Insights in the molecular epidemiology and antigenic characterization of influenza A viruses of pigs

Tesi doctoral presentada per Gerard Eduard Martin Valls per accedir al grau de Doctor en Veterinària dins del programa de Doctorat de Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del Dr. Enric Mateu.

Bellaterra, 2012

Enric M. Mateu de Antonio, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària,

Declara

Que la memòria titulada, "Insights in the molecular epidemiology and antigenic characterization of the influenza A viruses in pigs", presentada per Gerard Eduard Martín Valls per l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la seva direcció dins del programa de doctorat del Departament de Sanitat i Anatomia Animals, Opció Sanitat Animal.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 20 de Setembre de 2012.

Dr. Enric M. Mateu	Gerard Eduard Martín Valls
Director	Doctorand

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Per la Lolita i en Miquel

Per el Mique i la Meri

Per la Cathy i en Fred

I per la Débo

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Summary

In the first study of the present thesis, outbreaks of respiratory disease were investigated for the presence of swine influenza virus (SIV). In 14 cases the circulating influenzaviruses were isolated, fully sequenced and compared with other known SIV. H1N1 (including human pandemic H1N1) was the most common subtype involved in the outbreaks (n=6), followed by H3N2 (n=4) and H1N2 subtypes (n=4). In 11/14 cases the phylogenetic analyses indicated the occurrence of possible reassortment events. In the second part of the study, the genetic evolution of a H1N1 isolate was assessed over a six-month period in a longitudinal study in closed group of pigs. Sequencing of 22 isolates retrieved during that follow-up indicated the co-circulation of two different variants of the same virus. Also, the emergence of SIV reassortants at certain time-points was evidenced. These results indicate that reassortment events in SIV are common, and point towards the need for a better understanding of the epidemiology of SIV, particularly in endemic farms. In the second study of the present thesis, SIV isolates sequenced in the first study where analyzed by means of the haemagglutination inhibition assay (HI) using monospecific sera obtained from pigs immunized with the different isolates. Also, 100 serum samples obtained from seropositive and unvaccinated commercial farms were analyzed. Based on those analysis, a high antigenic diversity was found when comparing the H1N1 viruses. In contrast, HN2 and H3N2 viruses circulating in Spanish swine seemed to have less antigenic diversity regarding their cross-reactivity in the HI. Comparing the amino acid sequences of the haemagglutinin of the analyzed isolates, H1N1 viruses had more changes than the other subtypes. The causes behind this different behavior depending on the subtype are unknown and probably reflect a different epidemiology.

Resum

En el primer estudi d'aquesta tesi, es vàren estudiar diferents brots que presentàven patología respiratòria per tal de detectar-ne la presència de virus de la influença porcina. En catorze casos es vàren detectar virus de la grip circulants. Aquests van ser aïllats, seqüenciats a nivell de genoma complet i comparats amb altres virus de la influença coneguts. Els virus aïllats pertanyien als subtipus H1N1 (n=6, incloent un virus pandèmic H1N1 humà), H3N2 (n=4), i H1N2 (n=4). En 11/14 casos es van detectar possibles reorganitzacións genètiques mitjançant l'anàlisi filogenètica. En una segona part es va analitzar l'evolució genètica d'aïllats H1N1 obtinguts en un estudi longitudinal d'un lot de porcs durant 6 mesos. La seqüenciació de 22 aïllats obtinguts en aquesta explotació van indicar la co-circulació de dues variants del mateix virus, així com l'emergència de noves soques recombinants en diferents moments. Aquests resultats indiquen que les reorganitzacions genètiques són comuns i corroboren la importància de conèixer la epidemiologia dels virus de la influenza porcina, particularment en explotacions endèmiques. En el segon estudi d'aquesta tesi, els aïllats seqüenciats prèviament es varen analitzar mitjançant la inhibició de l'hemaglutinació. Aquesta anàlisi es va fer amb l'ús de sèrums mono-específics obtinguts de porcs immunitzats i amb 100 sèrums obtinguts d'explotacions porcines comercials seropositives a grip i no vacunades. Els resultats obtinguts van permetre detectar una gran diversitat antigènica dels virus H1N1. En canvi, els virus H1N2 i H3N2 circulants semblen ser més homòlegs per subtipus al analitzar la seva reactivitat creuada. Quan es van comparar les seqüències d'amino àcids del gen de la hemaglutinina es va observar que els virus H1N1 també presentaven una major quantitat de canvis de residus que els altres dos subtipus. Les causes d'aquestes característiques antigèniques diferents de cada subtipus no es coneixen i probablement són reflex una epidemiologia diferent.

Resumen

En el primer estudio de esta tesis, se estudiaron diferentes brotes de enfermedad respiratoria en cerdos para detectar la presencia del virus de la influenza porcina. En catorce casos se obtuvo el aislamiento de virus influenza A. Los aislados víricos se secuenciaron en todos sus genes y las secuencias se compararon con otros virus de la influenza porcina. Los virus aislados pertenecían a los subtipos H1N1 (n=6, incluyendo un virus pandémico H1N1 humano), H3N2 (n=4), y H1N2 (n=4). En 11/14 casos se detectaron posibles reorganizaciones genéticas en los genes examinados. En una segunda parte se analizó la evolución genética de aislados H1N1 obtenidos en un estudio longitudinal de un lote de cerdos durante 6 meses. La secuenciación de 22 aislados obtenidos en esa explotación indicaron la co-circulación de dos variantes del mismo virus, así como la emergencia de virus recombinantes en diferentes momentos. Estos resultados indican que las reorganizaciones genéticas son comunes y corroboran la importancia las situaciones endémicas. En el segundo estudio de esta tesis, los aislados secuenciados previamente se analizaron mediante la inhibición de la hemaglutinación (IHA). Este análisis se hizo con el uso de sueros mono-específicos obtenidos de cerdos inmunizados con los aislados obtenidos anteriormente y con 100 sueros obtenidos de explotaciones porcinas comerciales no vacunadas. Los resultados obtenidos permitieron detectar una gran diversidad antigènica entre los virus H1N1. En cambio, los virus H1N2 y H3N2 circulantes parecen ser más homólogos al analizar su reactividad cruzada. Cuando se compararon las secuencias de aminoácidos del gen de la hemaglutinina se observó que los virus H1N1 también presentaban una mayor cantidad de cambios en los residuos aminoacídicos que los otros dos subtipos. Las causas de estas diferentes características antigénicas de cada subtipo no se conocen y probablemente son reflejo de particularidades epidemiológicas.

Table of contents

Chapter 1. Introduction

1.1.Brief historical account of influenza A and its impact

Historically, influenza or, as commonly named, flu has been one of the most important viral diseases because of its impact in humans but also because of the great number of animal species that are affected (Figure 1) (Taugenberger and Morens, 2010). Descriptions of what could have been influenza in humans and animals are already found in classical texts. Thus, in the Chant I of *The Iliad* (circa VIIIth century B.C.), Homer describes a 9-day epidemic caused by the arrows that enraged Apollo shot first to mules and dogs and then to human. Four centuries later, Hippocrates described also a flu-like disease. Diodorus Siculus (first century B.C.) in his work *Bibliotheca Historica* described a disease affecting the Athenian army in Sicily in 412 B.C. that could have been influenza. Afterwards, and until the XVIth century, diseases resembling influenza and spreading in Europe (sources from other continents are less known) were reported in 876, 1173, 1293, 1323, 1357 and 1386 (Taubenberger and Morens, 2009). In fact, the term influenza was coined in 1357 in Italy and was applied also to the 1386 epidemic (or pandemic). However, the lack of clear medical descriptions of the cases and of a clear concept about nosology makes difficult to ascertain the nature of any of the abovementioned epidemics.

The two first descriptions that can be certainly assumed to be influenza epidemics dated in 1493 and 1510. The first one correspond to spread of seasonal or avian, equine or swine influenza to Taino people in the island of Santo Domingo during the second voyage of Colon to America. In this case, the description of the disease is very accurate and reports a four-to-five days respiratory disease that induced immunity in recovered individuals and devastated native Taino populations (Muñoz-Sanz, 2006). The 1510

episode probably is the first influenza pandemic recorded. The disease, that was thought to be originated in Asia, appeared in the summer of that year and in Asia but rapidly spread to the whole European continent and Africa along the trade routes (Morens *et al.*, 2010). In this case, there is a large number of descriptions and the signs reported correspond to influenza without doubt. After this first descriptions and up to XXth century at least nine possible influenza pandemics have been suggested (Taugenberger and Morens, 2009) of which at least five are almost beyond doubt: 1729-1733; 1781-1782; 1830-1833, 1847-1851 and 1889-1893. This last episode known as the Russian flu, probably involved an H3N8 (Morens *et al*., 2010). Three major influenza pandemics were reported in the XXth century: in 1918 ("the H1N1 Spanish flu"), in 1957 ("the H2N2 Asian flu") and in 1968 ("the H3N2 Hong Kong flu"). In 1976-77 a H1N1 virus circulating since the decade of 1950s re-emerged and although it spread worldwide (the Rusian flu episode), the high proportion of people still having immunity against the 1950s virus limited the importance of the spread.

Of all the XXth century flu pandemics, "Spanish flu" is the most important in epidemiological, social, economic and historical terms and has become a symbol of how a deadly disease can spread rapidly in the modern world. Most studies attribute about 20-40 million deaths to the 1918 pandemic (Patterson and Pyle, 1991; Johnson and Mueller, 2002; Taugenberger and Morens, 2006); however, it is difficult to calculate the real impact of the disease in terms of the number of deaths caused solely by the Spanish flu. This is so because this pandemic occurred before the antibiotic era and thus the effect of the concurrent infections was probably substantial at that time. Secondly, because hygienic conditions and medical resources were far from the current ones (Barry, 2005). One of the striking features of the 1918 pandemic was that fatality rates

peaked in young adults contrarily to what usually occurs in human influenza. This unusual pattern has been the subject of a large number of studies (reviewed by Taubenberger *et al.* 2001 and Taubenberger and Kash, 2011) and suggested an immune component in the high virulence of the 1918 strain. It is also relevant that soon after the beginning of the Spanish flu, a similar disease was detected in pigs (Koen, 1919). As a matter of fact, the first isolation of an influenza virus was carried by Richard Shope in 1931 (Shope, 1931) from pig samples. The isolated virus was a descendant of the 1918 H1N1 Spanish flu. The other XXth century pandemics did not have the same impact although they still were a cause of excess mortality in humans for several years (Louria *et al.*, 1959; Viboud *et al.*, 2005). Each flu pandemic counts its deaths from hundreds of thousands to millions around the world. For example, in the case of the last 2009 pandemic, resulted in 580,000 confirmed human deaths (World Health Organization, 2009a). In addition to the pandemics, influenza causes seasonal epidemics in humans every year with a toll between 250,000 and 500,000 deaths worldwide (World Health Organization, 2009a).

The impact of influenza in animal is also important. The more extreme case is that of the highly pathogenic influenza on domestic fowl that causes mortalities close to 100% of the infected animals. Beyond that, the cost of controlling influenza in animals is high because of the need for mobilizing economic and human resources aimed to the identification and elimination of infected animals and, eventually, the vaccination of susceptible animals at risk. Moreover, when influenza spreads in domestic animals, particularly chicken and hens it can cause a shortage in food supply or rises in the price of food.

Finally, current knowledge indicates that new human influenza A viruses are generated by reassortment between animal and human viruses or by adaptation of animal influenza viruses to humans (Smith *et al.,* 2009a; Smith *et al.,* 2009b)

Therefore, it is still an important threat for humans and animals that keeps the constant attention of the scientific community. Nevertheless, there are many questions to be solved regarding its epidemiology, genetic and antigenic evolution and control of the virus. Actually, influenza still is one of the most important infectious diseases and, in fact, the World Health Organization considers that lower respiratory infections, in which influenza plays an important role, is the fifth more important cause of death in high-income countries, and the third cause of death in low-income countries.

1.2. Etiology

1.2.1. Taxonomy and structure

Influenza A viruses are enveloped, single-stranded, negative sense, RNA viruses of about 80-120 nm of diameter belonging to the *Orthomyxoviridae* family, genus Influenzavirus A. Family *Orthomyxoviridae* also includes four other genera: *Influenzavirus B, Influenzavirus C, Isavirus* and *Thogotovirus* (MacCauley *et al.,* 2011). Influenza A viruses are further subdivided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Up to now 17 HA (Webster *et al.*, 1992; Rhom *et al.,* 1996; Fouchier *et al.*, 2005; Tong *et al.,* 2012), and 9 NA have been reported. Figure 1 shows the species where each HA and NA have been described.

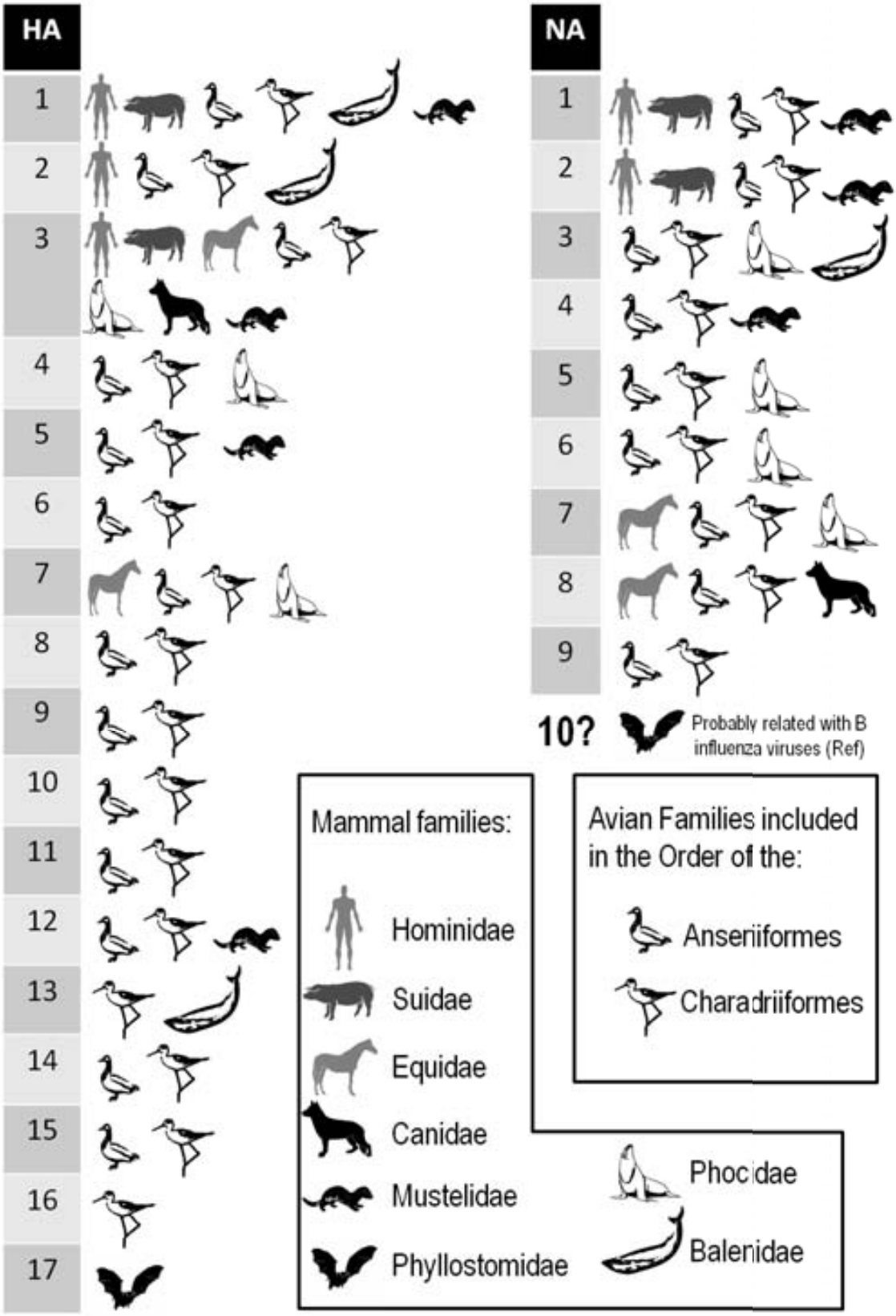

Figure 1. Haemagglutinin and neuraminidases detected in different mammals and birds. The existence of a tenth neuraminidase in bats is not confirmed yet.

