pigs. Later on this human-like swine H3N2 virus became adapted to the swine population and colonized the European pigs as demonstrated by virological and serological surveillance (Harkness et al., 1972; Tumova et al, 1976; Ottis et al., 1982). These viruses were antigenically related to early human strains such as A/Port Chalmers/1/73. It circulated at a low level for about ten years, causing clinical signs only sporadically. Since 1984 outbreaks characterised by severe clinical signs have associated with these H3N2 viruses throughout Europe and were first described in Italy (Castrucci et al., 1993) and France (Madec et al., 1984). A European reassortant human-like swine H3N2 virus resulted from a reassortment between the human-like swine H3N2 viruses and avian-like swine H1N1 viruses caused these cases. This virus was characterised by a HA and NA of human origin and all internal genes of avian origin (Campitelli et al., 1997). This reassortant swine H3N2 virus has replaced the original human-like H3N2 viruses in the European pig population. Although contemporary viruses are closely related to the prototype human viruses, antigenic variations was observed in the surface glycoproteins due to genetic drifts; this could be correlated with an increase in epizootics (De Jong et al., 1999). Significant differences in circulation of the

reassortant swine H3N2 occur across Europe. It is still very active in Southern Europe but appears to be absent in France and UK (Kyriakis et al., 2011).

H1N2 subtype

Influenza H1N2 viruses were first isolated in France in 1987 and were characterised by an HA gene deriving from classical H1N1 SIVs and a NA gene from the reassortant swine H3N2 viruses (Gourreau et al., 1994). Although these viruses were associated with clinical disease, they did not spread in the pig population and were replaced by another H1N2 virus. This new H1N2 SIVs was first reported in UK in 1994 and showed different genomic characteristics. It owned a HA gene closely related to the human H1N1 viruses, a NA gene deriving from the human H3N2 viruses and the internal genes of avian origin (Brown et al., 1998). This virus quickly spread to pigs in the rest of Europe (Van Reeth et al., 2000; Marozin et al., 2002; Maldonado et al., 2006) and became endemic. The circulation of H1N2 viruses in Eastern Europe is less documented and little data are available on their occurrence. The H1N2 SIVs have been reported in Poland together with H1N1 and H3N2 SIVs (Kowalczyk et al., 2007).

Novel reassortant strains

The continuous co-circulation of influenza viruses in the pig population gives the opportunity for continual genetic mixing that can lead to the detection of new genotypes or subtypes. However these new strains not always persist in pigs failing to establish stable genetic lineages. Evidences of new strains able to spread widely in pigs have been reported. First, the European reassortant human-like swine H3N2 virus originated from a reassortment between the human-like and avian viruses since

1983 (Castrucci et al., 1993). Second, the emergence of the H1N2 viruses in UK in 1994 and subsequent spread to pigs in continental Europe. These viruses derived from multiple reassortment events that involve influenza viruses of human and avian origin (Brown et al., 1998). Other new reassortant strains did not spread widely in pigs. In Italy, reassortment between H1N2 and avian-like H1N1 SIVs was reported with the isolation of H1N2 strains characterised by an avian-like H1 (Marozin et al., 2002). Similar H1N2 reassortant strains were reported in other countries (Kuntz-Simon and Madec, 2009). Other different reassortant H1N2 strains with HA closely related to H1N2 SIVs and NA deriving from H3N2 SIVs were reported in Germany (Zell et al., 2008) and Italy (Chiapponi et al., 2007). Unusual reassortant H1N2 strains were recently reported in Sweden (Balint et al., 2009; Meterveli et al., 2010). These strains resulted from a reassortment between avian-like H1N1 and human-like H3N2 SIVs. The occurrence of novel subtypes has been reported in different occasions but these viruses apparently fail to persist. They included the novel subtypes H3N1 in Italy (Moreno et al., 2009) and H1N7 in UK (Brown et al., 1994).

Clinical signs and pathogenesis

Swine influenza viruses are one of the primary respiratory pathogens in pigs that induce disease and lung lesions on its own. The typical clinical form is very similar to human influenza. It is characterised by a rapid onset after an incubation period of 1-3 days with anorexia, inactivity, prostration, huddling and high fever. Animals show an open-mouthed, laboured and abdominal breathing, especially when the animals are forced to move. Other symptoms are severe conjunctivitis, rhinitis, nasal discharge (figure n. 6), sneezing and considerable weight loss. Movements are also accompanied by severe paroxysm of coughing. Abortion, stillbirth and infertility could be observed in cases of acute swine influenza. Morbidity is very high, close to 100%

but the mortality is low (< 1%) unless there are secondary infections or pigs are very young. Recovery occurs in general within 7-10 days.

All the three subtypes are associated with disease and there are no differences in clinical forms and virulence between subtypes or strains. Subclinical infections are also very common and more than one influenza virus subtypes could infect pigs without showing detectable clinical signs. SIVs can also contribute to more chronic, multifactorial respiratory disease problems in combination with other viruses or bacteria.

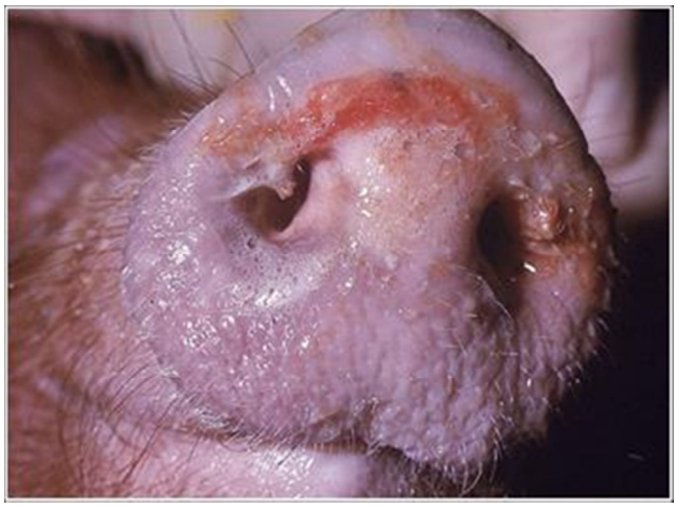


Figure n. 8 – Pig showing nasal discharge

The virus replicates in the epithelial cells of the entire respiratory tract such as nasal mucosa, tonsils, trachea and lungs. There is a massive infection in the bronchi, bronchiole and alveoli reaching more than 10^8 EID_{50} /gr tissue. Signs of typical respiratory disease are accompanied by an epithelial cell necrosis and infiltration of neutrophils, which cause obstruction of the airways and lung damage by release of their enzymes. The duration of virus replication in lungs and virus shedding through respiratory secretions is about 6-7 days. Virus isolation from non-respiratory tissues is very rare.

Lesions

Macroscopically, lungs of infected animals show a purple-red, multifocal to coalescing consolidation of predominantly the apical and cardiac lobes of the lungs. However in severe cases more than a half of the lungs may be affected. Generally, there is a sharp line of demarcation between the affected and normal lung tissue. The affected areas are purple and firm and some interlobular oedema could be evident. The airways are likely to be filled with blood-tinged, fibrinous exudate. In severe cases there may be fibrinous pleuritis.

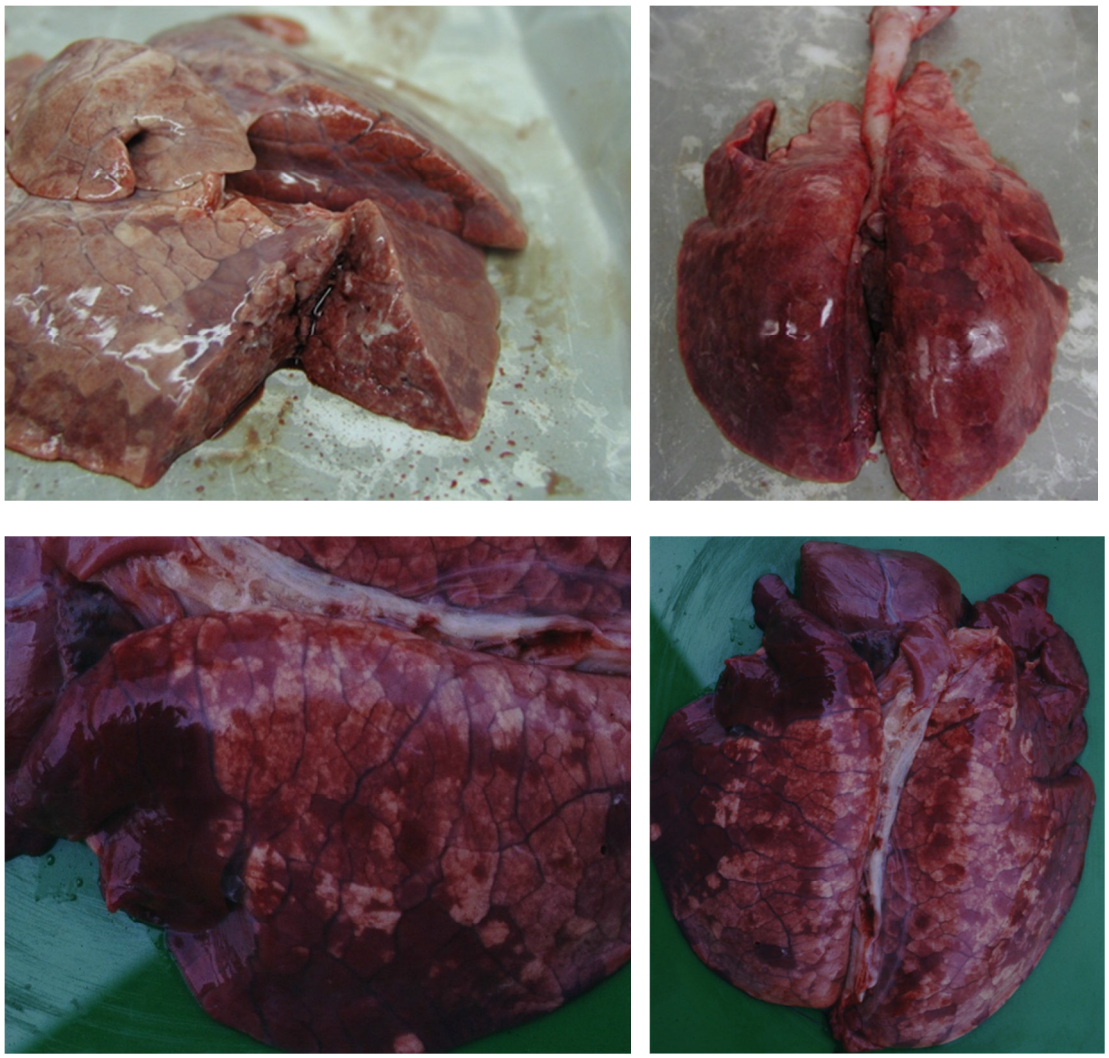


Figure n. 9- Lungs showing typical lesions

Microscopic changes in lungs in uncomplicated cases are consistent with viral pneumonia. They are degeneration and necrosis of the epithelial cells in bronchi and bronchioli and sloughing of these cells into airway lumen, which often contains cellular debris, proteinaceous fluid and a few leukocytes. This necrosis is accompanied by peribronchiolar lymphocytic infiltration and interstitial pneumonia of variable severity. Widespread alveolar atelectasis and emphysema accompany these lesions. In recovery, bronchiolar epithelium becomes proliferative and lymphocytic cuffing becomes more prominent. Viral antigen can be detected in many cells of the respiratory tract using inmunocytologic techniques.

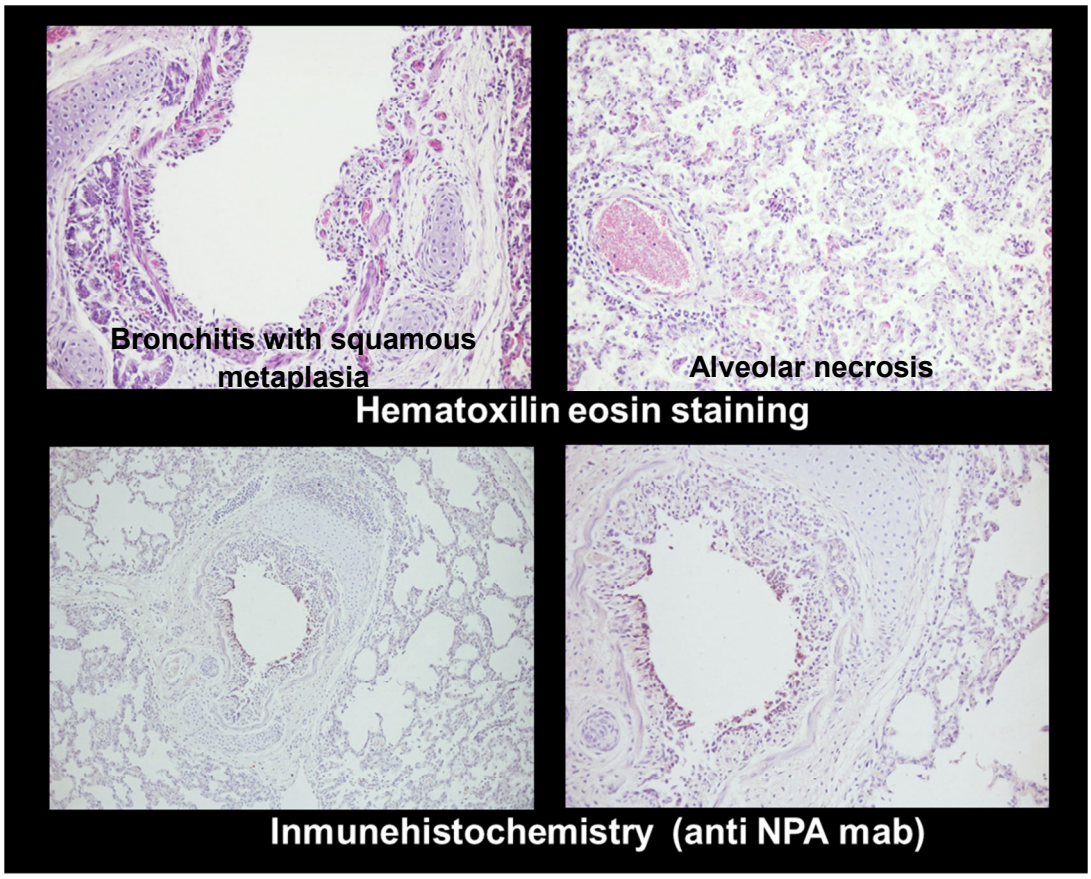


Figure n. 10 - Microscopic lesions using haematoxylin eosin staining and immunohistochemistry with an anti-NPA monoclonal antibody (Mab). (Laboratory of Histology, Milan – IZSLER)

Influenza viruses are part of the porcine respiratory disease complex (PRDC), acting in concert with other pathogens such as *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumonia, Pasteurella multocida*, *Porcine Reproductive and Respiratory Syndrome virus* (PRRSV), and *Porcine Circovirus type 2* (PCV-2) (Ellis et al., 2004; Thacker et al., 2001).

Diagnosis

Identification of the agent

Sample collection

Swab specimens can be used for virus isolation, genome detection by RT-PCR assays and antigen immunoassays. Nasal swabs are collected from clinical ill or dead animals in cell culture medium or phosphate buffered saline (PBS), supplemented with antibiotics. Lung tissues are collected from dead animals and processed for virus isolation and genome detection in different ways such as with a mortar and pestle or homogeniser. Processing of the tissue are done in cell culture medium with antibiotic supplement at a final concentration of 10-20% weight to volume. Nasal swabs and lung tissues are then centrifuged at 1500-1900g for 15-30 minutes at 4°C and the supernatant is collected and stored at -70°C until to be analysed. The supernatant can be filtered to reduce bacterial contamination but this may decrease virus titre.

Genome detection

RT-PCR methods

Molecular techniques (reverse-transcription polymerase chain reaction (RT-PCR), multiplex-RT-PCR and real-time RT-PCR) are based in the detection of the viral RNA

from culture fluids, swabs or tissue specimens. To detect the viral RNA, first it has to be reversed transcribed to cDNA using a primer complementary to the 3'end of all influenza viral RNA or a sequence specific primer. In the multiplex PCR more than one primer is included in the amplification reaction, then more than one gene or genome segment of the virus is detected [167]. All these methods enable the rapid detection of viral genomic nucleic acid sequences in a short period of time, obtaining also highly sensitive and specific results (Spackman and Suarez, 2008), being comparable to virus isolation procedures. In particular, the real-time RT-PCR enables to quantify the viral RNA. Tests for pan-type A influenza detection are often targeted to the conserved matrix (Spackman et al, 2002) which is able to detect any type A influenza virus from any species. Recently, Muradrasoli et al. (2010) reported the development of a TaqMan-based triplex real-time RT-PCR method that targets nucleoprotein gene of animal and human influenza viruses of all types: A, B and C.

Microarray techniques

Microarray technology proved to be a powerful tool for virus detection and subtyping. It allows simultaneous detection of a great diversity of genetic elements. For example, a microarray with clinical sensitivity of 95% and clinical specificity of 92% when validated against A/H5N1, A/H3N2, and A/H1N1 viral strains has been published (Dawson et al., 2007). It is important to mention that the detection limits here were from ten to one hundred times lower than that of real-time RT-PCR with respect to HA subtyping and pathotyping. However, the presence of multiple steps involved in these assays, like several amplification steps, probe labelling and incorporation of conjugated nucleotides into DNA makes them labour-intensive, time-consuming, and extremely costly. Additionally, the necessity of optimization of

parameters and numerous primers design presents scientists with a challenge. Therefore microarray techniques come second to RT-PCR.

Virus isolation

The influenza A virus is usually isolated and propagated by inoculating either swab or tissue samples from infected pigs in sensitive substrates such as cell cultures and SPF embryonated chicken eggs. Virus isolation can be conducted in cell lines and primary cells susceptible to SIV infection. Madin-Darby canine kidney (MDCK) and Caco-2 cells are the preferred cell lines but primary swine kidney, swine testicle, swine lung and swine trachea can be used. All the cell cultures, except Caco-2 cells, use a cell culture medium containing a concentration from 0.3 to 10 µg/ml of trypsin. Caco-2 cell line does not require the addition of trypsin to the medium as it already contains the proteases necessary for the cleavage of viral HA0 into HA1 and HA2 subunits and displays the characteristic cytopathic effect (Zhirnov and Klenk, 2003).

The comparison of the two preferred cell lines showed that the Caco-2 line was statistically more sensitive compared to the MDCK cells and embryonated chicken eggs for the isolation of H1N1 and H1N2 subtypes (Chiapponi et al, 2010).

Embryonated chicken eggs are considered the most sensitive method in the diagnosis of avian influenza viruses but are less sensitive in swine influenza diagnosis. Inoculation is performed in 9-11-day-old embryonated chicken eggs through allantoic cavity or amniotic sac route. Their main inconvenience is that not all influenza viruses grow well in chicken eggs. Out of the three subtypes currently circulating in pigs only the H3N2 subtype was isolated more readily in embryonated chicken eggs than in cultured cells (Chiapponi et al. 2010). The presence of influenza A virus should be confirmed by molecular techniques, agar gel immunodiffusion (AGID) assay for detection of the nucleocapsid or matrix antigens or antigen capture

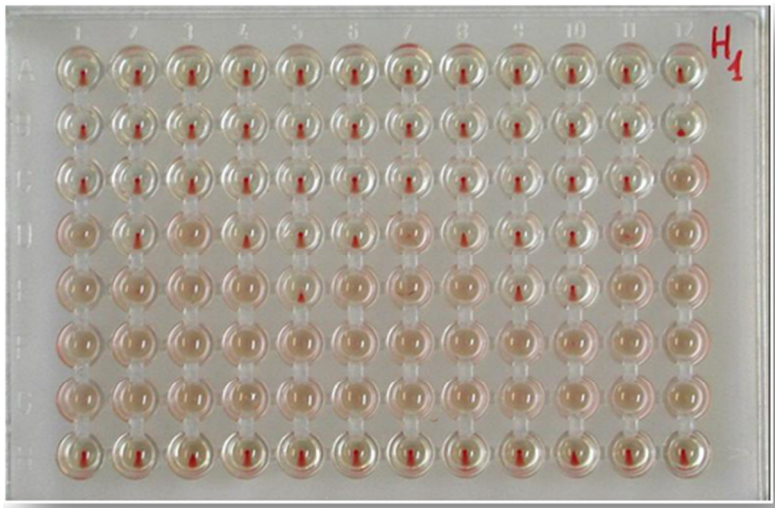
ELISAs or immunoassay kits for detection of the type A influenza. Influenza subtypes can be determined by haemagglutination inhibition and neuraminidase inhibition tests or by RT-PCRs with primers validated for sensitive and specific amplification of individual HA and NA genes (Swine influenza, 2010). A multiplex RT-PCR for subtyping H1 avian-like, H1 human-like, N1, N2 has recently developed and validated (Chiapponi et al., 2012). The presence of 2009 A/H1N1 pandemic can be demonstrated using CDC real time and traditional RT-PCR protocols (WHO, 2009).

Serological tests

Detection of subtype specific antibodies

Haemagglutination inhibition (HI) - The primary serological test for detection of SIV antibodies is the HI test and it is subtype specific. It should be conducted on paired sera collected 10-21 days apart. A four-fold or greater increase in titre between the first and second sample is suggestive of a recent SIV infection. HI test should be performed using different HA antigens (H1, H3, etc.) to a concentration of 4 or 8 HAU/25µl in 0.01M PBS. In order to remove the non-specific inhibitors and agglutinins sera can be treated by inactivation for 30 min at 56°C and/or using the receptor-destroying enzyme (RDE). The first dilution of serum is 1/10. The test use chicken or turkey erythrocytes (0,5% suspension).

Figure n. 11 - HI test Virology Department (IZSLER)

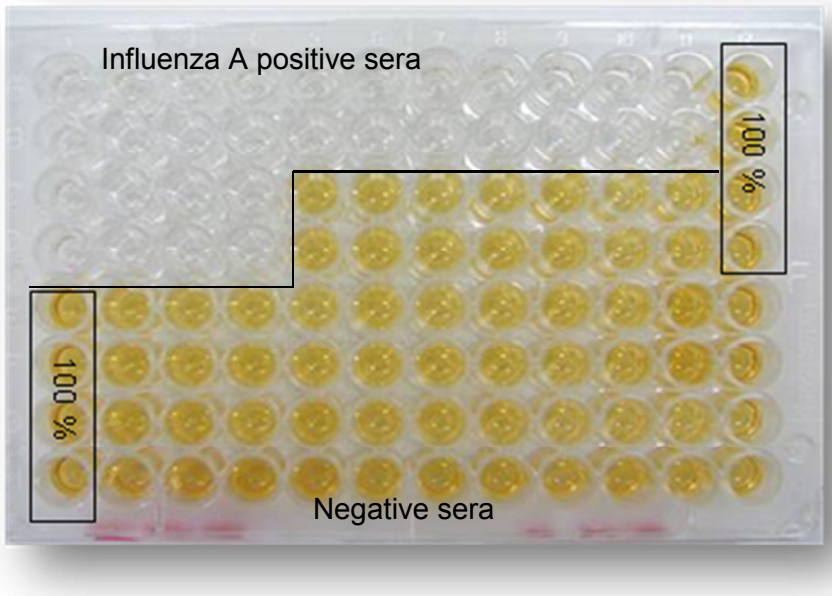


Virus neutralization (VN) - In the VN assay, serum dilutions were incubated (1 h, 37 °C) with 100 TCID50 of MDCK cell-adapted viruses belonging to different subtypes H1N1, H3N2 and H1N2 in microtitre plates. MDCK cells were then added to the virus–serum mixture at a concentration of 600,000 cells per ml. Virus-positive MDCK cells were demonstrated by immunoperoxidase staining, using monoclonal antibodies against influenza virus nucleoprotein (NP), biotinylated sheep anti-mouse IgG, and a streptavidin–biotin complex coupled to peroxidase.

Detection of type A influenza antibodies

Serological tests able to detect antibodies against type A influenza, which are common to all subtypes, are agar gel immunodiffusion test and ELISAs. Different ELISA formats such as Mab-based competitive ELISA and indirect ELISA are available.

Figure n. 12 – Mab-based competitive ELISA using an anti-nucleoprotein A Mab (HB65). Virology department, IZSLER



Hypothesis and objectives

Hypothesis

Swine influenza (SI) has remained of substantial importance to the swine industry throughout the world. Swine influenza is an economically important respiratory disease of swine caused by influenza A virus. Currently, three antigenic and genetically distinct subtypes (avian-like H1N1, human-like reassortant H3N2 and human-like reassortant H1N2) co-circulate in pig population in Europe in the last twenty years (De Jong et al., 2007; Marozin et al.,2002; Moreno et al., 2008b). In addition, new reassortant viruses between SIVs and seasonal human influenza have been occasionally reported worldwide (Ma et al., 2006; Moreno et al., 2009; Zell, 2008; Zhou, 1999).

Pigs play an important role as mixing vessels for animal and human influenza viruses, providing a place for reassortment and host adaptation to occur. The continuous co-circulation of influenza A viruses in pigs as well as the constant availability of immunologically naïve animals provide significant opportunity for persistence of strains and continuous genetic mixing with emergence of novel subtypes or genotypes. A better understanding of the evolution and adaptation of influenza A viruses to various hosts will provide more information on their capability to cross host barriers and become a pandemic strain. The reassortant viral strains with novel gene combinations and their ability to escape the immune system in other host species could satisfy the basic requirement needed to become a virus with pandemic potential. It is vital to continuously monitor the genetic content of circulating IAVs to detect new reassortants. Studying the process and patterns of viral reassortment is a key to better understanding influenza pandemics.

In Italy more than 70% of the swine industry and over 65% of the poultry farms are located in the Northern part of the country. Furthermore, in the last ten years, several

epidemics of H5 and H7 avian influenza occurred in the same area involving either domestic or wild birds (Alexander, 2007) .The simultaneous circulation of human, swine and avian influenza viruses in this area increases the risk of reassortment events and could be of great concern for their implications in human health. The detection and identification of SIVs in Italian pig population would be very interesting in order to better understand the complex epidemiology of SI and to give new data about the adaptation and circulation of novel reassortant viruses in pigs.

Objectives

Study n.1

To report the results of a swine influenza monitoring programme performed in Italy during the period 1998 -2011. This was based on control of all respiratory forms, genome detection, virus isolation and sequencing of the HA and NA genes.

To analyse the distribution of the swine influenza subtypes and evaluate the epidemiological situation in our country and to explore the genetic correlation between swine influenza viruses and influenza viruses of avian and human origin.

To decide the objectives of the further studies based on the results of the surveillance programme.

Study n. 2

To describe the isolation and complete genomic characterization of the first 2009 A/H1N1 pdm strain detected in a pig farm in Italy.

To carry out the phylogenetic and molecular analysis of this strains by comparing HA and NA sequences with selected Influenza A pandemic, swine and avian viruses.

To evaluate the serologic response versus homologous and heterologous antigens in the pigs presented in the farm.

Study n.3

To report the first isolation of a new H1N2 reassortant strain derived from 2009 A/H1N1 pandemic on an Italian pig farm by describing anamnestic data and clinical signs.

To perform the complete genome sequencing of the strain and the phylogenetic and molecular analysis of all genes comparing them with those retrieved in GenBank of human, swine and avian origin.

Study n.4

To perform the genomic characterization, phylogenetic and molecular analyses of SIVs belonging to H1N2 subtype isolated in Italy from 1998 to 2012 including a deeper investigation of reassortant strains.

To predict the three dimensional macromolecular structure (3D-MMS) of the HA protein.

To perform the evolutionary dynamics of IAVs of human and swine origin in order to estimate their past and recent epidemiology.

To investigate the site-by-site positive selection pressures by estimation of rates of nonsynonymous (dN) and synonymous (dS) substitutions in the HA and NA genes to identify the main mutations allowing viral immune-escape.

Study n. 1

Swine influenza viruses: virological surveillance in Italy during 1998 -2011

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. 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 come from the introduction of an avian virus in toto. These “avian-like” H1N1 viruses that emerged in European mainland pigs in 1979 replaced the previously circulating classical H1N1 strains. Classical H1N1 strains were reported for the first time in Europe in Italy with the isolation of a H1N1 SIV strain from USA imported pigs at IZSLER in 1976.

The H3N2 viruses present in Europe since 1984 are human-avian reassortants with the six internal genes deriving from the avian-like H1N1 viruses and the HA and NA genes from the earlier isolated human-like H3N2 viruses. The third subtype (H1N2) was first isolated in Great Britain in 1994 (Brown et al, 1995), subsequently spread to the swine population of continental Europe (Marozin et al, 2002) and was reported for the first time in Italy in 1998. 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 the last years in Italy, three subtypes (H1N1, H1N2, and H3N2) of swine influenza viruses have been circulating in the pig population.

In April 2009 a new virus, identified as novel 2009 A/H1N1 pandemic strain (H1N1pdm), was reported to cause a human influenza outbreak in Mexico. By June 2009 the H1N1pdm had rapidly spread among humans, leading the WHO to the declaration of the first influenza pandemic of the 21st century. The virus is a

quadruple reassortant and contains mostly genes from the triple reassortant H1N1 viruses found in swine in North America as well as two genes, NA and Matrix (M) from Influenza viruses found in swine in Europe and Asia.

Pigs have receptors in their respiratory tract, which will bind swine, human and avian influenza viruses. Consequently, pigs are considered mixing vessels for the development of new influenza viruses when swine, avian and/or human influenza viruses undergo recombination in pigs. Swine influenza infections are described as primary respiratory disease characterized by sudden onset and rapid recovery and by clinical sings such as coughing, sneezing, nasal discharge, conjunctivitis, fever, lethargy and depressed appetite. Morbidity rates can reach 100% while mortality rates are generally low however the economic impact is very high and is mainly related to grow depression resulting in an increase in the number of days to reach market weight.

Materials and Methods

Sampling

Samples submitted to IZSLER are lung tissues and relatives lymph nodes collected from pigs with gross lesions typical of respiratory diseases and nasal swabs from pigs showing respiratory sing.

Diagnostic procedures

All samples were screened for influenza type A by real time RT-PCR (rt RT-PCR) (Sapckman et al, 2002). Since 2009, responding to the emergence of the new pandemic virus and consequently to the recent reports on human to animal transmission, testing for the H1N1pdm was also performed on all the swine samples

resulted positive to influenza type A. H1N1pdm genome detection was carried out by rt RT-PCR, according to the CDC procedure.

In addition, samples were 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.

Samples positive to type A influenza PCR were further applied onto MDCK and Caco-2 cells and inoculated through the allantoic sac route of 9-11 day old SPF chicken embryonated eggs (CEE) for virus isolation. Culture supernatant (CS) following observed cytophatic effect and allantoic fluid (AF) were tested with haemagglutination (HA) assay using chicken erythrocytes and for influenza type A and H1N1pdm by real time RT-PCR assays. The subtype of the isolate was determined by three multiplex RT-PCR assays using primers to amplify H1, H3, N1 and N2 SIVs (Chiapponi et al., 2003; Chiapponi et al., 2012) and primers described by CDC to amplify fragment 4 of HA gene of H1N1pdm (WHO primers, 2009).

The diagnostic procedures routinely performed in IZSLER are summarized in figure n.1.

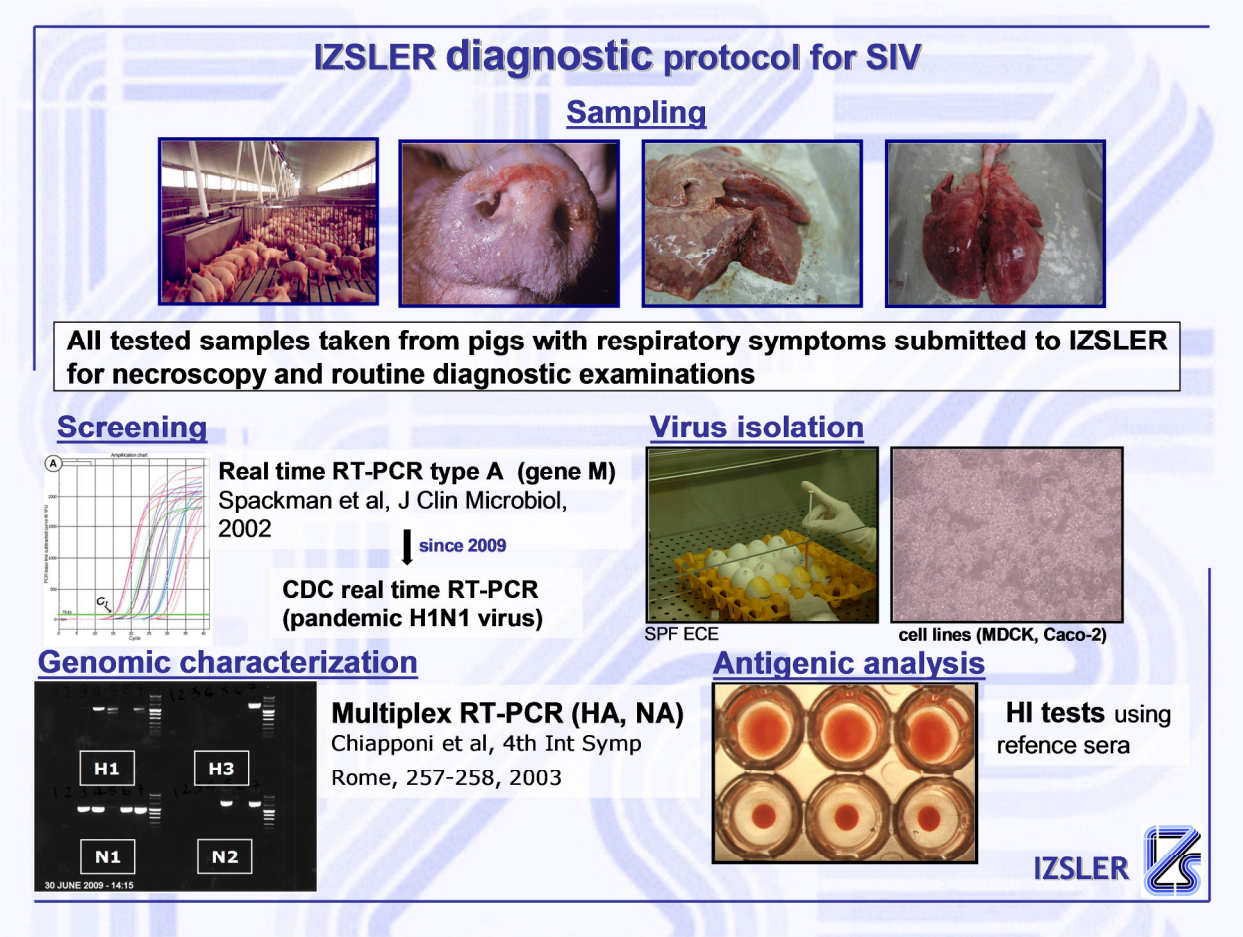


Figure n.1 – Procedures for swine influenza diagnosis

Genome sequencing

In order to better understand the epidemiology of SIV, the partial sequencing of HA and NA genes of all isolated SIV was performed. Viral RNA was extracted 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).

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. The results were verified using Neighbour joining and maximum likelihood analysis, which showed similar topologies. Gene sequences of the Italian strains were compared with swine, avian and human influenza viruses which sequences were retrieved from the NCBI Influenza Virus

Results

Virological surveillance

This study reports the results of swine influenza monitoring programme based on control of all respiratory forms, genome detection, virus isolation and sequencing performed in Italy during the period 1998 -2011. Investigated samples originated from the entire country, however the major part of them was from North Italy where more than 75% of the swine industry is located. Distribution of the pig population in Italy and comparison with the IZSLER jurisdiction area are reported in figure n. 2.

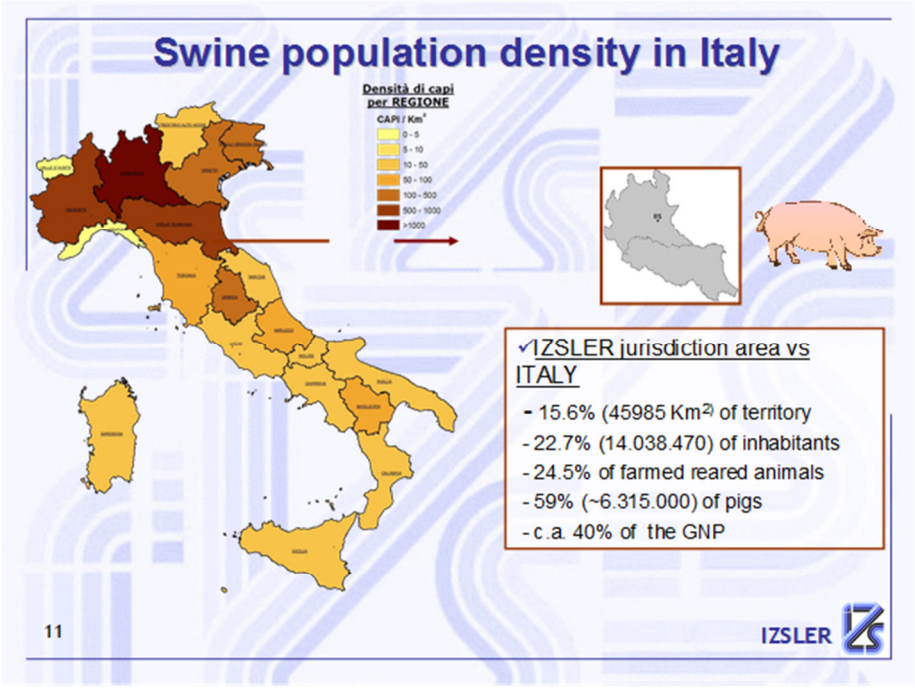


Figure n. 2 – Swine population density in Italy

In the investigated period, a total of 580 SIVs were isolated. The number of strains divided by subtype, year of isolation and geographical origin are reported in figure n.3.

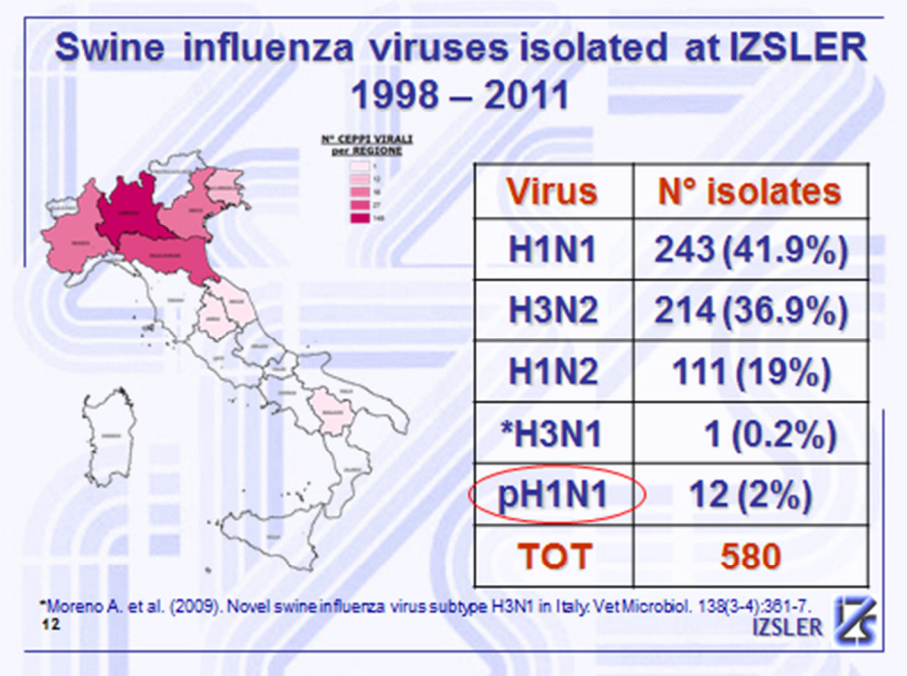
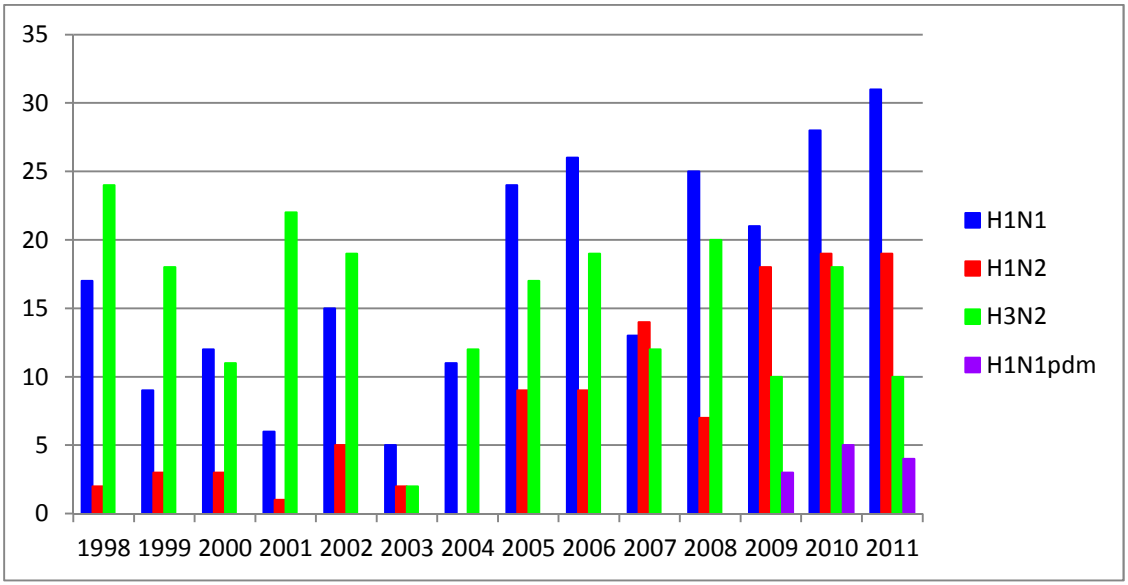


Figure n. 3 – SIV isolated at IZSLER in the period 1998-2011

The distribution per year of the SIV strains is showed in graphic n.1.



Graphic n.1 – Distribution per year of SIVs isolated in Italy

The results of the Italian surveillance programme revealed continuous circulation of H1N1, H3N2 and H1N2 viruses and isolation of the H1N1 pdm viruses in the pig farms starting in 2009. The avian-like H1N1 subtype was the most frequent in our country. The Italian epidemiological situation of this subtype was in line with that in Europe, where it was widely distributed in all countries. The second most frequent subtype in Italy was the H3N2. The circulation of the human-like H3N2 viruses in Europe evidenced significant differences with an extensive circulation in Italy and other countries in Southern Europe while almost absent in France and UK (Brown, 2012). The H1N2 subtype was rarely isolated in the first part of the investigated period but since 2005, its frequency increased becoming the second subtype in the last three years. Also in Europe the H1N2 viruses were becoming increasingly widespread (Kyriakis et al, 2011). Moreover, it is worth noting the isolation of 12 H1N1 pdm strains in 7 different pig farms also when these viruses did not circulate in the human population.

Phylogenetic analysis

Phylogenetic analysis of Italian strains isolated during the period 1998-2011 revealed that Italian H1N1 SIVs isolated in 2001-2011 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 several 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 four reassortant strains that showed a high similarity to the Italian H3N2 strains (Moreno Martin et al, 2008a). Interestingly the neuraminidase (NA) gene of the recent Italian strains showed different characteristics respect to the European H1N2 strains, deriving from the recent human H3N2 viruses.

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 (PCh-73 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 PCh-73 lineage. Moreover, it is worth noting the isolation and characterization of a novel H3N1 swine influenza virus in 2006. Genetic characterization suggested that the new isolate was a recombination between the H1N1 and H3N2 viruses circulating in Italy (Moreno et al., 2009)

Phylogenetic trees based on partial sequencing of H1, H3, N1 and N2 of Italian SIV isolates are reported in figures n. 4, 5, 6 and 7.

Discussion

Swine influenza monitoring programs, based on genome detection, virus isolation and sequencing of all respiratory forms, have been applied since the nineties, especially in North Italy where more than 75% of the swine industry is located.

Monitoring programs performed at IZSLER in the last ten years on pig farms with respiratory symptoms brought to the isolation of several strains of the three circulating subtypes. This high concentration of people and animals in the Northern

part of Italy provides an ideal opportunity for co-circulation of viruses and genetic reassortment. These results point out the homogeneity among the recent Italian H1N1 SIVs, distinguishing these from the earlier strains. It should be noted that while HA gene of recent H1N1 SIVs was clearly distinguishable from that of the early circulating strains, the NA gene seemed to be more related to them. Also for Italian H1N2 the presence of a homogeneous group, composed of the recent viruses, differing from earlier strains is reported. Finally, Italian H3N2 are gradually evolved from A/PCh/1/73-like human influenza virus, which is considered the most probable ancestor of the swine PCh-73 lineage.

The isolation of H1N1pdm from pigs in different countries, Italy included, raises further concerns on the possible establishment of H1N1pdm in the swine population. This could lead to a scenario with a co-circulation of influenza A viruses within swine that could act as mixing vessels for the reassortment between influenza viruses of both mammal and avian origin with unknown implications for public health. All these facts could have a great impact in the Northern part of Italy where over 70% of swine and 65% of poultry is raised and several epidemics of avian influenza H5 and H7 subtypes occurred in the past ten years (Alexander et al., 2007).

The immune selection is not considered important in pigs due to the continuous availability of susceptible pigs. Previous studies reported that human H1N1 and H3N2 viruses could be frequently transmitted to pigs but fail to persist (Van Reeth et al., 2007). This could be explained by the fact that virus strains with different antigenic characteristics could be disadvantaged compared to the well adapted established viruses which are circulating among the susceptible population. However some cases, such as the emergence of the H1N2 subtype in UK with a HA closely related to human H1N1 viruses and subsequently spread in the European pig

population, suggest that the genes of human viruses may persist after reassortment with one or more influenza viruses in pigs and following adaptation to pigs may often be associated with clinical disease. Moreover, the recent emergence and persistence of reassortant H1N2 strains in Italy since 2003 with a NA closely related to recent H3N2 human viruses (Moreno et al., 2010) could be another example supporting these hypotheses.

The evidence that pigs are more frequently involved in interspecies transmission of influenza A viruses than are other animals highlight the need for a stringent surveillance in pig populations in order to better understand the circulation of swine influenza viruses. Considering the results of this study special attention will be focussed on the H1N2 subtype and the H1N1 pdm viruses.