Virions can be either spherical or filamentous. The viral genome is segmented in 8 fragments which encode for twelve proteins (Wright *et al.*, 2005; Jagger *et al.*, 2012). Viral genes are designated as follows: PB2, that encodes for basic polymerase 2; PB1, encoding for basic polymerase 1 and with a frame shift for PB1-F2 (which seems to promote the apoptosis of the infected cell); PA encoding for the acid polymerase and the PA-X fusion protein; HA, encoding for the haemagglutinin (synthesized as a HA0 polypeptide precursor which becomes functional when it is cleaved into HA1 and HA2 subunits); NA, encoding for the neuraminidase, NP, encoding for the nucleoprotein; NS, encoding for non-structural proteins 1 and 2 (nuclear export protein), and M, encoding for matrix proteins 1 (capsid) and 2 (a ion channel protein). Each segment has common terminal sequences with RNA ends partially complementary. RNA segments are complexed with multiple monomers of the nucleoprotein and a single copy of the polymerase complex which is composed by the basic polymerases 1 and 2 and by the acid polymerase (PB1, PB2 and PA respectively). The whole structure made of the viral genome plus the nucleoprotein molecules and the polymerase is called ribonucleoprotein (RNP) (Figure 2).

1.2.2. Replication

The first step in the process of a viral infection is the attachment of the virion to the target cell. In this step, constitutive proteases present in the respiratory tract (for birds and mammals) and digestive tract (birds) will cleave HA0 precursos in the position arginine 338 – 340 (depending on the strain) into HA1 and HA2 subunits. The attachment of the virus is mediated by the interaction of HA1 with the sialic acid

receptors α-2,3 or α-2,6 in the cell surface. Once attached, the viral particle enters the cell via endocytosis.

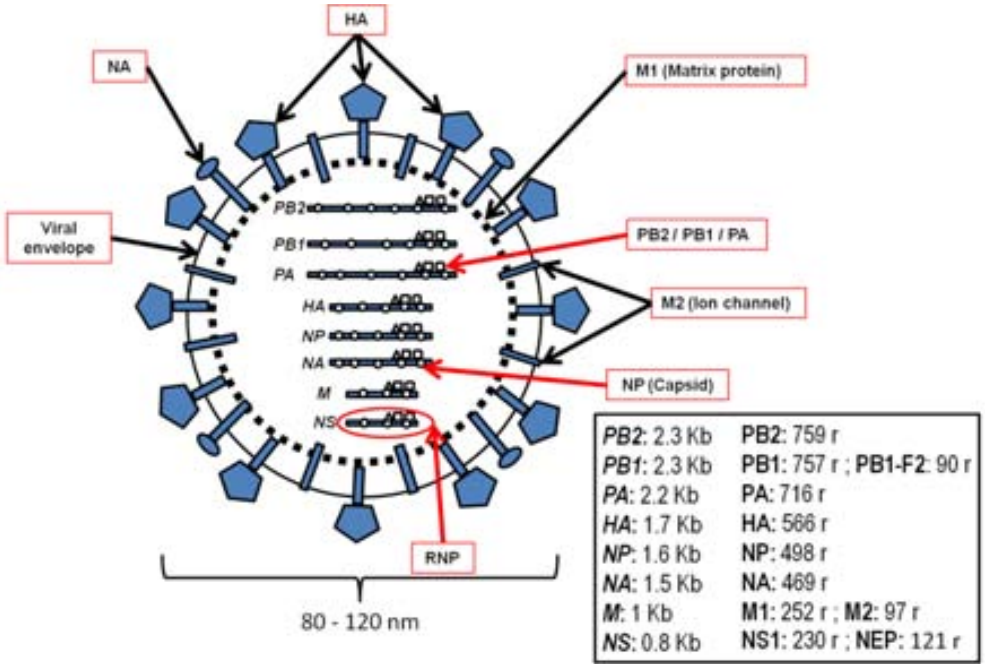

Figure 2. Structure of influenza A virus. Genes *PB2, PB1, PA, HA, NP, NA, M* and *NS* are indicated in italic. PB1-F2, PA-X, NS1 and NEP proteins are not shown in the figure.

The acidification of the endocytic vesicle –a physiologic mechanism- causes a conformational change in HA resulting in the activation of the HA2 subunit. Then, HA2 mediates the fusion of the viral membrane with the host cell membrane. At that point, H^+ ions are pumped into the virion through M2 causing its disruption and the release of the RNP to the cytoplasm. Viral RNA will be then actively transported to the nucleus where viral genome will be transcripted to mRNA. Once viral proteins have been translated, virions are packaged in the cytoplasm and then they are released from host cells thanks to the sialidase activity of the NA.

1.3. Evolutionary patterns of the A influenza viruses

Influenza A viruses are subject to two main evolutionary phenomena: a) antigenic drift, that is, the production -and eventually fixation- of minor but constant antigenic changes caused by the error-prone viral RNA polymerase (Figure 3a) and, b) antigenic shift; an abrupt change in the antigenic characteristics caused by the genetic reassortment between two or more different A influenza virus that might even result in a new subtype for a given host species (Figure 3b).

1.3.1. Antigenic drift

One common characteristic of most viral RNA polymerases is the lack of proof-reading abilities (Nelson and Cox, 2008). In the case of the influenza A polymerase, the mutation rate is estimated to be about 10^{-5} (Drake, 1993; Nobusawa and Sato, 2006). Taking into account that the influenza A genome is about 13 kb, in average, the chance for a new virion incorporating a mutation is about 0.1 - 0.01%. The relevance of a given mutation will depend on whether or not that mutation causes a change in the amino acid sequence of the protein. For example, non-synonymous mutations in the polymerases will affect the efficiency of viral replication and can be thus reflected in the virulence of a given strain (Seyer *et al.,* 2012; Zhu *et al.,* 2012). In NS1 and NS2/NEP, mutations would have relevance in terms of how the virus can modulate the immune response of the host, virulence and the efficiency of the replication (Ma *et al.,* 2010; Forbes *et al.,* 2012; Mänz *et al.,* 2012). Changes in NA are important in resistance to antiviral drugs (Abed *et al.,* 2011). Finally, changes in HA could affect the entrance of the virus into the cell and also may affect in the adaptation of a strain to different hosts, and its virulence and transmissibility (Seyer *et al.,* 2012; Herfst *et al.,* 2012).

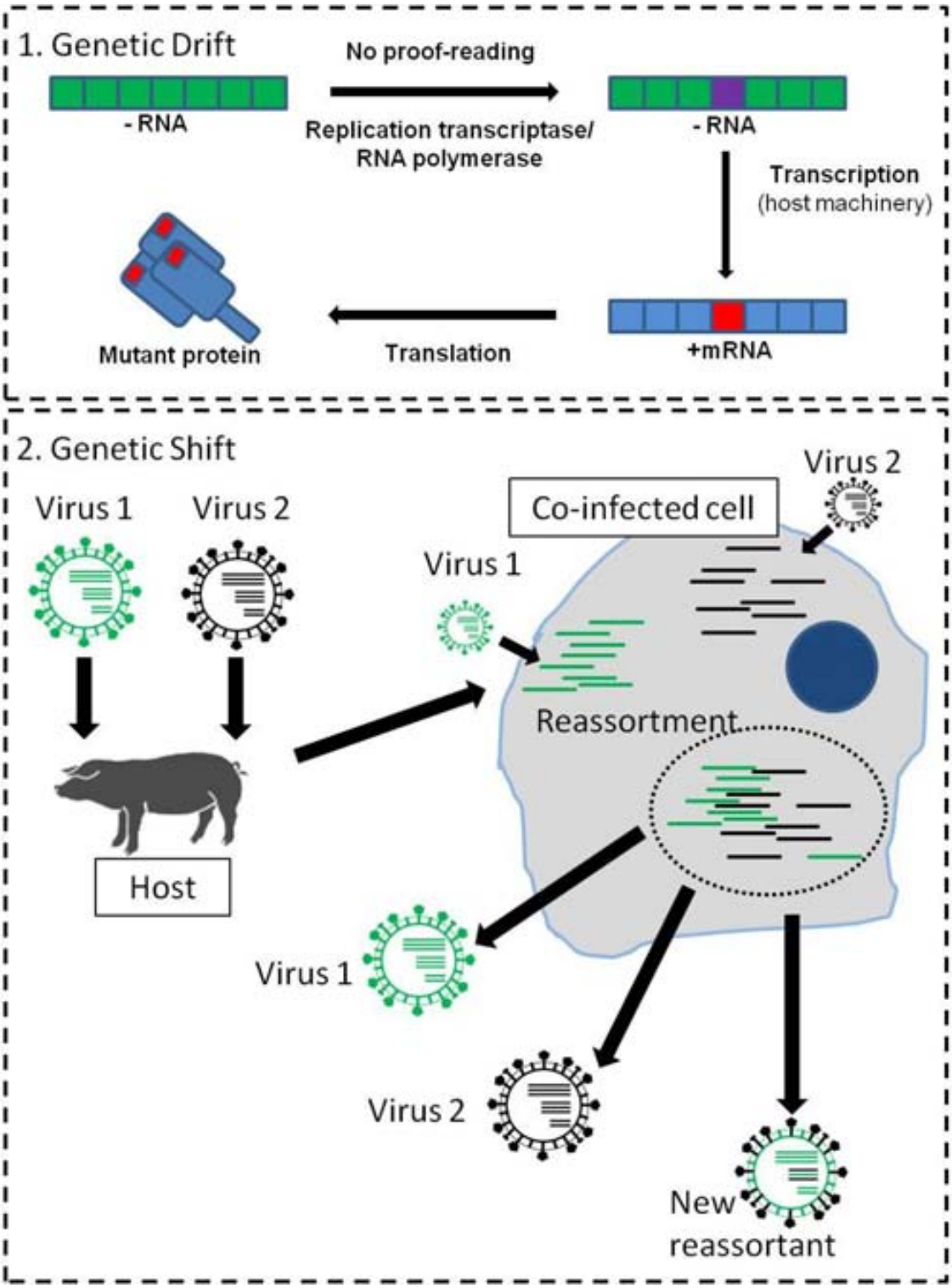

Figure 3. Mechanisms leading to genetic drift and genetic shift (a); genetic drift is a mutation due to the error prone of the RNA polymerases which can be causative of a mutation in the final translated protein, and (b); genetic shift is a major change due to the reassortment of gene segments of two viruses that infect in a same moment a same cell. The new reassortant virus will contain genes from both viral ancestors.

The phenomenon of antigenic drift is particularly important for HA and NA because they are directly exposed to antibodies with implication in the protection against the infection. So, amino acid mutations in the antigenic domains of both proteins may result in a mechanism for escaping pre-existing immunity in the host and may allow the spread of viral variants of a circulating subtype (Webster *et al.*, 1982). This antigenic drift and plays an important role in the ecology of influenza A viruses as will be discussed in the following sections.

1.3.2 Antigenic shift

Contrarily to the slow and gradual nature of the antigenic drift, antigenic shift, is a major and abrupt change that generates a completely new strain of influenza that never existed before through genetic reassortment between pre-existing influenza A strains (Murphy *et al.,* 1999). Antigenic shift can occur when two or more different influenza A strains infect simultaneously the same cell. During the replication of those strains, and because of the fragmented genome of influenza A viruses, a mixture of viral genome segments will exist in the cytoplasm of the cell. When virions are packaged, genes of different strains may package together and thus a new influenza strain harbouring genes of two or more viral ancestors may be created (Scholtissek, 1995).

1.4. Epidemiology of A influenza viruses

Influenza A viruses are present worldwide and can infect a wide variety of birds, particularly aquatic migratory species (such mallards) and domestic birds (quail, chicken, turkey, etc), aquatic mammals (whales, seals), terrestrial mammals (pigs,

horses, dogs, cats, etc.) and humans (Webster *et al.,* 1992). The lineages of these viruses are strongly related with the infected host being possible in many instances to establish philogenetically whether or not a given isolate circulates primarily in a given species (e.g. human viruses, avian viruses, etc.). Therefore, the epidemiology of influenza A viruses depends considerably on the ecology of its hosts.

Historically, it has been hypothesized that the restriction of host range for influenza A viruses depends firstly on the type of cell receptors, α-2,3 or α-2,6 predominant in a given species and, secondly, on the affinity of the HA for one type of receptor or the other (Rogers and Paulson, 1983). In the case of avian species and the horse, the predominant receptor for influenza A is the α-2,3, while in the case of other mammals, is the α-2,6 linked receptor. Pigs have both α-2,3 and α-2,6 receptors in the respiratory tract. Until recently, it was assumed that avian influenza viruses necessarily needed adaptation in pigs to become transmissible to humans (Kida *et al.,* 1994). Nowadays, it is know that humans possess both type of receptors in sufficient numbers to grant that at least some avian influenza A strains can be transmitted directly from birds to humans (Reviewed by Imai and Kawaoka, 2012). Actually, direct interspecies transmission of influenza A viruses have been reported from birds to humans and pigs. The episodes of avian H5N1 (Subbarao *et al.,* 2000) are a practical demonstration of this. Also, human viruses easily circulate in pigs and swine viruses can infect humans (Pensaert *et al.,* 1981; Subbarao, 2000; Fouchier *et al.,* 2004; Adiego Sancho *et al.,* 2008; Howden *et al.,* 2009; Smith *et al.,* 2009b)

Aquatic birds are the central elements in the epidemiology of influenza since they can be infected by any subtype of influenza A (Olsen *et al.,* 2006) acting thus as the main

reservoirs of influenza A in Nature. In contrast, only a few virus subtypes are able to establish in mammals (Figure 1 and 4). For example, for horses only two subtypes have been detected so far: H7N7 and H3N8 and, of these, H7N7 is thought to be extinct (Webster *et al.,* 1993; Bryant *et al.,* 2006). Marine mammals are mostly infected by avian viruses of different subtypes, for example: H3N3, H4N5, H7N7, H13N2, H13N9 (Webster *et al.,* 1981; Hinshaw *et al.,* 1986; Callan *et al.,* 1995; Anthony *et al.,* 2012). In the case of dogs, infections by H3N8 are the commonest with other human and avian viruses sporadically reported (Gibbs and Anderson, 2010; Rivailler *et al.,* 2010; Damiani *et al.,* 2012; Lee et al. 2012; Park *et al.,* 2012).

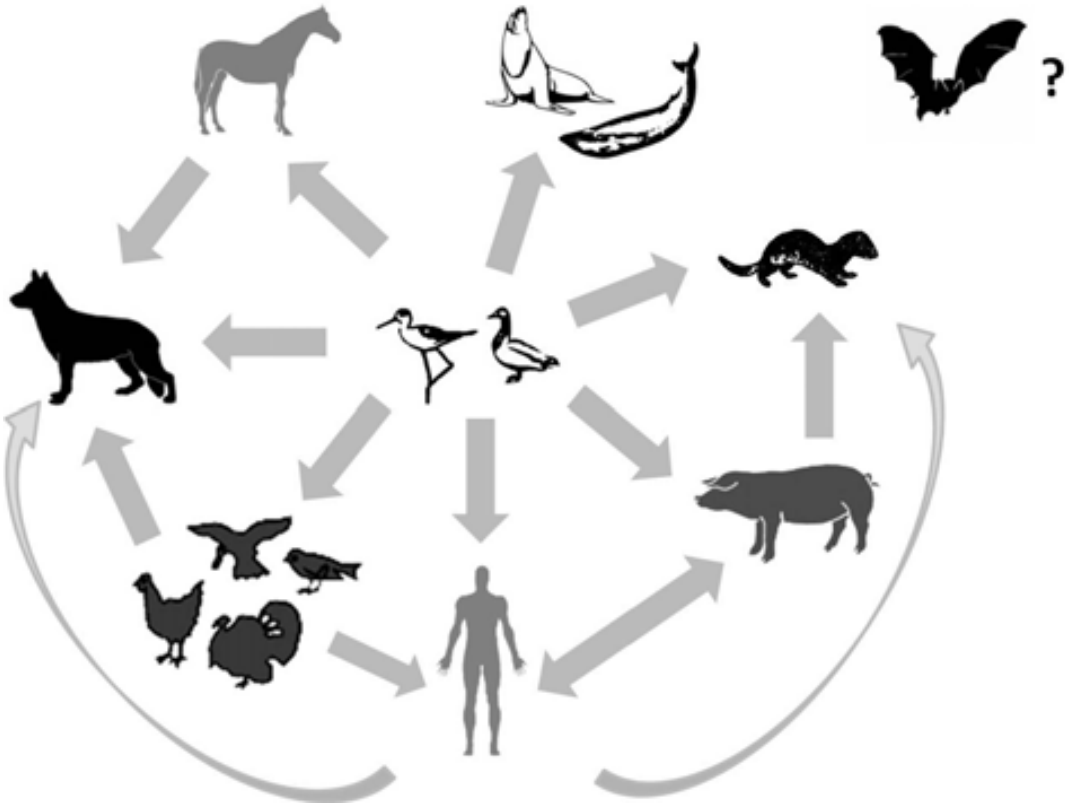

Figure 4. Influenza A virus reservoirs and transmission range. Aquatic birds are the reservoirs of influenza A in nature. Transmission from aquatic birds to other species has been reported. Also, direct transmission between human and pigs, poultry to human, domestic poultry and horses to dogs, pigs to minks and human to dog and minks can be found described in the literature.