Gene H1

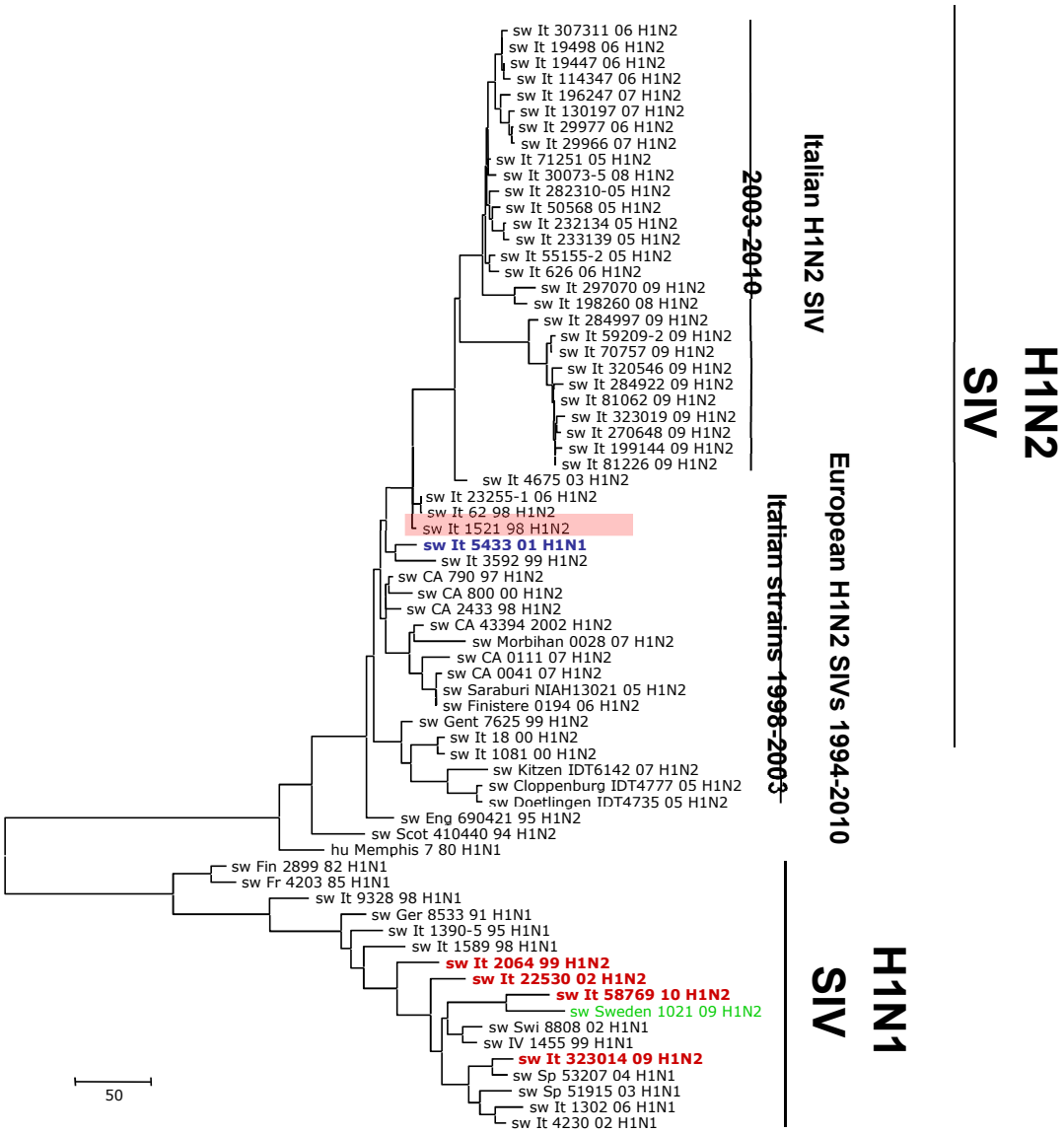


Figure n. 4 –Phylogenetic tree based on the HA gene (subunit H1) of the H1 swine influenza viruses.

Gene H3

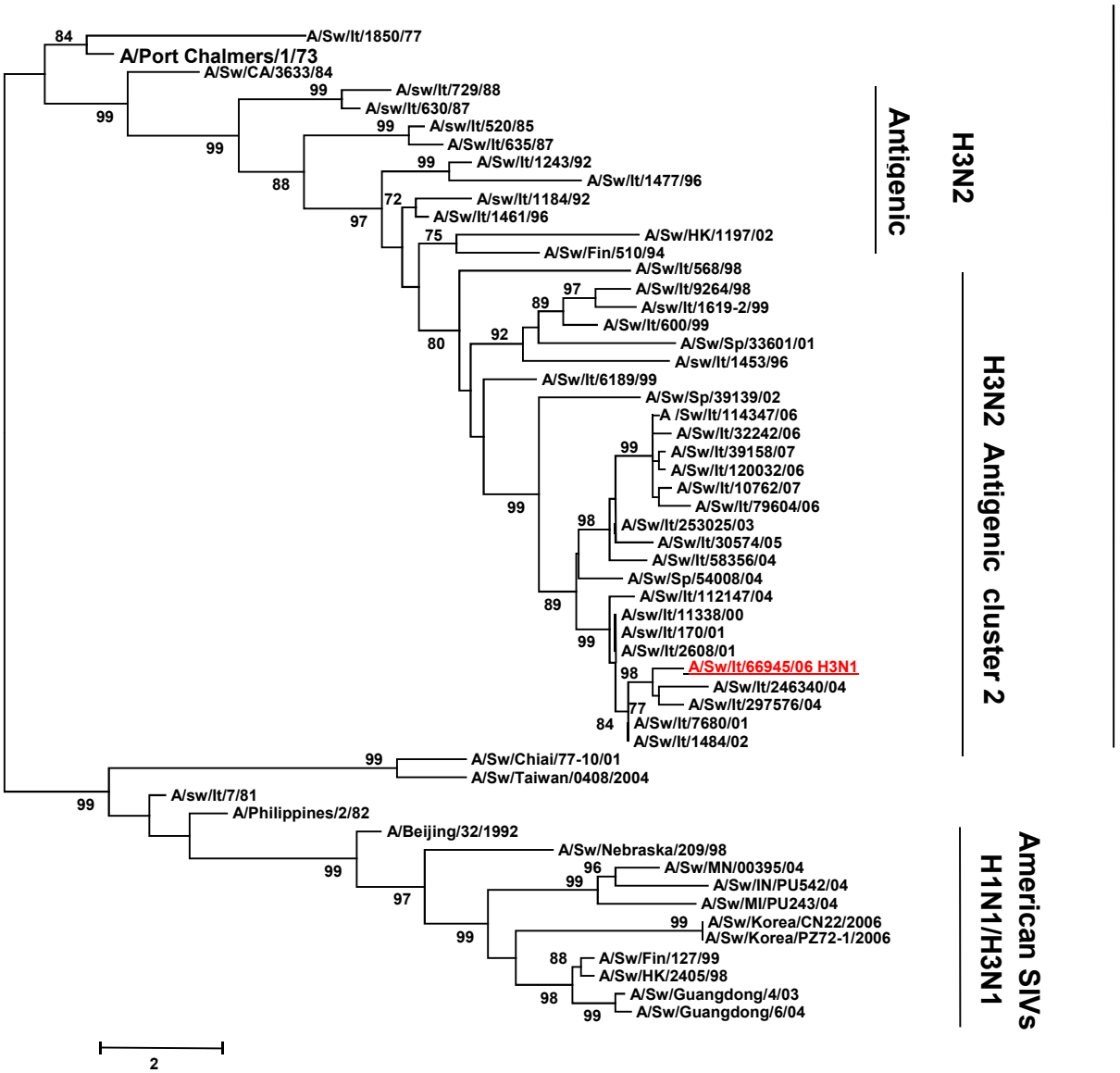


Figure n. 5 – Phylogenetic tree of the HA gene (subunit HA1) of the H3N2 swine influenza viruses.

Gene N1

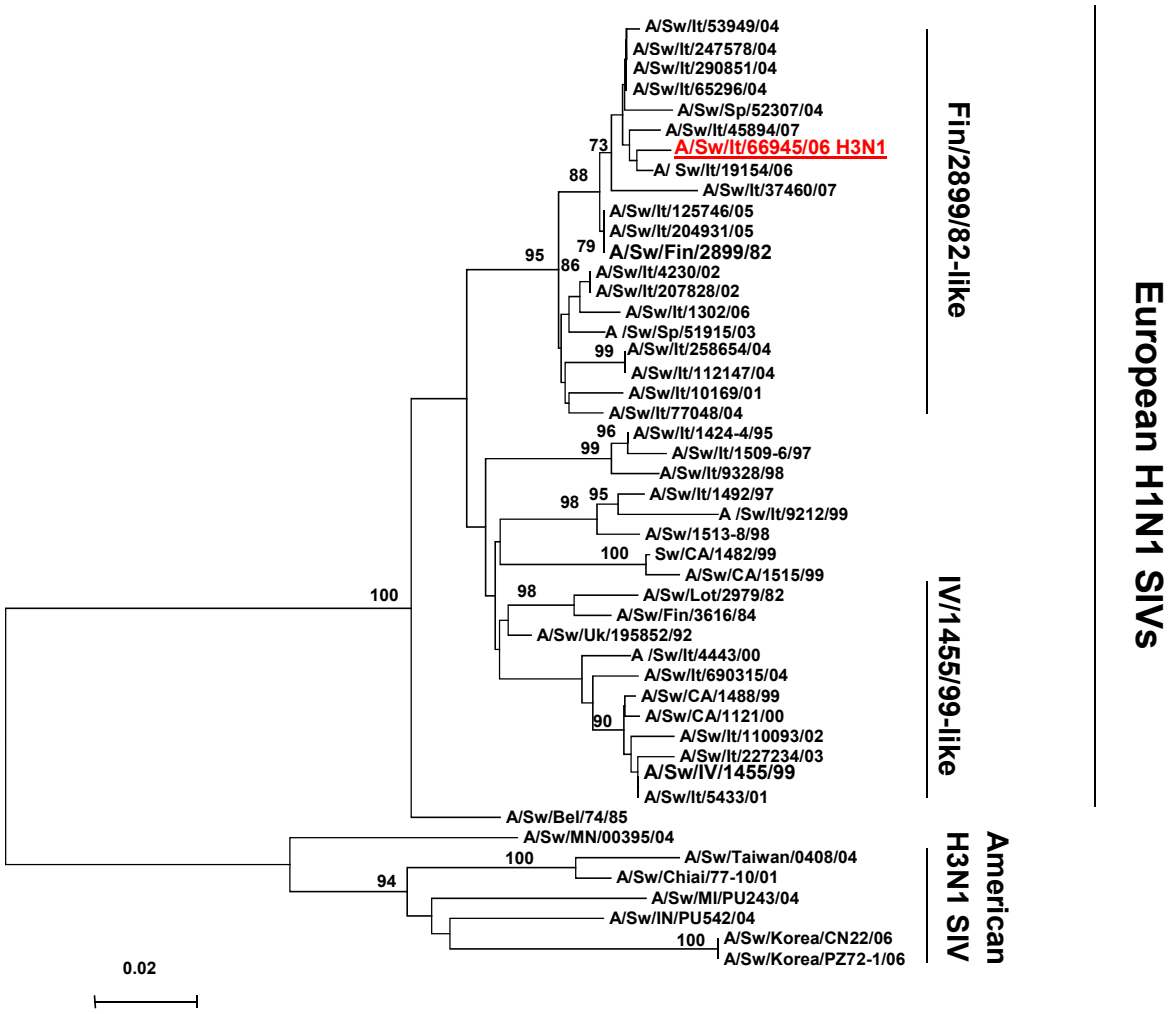


Figure n. 6 – Phylogenetic tree of the NA gene of H1N1 swine influenza viruses

Gene N2

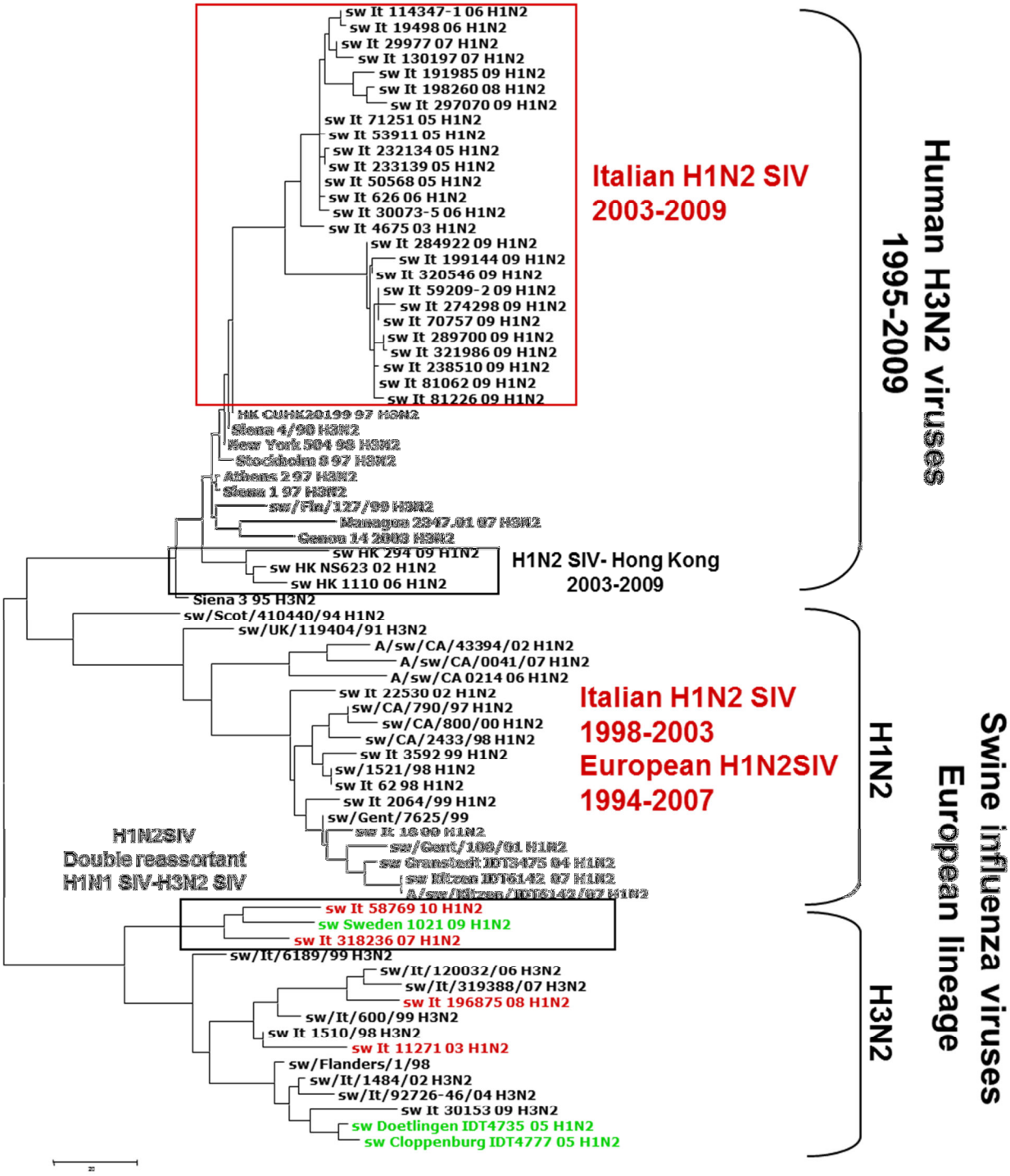


Figure n. 7 – Phylogenetic tree of the N2 gene of swine influenza viruses

Study n. 2

First pandemic H1N1 outbreak from a pig farm in Italy

Introduction

Influenza viruses cause annual epidemics and occasional pandemics spreading worldwide and infecting a large proportion of the human population. During April 2009, a novel 2009 A/ H1N1 pandemic virus (H1N1 pdm) was identified as the cause of the present flu pandemic (Center for Disease control and prevention, 2009). This virus was found to be genetically and antigenically unrelated to the seasonal human influenza and was generated as a quadruple reassortant, possessing genes from Euro-Asiatic and American lineages of swine influenza, as well as avian and human influenza genes (Schnitzler et al., 2009). In Italy, swine influenza monitoring programs, based on genome detection, virus isolation and sequencing of all respiratory forms, have been applied since the nineties, especially in North Italy where more than 75% of the pig population is located. Responding to the emergence of the new pandemic virus and consequently to the recent reports about human to animal transmission (Hofshagen et al., 2009; OIE World Animal Health Information Database, 2009) testing for the H1N1pdm was also performed on all the swine samples resulted positive for type A influenza.

This paper describes the isolation and complete genomic characterization of the first H1N1 pdm detected in a pig farm in Italy. The genome sequence of the isolate was determined and phylogenetic analysis was conducted by comparing HA and NA sequences with selected Influenza A pandemic, swine and avian viruses.

Material and Methods

Clinical Samples

In November 2009, a 1264 swine breeder farm located in Lombardia region (Northern Italy) experienced lower sow reproduction performances in farrowing units.

Clinical signs observed in sows included fever, depression, anorexia and agalactia, while in piglets diarrhoea and weight loss. The morbidity in sows was approximately 30% and the accumulated mortality rate was similar with those usually reported in piggeries (<10%). Swine influenza vaccination has not been applied. Nine dead piglets and four nasal swabs from sows were submitted to the diagnostic laboratory. Out of these, 8 piglets showed catarrhal enteritis whereas the last one presented pneumonia with a purple area of consolidation in the apical lobes. Lung homogenates from the 8 piglets without lung lesions were performed in two separate pools of 4 piglets each one, while lung from piglet 9 was tested alone.

Influenza type A antigen detection

Clinical specimens were screened for the presence of influenza A viruses by real time RT-PCR (Spackman et al., 2002). Positive samples were further tested for the H1N1 pdm by real time RT-PCR (rt RT-PCR), according to the CDC procedure (WHO, 2009).

Detecting swine respiratory pathogens

All samples were also cultured using different media for the most common swine respiratory bacterial pathogens and tested for detection of Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circoviruses type 2 (PCV2) and *Mycoplasma hyopneumoniae* by using RT-PCR, multiplex PCR and PCR assays respectively (Casamiglia et al., 1999; Ouardani et al., 1999; Suarez et al., 1994). The first pool (animals 1-4) and piglet 9 resulted positive to the type A influenza as well as for H1N1 pdm assay. Only PRRSV European strain was detected from the first pool, whereas other samples resulted negative for the tested pathogens.

Virus isolation

For virus isolation, positive samples were applied onto Madin-Darby canine kidney (MDCK) cells, Caco-2 cells and inoculated into SPF chicken embryonated eggs (CEE). The strains could be isolated from pool 1 and piglet 9 either on cell cultures or CEE (A/Sw/It/290271/09 and A/Sw/It/308288/09, respectively).

Genome sequencing and phylogenetic analysis

Viral RNA was extracted form allantoic fluid of A/Sw/It/290271/09 using QIAamp ViralRNA Mini Kit (Qiagen, Hilden, Germany). SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen Carlsbad, CA, USA) was used to perform RT-PCR of 46 amplicons representing the entire viral genome using the Influenza A H1N1 pdm genome Primer Set (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed using BigDye Terminator Cycle Sequencing kit v3.1 and resolved by an ABI 3130 DNA automatic sequencer. Multiple sequence alignments were made using ClustalW and maximum parsimony phylogenetic trees were created using MEGA4 (Tamura et al., 2007).

GenBank accession numbers of the eight genes are: CY053619 (HA), CY053621 (NA), CY053622 (M), CY053620 (NP), CY053618 (PA), CY053617 (PB1), CY053616 (PB2), CY053623 (NS1).

Results

Full genome sequencing of A/Sw/It/290271/09 confirms a very high similarity through the viral genome to the pandemic virus circulating in humans. Comparison with all H1N1pdm sequences available in GenBank shows in A/Sw/It/290271/09 three unique amino-acid (aa) changes in PB2 (S405T), PB1 (K386R) and PA (K256Q), not

yet associated to any well characterized phenotype markers of Influenza viruses. All eight aa at positions representing the so-called species-specific swine-human signatures (Chen and Shih, 2009), found in both swine and in the H1N1 pdm, are also present. The M2 protein displays the C55F and the PA protein the S409N substitutions, both corresponding to enhanced transmission phenotype markers (Squires et al., 2008).

Phylogenetic analysis was based on complete open reading frames of HA and NA sequences of A/Sw/It/290271/09 and representative H1 and N1 subtypes of human, avian and swine origin, mostly isolated in Italy in the last decade. (Fig.1 and Fig.2). The HA phylogenetic tree shows that the Italian avian and swine isolates from 1998to 2009, form a clearly distinct lineage including two different clusters of H1N1 (avian and swine viruses) and H1N2 swine strains. The N1 phylogenetic analysis revealed that NA genes closest to SW/IT/290271/09 and H1N1 pdm were all from “avian –like” sw/H1N1 viruses detected in Spain and Italy in the last decade (Moreno Martin et al., 2008).

Thirty days after H1N1 pdm diagnosis, sera from 40 sows were collected and tested by HI test performed as described (Swine influenza, 2012) using the following antigens: A/Sw/290271/99 (H1N1pdm), A/Sw/Fin/2899/82 H1N1 SIV, A/Sw/It/1521/98 H1N2 SIV and A/Sw/CA/3633/84 H3N2 SIV. Out of these, 20 samples resulted positive to HI test using H1N1pdm with HI titres from 1/10 to 1/80. All samples resulted negative by HI versus H1N2 and H1N1 SIVs. Only five animals resulted positive (HI titres from 1/20 to 1/80) to H3N2 SIV but were not from the same pigs positive to H1N1 pdm. Results of the HI test are reported in table n.1.

Discussion

This paper reports the first isolation and genomic characterization of H1N1 pdm from a pig farm in Italy. Full sequencing and phylogenetic analysis of A/Sw/It/290271/09 confirms a very high similarity throughout the viral genome to the H1N1 pdm viruses. During the epidemiological analysis, the hypotheses of human to pig transmission remained one of the most probable source for the spread of infection because several days before one of the pig farmers showed influenza-like symptoms. Diagnosis of H1N1 pdm in the farmer was not possible, due to late swabs sampling and consequently other additional routes of infection cannot be excluded. PRRS virus was also detected from the first pool of piglets; PRRS infection is extremely diffused in Italian swine farms and its association with swine influenza in weaning pigs is reported as a common event in the last years (Barigazzi et al., 2003).

Serological tests evidenced a circulation of H1N1 pdm in the farm since 20 to 40 animals showed antibodies (Ab) against H1N1 pdm although the HI titres were not very high. Some positive H3N2 sera were also observed in H1N1 pdm negative sows probably due to a H3N2 SIV circulation or an introduction of positive animals in the farm.

Until November 2009, an enhanced monitoring program for swine influenza conducted in Italy, led to the isolation of 53 Swine influenza viruses belonging to the three subtypes currently circulating in Europe (data not shown). Based on these results, it could be assumed that H1N1pdm was not circulating in the Italian pig population before this reported case. In Italy more than 70% of the swine industry and over 65% of poultry farms are located in the Northern part of the country. In addition, in the last ten years, several epidemics of avian influenza H5 and H7 subtypes occurred in the same area involving either domestic or wild birds

(Alexander, 2007). In this context several aspects must be considered also for their implications on human health.

First of all, simultaneous circulation of human, swine and avian influenza viruses in the same area increases the risk of reassortment events, in a susceptible host like swine that may function as a reservoir and play a vital role in interspecies transmission of influenza A viruses (Shen et al., 2009). Secondly, an improved surveillance programme should be implemented to undertake control measures for limiting the spread of H1N1 pdm in farms and the transmission from animals to humans. Moreover studies on H1N1 pdm sequences should be encouraged in order to monitor constantly any change in viral genotype and evolution of this virus in swine population.

Table n. 1- Results of the HI test performed on sera from sows using different antigens. Results are expressed as HI titre reciprocal. Negative sera are < 10.

Sera	HI antigens			
	A/Sw/It/290271/09 Pandemic H1N1	A/Sw/Fin/2899/82 H1N1 SIV	A/Sw/It/1521/98 H1N2 SIV	A/Sw/CA/3633/84 H3N2 SIV
1	20	N	N	N
2	N	N	N	N
3	N	N	N	N
4	N	N	N	80
5	20	N	N	N
6	N	N	N	N
7	20	N	N	N
8	N	N	N	N
9	N	N	N	N
10	10	N	N	N
11	10	N	N	N
12	10	N	N	N
13	40	N	N	N
14	N	N	N	N
15	10	N	N	N
16	80	N	N	N
17	10	N	N	N
18	20	N	N	N
19	20	N	N	N
20	N	N	N	N
21	N	N	N	80
22	N	N	N	N
23	N	N	N	80
24	N	N	N	N
25	10	N	N	N
26	80	N	N	N
27	N	N	N	80
28	10	N	N	N
29	N	N	N	N
30	20	N	N	N
31	N	N	N	N
32	20	N	N	N
33	N	N	N	N
34	N	N	N	N
35	N	N	N	N
36	10	N	N	N
37	80	N	N	N
38	20	N	N	N
39	N	N	N	80
40	20	N	N	N

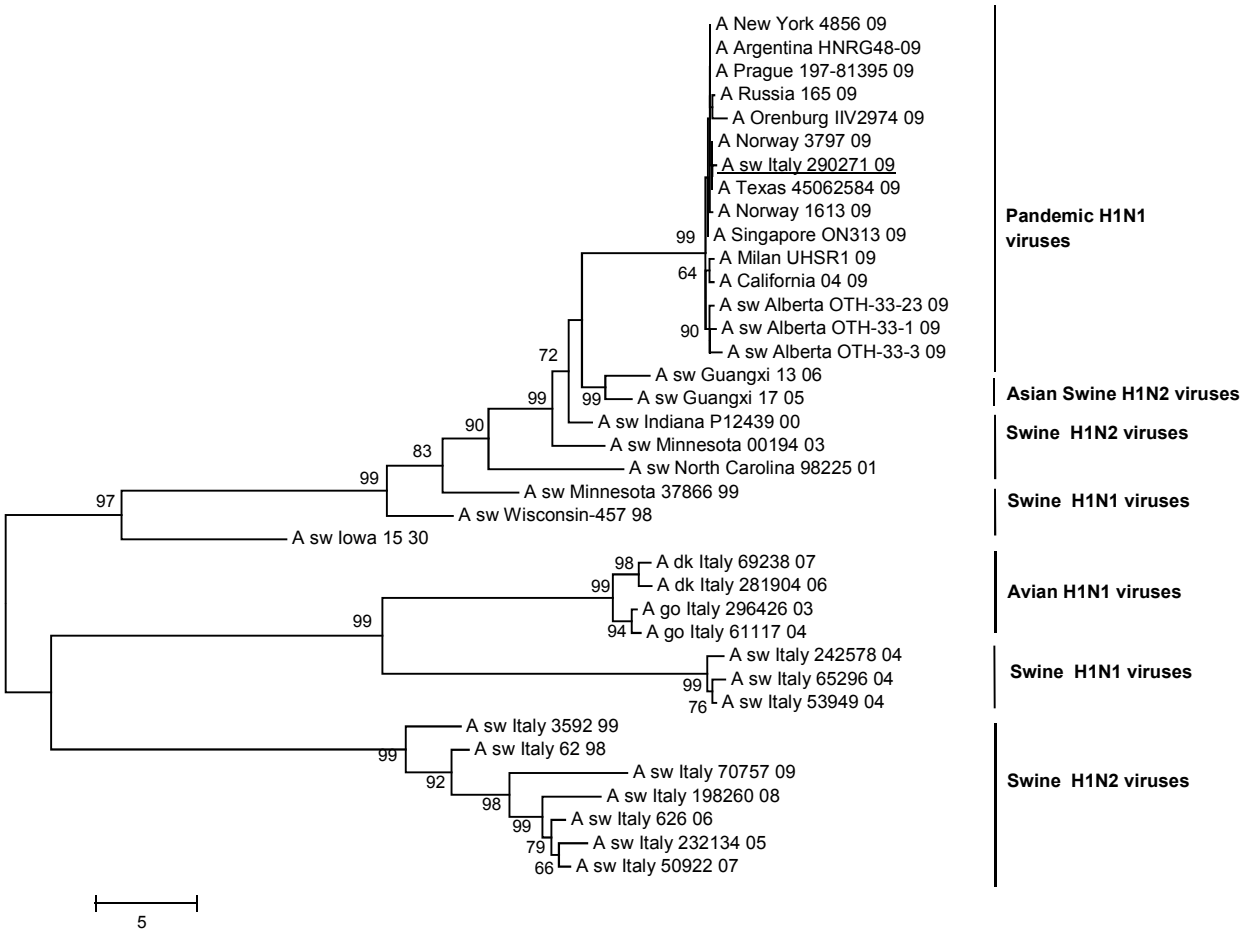


Figure n 1- Phylogenetic relationship of the HA gene of the swine H1N1 pandemic Italian isolate (A/sw/Italy/290271/2009 H1N1) with other Italian SIV and AIV viruses and database retrieved H1N1 and H1N2 swine and human viruses. The unrooted tree was created by maximum parsimony method and bootstrapped with 500 replicates. Only bootstrap values higher than 70% are shown.

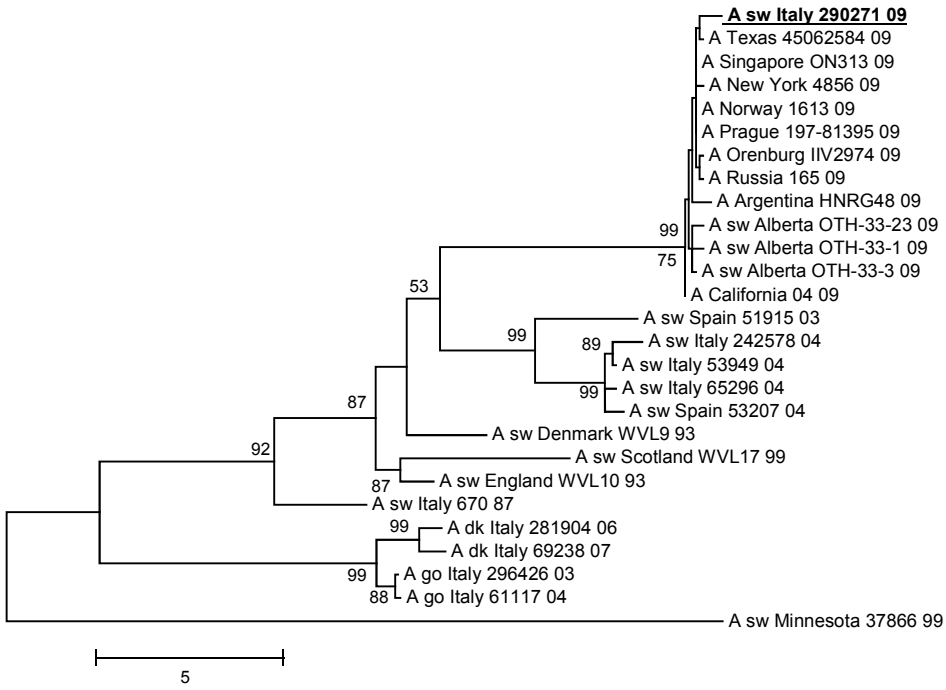


Figure n.2 - Phylogenetic relationship of the NA gene of the swine H1N1 pandemic Italian isolate (A/sw/Italy/290271/2009 H1N1) with other Italian SIV and AIV viruses and database retrieved H1N1 swine and human viruses. The unrooted tree was generated as described in the legend of figure n.1.

First Pandemic H1N1 Outbreak from a Pig Farm in Italy

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Abstract: The first outbreak of the pandemic H1N1 virus in a swine breeder farm in Italy in November 2009 was reported. Clinical signs observed in sows included fever, depression, anorexia and agalactia, while in piglets diarrhoea and weight loss. The morbidity in sows was approximately 30% and the accumulated mortality rate was similar with those usually reported in piggeries (<10%). Virus was isolated from piglets (A/Sw/It/290271/09) and the sequencing of the whole genome was then performed. Comparison with all (H1N1)v sequences available in GenBank shows A/Sw/It/290271/09 three unique amino-acid (aa) changes in PB2 (S405T), PB1 (K386R) and PA (K256Q), not yet associated to any well characterized phenotype markers of Influenza viruses. All eight aa at positions representing the so-called species specific swine-human signatures, found in both swine and in the pandemic H1N1v, are also present. The M2 protein displays the C55F and the PA protein the S409N substitutions, both corresponding to enhanced transmission phenotype markers. Phylogenetic analysis showed that the virus was genetically related to the pandemic H1N1 virus. In addition, serological samples were collected from 40 sows, of which 20 resulted positive to the pandemic H1N1 virus by HI test proving a virus circulation in the farm.

Keywords: Pandemic H1N1 virus, swine, genomic characterization, Italy.

Influenza viruses cause annual epidemics and occasional pandemics spreading worldwide and infecting a large proportion of the human population. During April 2009, a novel H1N1 (H1N1)v influenza A virus was identified as the cause of the present flu pandemic [1]. This virus was found to be genetically and antigenically unrelated to the seasonal human influenza and was generated as a quadruple reassortant, possessing genes from Euro-Asiatic and American lineages of swine influenza, as well as avian and human influenza genes [2]. In Italy, swine influenza monitoring programs, based on genome detection, virus isolation and sequencing of all respiratory forms, have been applied since the nineties, especially in North Italy where more than 75% of the pig population is located. Responding to the emergence of the new pandemic virus and consequently to the recent reports about human to animal transmission [3, 4], testing for the (H1N1)v was also performed on all the swine samples resulted positive for type A influenza.

This paper describes the isolation and complete genomic characterization of the first (H1N1)v detected in a pig farm in Italy. The genome sequence of the isolate was determined and phylogenetic analysis was conducted by comparing HA and NA sequences with selected Influenza A pandemic, swine and avian viruses.

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E-mail: anamaria.morenomartin@izsler.it

In November 2009, a 1264 swine breeder farm located in Lombardia region (Northern Italy) experienced lower sow reproduction performances in farrowing units. Clinical signs observed in sows included fever, depression, anorexia and agalactia, while in piglets diarrhoea and weight loss. The morbidity in sows was approximately 30% and the accumulated mortality rate was similar to those usually reported in piggeries (<10%). Swine influenza vaccination has not been applied. Nine dead piglets and four nasal swabs from sows were submitted to the diagnostic laboratory. Out of these, 8 piglets showed catarrhal enteritis whereas the last one presented pneumonia with a purple area of consolidation in the apical lobes. Lung homogenates from the 8 piglets without lung lesions were performed in two separate pools of 4 piglets each one, while lung from piglet 9 was tested alone. Clinical specimens were screened for the presence of influenza A viruses by real time RT-PCR [5]. Positive samples were further tested for the (H1N1)v by real time RT-PCR (rt RT-PCR), according to the CDC procedure [6]. All samples were also cultured using different media for the most common swine respiratory bacterial pathogens and tested for detection of Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circoviruses type 2 (PCV2) and *Mycoplasma hyopneumoniae* by using RT-PCR, multiplex PCR and PCR assays respectively [7-9]. The first pool (animals 1-4) and piglet 9 resulted positive to the type A influenza as well as for (H1N1)v assay. Only PRRSV European strain was detected from the first pool, whereas other samples resulted negative for the tested pathogens.

For virus isolation, positive samples were applied onto Madin-Darby canine kidney (MDCK) cells, Caco-2 cells and

1874-3579/10

Study n. 3

Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus

Introduction

Influenza type A viruses are currently circulating in the European pig population with high prevalence of H1N1, H1N2 and H3N2 subtypes. In April 2009 a new virus, identified as 2009 A/H1N1pandemic strain (H1N1pdm), was reported to have caused a human influenza outbreak in Mexico (Centers for Disease Control and prevention, 2009, pp. 400-2). By June 2009 the H1N1pdm had rapidly spread among humans, leading the WHO to the declaration of the first influenza pandemic of the 21st century (WHO, phase of pandemic alert, 2009). The virus is a quadruple reassortant and contains mostly genes from the triple reassortant H1N1 viruses found in swine in North America as well as two genes, Neuraminidase (NA) and Matrix (M) from Influenza viruses found in swine in Europe and Asia (Schnitzer et al., 2009). In Italy, swine influenza monitoring programs, based on genome detection, virus isolation and sequencing of all respiratory forms, have been in place since the nineties, especially in North Italy where more than 75% of the swine industry is located. Responding to the emergence of the new pandemic virus and consequently to the recent reports on human to animal transmission (Hofshagen et al., 2009; OIE, 2009), testing for the H1N1pdm was also performed on all the swine samples which resulted positive to influenza type A. In the last two years, systematic virological surveillance of influenza viruses in pigs has led to the isolation of 67 SIVs belonging to the following subtypes: 24 H1N1, 25 H1N2, 18 H3N2 SIVs (Moreno et al., 2010 a), 2 H1N1pdm viruses (Moreno et al., 2010 b) and one new reassortant strain derived from H1N1pdm. This study reports the first isolation of a new H1N2 reassortant strain on an Italian pig farm. The molecular characterization and phylogenetic analysis of the new reassortant strain revealed that all the genes except the NA belonged to the H1N1pdm viruses and the NA gene was closely related to two H1N2 SIV strains,

previously isolated in Sweden (A/Sw/Sweden/1021/2009) and Italy (A/sw/Italy/58769/2010).

Materials and methods

Clinical samples

In May 2010, a clinical outbreak was reported on a pig-fattening farm with 4000 heads located in the province of Mantua (North Italy). The clinical signs observed were coughing, dyspnoea, fever, anorexia and depression with 10% morbidity, 0,5% mortality. This farm used a continuous flow system of management with monthly introduction of animals and fortnightly departures to the slaughterhouse. SIV vaccination program had not been in place at the farm. One dead pig was submitted to the laboratory for respiratory disease diagnostic tests.

Influenza type A antigen detection

Lung homogenate was screened for the presence of influenza A viruses by M gene rt RT-PCR (Spackman et al., 2002). Positive samples were further tested for the H1N1pdm by rt RT-PCR, according to the CDC procedure (WHO, 2009).

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 circovirus 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; Suarez et al., 1994).

Virus isolation and subtype determination

The novel SIV isolate was obtained from a 10% lung homogenate applied onto Caco-2 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 haemagglutination assay (HA) using chicken erythrocytes performed as described (Swine influenza, OIE Manual, 2010) and for influenza type A and H1N1pdm by real time RT-PCR assays. The subtype of the isolate was determined from culture supernatant, allantoic fluid as well as from lung tissues by three multiplex RT-PCR assays using primers to amplify H1, H3, N1 and N2 SIVs (Chiapponi et al., 2003) and primers described by CDC to amplify fragment 4 of HA gene of H1N1pdm (WHO sequencing primers and protocol, 2009).

Genome sequencing and phylogenetic analysis

For genomic sequencing viral RNA was extracted from allantoic fluid of A/sw/It/116114/2010 using QIAamp ViralRNA Mini Kit (Qiagen, Hilden, Germany). SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen Carlsbad, CA, USA) was used to perform RT-PCR of 46 amplicons representing the entire viral genome using the Influenza A H1N1pdm genome Primer Set (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed using BigDye Terminator Cycle Sequencing kit v3.1 and resolved by an ABI 3130 DNA automatic sequencer. 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 500 bootstrap replicates.

The identification of the novel reassortant strain was confirmed by the full length sequencing of the HA and NA genes performed directly on lung homogenates.

Nucleotide sequence accession numbers

GenBank accession numbers of the complete genome of A/sw/Italy/116114/2010 were assigned CY067659 to CY067666. GenBank accession numbers of A/sw/Italy/58769/2010 were assigned HM771274 to HM771279, HQ168025 and HQ168026.

Results

Virus isolation and subtype determination

Lung homogenates from one pig carcase showing purple areas of consolidation in the apical lobes of the lungs, interlobular oedema, and fibrinous pleuritis resulted positive for influenza type A and H1N1pdm by real time RT-PCR assays. The clinical specimen was further inoculated in SPF CEE and onto Caco-2 cells for virus isolation.

The presence of influenza A and the H1N1pdm were detected on AF and Caco-2 CS by real time RT-PCRs. A multiplex RT-PCR specific for subtype determination was further used to subtype both AF and CS that unexpectedly resulted to be H1pdmN2. The presence of the H1pdmN2 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/116114/2010. Respiratory pathogens such as bacteria, *M. hyopneumoniae,* PRRSV or PCV2, which are involved in the porcine respiratory

complex, were not observed. Only a *Streptococcus suis* was isolated from the lungs and therefore could be the cause of the fibrinous pleuritis.

Genomic sequencing and phylogenetic analysis

HA and NA sequences were obtained from the clinical specimen and compared with the Influenza sequences available in Genbank, confirming the results of subtype determination obtained by the RT-PCR and real time RT-PCR assays. Full genome sequencing was also obtained from the virus isolate, confirming the results for HA and NA obtained from the original sample.

Phylogenetic analysis showed that all the genes of A/Sw/It/116114/2010, with the exception of neuraminidase (NA), belonged to the H1N1pdm clusters. In particular H1 showed more than 99% homology to H1pdm sequences. The phylogenetic tree of the HA gene shows that A/Sw/It/116114/2010 grouped together with H1N1pdm sequences in cluster 7, as most of the H1N1pdm human isolates in Italy (Potdar et al., 2010; Valli et al., 2010) (figure n.1). By contrast, NA was closely related to H1N2 SIV double reassortant viruses, isolated in Sweden (A/Sw/Sweden/1021/2009) (Bálint et al., 2009) and recently also in Italy (A/Sw/It/58769/2010) with 93,8% and 95,5% homology respectively. In the phylogenetic tree, the NA gene of these two H1N2 SIVs grouped together with A/Sw/It/116114/2010 and was located in the cluster of H3N2 SIVs (figure n.2). This Italian strain was isolated in 2010 from piglets with respiratory lesions of SI, raised on a breeder farm located in North Italy.

Full genome sequencing of A/Sw/It/116114/2010 confirms a very high similarity (from 99-100%) through the seven genes to H1N1pdm circulating in human and animal species. Comparison with the H1N1pdm sequences available in GenBank shows in A/Sw/It/116114/2010 three unique amino-acid (aa) changes respectively in HA

(N260K) (H3 numbering) and NP (A85V and G404S) proteins, not yet associated with any well characterized phenotype marker of influenza viruses. Two other aa changes of interest were also found. The first one in PB2 protein (K340N), frequently co-occurred with the potentially virulent HA-D222G mutation (WHO, 2009), although the latter is absent in A/Sw/It/116114/2010. The second aa change is the E67K substitution in NS2 protein, previously correlated with a possible modification of the protein function in viral nucleocapsid export from the nucleus (Wu et al., 2009). Comparison between NA proteins of A/Sw/It/116114/2010 and A/Sw/It/58769/2010 revealed changes in different phylogenetically important regions (PIRs) (Fanning et al., 2000): PIR-A-Y40C and C42R, PIR-F N339H, PIR-G D346N, PIR-K N401D. Combinations of HA and NA subtypes need to be functional to recognize the same type of sialic acid modifications to allow smooth interplay of the two proteins, which is important for the viral cycle (Wagner et al., 2002).

Complete genome sequences of the novel H1N2 strain were compared to those of A/California/4/2009 H1N1 pdm and A/Sw/It/58769/2010 H1N2 SIV and the amino acid differences were reported in table n.1.

Discussion

The identification of the new H1N2 reassortant virus demonstrates once again the important role played by pigs as mixing vessels for animal and human influenza, providing a place for reassortment and host adaptation to take place. These results show that the introduction of H1N1pdm in the swine population has provided opportunities for reassortment with the risk that the H1N1pdm virus could alter transmissibility and increase virulence. The success of interspecies transmission of influenza virus depends on the viral gene constellation. Successful transmission

between species can follow genetic reassortment with a progeny virus containing a specific gene constellation with the ability to replicate in the new host. The new reassortant virus could have the capability to replicate efficiently in humans, considering its specific gene constellation. This is characterized by seven genes belonging to H1N1pdm, well adapted to humans, and the NA gene closely related to H1N2 SIVs.

The isolation of H1N1pdm from pigs in different countries also raises further concerns about the possible establishment of H1N1pdm in the swine population. This could lead to a scenario where a co-circulation of influenza A viruses within swine that could act as mixing vessels for the reassortment between influenza viruses of both mammal and avian origin, with unknown implications for public health. All these facts could have a great impact in the Northern part of Italy where over 70% of swine and 65% of poultry are raised and several epidemics of avian influenza, H5 and H7 subtypes, occurred in the last ten years (Alexander et al., 2007).

The immune selection is not considered important in pigs due to the continuous availability of susceptible pigs. Previous studies reported that human H1N1 and H3N2 viruses could be frequently transmitted to pigs but fail to persist (Van Reeth, 2007). This could be explained by the fact that virus strains with different antigenic characteristics could be disadvantaged compared to the well-adapted established viruses, which are circulating among the susceptible population. However, some cases, such as the emergence of the H1N2 subtype in UK with a HA closely related to human H1N1 viruses (Brown et al., 1998) and subsequently spread in the European pig population, suggest that the genes of human viruses may persist after reassortment with one or more influenza viruses in pigs and following adaptation to pigs may often be associated with clinical disease. Moreover, the recent emergence

and persistence of reassortant H1N2 strains in Italy since 2003, with a NA closely related to recent H3N2 human viruses (Moreno et al., 2010, pp.73), could be another example supporting these hypotheses.

In the case we are reporting, after the isolation of the reassortant strain the three following groups of animals sent to the slaughterhouse were tested for influenza type A. Nasal swabs, collected from 60 pigs, were tested by M gene real time RT-PCR and resulted negative.

The unusual circulation of H1N1pdm in swineherds worldwide raises concern about the generation and transmission to humans of virus reassortants with key molecular determinants of pathogenicity lacking in the current H1N1pdm (Neumann et al., 2009).

Recently, swine influenza surveillance in Hong Kong has given raise to the isolation of a reassortant virus containing seven genes derived from SIVs circulating in China, and the H1N1pdm NA gene (Vijaykrishna et al., 2010).

These independent isolation events underline the importance of surveillance and complete genetic characterization of influenza viruses isolated in the swine population worldwide.

Table n.1 **-** Amino acid substitutions in HA and internal proteins of H1N1pdm viruses A/California/04/2009 - A/Sw/It/290272/09 and H1N2 reassortant A/Sw/It/116114/10.

Gene segment	Residue number	A/California/04/2009 (H1N1pdm)	A/Sw/It/290272/09 (H1N1pdm)	A/Sw/It/116114/10 (H1N2 reassortant virus)
PB2	**340**	K	K	**N**
	405	S	T	**S**
PB1	**386**	K	R	**K**
	535	I	V	**I**
	728	V	V	**I**
	736	K	G	**K**
PA	**14**	I	V	**V**
	224	P	S	**S**
	256	K	Q	**K**
HA	**100**	P	S	**S**
	214	T	A	**A**
	220	S	T	**T**
	277	N	N	**K**
	391	E	K	**E**
	544	V	V	**I**
NP	**85**	A	A	**V**
	100	V	I	**I**
	101	D	N	**D**
	375	D	D	**N**
	404	G	G	**S**
NS1	**123**	I	V	**V**
NS2	**67**	E	E	**K**

Figure n.1 - Phylogenetic relationship of the HA gene of the swine reassortant isolate (A/sw/Italy/116114/2010 H1N2) with database retrieved pandemic H1N1/2009 viruses. The unrooted tree was created by maximum parsimony method and bootstrapped with 500 replicates. Only bootstrap values higher than 50% are shown.