In human and swine only some subtypes have been detected, mainly H1 and H3, but a wide range of variants and reassortants can be found infecting human and pigs. Characteristics of avian, human and swine influenza A viruses are reviewed in detail in the following sections.

1.4.1. Influenza in avian species

As commented before, birds can be infected by all influenza A subtypes. However, it is in the Order *Anseriformes* (duck, geese, swan, etc.) and in the Order *Charadriiformes* (gulls, terns, waders, etc.) where the widest variety of influenza A subtypes have been detected (World Health Organization, 1980). Both orders constitute the major reservoirs of influenza A (Olsen *et al.*, 2006). Subtypes H13 and H16 seem to infect more specifically some *Charadriiformes* and it has been suggested that these subtypes belong to a genetically isolated branch of avian influenza A viruses (Fouchier *et al.*, 2005; Olsen *et al.*, 2006).

The spread of influenza in birds is related with the routes of the migratory species of the abovementioned orders (Olsen *et al.*, 2006). In summer and early fall the prevalences of influenza A in migratory birds is higher, probably because the season of births takes place in summer, and thus the bird population receives a flow of new susceptible animals during those months. In contrast, in spring the prevalence of influenza-infected birds is lower because the population comprises older animals that probably have been infected before and were thus immune. As a result, northwards migration in spring contribute little to the spread of influenza while southwards migration in autumm is a serious source of new influenza viruses (Olsen *et al.*, 2006).

As a result of long-term isolation of hosts depending on the migratory flyways avian influenza A viruses have evolved in two main lineages, the Eurasian and the American (Donis *et al.,* 1989). Nevertheless, viruses carrying genes from both lineages have been detected (Liu *et al.,* 2004; Wallensten *et al.,* 2005; Koehler *et al.,* 2008), indicating that this separation is partial and that the epidemiology of influenza A viruses of birds is probably more complex than thought.

1.4.2. Influenza in humans

The human influenza has by two main epidemiological presentations: seasonal epidemics and global pandemics. The seasonal form occurs when a human influenza virus (HuIV) circulates endemically. In that case, the pre-existing immunity of the population selects viral variants that harbor antigenic changes –because of the antigenic drift- allowing them to escape from the immune system. The time needed for the rise of a new variant of an already circulating strain in a non-naïve population is of about one year. Thus, after that period a seasonal epidemic will take place (White and Fenner, 1994). That is one of the reasons for the need of constant actualization of HuIV vaccines.

Pandemic influenza is a phenomenon representing the global spread of a new influenza A strain. that requires three conditions: 1) The generation of a new strain against which the population do not have any pre-existing immunity; 2) the adaptation of the new strain to replicate efficiently in the human host and, 3) the strain has to be easily transmitted between the hosts (World Health Organization, 2005).

Usually, the spread of the new epidemic strain is very fast and in about six months the virus can be detected worldwide. This has been observed in all pandemics since Spanish flu of 1918 (Cox and Subbarao, 2000; Taugenberger and Morens, 2006). After this first phase of global spread, secondary waves of spread will occur. These secondary waves are influenced by the previous development of immunity in the human population and the subsequent outbreaks will affect smaller numbers of people. At this point, the pandemic strain has usually displaced seasonal strains and later on becomes a seasonal virus in a new inter-pandemic phase.

At present, the three main subtypes of influenza A viruses circulating in humans are the H1N1, the H2N2 and the H3N2 (Morens *et al.,* 2010). In the last 100 years. Two H1N1 strains that have been involved in pandemics; the oldest is 1918 Spanish flu. It has been suggested that that virus was directly adapted from birds to humans (Smith *et al.,* 2009a). The second H1N1 strain is the 2009 virus originated in North America, which has been demonstrated to be closely related with triple reassortant H1N1 (trH1N1) (see section 1.4.3.7) swine viruses circulating in North American farms and that became adapted to human (Smith *et al.,* 2009b). Russian flu of 1977 was directly related with the seasonal H1N1 circulating in the 50's and derived from the 1918 strain.

In the 1957 a new pandemic occurred. In that case, was a H2N2 virus reassortant containing the HA, NA and PB1 genes from H2N2 avian viruses and the PB2, PA, NP, M and NS from the previously circulating H1N1. In 1968, the H3N2 virus causing the so-called Asian Flu pandemic was the product of a new reassortment between the H2N2 of 1957 an avian H3. The new virus acquired also the PB1 gene segment from this avian

virus. It is unclear in what species those reassortments took place although it has been hypothesized that pigs could have played a role in the generation of those pandemic strains (Ito *et al.,* 1998; Suzuki *et al.,* 2000).

Human infections with avian H5, H7 and H9 viruses directly transmitted from birds have been reported (Yuen *et al.,* 1998; Saito *et al.,* 2001; Fouchier *et al.,* 2004). In most cases, those infections have been mild except for the H5N1 strain spreading from Hong Kong area since 1997 which fatality rates for humans were very high (Yuen *et al.,* 1998).

1.4.3. Swine influenza

1.4.3.1. Diversity of swine influenza viruses

For a proper understanding of the epidemiology of swine influenza it is important to understand firstly the ecology of domestic pigs. In many countries, pig production is concentrated in commercial units managed under industrial criteria. Thus, pigs are often confined indoors in large groups and exportation of live animals between regions or even countries (e.g. Netherlands to Spain; Denmark to Hungary) take place between those commercial units where pigs are produced but live pigs rarely travel from continent to another. Nevertheless, familiar pig production still exists in substantial numbers in non-EU Eastern Europe countries, Asia, Latin America and Africa. In industrial units, new susceptible animals are continuously introduced because of the short life span of fattening pigs and the high replacement rates (30%-50%) of sows. These two factors explain at least partially why only three main subtypes of swine

influenza viruses (H1N1, H1N2 and H3N2) are found and also may explain the divergent evolution of swine influenza viruses in two different continents. Moreover, in spite that mutation rates are similar in human and swine influenza viruses, the antigenic drift is slower for pig viruses, probably because of the constant flow of the new naive animals, a fact that decreases the selective pressure created by antibodies against circulating subtypes (Noble *et al.,* 1993; De Jong; *et al*., 2007).

As long as new sequences of SIV are studied, genetic diversity of influenza viruses is found in a given geographical region (Vincent *et al.,* 2009; Kuntz-Simon and Madec, 2009; Moreno *et al.,* 2012; Vijaykrishna *et al.,* 2011). Thus, phylogenetic trees based on nucleotídic composition of the SIV show that, if compared with human influenza A viruses, in pigs there are more differentiated evolutionary lines but with less genetic drift regarding common ancestors. This genetic heterogenicity observed in SIV has been also reated with antigenic heterogenicity in H1N1 strains (de Jong *et al.,* 2001).

As mentioned above, antigenic shift plays an important role in the generation of new SIV strains. In fact, most of current SIV strains are products of reassortment events (Olsen *et al.*, 2002; Kuntz-Simon and Madec, 2009) and it has been demonstrated that swine influenza viruses circulating in Europe present a high reassortment rate (Lycett *et al.,* 2012).In the next sections the distribution of SIV in the different continents will be reviewed.

1.4.3.2. SIV in Europe

Genetic and antigenic diversity of SIV in Europe (Figure 5) have distinctive features. Earliest SIV isolations in Europe were done between 1938 and 1940 (Lamont, 1938; Blakemore and Gledhill, 1941). Those isolates were very close to the predominant 1918 Spanish flu-derived H1N1 and could be differentiated from the American H1N1 SIV, a fact that suggested different evolutionary lines (Neumeier *et al.,* 1994). That H1N1 remained predominant in European pigs until 1976, when the North American classical swine H1N1 (csH1N1, see section 1.4.3.4) was introduced in Southern Italy and spread to other countries of Europe (Nardelli *et al.,* 1978; Masurel *et al.,* 1983; Abusugra *et al.,* 1987; Roberts *et al.,* 1987). There are evidences that the 1977 Russian flu virus also spread in the European swine as seen in serological studies performed in several countries (Yus *et al.,* 1992; Brown *et al.,* 1993b). In 1979, an H1N1 strain of avian origin entered in swine of Belgium and Germany (Pensaert *et al.,* 1981), and spread across Europe. The new H1N1 strain displaced the previous csH1N1 (Schultz *et al.,* 1991) and established the avian-like H1N1 lineage (avH1N1) that currently is the predominant H1N1 in the continent (Kyriakis *et al.,* 2011). Other H1N1 strains of other origins have been isolated as well but did not establish in the swine population (reviewed by Kuntz-Simon and Madec, 2009)

With respect to the H3N2 subtype, this was detected firstly related to the 1968 human pandemic H3N2 (huH3N2) and is supposed to have been introduced in pigs from humans (Miwa *et al.,* 1987). In fact, the huH3N2 isolates obtained during the following 16 years maintained a high genetic and antigenic similarity with seasonal human H3N2 isolates (Aymard *et al.,* 1980; Ottis *et al.,* 1982; Castrucci *et al.,* 1994) suggesting a

constant introduction of human viruses to the pig population more than an adaptation of the virus in pigs.

In 1984, a reassortment event between the avH1N1 and the huH3N2 resulted in the generation of a strain containing the internal genes of the avH1N1 and the glycoproteins of the huH3N2 (Castrucci *et al.,* 1993). This virus, named "reassortant human like swine" H3N2 (rH3N2), was well adapted in pigs and spread in Europe. Thus in Spain, Italy, Denmark, Belgium, the Netherlands or Germany (Böttcher *et al.,* 2007; Van Reeth *et al.,* 2008; Simon-Grifé *et al.,* 2011) the seroprevalence of H3N2 is moderate to high while the virus is absent in France, Great Britain and Ireland and cannot be detected by serology in the Czech Republic (Franck *et al.,* 2007; Rosembergova *et al.,* 2007; Kyriakis *et al.,* 2011). In Poland, the introduction of these new H3N2 is recent since in 2008 could not be detected (Van Reeth *et al.,* 2008; Kowalczyk *et al.,* 2010). The last SIV subtype emerging in Europe is the H1N2. The diversity observed within H1N2 isolates strains when compared with either the H1N1 or H3N2 European is high, and it is thought to be the subtype which has been implicated more reassortment events in Europe (reviewed by Kuntz-Simon and Madec, 2009). In fact, the first SIV H1N2 isolate obtained in Britain (France) in 1987 (avH1N2) was a reassortant strain which contained the HA of the avH1N1 and the other genes came from the huH3N2 (Gourreau *et al.,* 1994). However, this SIV subtype was not established in the swine population until 1994. At that time emerged a new reassortant harboring HA related to the Russian flu H1N1 of 1977 (see section 1.4.2) and the rest of the gene segments comes from the rhsH3N2 virus (named "reassortant human-like swine" H1N2 (rH1N2) (Brown *et al.,* 1995). The HA of those H1N2 does not present any cross reactivity with the avH1N1 HA and both viruses can be differentiated by serological assays (Brown *et al.,* 1998)

while other H1N2 of different origin have been detected as well. For example, H1N2 viruses containing the NA segment of the "seasonal-human" H3N2 have been found in Italy (Moreno *et al.,* 2012), others carrying a HA related to the avH1N1 virus found in Denmark and France (Hjulsager *et al.,* 2006; Kuntz-Simon and Madec, 2009) and recently one H1N2 isolated in Sweden and Italy harboring the NA from the rH3N2 than the rH1N2 (Bálint *et al.,* 2009; Moreno *et al.,* 2012).

1.4.3.3. SIV in Asia

Epidemiology of Asian influenza viruses of pigs present a number of particularities compared to the infection occurring in other regions. This is probably attributable to the existence of a very large pig population often raised under extensive systems and in contact with migratory birds. The first particularity of Asian SIV is the existence of two main lineages within the H1N1 subtype (csH1N1 and avH1N1) as well as a wide variety of different reassortants belonging to this subtype (Vijaykrishna *et al.,* 2011; Choi *et al.,* 2012). The diversity of H3N2 strains in Asia is more complex than in Europe or North America. In fact, in Asia there are H3N2 reassortants between huH3N2, rH3N2 and the North American triple reassortant H3N2 (trH3N2; see section 1.4.3.4). As a matter of fact, H3N2 SIV isolated in China and Thailand have never been reported elsewhere (Chutinimitkul *et al.,* 2008; Takemae *et al.,* 2008; Yu *et al.,* 2008). To add complexity to this picture, since 2006 there are no evidences for the circulation of the H3N2 subtype in China (Vijaykrishna *et al.,* 2011) although that H3N2 circulates in Korea and other Asian countries(Song *et al.,* 2003; Lee *et al.,* 2008). Taking into account that China is the first pig producer of the world, this is not a negligible fact

In Asia H1N2 was firstly isolated in 1978 (Japan), earlier than in Europe and North America. In that case, the H1N2 virus was a reassortant between the csH1N1 and seasonal human H3N2 viruses. That reassortant established a lineage in Japan which has been circulating in that country since then (Yoneyama *et al.,* 2010). In Korea a triple reassortant H1N2 related to the North American viruses has been circulating since 2002 (Choi *et al.,* 2002; Pascua *et al.,* 2008). In China, strains belonging to the North American triple reassortant H1N2 (trH1N2, see section 1.4.3.4), rH1N2, and reassortants of both strains have been described (Yu *et al.,* 2009; Vijaykrishna *et al.,* 2011).

1.4.3.4. SIV in North America

Influenza virus isolated in North America or elsewhere (1931) was an H1N1 strain of pigs closely related with the Spanish flu H1N1 of 1918 (Shope , 1931) (Figure 6). In fact, in 1919 a first description of a flu-like disease was observed affecting pigs in North America close in time to the spread Spanish Flu (Koen *et al.,* 1919). The descendants of that H1N1 strain established what is called the classical swine H1N1 lineage that still circulates nowadays presenting different antigenic variants (reviewed by Olsen *et al.,* 2002).

The H3N2 subtype was first detected detected in pigs of North America in last years of the 1970 decade, having a low clinical impact (Hinshaw *et al.,* 1978). Introduction of H3N2 viruses probably took place as indicated by serologica evidences in Canada (Bikour *et al.,* 1995). Those Canadian H3N2 strains were close to the seasonal human H3N2 strains isolated in 1975 (Bikour *et al.,* 1994; Bikour *et al.,* 1995). In 1997, a

reassortment between the csH1N1 and the seasonal human H3N2 resulted in a new H3N2 strain which changed drastically the presence and the clinical impact of the H3N2 viruses in North American farms because of its higher virulence (Karasin et al, 2000a). From that moment, the new H3N2 strain suffered reassortments which resulted in the introduction of internal gene segments (all but HA and NA) from avian and human influenza viruses circulating in North America in that time (Zhou *et al.*, 1999; Karasin *et al.,* 2000a). Of these, a triple pig-avian-human reassortant H3N2 (trH3N2) was established in pigs (Van Reeth *et al.,* 2012).

The H1N2 subtype was firstly isolated in 1999 in North America. The original H1N2 SIV of North America harbored HA from the csH1N1 and the rest of the segments were of the trH3N2 virus. This virus has been described in several states of the USA and also in Canada and it is known as the triple reassortant H1N2 virus (trH1N2) (Karasin *et al.*, 2000b). Additionally to these triple reassortant H3N2 and H1N2, since 2000, viruses presenting the HA and NA from csH1N1 viruses have been detected (Yassine *et al.*, 2009) and are named as trH1N1 viruses.