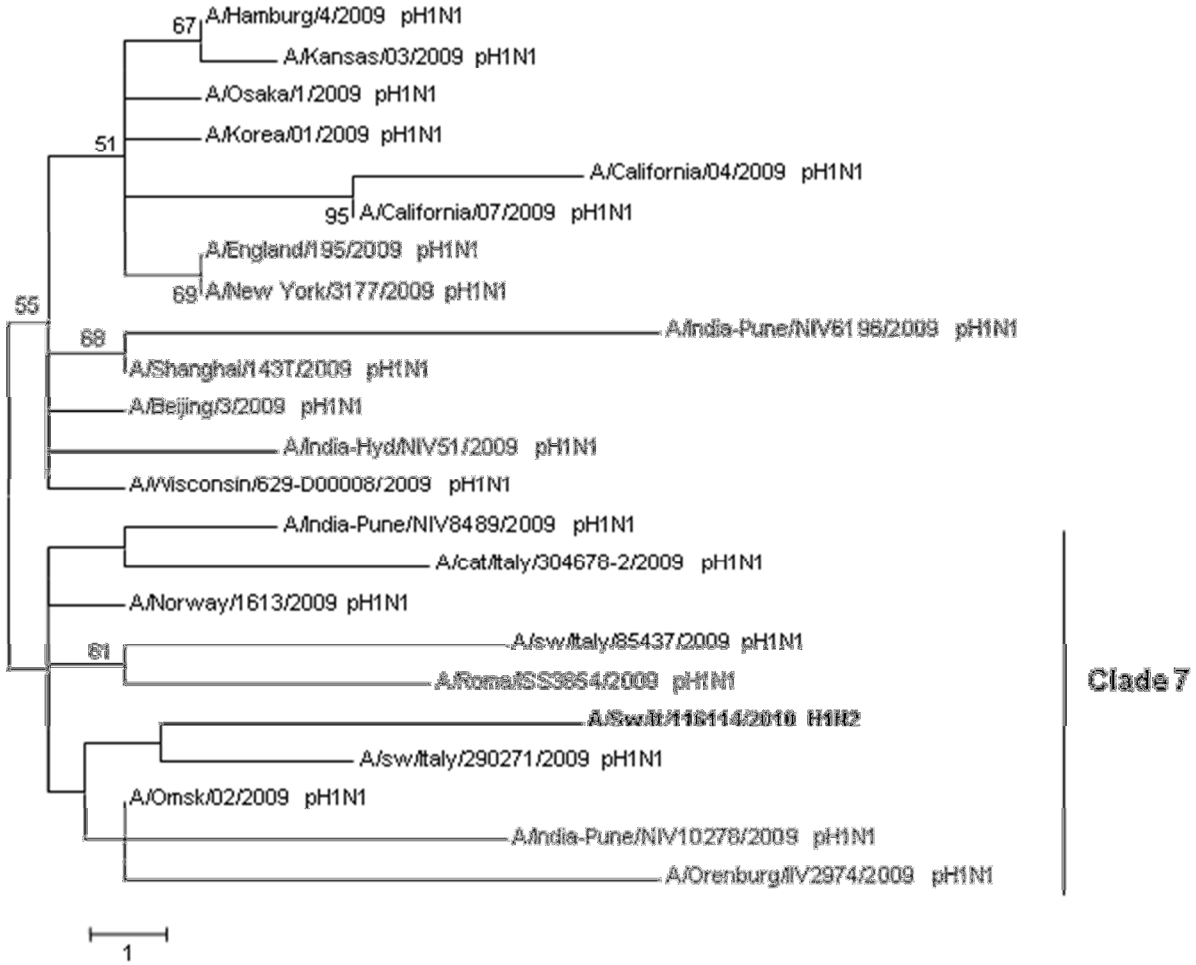
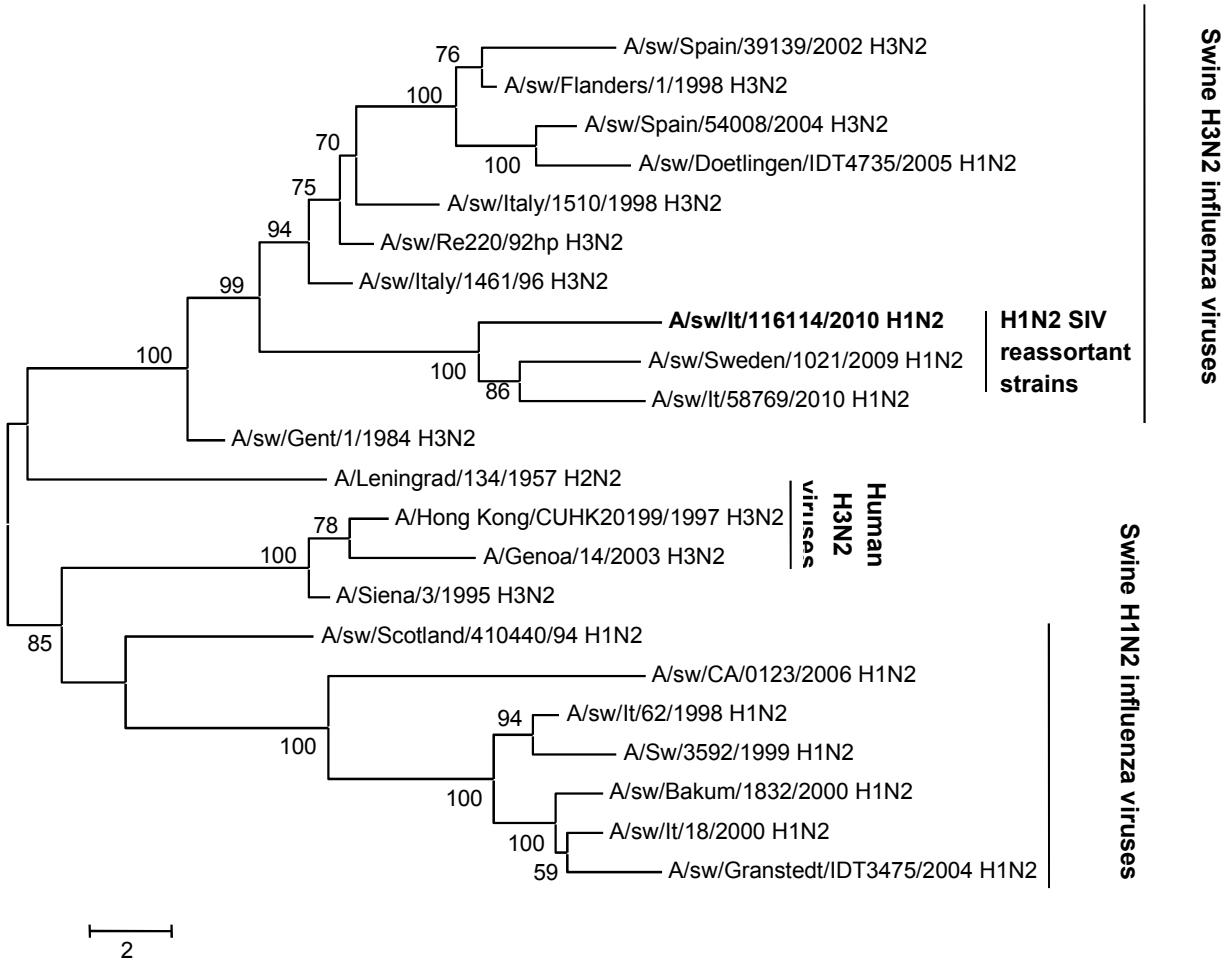


Figure n.2 - Phylogenetic relationship of the NA gene of the swine reassortant H1N2 Italian isolate (A/sw/Italy/116114/2010 H1N2) with other Italian SIV viruses and database retrieved swine H1N2 and H3N2 and human H3N2 influenza viruses. The unrooted tree was generated as described in the legend of figure n.1



Veterinary Microbiology 149 (2011) 472–477

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus

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ARTICLE INFO

Article history:
Received 21 September 2010
Received in revised form 18 November 2010
Accepted 7 December 2010

Keywords:
Swine influenza
Reassortant H1N2 strain
Pandemic H1N1/2009 virus
Pig
Italy

ABSTRACT

Swine influenza monitoring programs have been in place in Italy since the 1990s and from 2009 testing for the pandemic H1N1/2009 virus (H1N1pdm) was also performed on all the swine samples positive for type A influenza. This paper reports the isolation and genomic characterization of a novel H1N2 swine influenza reassortant strain from pigs in Italy that was derived from the H1N1pdm virus.

In May 2010, mild respiratory symptoms were observed in around 10% of the pigs raised on a fattening farm in Italy. Lung homogenate taken from one pig showing respiratory distress was tested for influenza type A and H1N1pdm by two real time RT-PCR assays. Virus isolation was achieved by inoculation of lung homogenate into specific pathogen free chicken embryonated eggs (SPF CEE) and applied onto Caco-2 cells and then the complete genome sequencing and phylogenetic analysis was performed from the CEE isolate.

The lung homogenate proved to be positive for both influenza type A (gene M) and H1N1pdm real time RT-PCRs. Virus isolation (A/Sw/It/116114/2010) was obtained from both SPF CEE and Caco-2 cells. Phylogenetic analysis showed that all of the genes of A/Sw/It/116114/2010, with the exception of neuraminidase (NA), belonged to the H1N1pdm cluster. The NA was closely related to two H1N2 double reassortant swine influenza viruses (SIVs), previously isolated in Sweden and Italy. NA sequences for these three strains were clustering with H3N2 SIVs. The emergence of a novel reassortant H1N2 strain derived from H1N1pdm in swine in Italy raises further concerns about whether these viruses will become established in pigs. The new reassortant not only represents a pandemic (zoonotic) threat but also has unknown livestock implications for the European swine industry.

1. Introduction

Influenza type A viruses are currently circulating in the European pig population with high prevalence of H1N1, H1N2 and H3N2 subtypes. In April 2009 a new virus, identified as pandemic H1N1/2009 strain of influenza A (H1N1pdm), was reported to have caused a human influenza outbreak in Mexico (Centers for Disease Control and Prevention, 2009a, pp. 400–402). By June 2009 the H1N1pdm had rapidly spread among humans, leading the WHO to the declaration of the first influenza pandemic of the 21st century (WHO, 2009c, phase of pandemic alert). The virus is a quadruple reassortant and contains mostly genes from the triple reassortant H1N1 viruses found in

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doi:10.1016/j.vetmic.2010.12.011

Study n. 4

Genomic characterization and evolutionary analysis of H1N2 swine influenza viruses in Italy

Introduction

Influenza A viruses have a wide range of hosts and have been isolated from avian species and mammals, including humans and pigs. In the swine population, three subtypes (H1N1, H1N2, and H3N2) are currently diffused worldwide. In Europe in recent years, the epidemiology of swine influenza viruses (SIVs) has considerably changed. The H1N1 viruses now prevalent in European countries are antigenically distinct from classical H1N1 strains apparently stemming 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 have been human-avian reassortants possessing six internal genes from the avian-like H1N1 viruses and HA and NA genes from the previously isolated human-like H3N2 viruses (Castrucci et al., 1993). The latest detected subtype is H1N2, which was introduced into the swine population in Europe in two different times. H1N2 SIVs were first isolated in France in 1987 and 1988 and were the result of a reassortment between avian-like H1N1 SIVs and human H3N2 viruses (Gourreau et al., 1994). These strains did not spread beyond their farms of origin. The H1N2 SIVs currently circulating in Europe derive from those isolated in Great Britain in 1994 (Brown et al., 1995) that subsequently spread to the swine population of continental Europe (Marozin et al., 2002) and were first detected in Italy in 1998. These 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). Recently, global and local molecular clock concept in a maximum likelihood framework were used to confirm that NA, HA and other internal protein

genes had been introduced to the European swine H1N2 lineage at the 1970s, early 1980s and late 1980s, respectively through reassortments (Lam et al., 2008).

Genetic reassortment between different influenza subtypes is considered one of the generating mechanisms for novel virus strains with pandemic potential for the human population. Swine were speculated as a reassortment vessel for human and avian viruses and as a reservoir for viruses with the potential to produce pandemics in the human population. This might be because pigs possess receptors for both the avian and the human influenza virus types (Ito et al., 1998; Thacker and Janke, 2008).

Investigation of the site-by-site positive selection pressure will be also performed by estimation of rates of nonsynonymous (dN) and synonymous (dS) substitutions in all genes characterized, in order to identify the main mutations allowing viral immune-escape. 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), yet few data regarding recently isolated H1N2 viruses are available. In order to better understand the epidemiology and molecular evolution of H1N2 SIVs in Italy, we investigated the genomic characterization of the 53 Italian H1N2 strains isolated from 1998 to 2012. Phylogenetic analysis was carried out in comparison with influenza viruses (IV) isolated from swine, humans and avian species, whose sequences were retrieved from GenBank. The presence of amino acid mutations in terms of drug-resistance, determinant of virulence and host species were also investigated. Furthermore, we reconstructed the evolutionary dynamics of influenza A viruses of human and swine origin using a Bayesian approach and the Markov chain Monte Carlo algorithm. The last part of the study was the investigation of the site by site positive selection pressures by estimation of rates of dN and dS

substitutions in the HA and NA genes characterised to identify the main mutations allowing viral immune-escape.

Materials and methods

Virus isolation and subtype determination

Nasal swabs or lungs were collected from pigs showing clinical signs and/or lesions related to swine influenza and tested for influenza A by a real-time RT-PCR performed as described by Spackman *et al.* (2002). For virus isolation, positive samples were then inoculated onto Madin-Darby canine kidney (MDCK) and CACO-2cells and through the allantoic sac route of 9-11 day old SPF chicken embryonated eggs (CEE). The cell culture supernatant and allantoic fluid were tested by haemagglutination assay (HA) using chicken erythrocytes using the standard procedure (Swine influenza, 2010). The presence of influenza type A was detected using a double antibody sandwich ELISA (NPA-ELISA) with an anti-NPA Mab (ATCC n. HB65 H16-L10-4R5) carried out using the Siebinga and de Boer method (1998). We then determined the subtype of the isolates with two multiplex RT-PCR assays (Chiapponi et al., 2003). Data of the strains analysed in this study such as production phases, gross lesions, presence of other pathogens and genes sequenced of are reported in Table 1.

Genome sequencing

Viral RNA was extracted from AF and CS using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, purified using QIAamp® ViralRNA Mini Kit (Qiagen, Hilden, Germany) and amplified by OneStep RT-PCR Kit (Qiagen, Hilden, Germany) (Bragstad et al., 2005).

The full length of the HA and NA and the internal genes were amplified using universal primers as described by Hoffman et al. (2001). Amplified products were then separated onto agarose gel and purified with Qiaquick® gel extraction kit (Qiagen, Inc, Valencia, CA, USA). Sequencing reactions were performed using BigDye® Terminator Cycle Sequencing kit v1.1 (Applied Byosistems, Foster City, CA, USA). Both strands of the amplicons were sequenced with 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. Distance-based phylogenetic trees were generated using the MEGA5 software (Tamura et al., 2011).

Phylogenetic analysis and dataset preparation

The phylogenetic trees were constructed with the Neighbour-joining method using the kimura-two-parameter model. The results were verified using maximum likelihood and maximum parsimony analysis, which showed similar topologies. Gene sequences of the Italian strains were compared with swine, avian and human influenza viruses which sequences were retrieved from the Influenza Virus Resource at the National Center for the Biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/).

For evolutionary analysis, two data sets were prepared taking into account only full-length sequences; one for the H1 gene with 120 HA nucleotide sequences and one

for the N2 gene with 161 NA sequences. Each data set was aligned by ClustalW first and further adjusted manually in BioEdit (Hall, 1999).

Phylogenetic inference, estimation of nucleotide substitution rate and times to common ancestors

For the HA and NA genes, distance-based phylogenetic trees were constructed with Maximum Likelihood (ML) method with the general time-reversible (GTR) model of base substitution using PhyML v.3.0 (Guindon et al., 2010) and MEGA5 software (Tamura et al, 2011). The results were verified using neighbor-joining and maximum parsimony analysis, which showed similar topologies. For each data set, the best-fit model of nucleotide substitution was determined using the jModelTest v.0.1.1 (Posada et al, 2008). All the models were compared using two different informatory criteria: the AIC (Akaike, 1973) and the BIC (Schwarz, 1978). For both of the HA and NA genes, the favoured models were closely related to the general GTR + Gamma + Inv model. Four substitution rate categories were used with the gamma distribution parameter estimated to account for variable substitution rates among sites.

Rates of molecular evolution (i.e. nucleotide substitutions per site per year) and the time of the most recent common ancestor (tMRCA) were estimated for the HA and NA genes using the Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in BEAST package v. 1.6.1 (Drummond and Rambaut, 2007). The marginal likelihoods of two different clock models, strict clock and relaxed uncorrelated lognormal clock (UCLD), were compared using a Bayes factor test for best fit (Drummond et al., 2006; Suchard et al., 2001). Both datasets were analysed using the GTR+G+Γ4 model of nucleotide substitution.

This test showed that for the two genes the UCLD model was the best fit for the sequence data. The UCLD model was further tested with different demographic models (constant population size, exponential growth, logistic growth and Bayesian skyline coalescent model). Convergence was assessed by effective sample sizes (ESS) values higher than 200 and 10% was discarded as burn-in. Uncertainty in parameter estimates was evaluated in the 95% highest posterior density (HPD95%) interval. The Bayes Factor was used to select the model that better fits to the data. The out-group sequences were not included in the BEAST analyses. Finally, Maximum Clade Credibility trees (MCCT) were estimated from the posterior distribution of trees generated by BEAST using the Tree Annotator software v.1.6.1 after the removal of an appropriate burn-in (10% of the samples). MCCT were visualized using the Fig-Tree software v 1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree/), which allowed estimating the tMRCA of each individual node on the trees.

Compilation of data sets and analysis of selection pressures

In addition to the sequences analysed in this study, data were also obtained from the Influenza Virus Resource database. Five data sets were compiled: 1- European SIVs H1 avian-like; 2- European SIVs H1 human-like; 3- European SIVs N2; 4- Human influenza viruses N2; 5- Recent Italian SIVs N2. In order to determine the selection pressures of the HA and NA genes, we estimated the rates of nonsynonymous (dN) and synonymous (dS) substitutions per site (ratio dN/dS) for each data set. The single likelihood ancestor counting (SLAC) and the fixed effects likelihood (FEL) methods available at the Datamonkey online version of the Hy-Phy package (http://www.datamonkey.org) were used (Kosakovsky et al., 2005a, 2005b). A best

fitting model of the nucleotide substitution was estimated for each data set according to the Akaike's Information Criterion. The ratio dN/dS was calculated using a codon model obtained by crossing MG94 and the best nucleotide model (Muse and Gaut, 1994).

Three-dimensional macromolecular structure (3D-MMS)

The prediction of the three dimensional macromolecular structure (3D-MMS) of the HA proteins was performed using the sequence-homology method based on sequences and structures released by the protein data bank (PDB) and visualized by Cn3D v4.3 software (Wang et al., 2000).

Nucleotide sequence accession numbers

The GenBank numbers assigned to the gene sequences determined in this study are listed in Table 1.

Results

Virus Isolation and subtype determination

Virus isolation was demonstrated on allantoic fluid and/or MDCK and CACO-2 cell culture supernatant through haemagglutination activity performed using the HA assay and influenza A antigen detection with NPA-ELISA. A multiplex RT-PCR, specific for HA and NA, was then used to subtype all the isolated strains. The complete HA and NA genes of fifty three Italian H1N2 strains isolated in the period 1998-2012 were sequenced and better investigated. Out of these, twenty-six H1N2 strains were completely sequenced in order to analyse the phylogenetic characteristics of the internal genes.

For the evolutionary analysis, we included the complete HA and NA genes of the fifty-three Italian H1N2 field strains used in this study.

Data regarding year of isolation, geographical origin, production phase and genes sequenced of these strains are reported in Table 1.

Phylogenetic analysis of Italian H1N2 viruses

The tree of the complete HA gene showed that the Italian H1N2 SIVs were closely related to the European human-like H1N2 strains located in a cluster, which was clearly distinct from the avian-like H1N1 SIVs. A distinguishable group formed by the more recent (2003-2010) Italian strains was seen within the European (EU) H1N2 cluster. This branches off from the A/Sw/It/1521/98 and A/sw/It/62/98 strains that were isolated in 1998. It is noteworthy the presence of ten Italian H1N2 strains that were placed in the cluster of the avian-like H1N1 SIVs. These strains were divided in two sub-clusters: one included two Italian strains (A/Sw/It/22530/02 and A/Sw/It/58769/10) that were closely related to two uncommon reassortant strains isolated in Sweden (Balint et al, 2009, Metreveli et al, 2011); the second was formed by the other eight Italian strains isolated recently in 2008-2012 (A/Sw/It/196875/08, A/Sw/It/310411/09, A/Sw/It/195639/10, A/Sw/It/195399/11, A/Sw/It/274551/11, A/Sw/It/329017/11 A/Sw/It/26654/12 and A/Sw/It/107798/12), which were related to the EU H1N1 SIVs. The last strain (A/Sw/It/116114/10) was a H1N2 reassortant strain derived from 2009 H1N1 pandemic viruses (H1N1pdm) (Moreno et al, 2012) and grouped together with H1N1pdm strains isolated from pig farms in Italy (Moreno et al, 2010) (Figure n.1).

Phylogenetic analysis of the NA gene demonstrated that the Italian H1N2 strains were located in three different clusters. EU H1N2 SIVs and the Italian strains isolated

until 2003 formed the first group. Interestingly, the other cluster included all the Italian strains isolated from 2003 to 2012 except six and were closely related to the recent human H3N2 viruses. BLAST analysis showed that the greatest homology (from 97.1% to 95.1%) was to A/Hong Kong/CUK20199/97 H3N2. Generally, the human H3N2 NA gene formed a seasonal phylogenetic cluster (Bagstrad et al., 2008) and the recent Italian H1N2 viruses clustered with human H3N2 viruses of the season 1997-1998. The last six strains showed an uncommon pattern as a result of an unusual reassortment event with an avian-like H1N1 HA SIVs and a EU H3N2 SIV-like NA. Out of these, four strains (A/sw/It/196875/08, A/sw/It/195639/10, A/sw/It/274551/11 and A/sw/It/317525/11) grouped in a separate cluster together with an Italian H3N2 strain isolated in 1998. The NA gene of these strains was closely related to two reassortant strains isolated in Germany in 2005 (Zell et al., 2008) (figure n.2).

The NA gene of the two remaining strains (A/sw/It/58769/10 and A/sw/It/116114/10) showed the greatest homology with two H1N2 reassortant strains (A/sw/Sweden/1021/09, A/sw/Sweden/9706/2010) isolated in Sweden (Balint et al., 2009; Metreveli et al., 2011) (figure n.2). The reassortment of these H1N2 strains was unusual, with an avian-like H1N1 HA SIVs and a European H3N2 SIV-like NA. The NA gene of these three strains was also closely related to a novel H1N2 SIV reassortant strain derived from the pandemic H1N1/2009 virus recently isolated in Italy (Moreno et al., 2011).

Phylogenetic trees using the nucleotide sequences of the PB1, PB2, PA, NP, M and NS genes available in GenBank were constructed and analysed. These results showed that all the Italian strains were located in each tree, together with the European avian-like SIVs (Figure 3, 4).

We should underline the high correlation observed between the complete genomes of A/sw/It/58769/10, A/sw/Sweden/1021/09 and A/sw/Sweden/9706/10, which are located together in each phylogenetic tree in a clearly differentiated branch within the European avian-like SIV cluster supported by high bootstrap values (Figure 3, 4).

Molecular analysis of Italian H1N2 viruses

To better investigate the differences between the Italian H1N2 SIVs, we aligned the deduced amino acid (aa) sequences of the HA gene human-like of the older (1998-2003) and more recent Italian strains (2003-2012) and the European H1N2 SIVs retrieved in GenBank. Eighteen phylogenetically informative regions (PIRs) (from A to R), previously reported by Fanning *et al.* (1999), were then highlighted. Many of these sites are involved in aspects of viral biology concerned with host specificity. Of these, 16 are located in the subunit HA1 whereas the other two are in the subunit HA2. Comparison between the older and more recent Italian strains did not reveal important aa changes in the reported sites. A deletion of two aa at positions 146-147 in the subunit HA1 were observed in all of the recent isolates and in the two strains isolated in 1998 located in the same cluster.