Additionally, different authors have proposed the existence of different clusters based in the HA phylogeny and antigenic recognition of the North American SIV. There are 4 main differentiated clusters for the H1 strains (α, β, δ, γ) and for the H3 strains (I-IV) (Vincent *et al.,* 2009; Lorusso *et al.,* 2011; Kumar *et al.,* 2011). However, whether this classification have impact in the epidemiology, pathogenesis and cross protection have not been deeply studied.

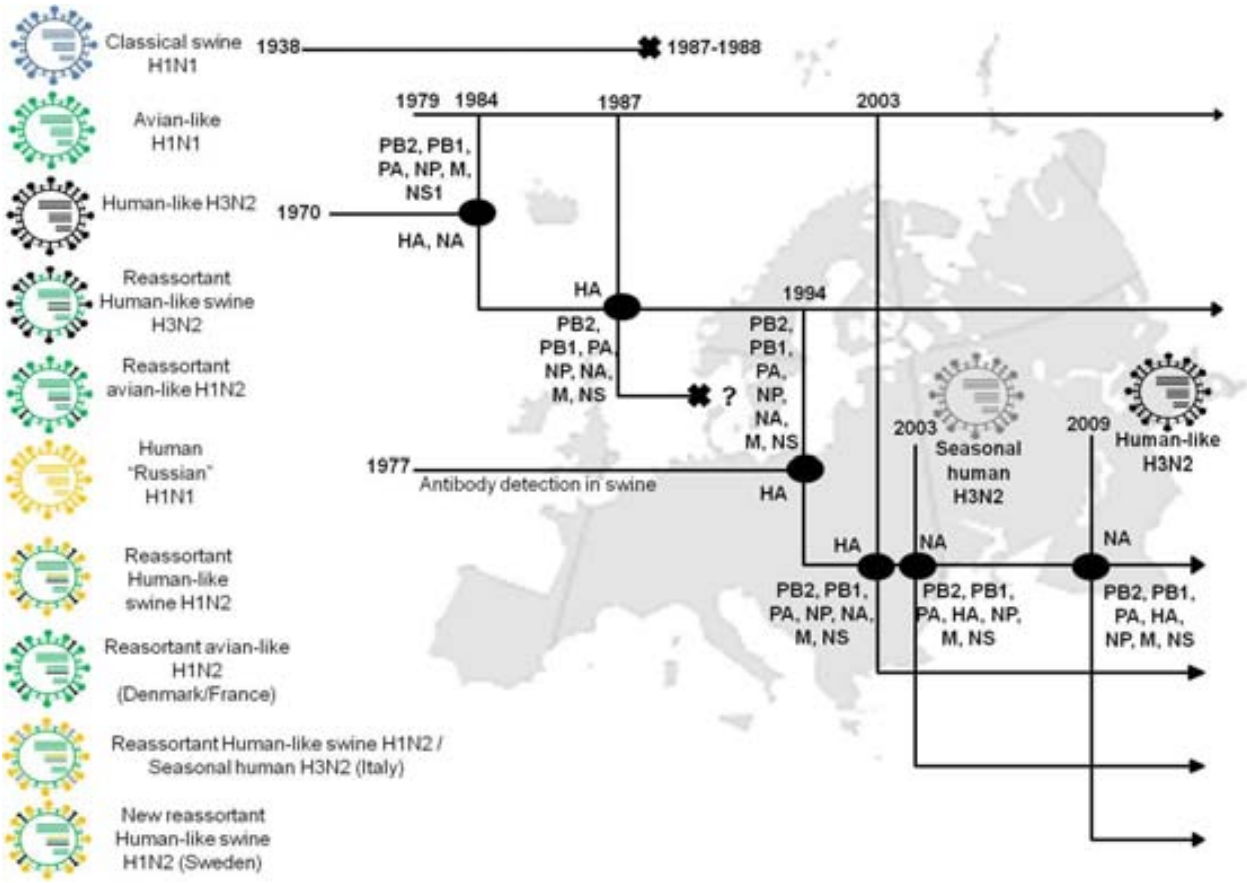

Figure 5. Reassortment events involved in the evolution of European SIV and year of detection of the reassortants. Black ovals indicate the reasssortment point. Gene abbreviations above and below the oval indicate the contribution of each virus involved in the reassortment event.

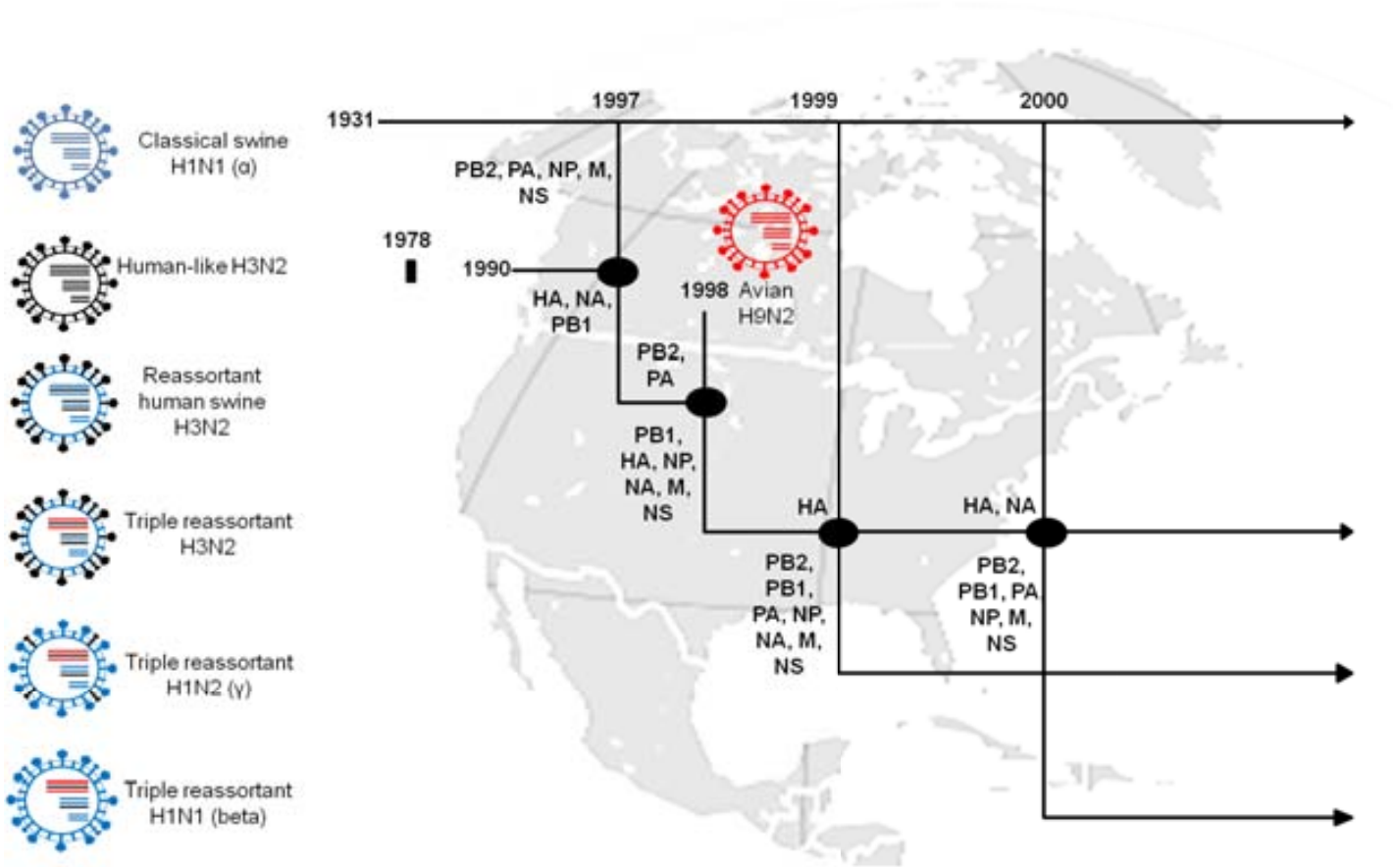

Figure 6. Reassortment events involved in the evolution of North American SIV and year of detection of the reassortants. Black ovals indicate the reasssortment point. Gene abbreviations above and below the oval indicate the contribution of each virus involved in the reassortment event.

1.4.3.5. SIV in Latin America

Little is known about the status of SIV infection in Latin America and most of the data account for serological analysis using the haemagglutination inhibition test (HAI). This is case for Chile (Vicente *et al.,* 1979) Brazil (Mancini *et al.,* 2006 ; Rajao *et al.,* 2011), and Venezuela (Boulanger *et al.,* 2004). These works indicated that H1N1 and H3N2 swine strains related with the North American lineages are circulating in Latin America. More recently, Cappuccio and coworkers (2011) described an H3N2 circulating in pigs in 2008. This virus was related with human seasonal H3N2 viruses circulating in 2003. However, the number of SIV sequences available from Latin America is scarce and this makes difficult to draw adequate conclusions.

1.4.3.6. Other influenza subtypes in pigs

Infections by subtypes other than H1N1, H3N2 and H1N2 have been also documented sporadically in pigs. For example H1N7, H3N1, H3N3, H4N6, H4N8, H6N6, H7N2, H7N7 viruses have been reported in pigs either by isolation, PCR or by serology (Brown *et al.,* 1994; Karasin et al., 2000c; Karasin *et al.,* 2004; Lekcharoensuk *et al.,* 2006; Moreno *et al.,* 2009; de Jong *et al.,* 2009; Kwon *et al.,* 2011; Zhang *et al.,* 2011; Su *et al.,* 2012). In none of those cases the viruses had any particular virulence. Other subtypes such H2N3, H9N2 and H5N1 deserve a more detailed comment.

The H2N3 subtype was isolated from two swine herds in Missouri (USA) (Ma *et al.,* 2007). Under experimental conditions the virus was able to infect pigs, was transmissible among pigs and ferrets without prior adaptation and produced an overt disease. For H9N2 viruses, two main type of strains have been described in China

infecting pigs from 1997 to 2007 (Yu *et al.,* 2011). The first type contained strains of bird origin, indicating direct transmission from birds to pigs. The second type of H9N2 were reassortants including one to four genes from H5 viruses.

Finally, the highly pathogenic avian H5N1 strain has been detected in pigs of Asia (Choi *et al.,* 2004; Choi *et al.,* 2012) without causing clinical signs. This has been confirmed under experimental conditions, where the avian H5N1 replicated in pigs but failed to cause disease (Shortridge *et al.,* 1998; Isoda *et al.,* 2006).

1.4.3.7. The 2009 H1N1 pandemic and its impact in SIV

Soon after the beginning of the H1N1 pandemic of 2009 the virus was found in a swine farm from Canada suffering an Outbreak (Howden *et al.,* 2009). Afterwards, the H1N1 strain (pH1N1) has been detected in pigs of many countries: Argentina, Italy, Korea, United States, etc. (Pereda *et al.,* 2010; Moreno *et al.,* 2010; Kim *et al.,* 2011; Ducatez *et al.,* 2011). Interestingly, countries which pigs were previously free of SIV such as Norway or Australia became infected by the pH1N1 strain, with a high number of affected farms (Hofshagen *et al.,* 2009; Deng *et al.,* 2012).

Reassortants between previously circulating SIV strains and the 2009 pH1N1 have been reported in different continents (Vijaykrishna *et al.,* 2011; Han *et al.,* 2012; Hiromoto *et al.,* 2012; Moreno *et al.,* 2012; Starick *et al.,* 2012; Tremblay *et al.,* 2011; Ali *et al.,* 2012; Liu *et al.,* 2012). For example, trH3N2 and rH1N2 reassortants containing the M gene from pH1N1 have been detected infecting in pigs of Canada and Italy (Tremblay *et*

al., 2011; Moreno *et al,* 2012). Moreover, a reassortant virus containing genes from the swine trH3N2 and pH1N1 was identified in 12 human cases in the United States (Nelson *et al.,* 2012; Kitikoon *et al,* 2012). The isolated virus did not cross-react with seasonal H3N2 human viruses. Human-to-human transmission of this strain has been suggested.

1.4.4. Transmission of Influenza A viruses between individuals

The main routes of transmission of influenza viruses depend on the type of host and the environmental conditions. So, in birds, where the virus replicates in the gastrointestinal tract, transmission mainly occurs by the faecal-oral route, being particularly facilitated by the humid environments where most migratory birds live (Webster *et al.,* 1978; Ellström *et al.,* 2008; Stallknecht *et al.,* 2009). Also, closed environments, with high density population or the use of common feeders and watering holes -as the case of domestic poultry- make transmission much easier.

In the case of humans, it is a common idea that transmission takes place by Pflüger droplets in aerosols. However, the low R_0 (1-2) indicates that a closer contact is needed for the transmission to occur and it is believed that contaminated hands may be one of the main sources of infection (Bean *et al.,* 1985; Boone *et al.,* 2005). Thus, in crowded spaces (underground transportation system, airports, schools or commercial surfaces) the transmission would be optimal.

In swine, transmission probably occurs similarly by aerosols but also by nose-to-nose contact or by contact with contaminated surfaces. Infection by contaminated water could also occur since viral particles remain infective in the water for long periods (Stallknecht *et al.,* 2009). The role of arthropods acting as mechanical vector has not been studied properly. However, transmission by biological vectors is negligible due to the lack of infectiveness to mammals in blood of influenza A viruses (White and Fenner, 1994).

The routes of entry of influenza A viruses in a swine herd are diverse. In a work carried out in Spain (Simon-Grifé *et al.,* 2011) where risk factors for higher seroprevalence of influenza A viruses were analyzed, the higher the replacement rate of sows and an uncontrolled access to the farm were considered a risk for a higher seroprevalence. However, other factors as the use of birdproof nets, the lack of insect or rodent control programs or the existence of other farms in the nearby represented an increasing risk. In fact, most of the isolates obtained from pig herds are viruses of swine origin and some others are of human origin. Avian strains are rarely detected in pigs. These data are indicative that influenza A viruses are mostly introduced by the entrance of foreign pigs or personal such as veterinarians or sales representatives visiting pig farms.

1.4.5. Epidemic and endemic presentation of influenza infections in pigs

It is a common assumption that when a new influenza strain enters in a pig herd the infection will spread rapidly affecting almost the 100% of the animals but with a low fatality rate (Van Reeth *et al.,* 2012). After this initial outbreak, immunity against the infecting SIV strain will be generated and the virus would disappear from the

population. This form of the disease can be named a "classical outbreak" or a"epidemic" presentation.

Endemic forms of SIV infection in pigs have been also described (Madec *et al.*, 1985; Simon-Grifé *et al.*, 2012). In those cases, incidence is lower and most of the infected pigs suffer subclinical infections with an undetermined impact in the porcine respiratory complex (PRC). It is still uncertain which form of the infection is the commonest, but serological studies carried out in apparently healthy and unvaccinated herds resultedin high seroprevalences of SIV, suggesting that the endemic and subclinical forms are frequent (Simon-Grifé *et al.*, 2012). These endemic presentation is the ideal scenario for allowing long-term circulation of influenza virus. Under these circumstances evolution of new variants or reassortants is easily underestimated.

1.4.6. Prevalence of SIV in swine herds

In Spain, two studies carried out in the last 10 years (Maldonado *et al*, 2006; Simon-Grifé *et al.*, 2011) demonstrated serologically that all three common SIV subtypes are present in pigs with high prevalences. In the most recent study (Simon-Grifé *et al.*, 2011) 40% of the studied pigs were seropositive against more than one subtype. Also, prevalence of seropositive sows was significantly higher in sows than in fattening pigs suggesting that SIV infections are common. More importantly, only a low percentage of the studied farms were reported to present a recent record of clinical signs compatible with flu indicating that either SIV infection was subclinical or, alternatively, that swine veterinarians fail to recognize the disease (see section 1.5.2). Similarly, Loeffen and co-workers (2009) reported that incidence of respiratory outbreak, was much lower than that of SIV seroconversion.

In other countries of Europe with intensive swine production high seroprevalences (>30%) have been reported (Van Reeth *et al.*, 2008). Nevertheless, in countries where pig production is less intensive such as Ireland, Poland or the Czech Republic, seroprevalences were lower, ranging from 0% to 11%, with H1N1 as the predominant subtype and lacking evidences for H3N2 circulation. A recent virological surveillance carried out in five European countries, failed to find evidences for the circulation of H3N2 viruses in France and in the United Kingdom. Finally, it is worth to note that in regions as Southern China, serological analyses have demonstrated circulation of a high diversity of influenza A viruses of different serotypes, for example H4, H5, H6, H7 or H9 (Ninomiya *et al.*, 2002; Zhang *et al.*, 2011; Choi *et al.*, 2012; Kwon *et al.*, 2012). In that area, extensive pig production systems allow a greater contact with wild and domestic birds that can be the source of those different subtypes (Shortridge and Stuart-Harris, 1982).

1.5. Pathogenesis, signs and lesions of influenza in different species

1.5.1. Avian influenza

Based on the virulence for avian species, influenza A viruses in birds are classified in two groups: the low pathogenic avian influenza viruses (LPAIV) and the highly pathogenic avian influenza viruses (HPAIV). LPAIV infection in aquatic birds is mostly asymptomatic, while in poultry causes a mild condition with diarrhea and/or respiratory signs (Akey, 2003; Suarez, 2010). In contrast, HPAIV can cause severe disease in wild and particularly, in domestic birds. Up to date, all HPAIV isolates have been classified within the H5 and H7 subtypes. They cause a systemic disease with hemorrhagic lesions that are most prominent in guts, proventricle, aerial sacs and lungs but also causing

lesions in brain, pancreas, heart and other organs (Tang *et al.,* 2009). Morbidity and mortality in poultry may reach 100% while virulence is lower for aquatic birds (Iglesias *et al.,* 2009). The reason explaining the systemic nature of HPAIV lies in the presence of a poly-arginine chain present in the cleavage site of the HA0 precursor that render the haemagglutinin susceptible to the cleavage by almost all body proteases and not only respiratory and digestive proteases as occur with the LPAIV cleavage site (Neumann and Kawaoka, 2006; Garten *et al.,* 2008).

1.5.2. Influenza in swine and other mammal species

In mammals, influenza A is purely a respiratory process and the virus does not spread systemically. Even in severe cases of infection of humans by the highly virulent avian H5N1 systemic dissemination did not take place (Yuan *et al.,* 1998). In all mammal species including humans, influenza courses as a mild to severe respiratory disease with conjunctivitis, sneezing and cough. High fever, muscular pain and prostration are common signs in affected animals. The disease usually resolves within a week if secondary infections are not present or are adequately treated, and complete recovery is the common evolution (White and Fenner, 1994). In the case of pigs, SIV is generally included in the group of components of the Porcine Respiratory Complex (PRC) –the respiratory syndrome of weaned and growing pigs- along with other swine pathogens such as *porcine reproductive and respiratory syndrome virus*, *porcine circovirus* type 2, *Mycoplasma hyopneumoniae*, *Pasteurella multocida* or *Actinobacillus pleuropneumoniae* among others. Its role is a subject of some debate. In a recent work it has been shown that the clinical outcome of the infection could be different depending on the strain used for inoculation. Thus, the disease was reprudiced with rH1N2 but this

was not achieved with an avH1N1. However, when the experimental infection with SIV was combined with *M. hyopneumoniae*, classical respiratory signs of flu developed with both strains (Deblanc *et al.,* 2012). In any case, the main lesion observed in lungs of SIV infected pigs is a bronchointerstitial pneumonia (BIP) (Van Reeth *et al.,* 2012) characterized by the presence of a diffused pulmonary consolidation mainly, but not exclusively, in cranial lobes. Another particularity of SIV infections in swine is that they are often associated with abortions in pregnant sows. Abortion cases are usually attributed to the high fever since no dissemination of the virus to the placenta or fetuses has been proved (Van Reeth *et al.,* 2012).

1.6. Diagnosis of swine influenza

Diagnosis of SIV in pigs is usually done firstly by the observation of clinical signs and by recording the epidemiology (an acute respiratory syndrome affecting a high proportion of the present animals). However, since many other respiratory pathogens are often circulating in the farm and because of the different virulence of different SIV strains the clinicoepidemiological approach cannot be used as a definitive diagnostic of SIV infection. Therefore, laboratory confirmation is needed. This confirmation can be obtained either by the direct detection of the virus in biological samples or by the detection of antibodies. In the second case, it is important to note that during the acute phase of the disease animals are seronegative against the infecting strain.

In the event of an outbreak, samples to be collected for detection or isolation of the virus from live animals are nasal swabs or oral fluids. Lungs should be collected as well when possible from naturally dead or euthanized pigs. However, due to the short

duration of the (Van Reeth *et al.*, 2012) -7 to 10 days-, the moment of the sampling is crucial for a good detection of the pathogen, particularly if nasal swabs are to be taken since the virus is shed in nasal mucus only in the first days of the infection (Brown *et al.*, 1993a). Oral fluids perform worse than nasal swabs in terms of virus recovery by isolation, but in contrast, the sampling is easier and the sensitivities are similar or even higher for PCR (Romagosa *et al.*, 2012a).

A pretty convenient system for performing a rapid in-farm diagnosis of SIV is the ready-to-use kits based on immunochromatographic methods (for example, Flu-Detect by Synbiotics). In this case, acutely diseased animals with high fever (>40ºC) and clear respiratory signs should be selected for sampling (nasal swabs). This rapid system of detection indicates the presence of SIV in the samples but, usually, does not permit the identification of the subtype involved. Thus, additional tests are usually required for a complete diagnosis of the outbreak. Finally, lung sampling is recommended if animals representative of the outbreak are available for necropsy, avoiding pigs with chronic disease.

Characterization of the influenza virus involved in one outbreak can be carried out by means of molecular or serological approach. For subtyping, both conventional and real time RT-PCR have been used (Foni et *al.*, 2003; Alvarez *et al.*, 2008; Gall *et al.*, 2008; Chiapponi *et al.*, 2012). If the phylogeny of the strain has to be determined (e.g. epidemiological surveys or determination of the origin of the strain), conventional sequencing can be used. For these purposes, primer sets can also be found in the literature (Hoffman *et al.*, 2001; Simon-Grifé *et al.*, 2012; World Health Organization,

2009b). More recently, high-throughput sequencing has been used in phylogenetic studies (Lorusso *et al.,* 2011). However, these methodologies are rarely used for diagnostic purposes due to its elevated cost.

Serological identification requires the isolation of the virus in either MDCK cells or chicken embryonated eggs and the confrontation of the isolate with specific antisera against different subtypes –or different variants of a subtype- using the hemagglutination inhibition assay (HI) (OIE, 2008). Classically, HI has been used to determine the antigenic relationships between swine influenza viruses (de Jong *et al.*, 2001; de Jong *et al.,* 2007; Vincent *et al.,* 2009; Kyriakis *et al.,* 2011)

Another way to approach the diagnostic of influenza is through the serological analysis of affected individuals. Both blood samples and oral fluids can be used for serology. HI still is the reference technique for serological diagnosis of influenza and is useful to differentiate subtypes or even strains belonging to different variants of a subtype (e.g. H1N1, avH1N1 and H1N2) (OIE, 2008). However, the test lacks some sensitivity and specificity and laboratories performing HI have to update constantly the panel of SIV strains used. It is generally assumed that titres >1:160 are the product of infections caused by a strain close to the used in the test (OIE, 2008). Yet, different studies have shown that after consecutive infections or repeated vaccination, antibodies recognizing heterologous strains and even other subtypes could be generated and detected by HI (Van Reeth *et al.,* 2006; Kyriakis *et al.,* 2011). This indicates that interpretation of HI titers should be done very carefully to avoid false positive results (Figure 7 summarizes the timing and use for each type of sampling and technique).

Alternatively, several ELISAs are available in the market. The four most commonly used for diagnosis of influenza in pigs are CIVTEST influenza (HIPRA), IDscreen (ID.vet), Idexx Influenza A Ab Test (Idexx) and Idexx Herdcheck H1N1 and H3N2 (Idexx). The first three are based in the detection of antibodies against influenza A viruses with no precise distinction of subtypes. The fourth ELISA can detect specifically anti-H1 or anti-H3 antibodies. These ELISA are useful to detect SIV seroconversion or for seroprofiling swine herds but are of little use in the diagnosis of SIV outbreaks in endemic areas. In addition, the sensitivity of these tests is different for the different subtypes (Maldonado et al., 2007; Barbé et al., 2009).

1.7. Control and prevention of SIV infection in pigs

Measures implemented for SIV control and prevention vary depending on perception of the importance influenza as a problem for pig herds. For example, in the USA and Canada, swine influenza is considered an important economic threat for swine and by vaccination is a common practice in the areas where pigs are produced intensively. Contrarily, in Europe vaccination against SIV is less frequent and is mostly restricted to sows of farms suffering repeated influenza outbreaks that resulted in abortions. Biosecurity measures also contribute to create a firewall for the entrance of SIV in a farm. In the following sections, the major measures for treatment, control and prevention of SIV infections will be summarized.

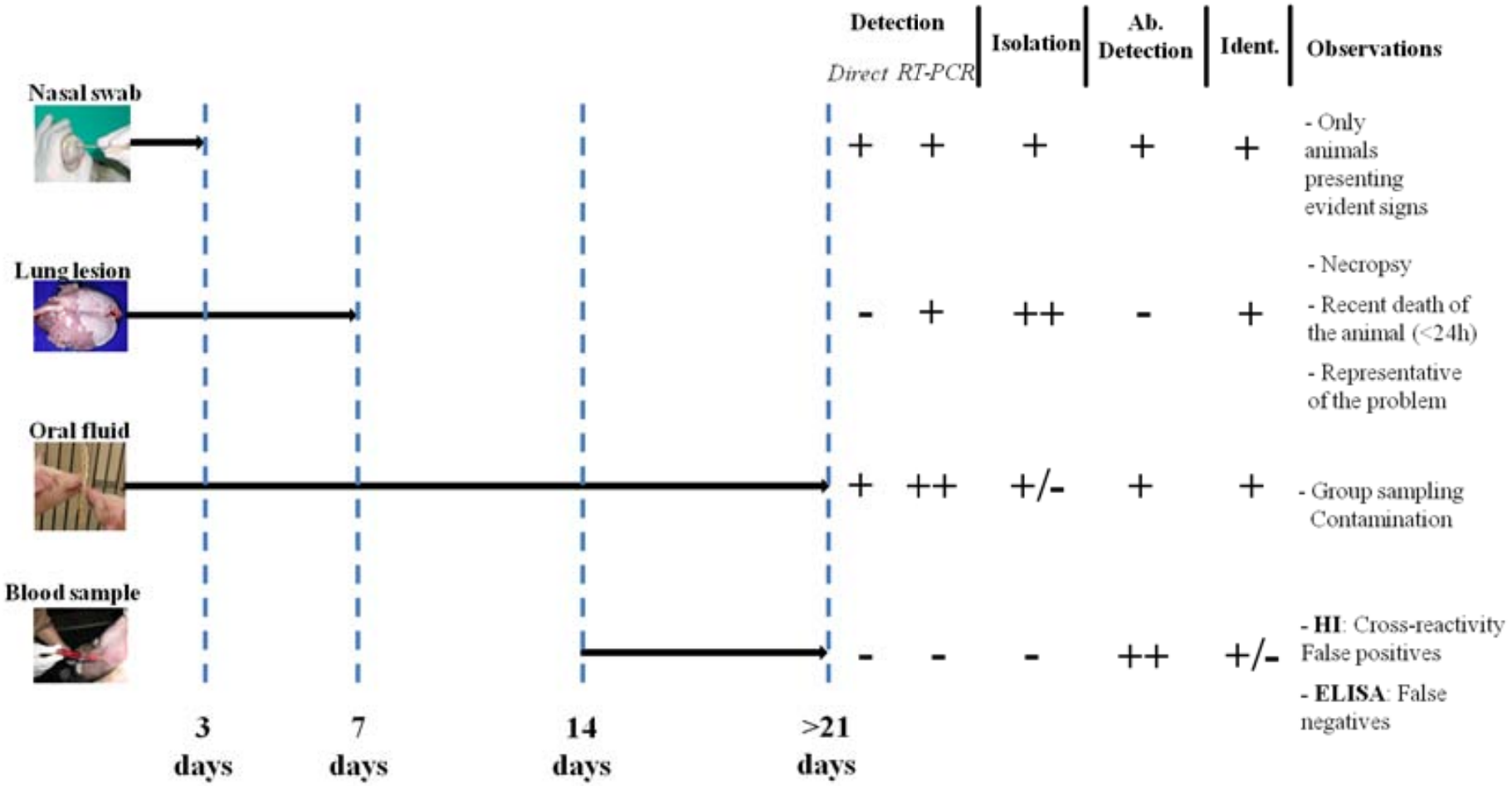

Figure 7. Adequate timing in days after the onset of the infection for sampling pigs and diagnostic methodologies used for the laboratory diagnosis of an outbreak of influenza.

1.7.1. Commercial vaccines

Vaccines marketed in Europe and North America are made of inactivated and adjuvanted SIV. These vaccines are based on the lineages of the different subtypes circulating in each continent although they may contain either "old" or "current" strains of SIV. In Europe, the most recent influenza vaccine is Gripovac 3® (Merial), containing H1N1, H1N2 and H3N2 strains from 2000 to 2003 (Table 1).

Productor	**Product name**	**Strains used for each vaccine**
Merial	Gripovac ®	A virus/New Jersey/8/1976 (H1N1) A virus/Port Chalmers/1/1973 (H3N2)
Fort Dodge	Suvaxyn Flu®	A virus/swine/Netherlands/25/1980 (H1N1) A virus/Port Chalmers/1/1973 (H3N2)
Hipra	Gripork®	A virus/swine/Olost/1984 (H1N1) A virus/Port Chalmers/1/1973 (H3N2)
IDT-Biologika	Respiporc Flu®	A virus/swine/Belgium/230/1992 (H1N1) A virus/swine/Belgium/220/1992 (H3N2)
Merial	Gripovac 3®	A virus/swine/Haselünne/IDT2617/2003 (H1N1) A virus/swine/ Bakum/1832/2000 (H1N2) A virus/swine/ Bakum/IDT1769/2003 (H3N2)

Table 1. Vaccines commonly used in Europe.

The efficacy of commercial SIV vaccines is difficult to ascertain given the diversity of SIV that circulate in pigs. Studies carried out with vaccines marketed in the USA, showed that vaccination partially protected pigs and contributed to decrease viral shedding after an experimentally challenge. However, lung lesions were still reproduced in vaccinated pigs (Macklin *et al.,* 1998). Another study showed that a H3N2 vaccine provided cross-protection against a heterologous H3N2 isolate (Rapp-Gabrielson *et al.*, 2000). In another experiment, partial cross-protection between heterologous strains was

demonstrated in spite that cross-reacting antibodies were not detected in HI (Platt *et al.*, 2011).

A recent experimental vaccination/challenge study has shown different results depending on the homology of the HA between the vaccine strain and the challenge strain. Thus, when the challenge and the vaccine strains where the same, protection was complete; in contrast, unvaccinated pigs became infected and shed enough virus to be able to transmit the infection (R_0>10 for a 5-day period). Pigs vaccinated with a commercial vaccine which HA was only 92% homologous to the challenge strain, had only partial protection (R_0=1) but in a 14-day period 40% of the contact animals became infected (Romagosa et al., 2012b). In any case, it is known that few changes in antigenic positions of the hemagglutinin can generate lack of serological recognition under experimental conditions (O'Donnell *et al.*, 2012), suggesting that similarity of HA is not enough to predict protection.