Furthermore, several PIRs were identified in the NA proteins of the Italian strains (Fanning et al., 2000). Twelve PIRs (from A to L) were previously identified in the N2 neuraminidases. Regions D, F, G, I, K are antigenic sites (Air et al., 1985, Colman et al., 1983). Region C contains a glycosylation site in nearly all the N2 sequenced to date. Regions A, B, E, H, J, and L have reported not to be associated with any function of the N2 protein. Inside these regions, several phylogenetically important aa positions (PIPs) were identified (Fanning et al., 2000). Comparison of the deduced aa NA sequences of both the older and the more recent Italian H1N2 strains was

carried out revealing the presence of aa changes in the PIPs and PIRs (Table 2). Only A/sw/It/58769/10 was excluded as it showed a different NA pattern closely related to H3N2 SIVs.

The close correlation between the internal genes of Italian isolates and European avian-like SIVs was confirmed by the aa analysis, also supported by the key amino acid changes reported by Dunham *et al.* (2009).

The PB1-F2 protein encoded by an alternative ORF of PB1 (Zell et al., 2007) showed that 10/11 Italian strains carried a full length PB1-F2 protein, whereas only one (A/sw/Italy/626-2/06) had a truncated (57aa) protein. Furthermore, five strains isolated in the last two years (A/sw/Italy/70757/09, A/sw/Italy/81226/09, A/sw/Italy/289700/09, A/sw/Italy/320546/09 and A/sw/Italy/58769/10), exhibited the single mutation N66S, which characterized a very low number of SIVs. The analysis of 450 PB1-F2 protein sequences of SIV retrieved from the NCBI Influenza Virus Resource revealed that this mutation is presented in only 1,6% of strains. Moreover, it has been demonstrated that this aa change contribute to increase the virulence of the 1918 H1N1 pandemic and the 1997 H5N1 Hong Kong influenza viruses (Conenello at al., 2007, 2010, Ramakrishnan et al., 2009).

As with the prevalent EU SIVs, amantadine resistance was seen in all the Italian H1N2 strains through the presence of several aa substitutions in the M2 protein (Krumbhloz et al., 2009). Of these, two aa mutations (S31N and R77Q) were present in all the Italian strains.

NS1 protein could vary in length in human and swine influenza viruses (Parvin et al., 1983); however an apparent selection pressure leads to the maintenance of an NS1 protein of 230 aa in length in bird and in mammalian isolates of recent avian origin. All the isolates except three recent strains (A/sw/It/626-2/06, A/sw/It/198260/08 and

A/sw/It/70752/09) have a full-length protein; the other three isolates have a 217aa long truncated protein that was previously reported in two avian influenza strains (Suarez and Perdue, 1998).

Nucleotide substitution rates and times to common ancestors

Based on the results of the Bayes factor, the model that better fits to the data sets was the uncorrelated lognormal relaxed molecular clock with Bayesian skyline coalescent demographic model and the GTR+G+Γ_4 model of nucleotide substitution. The highest rate of evolution was observed in the HA segment ($4{,}09 \times 10^{3}$ substitutions/site/year; 3,29- 4,90x 10^{3} 95%HPD) whereas in the NA was $3{,}74 \times 10^{3}$ ($3{,}32\text{-}4{,}17 \times 10^{3}$ 95%HPD). Based on these evolutionary rates, our tMRCA estimations suggest that an European H1N2 precursor acquired in a first reassortment event the NA gene from an old human H3N2 IVs in 1978 (1975-1982 95%HPD) and after the HA gene from the human H1N1 IVs in 1986 (1983-1989 95%HPD). These two reassortment events would give rise to the EU H1N2 SIVs and the older Italian strains, which were closely related and originated from the same common ancestor. Furthermore, our analysis evidence other three different reassortment events (named 3, 4 and 5) between circulating swine and human influenza viruses occurred at different times: 3- The introduction of a NA gene deriving from the EU H3N2 SIVs in 1987 (1985-1989 95%HPD); 4- The introduction of a H1 avian-like gene typical of the EU H1N1 SIVs in 1996 (1994-1998 95%HPD); 5- The introduction of a NA from the human H3N2 of the later 90's in 2000 (1999-2002 95%HPD). An scheme of the reassortment events leading to the H1N2 SIVs currently circulating in Europe are reported in figure n. 5. Molecular clocks of the HA and NA genes are reported in figures n. 6 and 7.

In addition, it is noteworthy the presence of a separate cluster within the EU H1N2 SIVs in the HA tree formed by the more recent Italian strains having a H1 human-like and an Italian strain of 1998 (A/sw/62/1998). This suggested a common ancestor circulating around 1997 (1996-1998 95%HPD) (Figure n.6).

This separate cluster was characterised by an interesting deletion of two aa at positions 146 and 147 equivalent to 133 and 133A in H3 numbering (Ha et al., 2002). The predicted 3D-MMS of the HA protein showed that these positions are located in the membrane-distal globular portion of the molecule within the receptor-binding subdomain (Ha et al., 2002). Three secondary structure elements: the 190 helix (from R190 to R198), the 130 loop (from R135 to R138) and the 220 loop (from R221 to R228) form the sides of each site, with the base made up of the conserved residues Y108, W167, H197 and Y209 (Y98, W153, H183 and Y195 in H3 numbering) (Skehel and Wiley, 2000). The two aa deletions observed in the Italian strains are located near the 130 loop (figure n.8).

Selection pressure analysis

Five datasets were prepared to perform the selection pressure analysis: H1 avian-like of European H1N1 SIVs; H1 human-like of European H1N2 SIVs; N2 of European H3N2 and H1N2 SIVs; N2 of recent human H3N2 IVs; N2 of the recent Italian H1N2 SIVs closely related to the recent human H3N2.

The analysis of selection pressures revealed that most codons were subject to purifying selection. For each dataset, values of mean dN/dS ratio (ω) and individual codons subjected to positive selection are reported in table n.3 The ω values were below 1.0 for all datasets, which shows that there is no detectable positive selection

on the gene as a whole. The ω of the N2 human dataset was the higher whereas the lower was evidenced for the H1 avian-like dataset.

Site-by-site tests of positive selection helped to identify the specific sites that were not detected by the global positive selection analysis. Results obtained by SLAC and FEL methods are evaluated and the SLAC analysis showed in all datasets fewer sites under positive selection than the FEL analysis (table n.3). Specifically, eight positively selected residues were estimated with FEL in the H1 avian-like dataset and out of these, six (R121, R131, R136, R146, R156 and R223 in H3 numbering) are located in the receptor-binding site. Substitutions in these positions may affect virus interactions with cell surface receptors. In the H1 human-like dataset six codons were identified as subjected to positive selection, of which four (R133, R144, R199 and R256) were located at the receptor-binding site.

The FEL analysis of the human N2 gene showed five sites under positive selection. Although the N2 of the recent Italian strains was highly related to the human N2 gene, no sites were detected to be under positive selection by both SLAC and FEL analysis.

Discussion

The H1N2 subtype was detected for the first time in UK in 1994 and in Italy in 1998. Following the introduction of these H1N2 viruses in pigs, a process of adaptation and establishment of the new lineage in the swine population occurred due to the continuous availability of susceptible animals. Indeed, the number of H1N2 isolations has increased in Italy over the last five years making it one of the most frequently detected subtypes between 2009 and 2010 representing 37% of all the isolations compared to the 35% and 28% of H1N1 and H3N2, respectively. In this study, we

investigated the genomic evolution of 26 H1N2 SIVs in Italy from 1998 to 2010. All samples originated from animals with the typical clinical signs of influenza and no differences in pathology between the older and the more recent Italian isolates was observed. In 2004, no isolates belonged to the H1N2 subtype therefore no sequences were available. Regarding the geographical origin, 75% of the swine industry is concentrated in Northern Italy and of this over 50% is located in Lombardy, with most strains originating from this region.

Phylogenetic analysis revealed interesting findings: 1- a clear difference between NA gene of the older Italian strains (1998-2003) and the more recent ones (2003-2010). Recent Italian strains showed a different NA gene from the contemporary European H1N2 SIVs and grouped with the recent human H3N2 viruses.

2- deletion of two aa at positions 146 and 147 within the HA1 subunit of the recent strains and the two closely related strains isolated in 1998, which appeared, as far as we know, only in the Italian strains. Furthermore, in some recent SIVs isolated in Germany (Zell et al., 2008) deletion of only one aa at position 147 was observed. Interestingly the HA of the strain isolated in 2003 with NA related to the European SIVs did not show these deletions.

3- the presence of the uncommon single aa change (N66S), in the PB1-F2 protein, in five strains isolated from 2009-2010, which is said to have contributed to the increased virulence in the 1918 pandemic and H5N1 influenza viruses (Conenello at al., 2007, 2010, Ramakrishnan et al., 2009). An increased viral pathogenicity of the 1918 virus and the 1997 Hong Kong H5N1 viruses in mice has been reported, yet further investigation is needed to confirm whether this single mutation could also be a contributing factor to the higher virulence of H1N2 SIVs in pigs.

4- the presence of ten H1N2 strains with an H1 avian-like, which is located in the cluster of EU H1N1 SIVs. Similar atypical H1N2 viruses were previously described in Italy (Marozin et al., 2002), in Denmark (Hjulsager et al., 2006) and France (Kuntz-Simon and Madec, 2009). Of these, three were characterised by NA not related to the EU H1N2 SIVs but deriving from the EU H3N2 SIVs. Other strain (A/sw/It/58769/10) possesses the complete genome that is closely related to two uncommon reassortant Swedish strains (Balint et al., 2009; Metreveli et al., 2011). Further surveillance investigations are needed to better understand the possible correlation between the strains isolated in Italy and in Sweden.

These results show that reassortment events involving human influenza viruses occur among pigs in Italy and also demonstrate the important role played by pigs as mixing vessels for animal and human influenza. However, previous studies have reported that human H1N1 and H3N2 viruses could frequently be transmitted to pigs, but fail to persist (Van Reeth, 2007). This fact could be explained by considering that virus strains with different antigenic characteristics may be at a disadvantage compared to well-adapted established viruses, which circulate among the susceptible pig population (Brown, 2000). However, some cases such as the emergence of the H1N2 subtype in the UK, with an HA closely related to human H1N1 viruses (Brown et al., 2000) and subsequently spread in the European pig population, suggest that the genes of human viruses may persist after reassortment with one or more influenza virus in pigs and the following adaptation to pigs may often be associated with clinical disease.

The success of influenza virus interspecies transmission depends on the viral gene constellation. Successful transmission between species can follow genetic reassortment with a progeny virus that contains a specific gene combination with the

ability to replicate in the new host. The emergence and persistence of the recent Italian H1N2 strains here reported, with an NA closely related to the recent H3N2 human viruses, suggested the presence of a particular gene constellation that contributes to efficient replication and successful transmission among pigs. Mitnaul *et al*. (2000) reported that the HA and NA proteins recognized the same molecule (sialic acid) with conflicting activities and a balance of the protein performance was essential to ensure an efficient replication of the virus. Since these recent reassortant strains are characterized also by an HA gene with different features than the circulating European H1N2 strains, a better balanced HA-NA combination able to ensure a more efficient viral replication could be hypothesized. Up to now, these new reassortant strains have apparently been reported only in Italy. However, this could be due to the lack of data from other countries, particularly Eastern Europe. Furthermore, the finding that the NA of the recent Italian strains is closely related to the human H3N2 NA of the 1997-1998 season highlights a six-year period during which these swine isolates were not detected in the Italian pig population.

The isolation of several reassortant strains in Italian pigs also raises further concern on the cross-species transmission of influenza viruses, especially in Northern Italy where over 70% of swine and 65% of poultry production is raised, and where several epidemics of the avian influenza H5 and H7 subtypes have occurred during the last ten years (Alexander, 2007). This could lead to a scenario of a co-circulation of influenza A viruses within swine acting as mixing vessels for the reassortment between influenza viruses of both mammal and avian origin with unknown implications for public health. People working in swine and poultry industry, especially those with intense exposure are at increased risk of zoonotic infection with influenza viruses endemic in these animals and could represent a population of

human hosts in which reassortment and zoonosis can co-occur. An important public health measure may therefore be vaccination of these workers against influenza in order to reduce the risk of co-infections with influenza viruses of different species.

Vaccination of professions at high risk of infection is considered one of the recommendations of the influenza vaccination campaign by many countries, Italy included.

The evolutionary analysis showed that a European H1N2 precursor acquired in a first reassortment event the NA gene from an old human H3N2 IVs in 1978 (1975-1982 95%HPD) and later the HA gene from the human H1N1 IVs in 1986 (1983-1989 95%HPD). The older Italian strains were closely related to the European H1N2 viruses and originated from the same common ancestor. Furthermore the tMRCAs estimated for the recent strains suggest a common ancestor that acquired the NA gene from the recent human H3N2 viruses around 2000 (1998-2002 95%HPD) and with an HA gene closely related to two Italian strains of 1998 (characterised by two aa deletions). It is worth noting the deletion of the two aa at position 146 and 147 in the receptor binding site of the HA protein. To our knowledge, deletion of one aa at position 147 has been observed in 4/285 (1,6%) of the H1 European SIVs available in GenBank whereas the deletion of two aa is presented only in the Italian strains.

These results highlighted the different evolutionary trend of the recent Italian strains compared to circulating European viruses, showing the presence and establishment of reassortant strains involving human viruses in pigs in Italy.

Strains	Year	Prov	Region	Production phases	HA GeneAcc Number	NA GeneAcc Number	Internal genes GeneAcc Number	Gross lesions	Other Pathogens
62	1998	MN	Lombardia-N+	Fattening	HQ709201	HQ709202	PB2 HQ845020, PB1 JF317563 PA HQ709215, NP HQ709205 M HQ845029, NS HQ850080	n.a.	
3592	1999	MN	Lombardia-N	Fattening	HQ660233	HQ658492	PB2 HQ845021, PB1 JF317564 PA HQ709220, NP HQ709206 M HQ845030 , NS HQ850078	n.a.	
18	2000	FC	Emilia Romagna-N	Weaning	HQ709203	HQ709204	PB2 HQ845022, PB1 HQ829648 PA HQ709216, NP HQ709207 M HQ845031 , NS HQ850074	pneumonia with purple areas of consolidation	
22530	2002	PV	Lombardia-N	Weaning	HQ658491	HQ660234	PB2 JN596946, PB1 JN596947 PA JN596944, NP JN624324 M JN596945, NS JN596940	pneumonia with purple areas of consolidation	PRSV
4675	2003	MN	Lombardia-N	Sows	HM996942	HM996957	PB2 JN596938, PB1 JN596937 PA JN596935, NP JN596933 M JN596931, NS JN596940	pneumonia	*P. multocida*, PRRSV, PCV2, *M. hyopneumoniae*
259543	2003	PV	Lombardia-N	Fattening	JN022470	JN022471	PB2 JN596939, PB1 JN596936 PA JN596934, NP JN596942 M JN596932, NS JN596941	pneumonia with purple areas of consolidation, pleuritis, interstitial oedema	*M. hyopneumoniae*
50568	2005	CR	Lombardia-N	Fattening	HQ660235	HQ660236	PB2 HQ845023, PB1 HQ829651 PA HQ709221, NP HQ709208 M HQ845033, NS HQ850079	pneumonia with grey areas of consolidation in the apical and cardiac lobes	
53991	2005	BS	Lombardia-N		submitted	submitted.	n.a..		
203047	2005	BS	Lombardia-N	Fattening	submitted	submitted	n.a..	pneumonia with grey areas of consolidation in the apical and cardiac lobes, pleuritis	PRSV, PCV2
232134	2005	CR	Lombardia-N	Fattening	HQ660249	HQ660250	n.a.	pneumonia with grey areas of consolidation in the apical and cardiac lobes	
233139	2005	PU	Marche- C°	Weaning	HQ660251	HQ660252	PB2 HQ845024, PB1 HQ829650 PA HQ709217, NP HQ709209 M HQ845032, NS HQ850076	pneumonia with purple areas of consolidation	
267010	2005	VE	Veneto-N	Weaning	submitted	submitted		interstitial pneumonia	PCV2
626/2	2006	CR	Lombardia-N	Fattening	HQ658489	HQ658490	PB2 HQ845025, PB1 HQ829652 PA HQ709222, NP HQ709210 M HQ845038, NS HQ850081	pneumonia with grey areas of consolidation in the apical and cardiac lobes	PCV2, *A. pleuropneumoniae 1, P. multocida*
20333	2006	CR	Lombardia-N	Piglet	submitted	submitted	n.a.	pneumonia with areas of consolidation in the cardiac lobes	
114347/1	2006	BG	Lombardia-N	Fattening	HQ658487	HQ660244	M JN596926, NS JN596928	pneumonia with grey areas of consolidation in the apical and cardiac lobes	
226846	2006	MN	Lombardia-N	Fattening	submitted	submitted	n.a.	pneumonia with grey areas of consolidation	PRSV, PCV2
269578	2006	MN	Lombardia-N	Fattening	submitted	submitted	n.a.	bronchitis and pneumonia with areas of consolidation	PRSV

Strains	Year	Prov	Region	Production phases	HA GeneAcc Number	NA GeneAcc Number	Internal genes GeneAcc Number	Gross lesions	Other Pathogens
29141	2008	BS	Lombardia-N	Fattening	submitted	submitted	n.a.	Suppurative broncopneumonia with grey areas of consolidation in the apical lobes	*A. pyogenes*
196875	2008	CN	Piemonte-N	Weaning	submitted	submitted	n.a.	interstitial pneumonia with areas of consolidation in the apical lobe	
198260	2008	BS	Lombardia-N	Piglet	HQ660247	HQ660248	PB2 HQ845026, PB1 HQ829649 PA HQ709218, NP HQ709211 M HQ845037, NS HQ850075	interstitial pneumonia, white necrotic foci in the myocardium	ECMV
59209/2	2009	BS	Lombardia-N	Fattening	HQ660237	HQ658488	n.a.	pneumonia with grey areas of consolidation and pleuritis	*P. multocida*
70757	2009	BS	Lombardia-N	Fattening	HQ660238	HQ660239	PB2 HQ845028, PB1 HQ829653 PA HQ709223, NP HQ709212 M HQ845035, NS HQ850082	pneumonia with grey areas of consolidation and pleuritis	PCV2 , *P. multocida*
81062	2009	BS	Lombardia-N	Piglet	HQ660240	HQ660241	n.a.	pneumonia and fibrinous pleuritis and pericarditis	PRSV, *H. parasuis*
81226	2009	PR	Emilia Romagna-N	Weaning	HQ660242	HQ660243	PB2 HQ845019, PB1 HQ829654 PA HQ709219, NP HQ709213 M HQ845034, NS HQ850083	pneumonia with monolateral purple areas of consolidation	PRSV, *H. parasuis*
191985	2009	LO	Lombardia-N	Fattening	HQ660245	HQ660246	n.a.	pneumonia and fibrinous pleuritis	*A. pleuropneumoniae 1*
274298	2009	BG	Lombardia-N	Fattening	HQ709193	HQ709194	n.a.	pneumonia with purple and grey areas of consolidation, interstitial oedema	PCV2, *M. hyopneumoniae*
289700	2009	BS	Lombardia-N	Weaning	HQ709195	HQ709196	PB1 JF432091	pneumonia and fibrinous pleuritis	PRSV, *P. multocida*
310411	2009	BS	Lombardia-N	Fattening	submitted	submitted	n.a.	interstitial pneumonia	PRSV
320546	2009	MN	Lombardia-N	Fattening	HQ709197	HQ709198	PB2 HQ845027, PB1 JF432092 PA HQ709224, NP HQ709214 M HQ845036, NS HQ850077	interstitial and fibrinous pneumonia	PRSV, PCV2, *P. multocida*
321986	2009	BS	Lombardia-N	Fattening	HQ709199	HQ709200	PB1 JF432093	pneumonia with purple areas of consolidation, fibrinous pleuritis and pericarditis	PRSV, PCV2, *H. parasuis*
38272	2010	BS	Lombardia-N	Weaning	JN596916	JN596917	n.a.	pneumonia with grey areas of consolidation in the apical lobes	PRSV, PCV2
58769	2010	VR	Veneto-N	Piglet	HM771276	HM771275	PB2 HQ168026, PB1 HM771279 PA HM771274, NP HM771278 M HM771277, NS HQ168025	pneumonia with areas of consolidation in the apical lobes, fibrinous pleuritis	*A. pleuropneumoniae 1*
63580	2010	BS	Lombardia-N	Weaning	submitted	submitted	n.a.	poly-serositis and catarrhal enteritis	PRSV, *H. parasuis, M. hyopneumoniae*
76687	2010	CR	Lombardia-N	Weaning	submitted	submitted	n.a.	pneumonia with purple areas of consolidation in the apical and cardiac lobes	PRSV, PCV2
85218	2010	BS	Lombardia-N	Weaning	submitted	submitted	n.a.	interstitial pneumonia	
116114	2010	MN	Lombardia-N	Fattening	CY067662	CY067664	n.a.	pneumonia with red areas of consolidation in the apical lobes, , interlobular oedema, and fibrinous pleuritis	*S. suis*

Strains	Year	Prov	Region	Production phases	HA GeneAcc Number	NA GeneAcc Number	Internal genes GeneAcc Number	Gross lesions	Other Pathogens
118616	2010	PC	Emilia Romagna-N	Weaning	JN596920	JN596921	n.a.	pneumonia with areas of consolidation in the apical lobes	PRSV, PCV2
149992	2010	BS	Lombardia-N	Fattening	JN596922	JN596923	n.a.	pneumonia and fibrinous pleuritis	PRSV, P. multocida
166015	2010	BS	Lombardia-N	Weaning	submitted	submitted	n.a.	suppurative broncopneumonia	*A. pyogenes*
170177	2010	CR	Lombardia-N	Weaning	submitted	submitted	n.a.	pneumonia and fibrinous pleuritis and pericarditis	PRSV, *H. parasuis*
195639	2010	CN	Piemonte-N	Weaning	submitted	submitted	n.a.	pneumonia with red areas of consolidation in the apical lobes, interstitial oedema	PRSV, PCV2
282964	2010	MN	Lombardia-N	Fattening	JN596924	JN596925	n.a.	pneumonia, fibrinous pleuritis, interstitial oedema	PRSV, *P. multocida, M. hyopneumoniae*
254261	2010	BS	Lombardia-N	Piglet	submitted	submitted	n.a.	nasal swabs , monitoring program	
16959	2011	MN	Lombardia-N	Weaning	submitted	submitted	n.a.	nasal swabs , monitoring program	
134110	2011	MN	Lombardia-N	Weaning	submitted	submitted	n.a.	interstitial pneumonia, catarrhal enetritis	*E. coli O159*
186822	2011	RA	Emilia Romagna-N	Fattening	submitted	submitted	n.a.	pneumonia with red areas of consolidation, pleuritis	PCV2
195399	2011	CR	Lombardia-N	Weaning	submitted	submitted	n.a.	nasal swabs , monitoring program	
274551	2011	CN	Piemonte-N	Fattening	submitted	submitted	n.a.	nasal swabs , monitoring program	
308725	2011	CN	Piemonte-N	Weaning	submitted	submitted	n.a.	pneumonia with grey areas of consolidation in the apical lobes	
315977	2011	MO	Emilia Romagna-N	Weaning	submitted	submitted	n.a.	pneumonia with purple areas of consolidation	PRSV, PCV2
317525	2011	CR	Lombardia-N	Fattening	submitted	submitted	n.a.	nasal swabs, monitoring program	
329017	2011	BS	Lombardia-N	Weaning	submitted	submitted	n.a.	nasal swabs, monitoring program	
26654	2012	CR	Lombardia-N	weaning	submitted	submitted	n.a.	pneumonia with red areas of consolidation, fibrinous arthritis	PRSV, *H. parasuis*
107798	2012	BS	Lombardia-N	Weaning	submitted	submitted	n.a.	Interstitial pneumonia	PRSV, *H. parasuis*

[+]Northern Italy, [°]Central Italy

n.a. no sequences or data available

submitted: sequences submitted to GenBank

Table 1 – Data on the Italian strains investigated in this study

A Amino acid residue*	Old Italian H1N2 SIV 1998-2003	Recent Italian H1N2 SIV 2003-2012	PIRs
16	N	S	
43	G/S	N/S	PIRA
46	A	P/S	
81	T	L	PIRB
141	D	N	
143	K	R	PIRC
149	I	V	
199	K	E	PIRD
220	M	K	
248	K	R	
308	E	K	PIRE
328	N	K	
331	I/R	S	PIRF
338	R	L	
344	K	E/D	PIRG
369	D	K	PIRF
385	T	K	PIRI
400	S	K	PIRK
401	N	G	

*Numbering is based on defining the first amino acid of the open reading frame as amino acid 1

Table n. 2- Sites of putative amino acid sequence differences in the NA protein of the old and recent Italian H1N2 SIVs. Only differences located in phylogenetically important positions (PIPs) are reported. Some of these are placed within phylogenetically important regions (PIRs), which are reported in the table. PIPs and PIRs are identified according to Fanning et al. (2000).

Figure n. 1- Phylogenetic tree of the HA gene of Italian H1N2 SIVs with database retrieved swine, avian and human influenza viruses. The unrooted tree was generated with MEGA5 program using Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method. Bootstrap values were calculated on 1000 replicates and only values higher 70% are shown. Viruses used in this study are underlined.

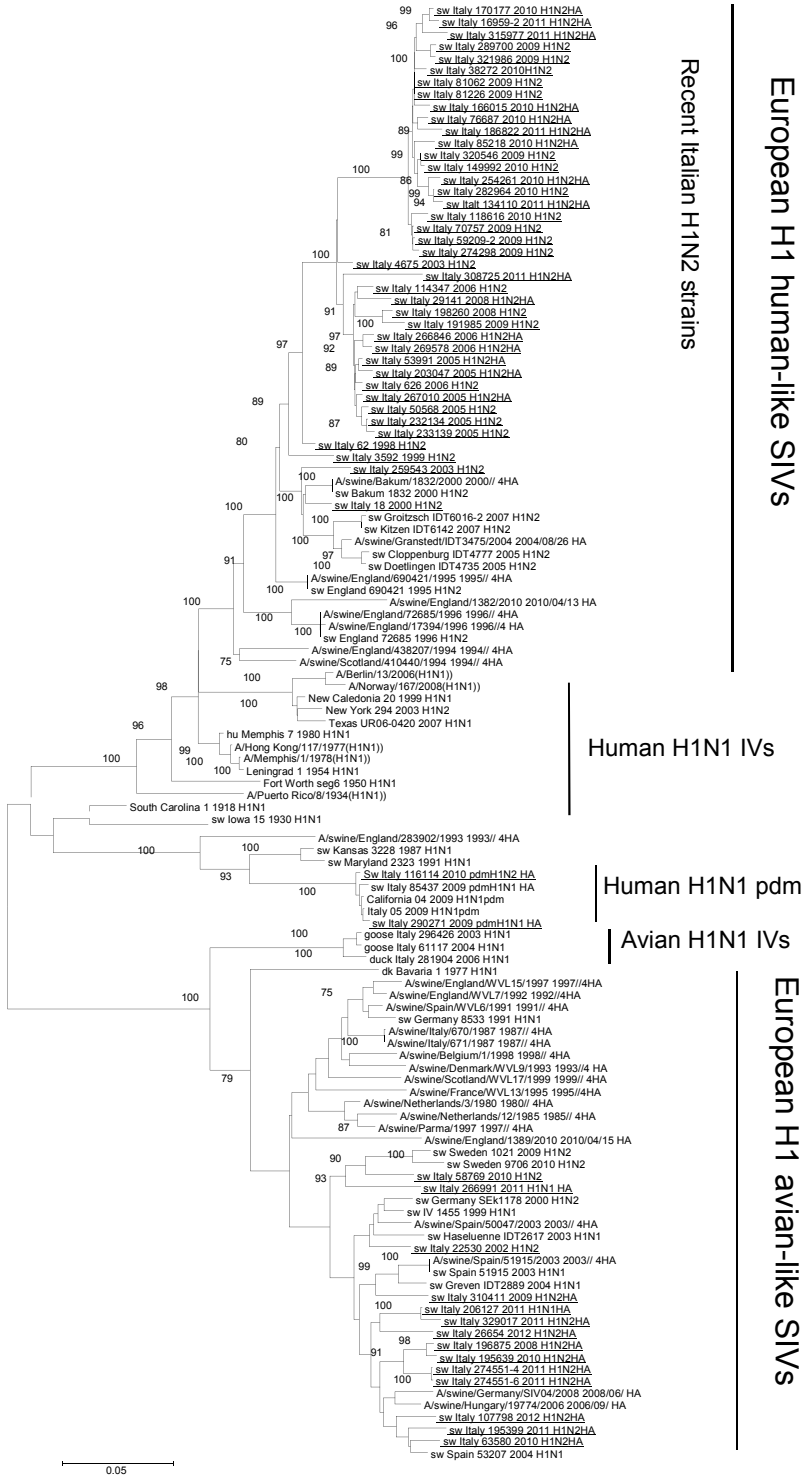
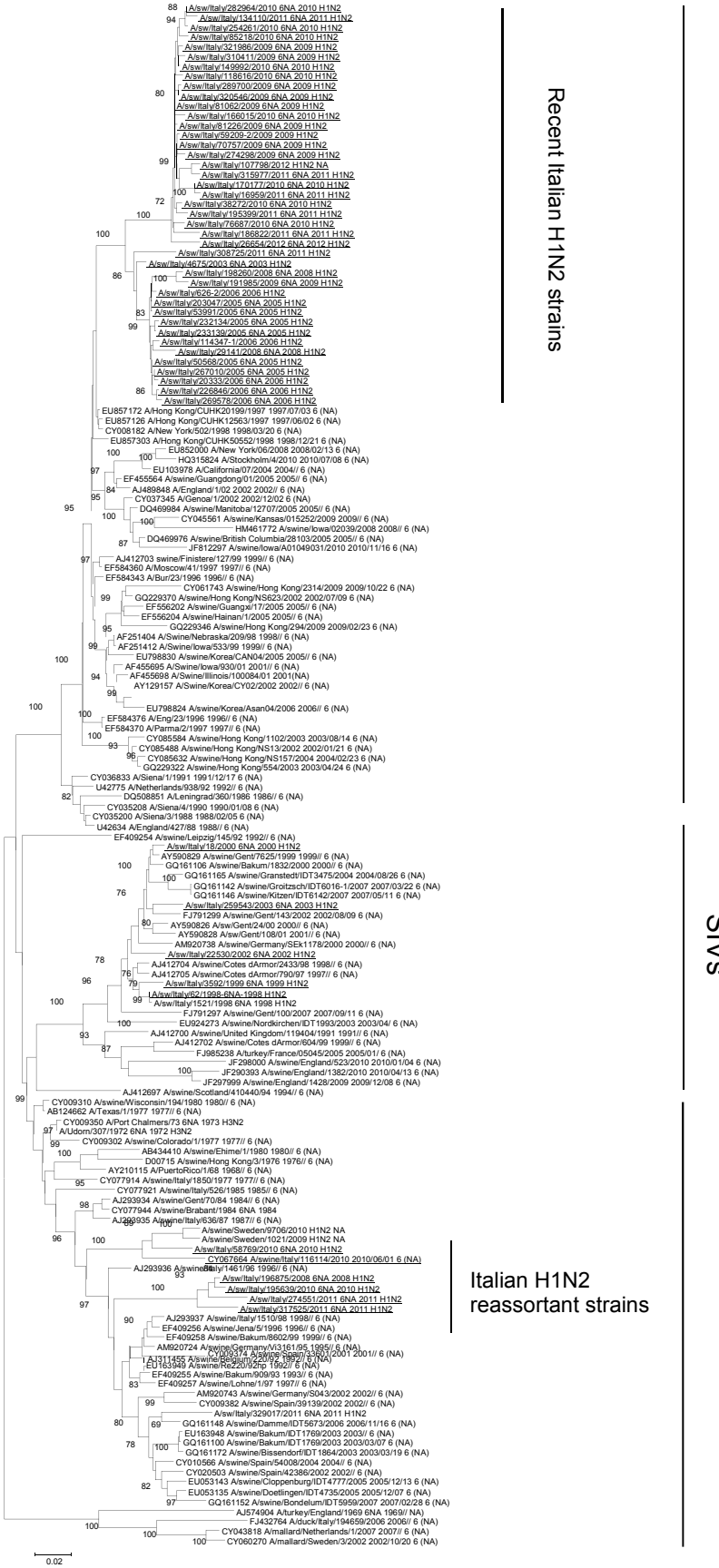
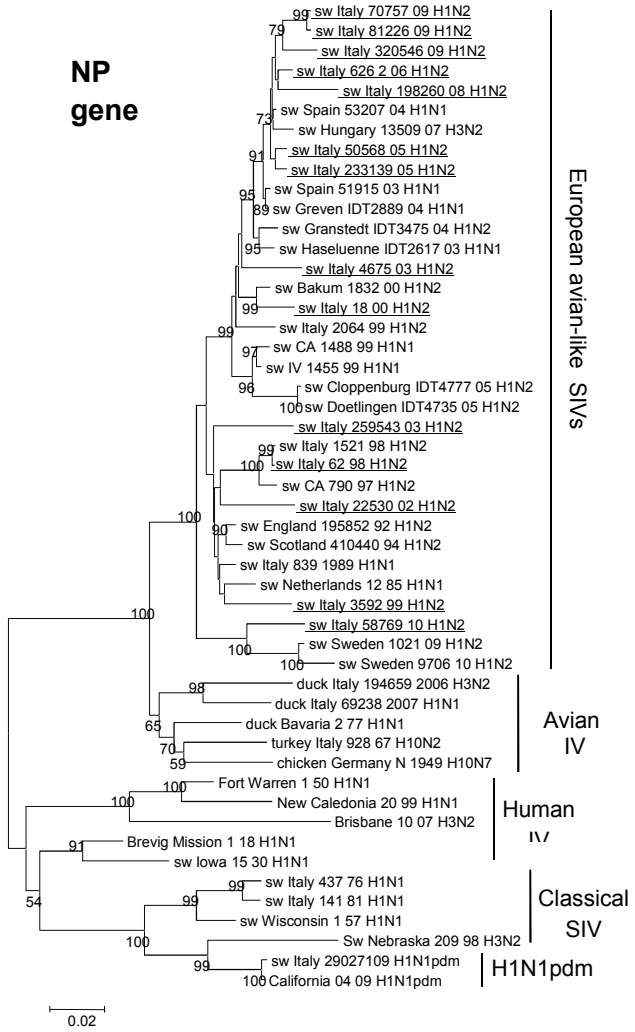
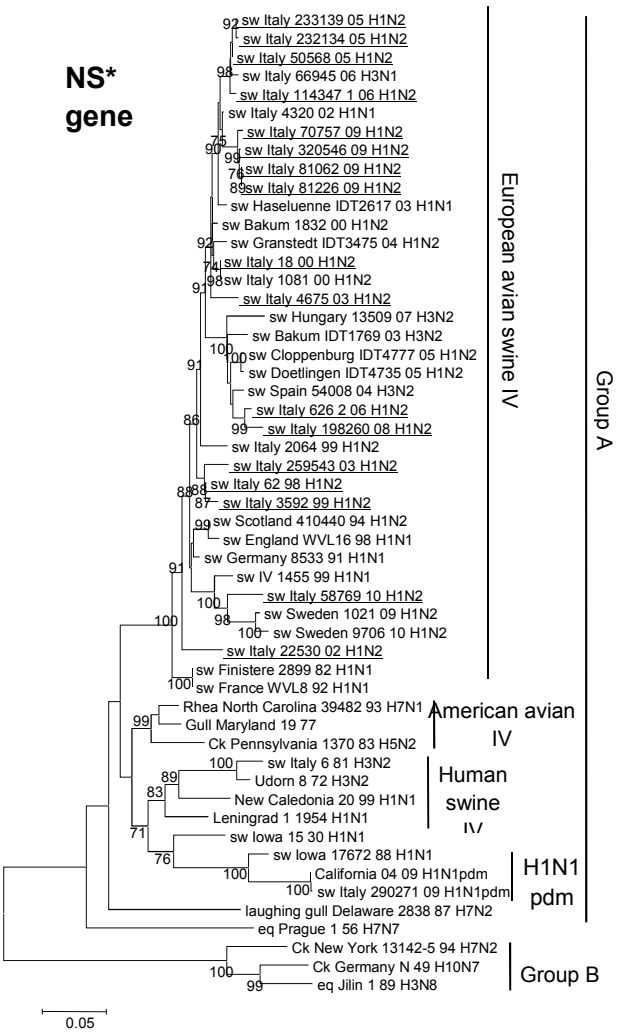


Figure n. 2. Phylogenetic tree of the NA gene of Italian H1N2 SIVs with database retrieved swine, avian and human influenza viruses. The unrooted tree was generated as described in figure n.1.







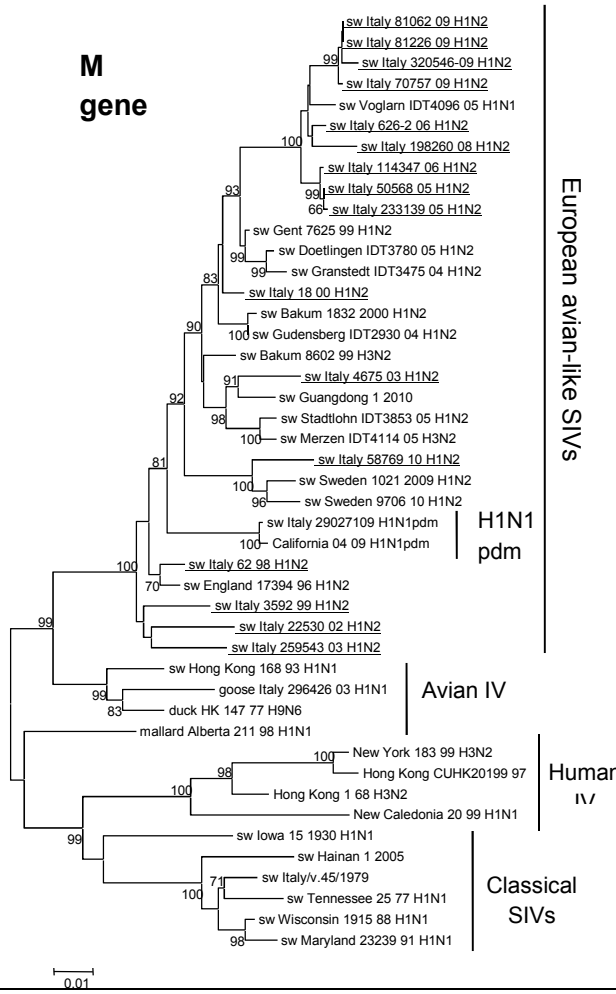
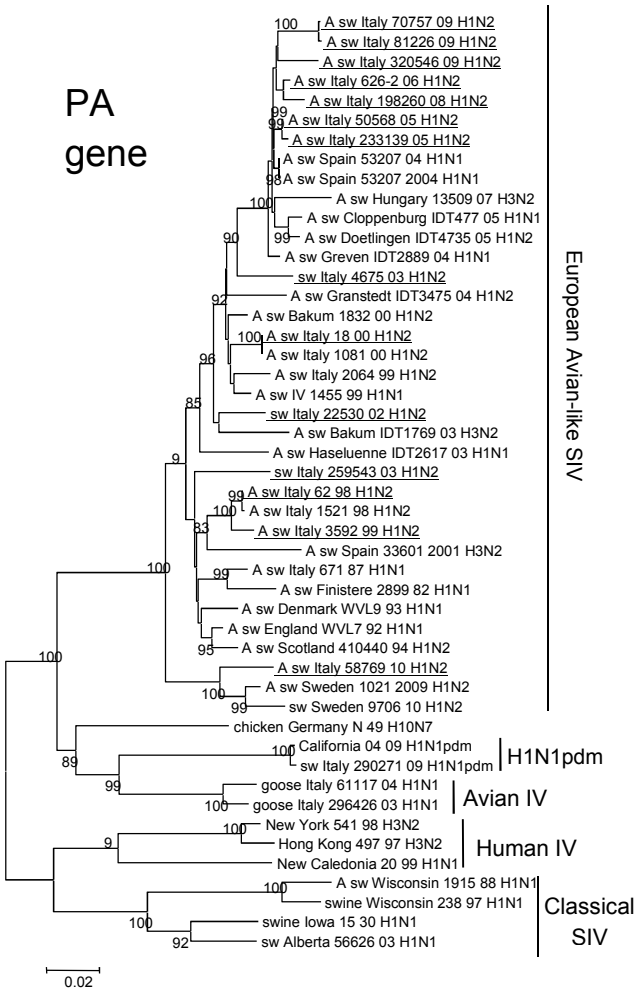
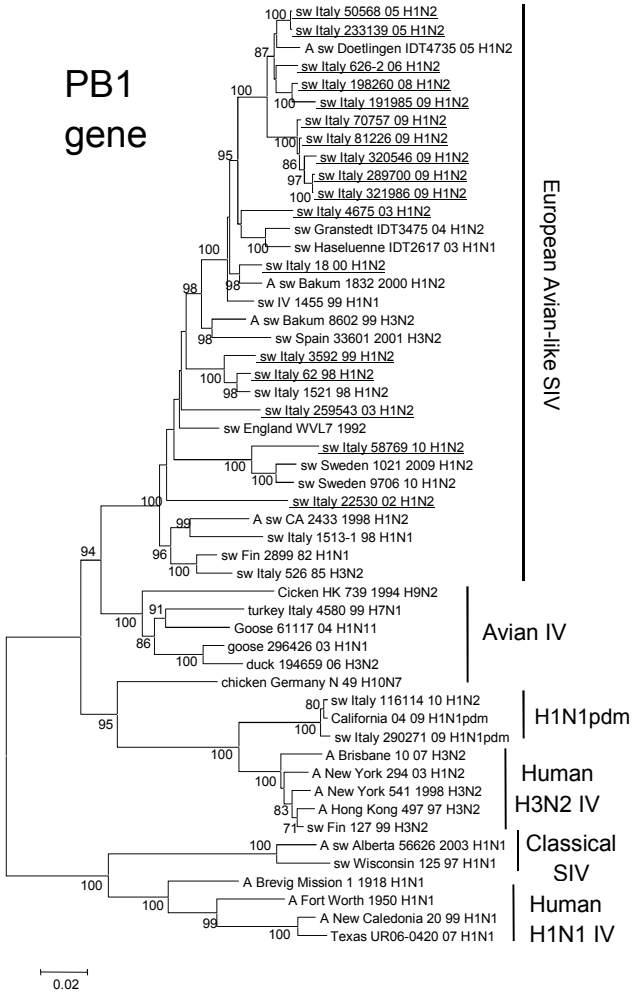


Figure n.3 – Phylogenetic trees of NP, NS and M genes of Italian H1N2 SIVs. The method used is described in figure n.1.

* Clusters previously reported by Suarez and Perdue, 1998.





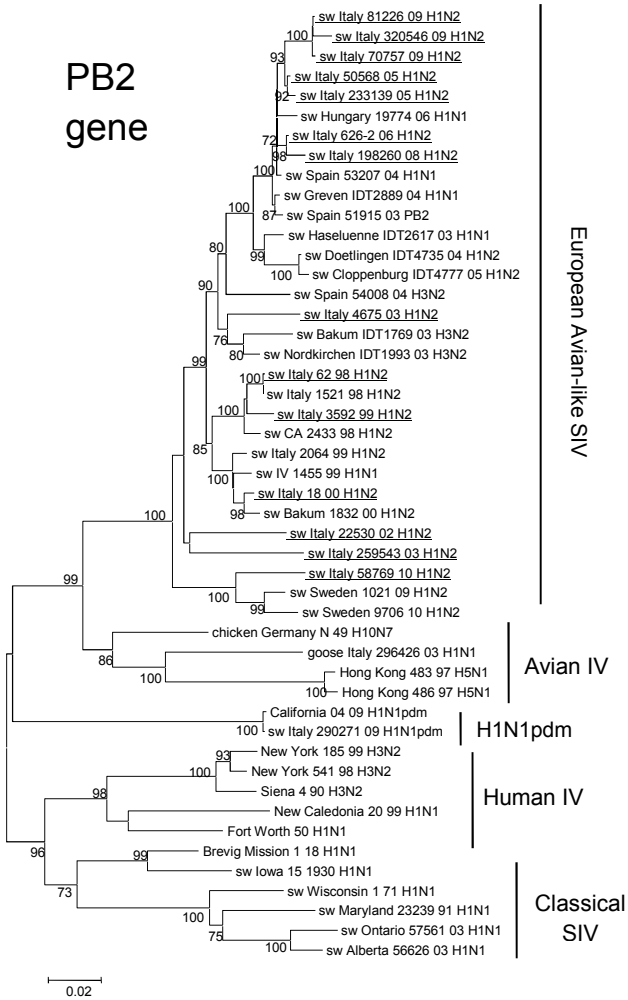


Figure n.4 – Phylogenetic trees of PA, PB1 and PB2 genes of Italian H1N2 SIVs. The method used is described in figure n.1.