One of the practical difficulties in assessing the extent of the protection afforded by a given SIV vaccine is the lack of adequate correlates of protection. In Europe, several studies demonstrated that after vaccination HI titers >1:160 were able to protect pigs against the development of clinical disease and against the replication of the virus in lungs (Van Reeth *et al.*, 2001). However, lower titers may be effective against challenge with a lower virus dose or under field conditions (Bikour *et al.*, 1996)

In most situations, vaccination is only performed in sows with the aim of minimizing the reproductive impact of influenza should an outbreak occur. Vaccination of sows results in the transfer of maternal antibodies to piglets. It has been shown that hiperimmune sows may transfer maternal antibodies that will last until the 16th week of age at titres >1:40 as determined in HI (Van Reeth *et al.*, 2012). However, under natural conditions colostral-transferred immunity can wane before than 6 weeks of age (Van Reeth *et al.*, 2012; Simon-Grifé *et al.*, 2012). Vaccination in growers or finishers is rarely used, being implemented only when SIV outbreaks are recurrent. However, if sows have been vaccinated, interactions between the maternal antibodies and the vaccination may occur (Vincent *et al.*, 2008; Gauger *et al.*, 2011).

1.7.2. New insights on SIV vaccination

Vaccines available for swine only give partial protection against heterologous strains. Under this situation, protection when new a new influenza strain or variant appears is uncertain and there is a need for developing new vaccine platforms providing a broader protection. Several new SIV vaccine formulations are being developed and tested.

Experimental DNA vaccines based in the HA of SIV have been tested. According to Maclin and co-workers (1998), the use of a DNA vaccine afforded partial protection consisting of a a reduction in the extent and duration of viral shedding. However, other studies with DNA vaccines failed to demonstrate protection (Larsen *et al.*, 2002) although priming was observed.

Live vaccines have been recently tested and demonstrated to be able to induce higher protection against heterologous influenza viruses than inactivated vaccines (Vincent *et al.*, 2012). However, in that study, animals vaccinated in the presence of maternal derived antibodies (MDA) showed an enhanced respiratory disease compared to controls (Vincent *et al.*, 2012), indicating that some type of interaction of the vaccine efficacy by colostrum-derived antibodies.

Another strategy of vaccination recently tested in pigs is the use of replication defective adenovirus-5 encoding influenza hemagglutinin (Braucher *et al.*, 2012). In this case, the vaccination elicited protective immunity against the homologous challenge and partial protection to heterologous challenge.

Recently, an H3 alphavirus replicon-based vaccine has been tested in pigs and has been shown to induce anti-H3 antibodies at titers high enough to provide protection. This strategy could be interesting for the fast generation of new vaccines that can be adapted easily to the changes in the variants of circulating influenza strains (Vander Veen *et al.*, 2012). Vergara-Alert and co-workers (2012) induced both humoral and cell-mediated immune responses in pigs against pH1N1, HPAIV H5N1 and two swine isolates (H1N1 and H1N2) by using one conserved HA peptide as a vaccine although they fail to show protection in the case of pigs.

1.7.3. Biosecurity measures and other strategies for SIV control in swine herds

Implementation of biosecurity measures can be useful not only for the control of SIV but for other respiratory pathogens as well. As seen in section 1.4.4, high replacement

rates have been suggested to be a risk factor for having a higher within farm seroprevalence. This result could be explained by the introduction of new SIV subtypes or variants with the introduction of animals or by the maintenance of the virus due to the entrance of new susceptible animals in the farm. In any case, quarantine and vaccination are useful to minimize the impact of new virus entrances (Lee *et al.*, 2007; Torremorell *et al.*, 2009). Besides this, hygienic measures for farm workers, visitors and veterinarians –taking a shower, frequent handwash, change of clothes, etc- may help to reduce introduction of new SIV strains or to hamper the dissemination of the virus within the farm. All in/all out management systems accompanied by cleaning and disinfection of the facilities should prevent the maintenance of SIV in the farm and later circulation of the virus in new batch susceptible animals by environmental contamination. A work flow that considers the different production stages of pigs can be also implemented to prevent enhanced circulation of pathogens.

Unfortunately, the lack of controlled studies makes difficult to assess the precise efficacy of all the above mentioned measures. A study carried out by Torremorell and co-workers (2009) accomplished efficient elimination of an H3N2 strain through the application of changes in the management of pigs, changes in the quarantine procedure and on the origin of replacement gilts and a total depopulation of nurseries and finishing units. Elimination of SIV resulted in an improved growth rate of 123 g/day and a 2% decrease of mortality in nurseries. However, the impact of these measures are probably not only attributable to SIV elimination but to the elimination of other respiratory pathogens as well.

The recent discovery in pigs herds of new H1N1 reassortant viruses including genes from the pH1N1 (see section 1.4.3.7) indicate that the entrance of human viruses can occur frequently in swine. Another measure that could have significance to prevent the entrance of new influenza viruses in herds is the vaccination of farm workers or other personnel related with pig the production at any level. This measure is especially important, not only from an economical point of view but due to its implication on the generation of new influenza variants with zoonotic potential.

Chapter 2. Hypotheses and objectives

Pigs have been proposed to act as a mixing vessel where avian and mammalian influenza A viruses reassort to produce new influenza A strains. Nevertheless, the knowledge on the epidemiology of influenza A viruses in pigs is relatively scarce compared to what is known for birds or humans. As a matter of fact, for many years influenza in pigs has been described almost solely as a disease appearing in an epidemic form that strikes the farm rapidly and vanishes as rapidly as it appeared. Although epidemic outbreaks are relatively easy to recognize there are also endemic situations in which a given influenza virus persists in the farm for a long time. Thus, while epidemics may reflect the introduction of new and virulent influenza strains in a naïve population, endemic situations are the field where drift and shift more probably occur. It has been suggested that genetic diversity of influenza viruses of swine is high; however, since antigenic drift seems to be slower in pigs than in human, it is difficult to foresee whether or not this genetic diversity can impact in the antigenic diversity of swine influenza viruses (SIV). When looking at the Spanish situation, almost nothing is known on the molecular epidemiology of SIV or on the antigenic cross-reactivity of circulating influenza viruses. While the first point can provide valuable information on the diversity, sources and spread of SIV; the second point would contribute to foresee the potential circulation of viruses of a given subtype in partially immune populations.

Based on the abovementioned previous knowledge and considerations, three hypotheses were proposed in the present thesis:

1) Reassortment will be a frequent phenomenon in currently circulating Spanish SIV.

2) In an SIV endemic pig farm the generation of new SIV variants, either by reassortment or by drift could be observed.

3) Genetic diversity of currently circulating Spanish SIV will have a reflect on the antigenic diversity of these viruses.

With the aim to provide answers to the abovementioned hypotheses, three objectives were formulated:

1) To determine the existence of reassortment events in SIV isolated from respiratory disease cases in pig farms and to establish the phylogenetic relatedness of each viral gene of the obtained isolates.

2) To determine the evolutionary patterns at a whole genome level of SIV present in an endemically infected farm.

3) To determine how genetic diversity of SIV isolated in Spanish pig farms correspond to an antigenic diversity based in the results of the haemagglutination inhibition assay.

Chapter 3. Study 1.

Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm.

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Abstract: In the present study, outbreaks of respiratory disease were investigated for the presence of swine influenza virus (SIV). In 14 cases the circulating SIV strains were isolated, fully sequenced and compared with other known SIVs. The viruses causing the outbreaks belonged to the H1N1 (including human pandemic H1N1), H3N2 and H1N2 subtypes. In 11/14 cases the phylogenetic analyses indicated the occurrence of probable reassortment events. In the second part of the study, the genetic evolution of H1N1 SIV was assessed in a longitudinal study in closed groups of pigs over six months. Sequencing of the 22 isolates indicated co-circulation of two different variants for the same virus, as well as the emergence of SIV reassortants at certain time-points. These results indicate that reassortment events in SIV are common, and point towards the need for a better understanding of the epidemiology of SIV, particularly in endemic farms.

Supplementary information (in Chapter 5): Table S1; correspondences between the strains from the Chapter 3 with the Chapter 4. Tables S2 and S3; accession numbers of the sequences used in the present chapter. Figures S1 a – f; phylogenetic trees obtained in the present work; Figure S2: Scheme of the results obtained in the longitudinal study; Figure S3; phylogenetic tree obtained with the high throughput sequencing.

Introduction

Swine influenza viruses (SIV) belong to the genus *Influenzavirus* (type A) within the *Orthomyxoviridae* family. Influenza viruses are enveloped single stranded negative sense RNA viruses in which the genome is organized in 8 different segments encoding 12 different proteins. One of the key characteristics of influenza viruses is their potential for rapid evolution. On the one hand, evolution of influenza viruses is based on the lack of proof-reading activity of the viral RNA-polymerase, allowing the continuous generation of mutations that are responsible for the genetic and antigenic drift (1). In pigs, antigenic drift phenomena have been said to play a minor role in SIV evolution when compared with human viruses (2, 3). This has been attributed to the high replacement rate in pig herds that implies a high and constant flow of susceptible animals, resulting thus in a low selective pressure for the virus. On the other hand, antigenic shift, that is the arising, by genetic reassortment, of new influenza viruses containing genes of different subtypes, is considered to be a major force in the generation of influenza pandemics. For example, the avian-like H3N2 and the human-like H1N2 SIV circulating in Europe are the product of the reassortment between European avian-like H1N1 and human H3N2, and between human H3N2 and human H1N1, respectively (4).

It is also known that pigs can be infected with avian, swine and human influenza A viruses, and for that reason, pigs have been classically proposed to be the mixing vessel where reassortant "humanized" influenza strains can arise (5). The reason behind this concept is related to the fact that avian influenza viruses have a high affinity for α-2,3 sialic acid receptors while mammalian influenza viruses usually bind to α-2,6 receptors.

Pigs have both types of receptors. It is now known that avian viruses can infect humans without previous adaptation in pigs, as is the case with the highly virulent avian H5N1 (6). Nevertheless, the recent emergence of a human pandemic influenza A H1N1 virus (pH1N1) harbouring SIV genes and the arising of new reassortants between an avian H3N2, a human H1N2 and the pH1N1 isolated from pigs and minks are evidence favouring the notion that reassortment occurs frequently in SIV (7,8).

The entry of a new influenza virus into a swine herd is classically considered to cause a clinical outbreak with common flu signs: fever, lethargy, conjunctivitis, nasal discharge, coughing, laboured breathing and eventually abortions (9). However, increasing evidences indicate that SIV infections are often endemic and may remain as a subclinical or insidious problem (10). Thus, endemic situations where viral circulation keeps on going for prolonged periods are optimal for studying drift and shift phenomena. The objective of the present study was to characterize influenza A virus isolated in clinical outbreaks in Spanish swine herds and to establish their genetic relationship with other SIV, as well as to assess the evolutionary events in SIV circulating in an endemically infected pig farm.

Material and Methods

Ethics Statement

The present study was carried out in accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union, and with the recommendations approved by the Animal and Human Ethics experimentation

Committee (CEEAH) of the Universitat Autònoma de Barcelona, that ensures the protection and welfare of the animals used in research, in agreement with the current European Union legislation.

Sampling

a) Outbreaks of respiratory disease suspected to be swine influenza

This study was conducted from January 2010 to August 2011 in a NE Spain area that accounts for more than 40% (>10 million pigs) of the Spanish pig production, with the collaboration of swine veterinarians who reported the cases. Twenty-two reports of respiratory disease outbreaks compatible with SIV were followed-up. For each suspected case, clinical data and the age group of affected animals were recorded. In each case, nasal swabs from 20 animals showing clinical signs were collected. These swabs were immediately suspended in 1ml of transport medium (PBS, 0.15 M, pH 7.2 70%; glycerol 20% and 10% of penicillin/streptomycin solution) and sent to the laboratory at 4°C where they were processed (<24 h after collection). When possible, lungs from dead or euthanized pigs were also sent to the laboratory. Two additional cases with no clear signs of influenza were included in the study because of in-field positive influenza results (Flu Detect Swine test; Synbiotics, Lyon, France) reported by the veterinarian.

b) Longitudinal study carried out in a farrow-to-finish farm.

Infection dynamics, clinical outcome and subtype characterization of the viruses isolated in the farm are described in Simon-Grifé et al. in 2012 (10). Briefly, a whole

batch of 3-week-old piglets (n=121; 11 litters) was followed up during the whole productive period until pigs were sent to the slaughterhouse. Animals were ear-tagged at the beginning of the study in order to follow them individually, and were sampled (nasal swabs) weekly form 3 to 13 weeks of age. The pigs were then sampled at 15, 17, 20 and 24 weeks of age.

Processing of samples

Viral RNA was extracted with a commercial kit (Qiamp, Qiagen) according to the instructions of the manufacturer, and detection of SIV was performed by means of a Taq-Man real time reverse transcriptase/polymerase chain reaction (RRT-PCR) aimed at detecting the *M* gene of influenza A viruses (11). Samples yielding RRT-PCR positive results were inoculated into specific pathogen free (SPF) embryonated chicken eggs (ECE) in an attempt to isolate SIV. Briefly, nasal swab suspensions were centrifuged, and 100 µl of the supernatant were inoculated into the allantoic cavity of 9–11-day-old ECE. Allantoic fluid was harvested 3 days after inoculation and viral growth was detected by the haemagglutination assay using chicken red blood cells. Negative allantoic fluids in the first passage were inoculated in ECE again before being discarded. At the same time, nasal swab suspensions were inoculated into Madin-Darby Canine Kidney (MDCK) cells cultured with added trypsin (2µg/ml)(Sigma-Aldrich). Cell culture supernatants were collected at approximately 75% cytopathic effect, centrifuged and later tested as above. Samples were discarded if negative after the second passage in cell culture.

Sequencing and phylogenetic analysis

All SIV genes were sequenced. Primers used and the segments of the genome amplified are shown in Table 1. Sequence analysis of isolates retrieved from outbreaks was carried out initially to determine the grouping of each viral gene for each isolate. For that purpose a database was constructed for each viral gene. The database initially included sequences from SIV isolates contained in the Influenza Sequence Database (ISD; http://www.flu.lanl.gov) that had been sequenced in full (all the available sequences of European H1N1"avian-like", European H3N2 and H1N2 "human-like" SIV were included). Also included was a selection of sequences representative of the different Asian SIV reported by Vijakrishna et al. 2011 (12) including "avian-like", "classical" H1N1 and triple reassortant H1N2 strains. Additional American and Asian H3N2 SIV isolates from ISD corresponding to different years were also included for a total of 229 sequences for each gene. This set of sequences was aligned using Clustal W (14) and compared in a maximum likelihood analysis (MLA, 100 iterations for bootstrapping) with a generalised time-reversible model with both MEGA 5.0 and PhyML 3.2 software (13, 14). In order to produce trees reflecting the maximum diversity with the minimum number of isolates, redundant sequences (≥99.5% similarity) were eliminated as long as the grouping of isolates was preserved.