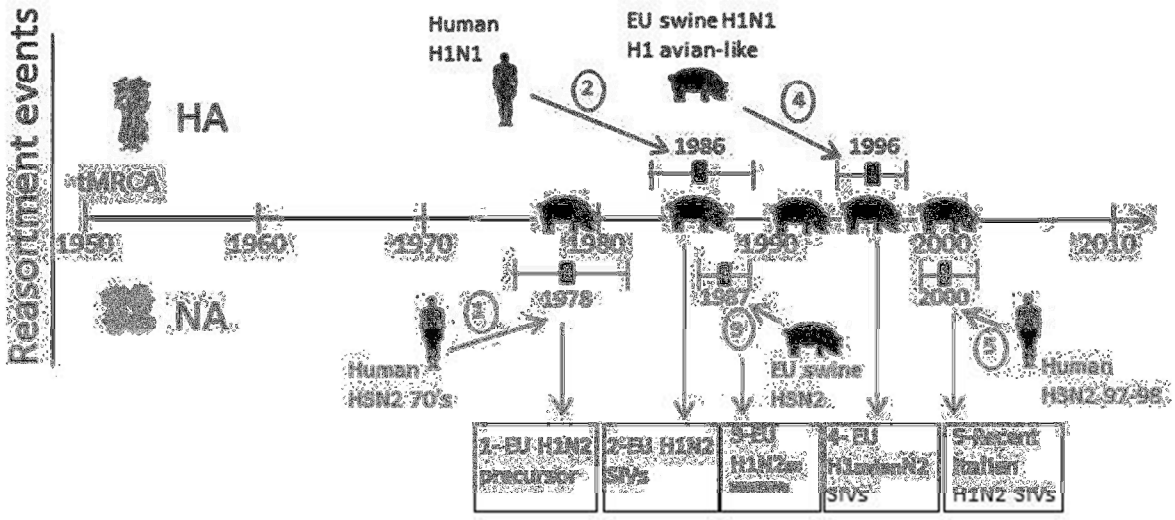
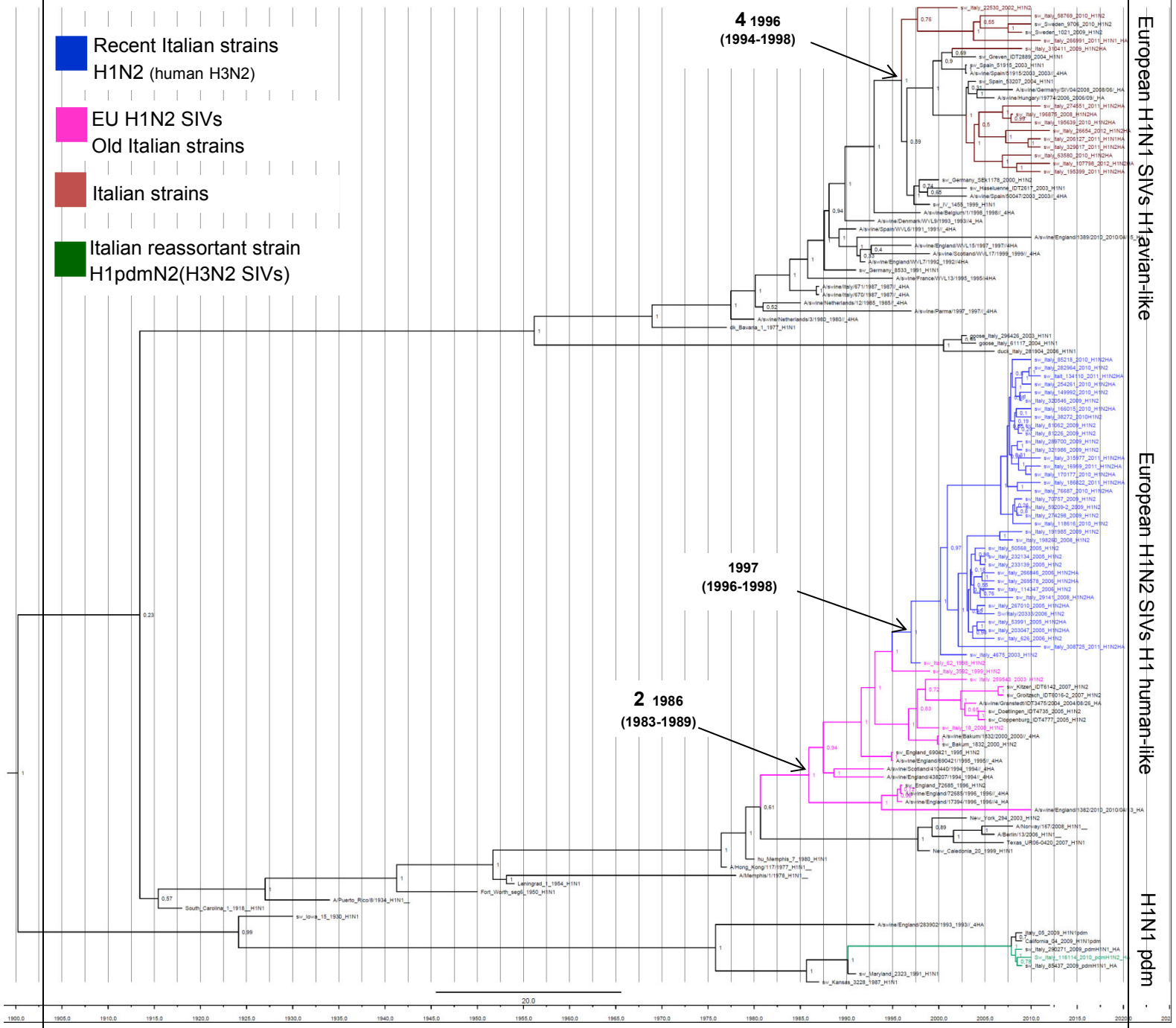


Figure n.5 - Scheme of the reassortment events leading to the H1N2 SIVs currently circulating in Italy

Figure n. 6 and 7 – MCC phylogeny and timing for the HA and NA genes. The most interesting internal nodes, which reconstructed common ancestors with 95% credible intervals, are reported. Numbers from 1 to 5 are referred to the reassortment events described in figure n.5. Branches are coloured to indicate different groups of H1N2 strains. For each node posterior probability are reported.

HA gene



NA gene

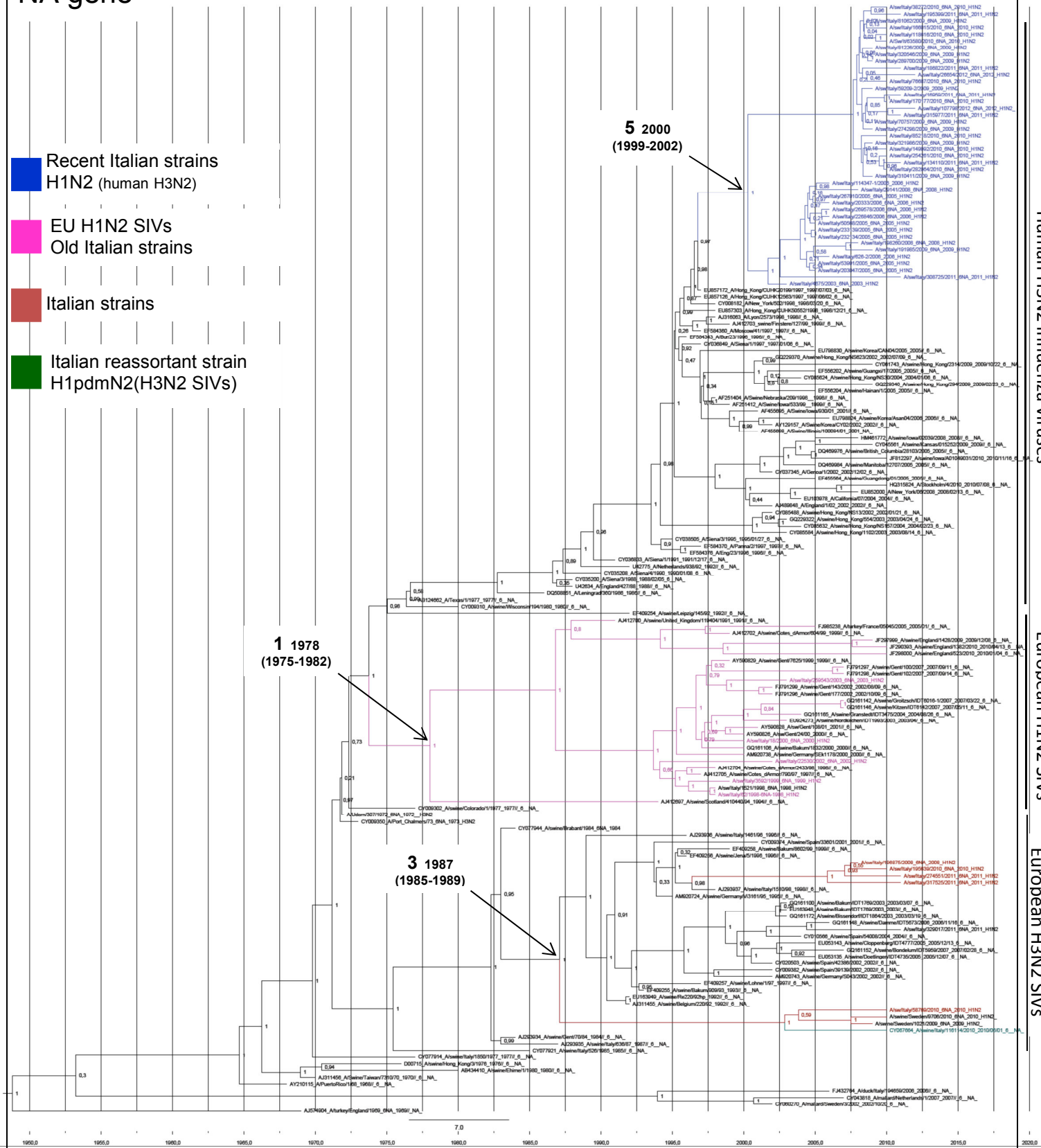
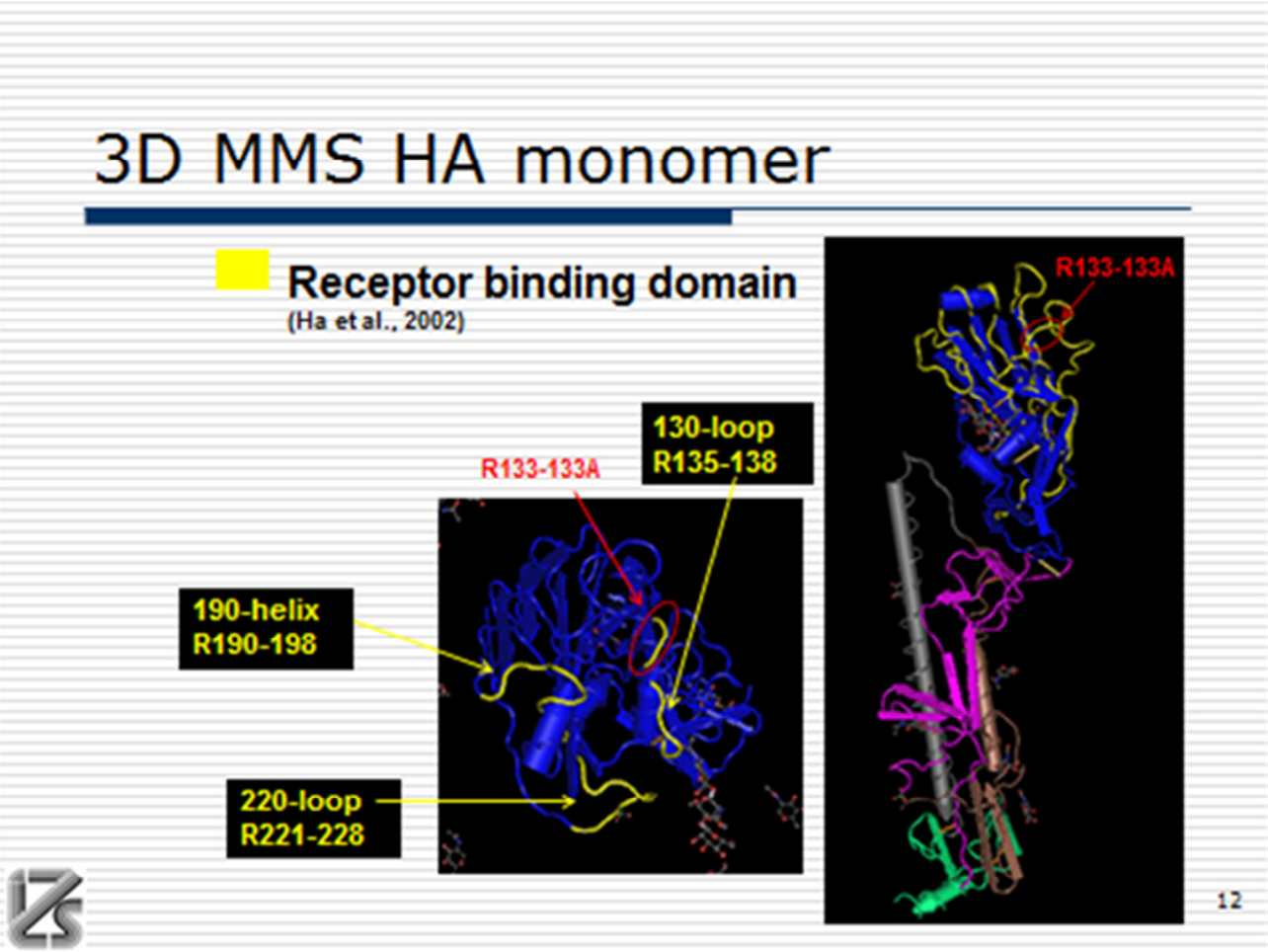

Recent Italian strains
H1N2 (human H3N2)
EU H1N2 SIVs
Old Italian strains
Italian strains
Italian reassortant strain
H1pdmN2(H3N2 SIVs)
5 2000
(1999-2002)
1 1978
(1975-1982)
3 1987
(1985-1989)
Human H3N2 influenza viruses
European H1N2 SIVs
European H3N2 SIVs
7,0
1950,0
1955,0
1960,0
1965,0
1970,0
1975,0
1980,0
1985,0
1990,0
1995,0
2000,0
2005,0
2010,0
2015,0
2020,0

Figure n. 8 - Monomer of the H1 protein (top and lateral view). Amino acid residues R133 and 133A, which are deleted in the recent Italian H1N2 strains, are evidenced in red. Numbering is expressed in H3 numbering. The receptor-binding subdomain located in the globular part of the molecule and the three secondary structures units making up the site are reported in yellow (Ha et al., 2002).



Gene	Number of sequences	Length of alignment (aa)	Mean dN/dS (95%CI)	Positively selected sites P=0.1 (H3numbering)	
				SLAC	FEL
H1 avian like	66	539	0.173 (0.16-0.19)	137, 159, 175, 239	60 (53), 137(124), 147(133), 152(138), 159(145), 175(158), 239(226), 338(323)
H1 human like	65	555	0.225 (0.21-0.24)	102, 271, 550	102(94), 146(133), 158(144), 213(199), 271(256), 550(533A)
N2 swH1N2	84	469	0.185 (0.17-0.20)	0	358, 381, 455
N2 human H3N2	240	459	0.265 (0.24-0.29)	151, 221, 267, 370	43, 151, 221, 339, 370
N2 Recent Italian strains	42	461	0.181 (0.15-0.22)	0	0

Table n. 3 – Positive selected sites and mean dN/dS ratios for each data set. Numbering is based on defining the first amino acid of the open reading frame as amino acid 1; in parenthesis are reported the equivalent in H3 numbering.

Veterinary Microbiology 156 (2012) 265–276

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Genomic characterization of H1N2 swine influenza viruses in Italy

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ARTICLE INFO

Article history:
Received 4 October 2011
Received in revised form 25 October 2011
Accepted 1 November 2011

Keywords:
H1N2 swine influenza viruses
Genomic characterization
Reassortant strains
H3N2 human influenza viruses
Italy

ABSTRACT

Three subtypes (H1N1, H1N2, and H3N2) are currently diffused worldwide in pigs. The H1N2 subtype was detected for the first time in Italian pigs in 1998. To investigate the genetic characteristics and the molecular evolution of this subtype in Italy, we conducted a phylogenetic analysis of whole genome sequences of 26 strains isolated from 1998 to 2010. Phylogenetic analysis of HA and NA genes showed differences between the older (1998–2003) and the more recent strains (2003–2010). The older isolates were closely related to the established European H1N2 lineage, whereas the more recent isolates possessed a different NA deriving from recent human H3N2 viruses. Two other reassortant H1N2 strains have been detected: A/sw/It/22530/02 has the HA gene that is closely related to H1N1 viruses; A/sw/It/58769/10 is an uncommon strain with an HA that is closely related to H1N1 and an NA similar to H3N2 SIVs. Amino acid analysis revealed interesting features: a deletion of two amino acids (146–147) in the HA gene of the recent isolates and two strains isolated in 1998; the presence of the uncommon aa change (N66S), in the PB1-F2 protein in strains isolated from 2009 to 2010, which is said to have contributed to the increased virulence. These results demonstrate the importance of pigs as mixing vessels for animal and human influenza and show the presence and establishment of reassortant strains involving human viruses in pigs in Italy. These findings also highlighted different genomic characteristics of the NA gene the recent Italian strains compared to circulating European viruses.

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1. Introduction

Influenza A viruses have a wide range of hosts and have been isolated from avian species and mammals, including humans and pigs. In the swine population, three subtypes (H1N1, H1N2, and H3N2) are currently diffused worldwide. In Europe in recent years, the epidemiology of swine influenza viruses (SIVs) has considerably changed. The H1N1 viruses now prevalent in European countries are antigenically distinct from classical H1N1 strains apparently stemming 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 have been human-avian reassortants possessing six internal genes from the avian-like H1N1 viruses and HA and NA genes from the previously isolated human-like H3N2 viruses (Castrucci et al., 1993). The latest detected subtype is H1N2, which was introduced into the swine population in Europe in two different times. H1N2 SIVs were first isolated in France in 1987 and 1988 and were the result of a reassortment between avian-like H1N1 SIVs and human H3N2 viruses (Gourreau et al., 1994). These strains did not spread beyond

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0378-1135/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetmic.2011.11.004

General conclusions

General conclusions

This research has been divided in four different studies on the epidemiology, genomic characterization and evolutionary analysis of swine influenza viruses in Italy. The objectives of this research were to better investigate the epidemiology of SIVs in our country, contribute to a better understanding of the emergence and transmission of pathogens with pandemic potential and help to identify the factors that render IAVs prone to cross the species-barrier.

Genetic reassortment plays a vital role in the evolution of IAVs and has been historically linked to the emergence of pandemic strains (Taubenberger, 2006). All known pandemics in recent human history, except for the 1918 flu pandemic, have been attributed to reassortment of genes between different viruses. In 2009, a new H1N1 pandemic virus (H1N1 pdm) spread worldwide leading the WHO to declare the first influenza pandemic of this century. The virus is a quadruple reassortant with genes from North American H1N1 and from European swine IAVs. There is extensive evidence that H1N1pdm has reassorted again in swine, possibly giving it a chance to escape herd immunity in humans (Bradley et al., 2011). H3N2 variant IAVs with an M gene derived from H1N1pdm have been recently detected in USA in pigs and subsequently in people. Human to human transmission has been demonstrated but no efficient community spread (CDC, 2012).

A better understanding of the evolution and adaptation of IAVs to various hosts will provide more information on their capability to cross host barriers and become a pandemic strain. The reassortant viral strains with novel gene combinations and their ability to escape the immune system in other host species could satisfy the basic requirement needed to become a virus with pandemic potential (de Silva et al., 2012). It is vital to continuously monitor the genetic content of circulating IAVs to

detect new reassortants. Studying the process and patterns of viral reassortment is a key to better understand influenza pandemics.

The simultaneous circulation of human, swine and avian influenza viruses in Italy, mainly in the North area, with the risk of reassortment events could be considered of great concern for their implications in human health. In this context, the detection and identification of SIVs in the Italian swine population has contributed to a better understanding of the complex epidemiology of SI and to give new data on the adaptation and circulation of novel reassortant viruses in pigs.

The first study reported the results of the swine influenza surveillance programme carried out in our country in the period 1998 – 2011. This programme was based on genome detection, virus isolation, sequencing and genomic characterisation of all the respiratory forms with particular attention to the detection of reassortant strains. It revealed continuous circulation of the three subtypes, H1N1, H3N2 and H1N2 and H1N1pdm starting from 2009. Genomic characterization was performed comparing Italian viruses with other European swine influenza viruses and influenza viruses form other species (avian and humans), which sequences were available in public databases. Phylogenetic analysis evidenced several reassortment events involving swine and human influenza viruses with no involvement of avian viruses. The most interesting results were observed in the H1N2 viruses, which showed different genomic characteristics respect to the circulating EU H1N2 viruses. Indeed the recent Italian strains isolated since 2003 exhibited a different pattern characterised by a new HA and NA combination with a NA gene deriving from the recent human H3N2 influenza viruses. Moreover, other different reassortant H1N2 strains were detected. The other interesting result was the isolation of 12 H1N1pdm strains in 7 different farms since 2009 also with no evidence of H1N1 pdm circulation in humans.

The re-isolation of H1N1pdm in the same pig farms as well as the constant availability of immunologically naïve animals could provide significant opportunity for persistence of strains and continuous genetic mixing with emergence of novel subtypes or genotypes. Considering these results, the other studies were focussed on the investigation of H1 SIVs, mainly the H1N2 subtype and the H1N1 pdm viruses.

The second study reported in detail the isolation and characterization of the first H1N1 pdm strain isolated in a pig farm in Italy in 2009. Anamnestic data, clinical sings and lesions were described. In order to investigate the serologic response against homologous and heterologous antigens, HI tests were performed using H1N1 pdm and SIVs of different subtypes as antigens. The complete genome sequencing of the H1N1 pdm strain was carried out and confirms very high similarity to the viral genome of the H1N1 pdm viruses. Based on the epidemiological analysis, the hypotheses of human to pig transmission remain one of the most probable sources of infection. The isolation of this virus in November 2009 was one of first cases of H1N1 pdm infection in pigs in Europe.

The third study described the isolation and complete genome characterization of a very interesting reassortant H1N2 strain. The molecular characterisation and phylogenetic analysis revealed that all the genes except the NA belonged to the H1N1 pdm viruses and the NA gene was closely related to two uncommon reassortant H1N2 strains isolated in Sweden and Italy. This case is evidenced of reassortment between H1N1pdm and SIVs; moreover it demonstrated once again the important role played by pigs as mixing vessels for animal and human influenza, providing a place for reassortment and host adaptation to occur.

Finally the last study was focussed on the investigation of the H1N2 subtype in Italy since 1998 (year of the first isolation) to 2012. Phylogenetic and molecular analyses of the complete genome of fifty three Italian H1N2 strains were performed comparing them with other swine influenza viruses and influenza viruses of human and avian origin. These analyses revealed interesting findings. First, a clear difference between the older Italian H1N2 strains closely related to the European H1N2 strains and the more recent ones that showed a different HA-NA combination. This is characterised by an HA deriving from two H1N2 strains isolated in Italy in 1998 with two aa deletions within the receptor binding site in the HA protein and a NA gene closely related to the human H3N2 viruses of 1997. Second the detection of other reassortant strains showing different characteristics but not identical to each other. Third the presence of the uncommon aa change (N66S) in the PB1-F2 protein in some H1N2 strains isolated in the last four years related to the increased viral pathogenicity in 1918 pandemic H1N1 and H5N1 human influenza viruses.

Evolutionary analysis evidenced that EU H1N2 SIVs originated as result of different reassortment events occurred at different times. A European H1N2 precursor acquired first the NA gene from the old human H3N2 IVs and later the HA gene from the human H1N1 IVs. The Old Italian strains originated form the same common ancestor. Moreover, this analysis showed also other three different reassortment events occurred at different times between circulating Italian SIVs and human influenza viruses and confirm that the recent Italian H1N2 SIVs, characterised by the new HA-NA gene combination, form a homogeneous group deriving from a common ancestor.

Reassortment events between human and swine influenza viruses could frequently occur but fail to persist in the pig population (Van Reeth, 2007). Indeed virus strains

with different antigenic characteristics may be at a disadvantage compared to well-adapted established viruses already circulating in pigs. However, in some cases the new viruses may persist in pigs and, following adaptation, could be associated with clinical disease (Brown, 2000). The successful transmission of IAVs depends on a specific gene constellation (Brown, 2000) and a better balanced HA-NA gene combination (Mitnaul et al, 2000). The emergence and persistence of the recent Italian H1N2 strains suggested the presence of a particular gene constellation and a well-balanced HA-NA combination that contributes to efficient replication and successful transmission among pigs.

The frequent reassortment events between human and swine influenza viruses in pigs provide the conditions to investigate adaptive evolution IAVs in different hosts and identify instances of parallel adaptive evolution, and to compare rates of evolutionary changes and selection pressure along the branches that define human-to-swine transmission events. These highlight the need of continuous swine influenza surveillance.

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RINGRAZIAMENTI

A CONCLUSIONE DEL LAVORO SVOLTO E' DOVEROSO PORGERE IL PRIMO RINGRAZIAMENTO AL DR. GUIDO GRILLI PER LA FATTIVA COLLABORAZIONE ED IL SUPPORTO PRESTATO COME TUTOR. DESIDERO ANCHE RINGRAZIARE IL PROF. CLAUDIO GENCHI E IL DOTT. GIUSEPPE SIRONI COORDINATORI DEL DOTTORATO PER LA LORO DISPONIBILITÀ ED ATTENZIONE.

UN GRAZIE PARTICOLARE VA A TUTTO IL PERSONALE ED AI COLLEGHI VETERINARI DEL REPARTO DI VIROLOGIA DELL'ISTITUTO ZOOPROFILATTICO SPERIMENTALE DI BRESCIA PER IL LORO SUPPORTO ED AIUTO ED SPECIALMENTE IL DOTT. PAOLO CORDIOLI CHE È STATO UN PUNTO DI RIFERIMENTO IN TUTTI QUESTI ANNI.

UN RINGRAZIAMENTO MOLTO SPECIALE AI MIE GENITORI ED ALLA MIA FAMIGLIA PER IL LORO SOSTEGNO E PER ESSERMI STATI VICINO SIA NEI MOMENTI DIFFICILI CHE NEI MOMENTI FELICI