Once each of the isolates of the present study was assigned to a given cluster for a given gene, the database was further enriched with individual gene sequences (HA, NA; M, etc...) available at ISD and Genbank, and clustering with each of the genes of the isolates of the present study. A new MLA (100 iterations for bootstrapping) (15) was

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
PB2	1-AGCAAAAGCAGGTCAA-16	2341-AGTAGAAACAAGGTCGTTTTTAAAC-2316
PB2	533-ATGGAAGTTGTTTTCCC-550	1622-CTCCCACATCATTGACGATG-1602
PB2	974-ATATGCAAGGCTGCAATGGG-994	
PB2	1640-TCATCGTCAATGATGTGGGA-1660	
PB2	3-CRAAAGCAGGTCAAWTATATTC-22	
PB1	1-AGCGAAAGCAGGCAAACCATTTGAATG-27	785-CTTTTGTCATTGTGTTCAGTGTCAGTGC-712
PB1	598-AGGGACAACATGACCAAGAAAATG-621	1092-AGCTTCATGCTCTTACTTTCGAAC-1115
PB1	1036-AGCATTGCTCCTATAATGTTCTC-1058	1708-GTTTGAATTTGTGTGTCACCTCTGTC-1733
PB1	1621-AATATGATAAACAATGACCTTGG-1643	2320-AGTAGAAACAAGGCATTTTTTC-2341
PA	1-AGCGAAAGCAGGTACTGATCCAAAATGG-28	653-GTTCCTGTGATTTCAAATCTTTCTTC-628
PA	466-TTCTCATTCACTGGGGAGGAAATGGC-491	1260-GTTGAATTCATTCTGGATCCAGCTTG-1235
PA	1123-AAGTGGGCACTTGGTGAGAATATGGC-1148	1777-GGCAGCGCCTCATTTCCATTCCCC-1754
PA	1570-GATGTGGTAAACTTTGTGAGTATGG-1594	2233-AGTAGAAACAAGGTACTTTTTTGGAC-2208
HA	1-AGCAAAAGCAGGGG-14	1743-AGTAGAAACAAGGGTGTTTT-1724
HA	773-AATAACATTCGAAGCAACTGGAA-795	857-TCTGAAATGATAATACCAGATCC-835
HA	7-CTACGTCTCTGGGGAGCAAAAGCAGG-21	979-GTGATGGGATGTACATTCTG
NP	1-AGCAAAAGCAGGGT-14	1565-AGTAGAAACAAGGGTATTTTTC-1544

NA	1-AGCAAAAGCAGGAGT-15	1467-AGTAGAAACAAGGAGTTTTTT-1447
NA	680-TGAGAACACAAGAGTCTGAATGTG-700	1140-TTCGGATCCCAAATCATCTC-1120
MA	1-AGCAAAAGCAGGTAGAT-17	1027-AGTAGAAACAAGGTAGTTTTTTACTC-1002
MA deep	5-AAAGCAGKTAGATRTRTTGAAARATG	564-ACCATTCTGTTYTCATGYCTG
NS	1-AGCAAAAGCAGGGTG-15	AGTAGAAACAAGGGTGTTTTTTA

Table 1. Primer set used to amplify each segment of the SIV. Primer set used to amplify each segment of the SIV and information about the begin and end positions of each one. Abbreviations: polymerase genes, PB2, PB1, PA; hemagglutinin gene, HA; nucleoprotein gene, NP; neuraminidase gene, NA; matrix gene, MA; non-structural gene, NS.

conducted for each enriched database of genes and clusters. In a final step, and in order to determine potential reassortment events between human and porcine viruses, sequences belonging to the internal genes of European influenza strains isolated from humans (n=64) were compared with the internal genes from SIV isolates retrieved in the outbreaks. Supplementary Table S1 shows the identification of SIV sequences used for comparison

In the longitudinal study, sequences of the SIV genes of the isolates retrieved during the follow up were compared by means of MLA (1000 iterations for bootstrapping) using Phyml 3.2 software. Deep sequencing of the first 500 nt of the viral matrix gene was performed on six isolates. RNA obtained directly from six original nasal swab samples from 3 week-old positive piglets was selected for the high throughput sequencing. The first five-hundred nucleotides from the M gene were amplified using an RT-PCR (primers are summarized in Table 1). DNA concentration was determined using the Nanodrop Spectrophotometer 1000 (Thermo Fisher Scientific) and 168 ng from each sample were used for the sequencing. The DNA library was prepared by the ligation of different adaptors for each amplified sample. Procedures of library preparation, emulsion PCR and sequencing methodologies were carried out according the manufacturer's instructions (Rapid Library Preparation Method Manual, emPCR Amplification Method Manual –Lib-A and Sequencing Method Manual for GS Junior Titanium Series. Roche) and sequencing was carried out in a 454 GS Junior Titanium Series (Roche). For the output data analysis, CLC genomics workbench software was used (CLC bio, 2005, Denmark). Finally, maximum likelihood trees were obtained using PhyML 3.2 and were repeated with Mega 5 software.

Results

Study of respiratory disease outbreaks

Twelve out of the 22 studied outbreaks yielded positive results for SIV. Further characterization by sequencing revealed five H1N1 strains (designated as /01 to /05), four H3N2 (/01 to /04) and three H1N2 (/01, /02 and /03). H1N1/01 and /02 were detected in fattening units while H1N1/03, /04 and /05 were detected in farrow-to-finish farms. In all cases, respiratory disease was evident, mainly in fatteners. As regards H3N2 isolates, H3N2/01 and /03 were detected in farrowing farms (only sows) while H3N2/02 and H3N2/04 were retrieved in a farrow-to-weaning farm. In these cases, the disease was only seen in sows, with abortions and sudden death of pregnant sows being reported by veterinarians. H1N2/01 and /02 with overt respiratory disease were detected in farrow-to-finish farms. Besides the outbreaks, samples of two farms were added because of previous on-farm detection of SIV. This resulted in 2 SIV isolates, one H1N1 (H1N1/06) and one H1N2 isolate of (H1N2/04) were found. In both cases, respiratory disease was mild and restricted to weaners.

All examined genes for all isolates belonged to European lineages of SIV, with the exception of two. H1N1/04 was identified as a pH1N1 closely related to human isolates, and the NA of H1N2/03 clustered with some American strains. Four of the five remaining SIV H1N1 isolates were identified as avian-like. For one (H1N1/05) the HA clustered with H1 of reassortant “human like” European H1N2. All H3N2 isolates belonged to the reassortant human-like European clade (Figures 1 to 4).

The four H1N2 isolates of the present study belonged to the reassortant human-like H1N2 clade and formed a separated and statistically significant cluster within that group for HA. The HA of H1N1/05 was also included in this cluster. Three of the four H1N2 also clustered together, but the fourth (H1N2/03) contained an NA closely related to a group of H3N2 and H1N2 SIV isolates, mainly formed by American and Asian strains, but also containing some swine isolates of Italy retrieved from 2005 onwards.

Interestingly, for most isolates (n=11) except pH1N1 and H3N2/02 and 03, when clustering of internal viral genes was examined it was seen that the same strains could cluster in different groups depending on the examined gene, a further indication of the occurrence of reassortment (Figure 5).

As expected, the percentage of similarity among isolates of the present study clustering together was high for internal genes, and much lower for *HA* and *NA*. Table 2 shows the similarity matrices and the closest known isolate for *HA* and *NA*.

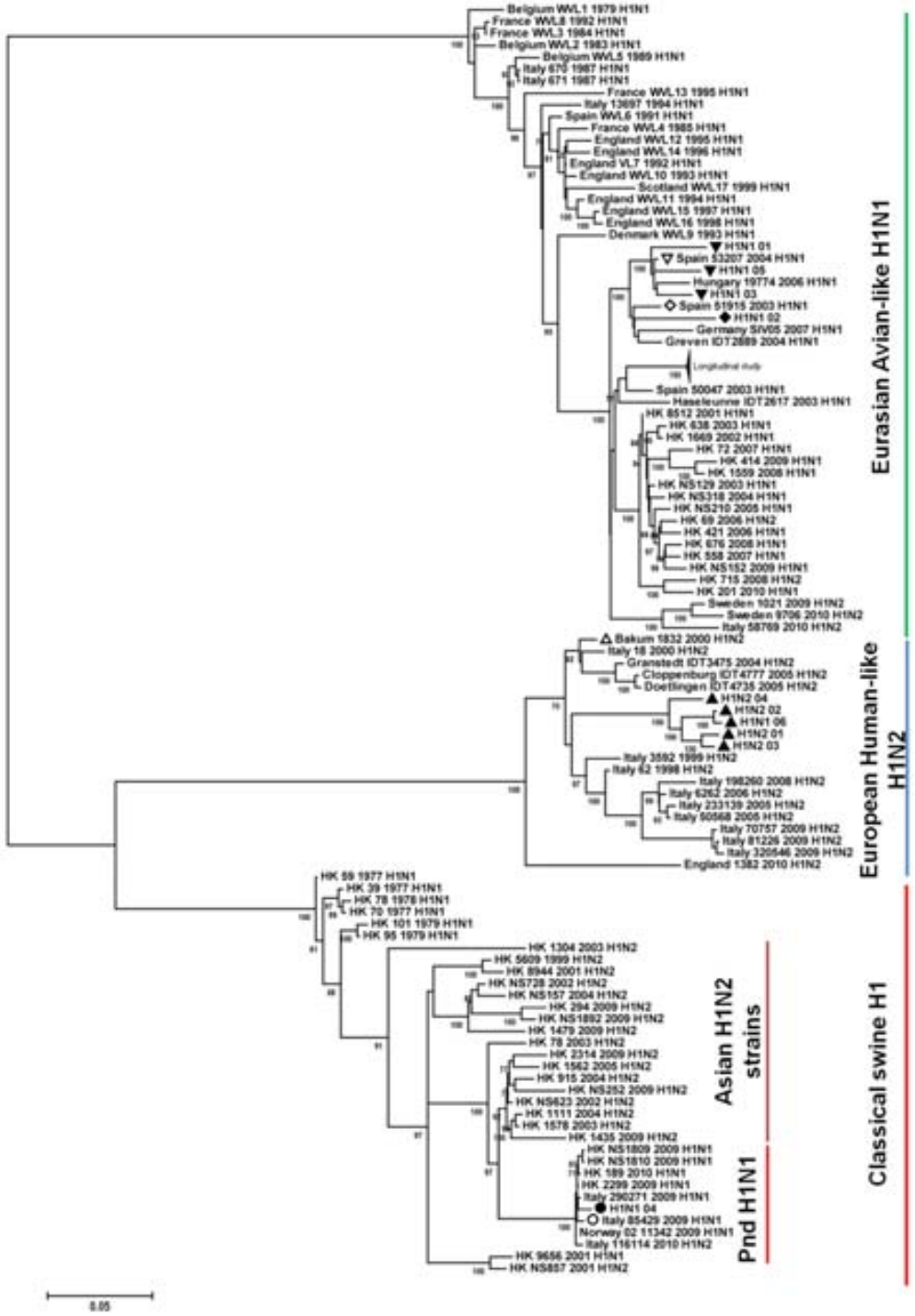

Figure 1. Maximum likelihood tree including H1N1 and H1N2 hemagglutinin from swine strains. Black symbols indicates the strains obtained in the present study. White symbol indicates the closest relative strain for each isolate. Bootstrap values lower than 70 are omitted.

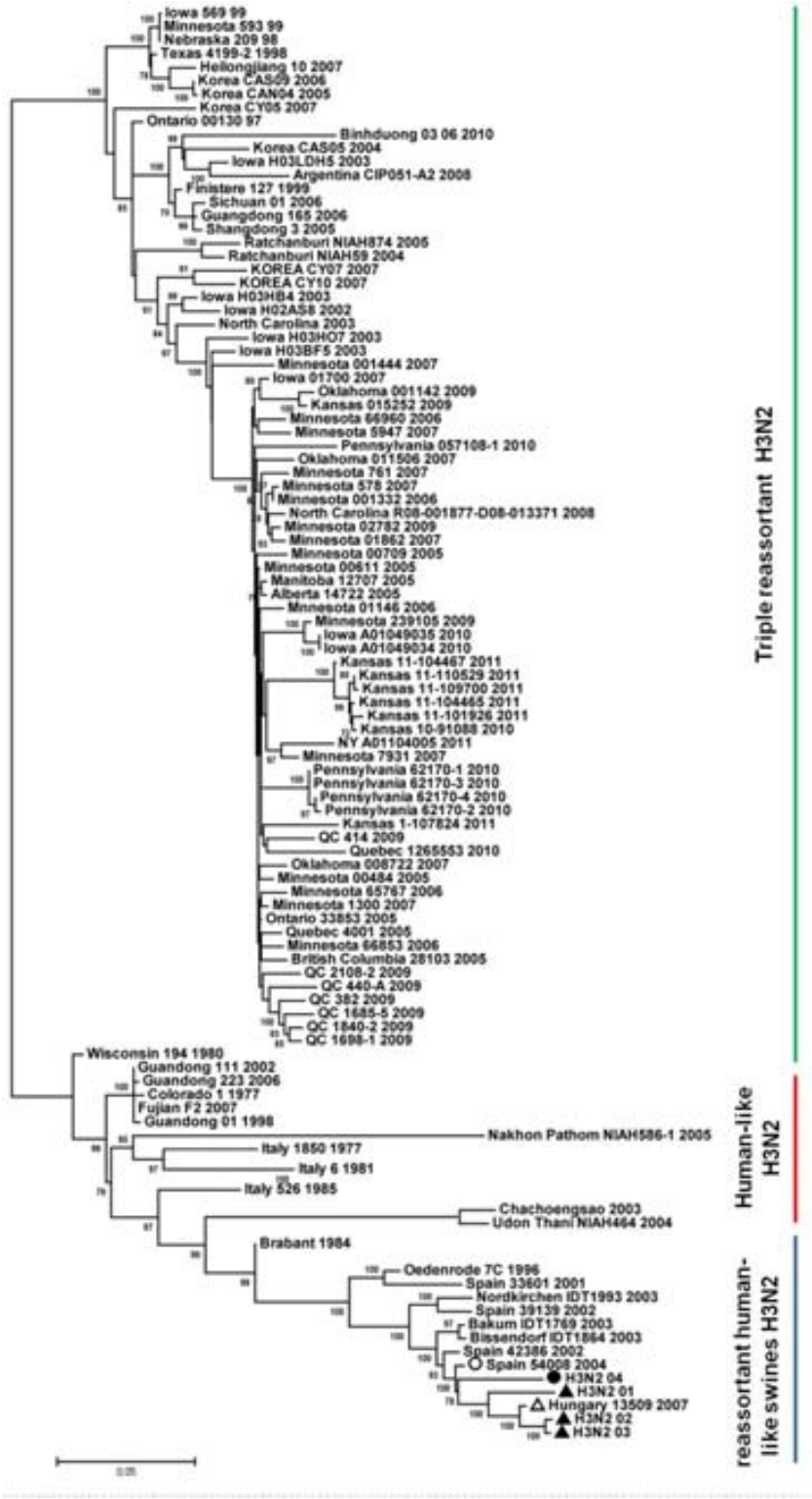

Figure 2. Maximum likelihood tree including H3N2 hemagglutinin from swine strains. Black symbols indicates the strains obtained in the present study. White symbol indicates the closest relative strain for each isolate. Bootstrap values lower than 70 are omitted.

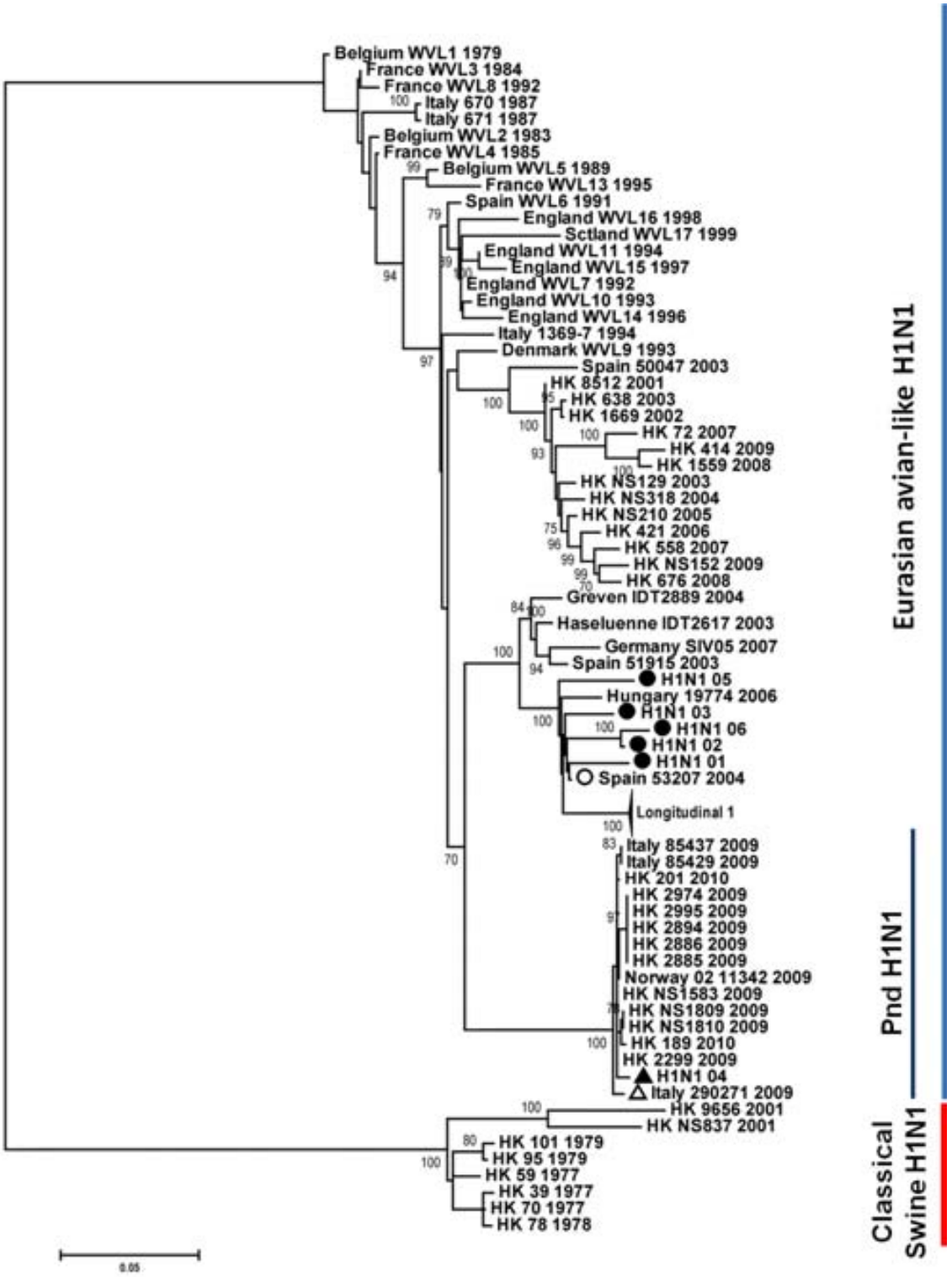

Figure 3. Maximum likelihood tree including H1N1 neuraminidases from swine strains. Black symbols indicates the strains obtained in the present study. White symbol indicates the closest relative strain for each isolate. Bootstrap values lower than 70 are omitted.

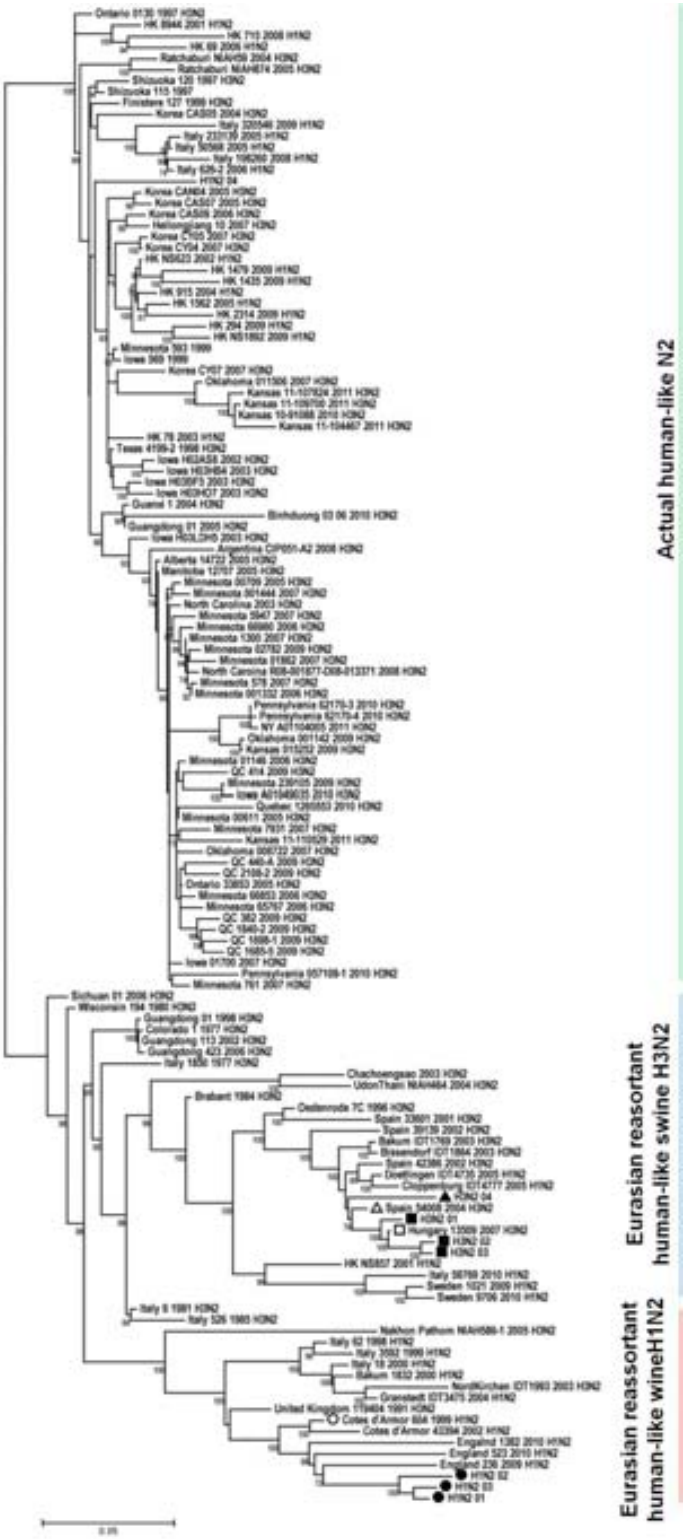

Figure 4. Maximum likelihood tree including H1N2 and H3N2 neuraminidases from swine strains. Black symbols indicates the strains obtained in the present study. White symbol indicates the closest relative strain for each isolate. Bootstrap values lower than 70 are omitted.

H1N1

HA	01	02	03	05
01	**ID**			
02	**92**	**ID**		
03	**95.3**	**92.8**	**ID**	
06	**94.8**	**92.4**	**95.6**	**ID**

Average similarity: 93,82%; SD: 1,6%
CKS: A/Sw/Spain/53207/2003 H1N1
Average similarity with CKS: 96,47%; **SD** : 1,7%

NA	01	02	03	05	06
01	**ID**				
02	**95.9**	**ID**			
03	**96**	**96.3**	**ID**		
05	**94.9**	**95.1**	**95.3**	**ID**	
06	**95.4**	**98.8**	**95.5**	**94.7**	**ID**

Average similarity: 95,58%; SD: 1,16%
CKS: A/Sw/Spain/53207/2003 H1N1
Average similarity with CKS: 96,4%; **SD**: 2,6%

H1N2

HA	01	02	03	04
01	**ID**			
02	**95.8**	**ID**		
03	**95.8**	**99.5**	**ID**	
04	**94.1**	**94.3**	**94.4**	**ID**

Average similarity: 95.65%; SD=2%
CKS:
A/Sw/Hungary/13509/2007 H3N2
A/Sw/Spain/54008/2004 H3N2
Average similarity with CKS: 97,8% - 96,5%;
SD: 1,3%

NA	01	02	03	04
01	**ID**			
02	**97**	**ID**		
03	**97**	**99**	**ID**	
04	**94.4**	**93.2**	**93.1**	**ID**

Average similarity: 95.6%; SD = 2,4%
CKS:
A/Sw/Hungary/13509/2007 H3N2
A/Sw/Spain/54008/2004 H3N2
Average similarity with CKS: 96,5% - 95,6%;
SD: 0,7%

H3N2

HA	01	02	03	04	H1N1 /05
01	**ID**				
02	**96.2**	**ID**			
03	**95.8**	**95.9**	**ID**		
04	**98.4**	**96.5**	**95.9**	**ID**	
H1N1/05	**96.2**	**99.4**	**95.8**	**96.3**	**ID**

Average similarity: 96,65%; SD= 1,2%
CKS: A/Sw/Bakum/1832/2000 H1N2
Average similarity with CKS: 92,04% **SD:** 0,2%

NA	01	02	03	04
01	**ID**			
02	**96.4**	**ID**		
03	**83.8**	**83**	**ID**	
04	**98.4**	**96.7**	**83.7**	**ID**

Average Similarity: 97,2% - 83,5% ; **SD**: 1,1% - 0,4%
CKS:
A/Sw/Cotes d'Armor/604/1999 H1N2
A/Sw/Minnesota/593/1999 H3N2
Average Similarity with CKS: 95,8% - 95,1%
SD: 2,8%

Table 2. Nucleotide identity of within all but PH1N1 isolate (H1N1 04) by subtype and glycoprotein segment (HA and NA). The Closest Known Strain (CKS) for each group of strains, average similarity and standart desviation (SD) are also shown.

Ref H1N1	PB2	PB1	PA	NP	MP	NS1
H1N1/01	97.9	96.6	97	94.2	97.5	92.5
H1N1/02	97.3	96.9	97	94.6	95.1	97.2
H1N1/03	97.4	98.1	97.3	94.9	98.7	93.2
H1N1/04	83.4	85.2	85.3	82.7	92.9	80.5
H1N1/05	97.2	97.1	96.6	94.6	94.8	96.6
H1N1/06	96.8	97.3	97.5	95	98.3	98
H1N2/01	96.8	96.6	96.2	94.9	94.4	92.9
H1N2/02	96.2	97.4	96.5	94.9	97.4	97.9
H1N2/03	97	97.2	97.1	95.3	97	92.1
H1N2/04	97	96.6	97.1		95.1	93
H3N2/01	96.8	97.3	98.2	95.2	98.1	98.1
H3N2/02	97.1	97.4	96.7	95	94.9	94.5
H3N2/03	97	97.1	96.3	94.9	95	92.8
H3N2/04	96.8	97	95.8	94.3	97.4	97.7
Long 1 (a)			92			93.9
Long 1 (b)				94.7	94.4	

Figure 5. Percentage of nucleotidic similarity of each gene segment and strain when compared with the virus A/Swine/Spain/51915/2003 is shown in the figure. The different colors indicate different clustering of the strains supported by bootstrap values >70. Trees are shown in the supplementary figures S1 a-f.

Longitudinal study

The sequence analyses for the 22 examined isolates revealed that *HA*, *NA*, *PB1*, *PB2*, *PA* and *NS* genes were highly conserved among all isolates (average similarity >99%). Two different variant genes were present for *M* and *NP*, designated from now on as M_a, M_b and NP_a, NP_b with a similarity of 94.7% and 97.1%, respectively. Ma was present in most of the isolates (20 out of 22) while M_b was only detected in two samples of 3-

week-old piglets. Most isolates harboured NP_a (21 out of 22), and one sample from a 7-week-old pig contained the NP_b gene. Interestingly, the isolate harbouring the rare NP_b had the predominant M_a gene. The closest known sequences to the different *M* and *NP* genes were A/H1N1/Sw/Spain/53207/2004 (M_a), A/H3N2/Sw/Seesen/IDT3055/2004 (M_b), A/H1N1/Sw/Spain/51915/2003 (NP_a) and A/H1N2/Sw/Bakum/1832/2000 (NP_b) with which they share a similarity of between 97.6% and 98.6% in MP, and 98% and 98.6% % in NP.

A more detailed analysis of the *HA* indicated the potential existence of two variants (<1% of difference) among circulating isolates (bootstrap value 97%, tree not shown). In order to further check the existence of those two potential variants, sequences of *HA, NA, PB1, PB2, PA* and *NS* were concatenated and re-analysed as above. *M* and *NP* sequences were excluded. The analysis of concatenated segments (Figure 6) showed the actual existence of two variants of the same strain. Those two branches arose because of some nucleotide mutations (12 in *HA*, 8 in *PA*, 7 in *PB1* and *NS*, 6 in *PB2* and *NA*). These changes represented non-synonymous mutations in HA (positions 78 I→V; 151 A→S; 158 A→S; 159 R→N; 214 A→T; 417 I→L), NS1 (52 M1→L; 79 T→I, 84 V→M; 176 I→N; 209 D→N), PA (positions 29 K→R; 204 K→N), PB1 (Positions 336 V→I; 384 S→L), PB2 (Position 461 V→I). Interestingly, all changes in NA were synonymous and HA A158S and R159N changes are both included in the Ca antigenic site (16).

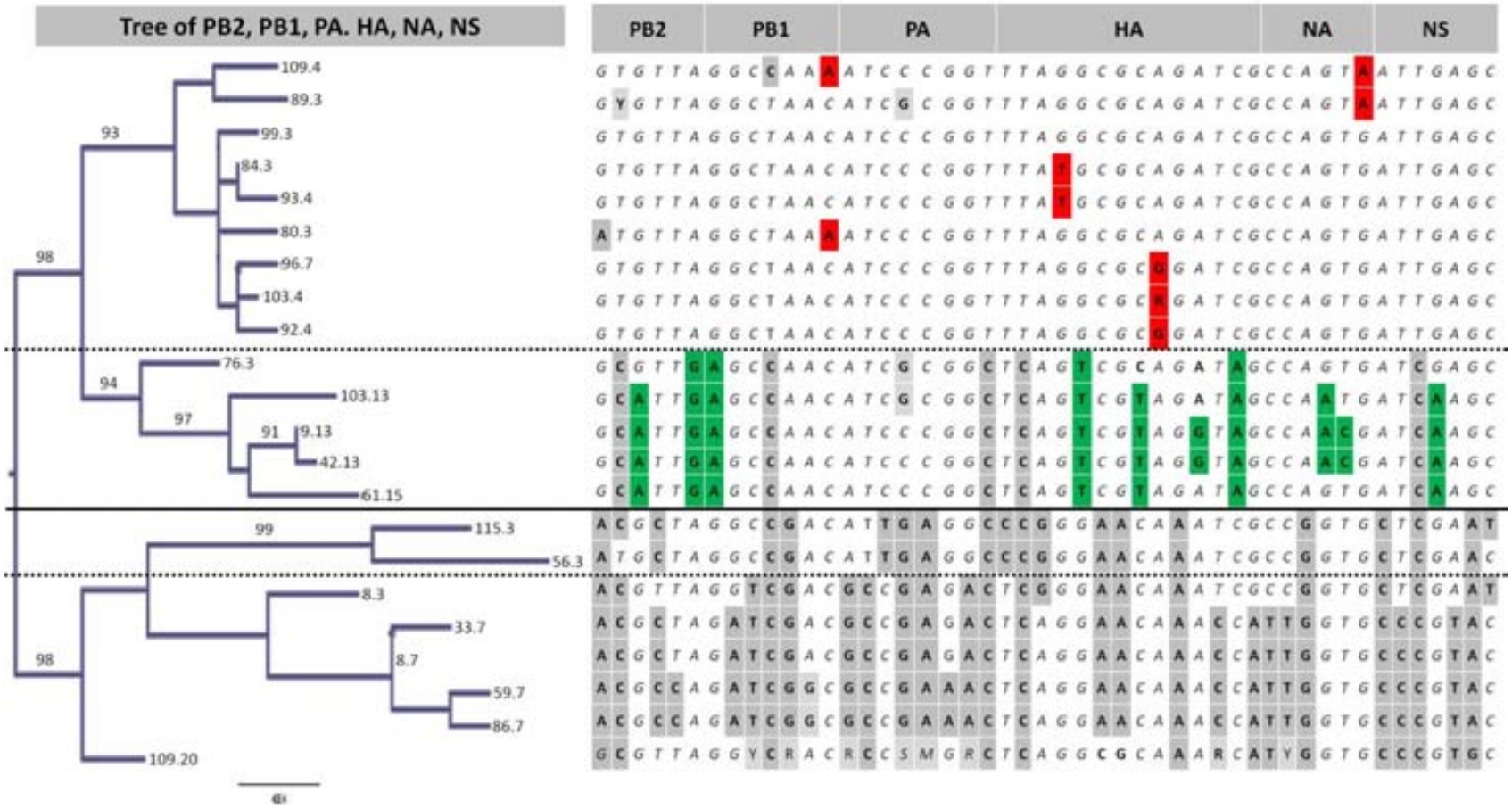

Figure 6. 100 bootstrap maximum likelihood tree obtained from the concatenation of the genes PB2. PB1. PA. HA. NP. NA. and NS. In the figure it is also shown all the mutation sites observed in more than one isolate. Black line separates the two main branches observed in the longitudinal study. Discontinuous line separates the subclades that are not observed in all the segments.

Deep sequencing of the *M* gene was carried out on six samples of 3-week-old piglets. Those six animals included one harbouring the M_b gene, and five in which isolate harboured the M_a gene. Analysis of M_a isolates did not provide any more evidence of the existence of a number of quasi-species differentiable by just a few mutations (up to three). Two different matrix segments were identified in M_b and thus, 275/280 sequences clustered with the consensus sequence M_b obtained by conventional Sanger sequencing from this animal (similar to A/H3N2/Sw/Seesen/IDT3055/2004), but 5/280 yielded a sequence with between 99.9% and 100% similarity to the consensus M_a.

Discussion

The intensive research on the epidemiology of influenza viruses carried out in recent years has shown that the complexity of the natural history of these viruses is much higher than previously thought. In the present work we aimed to determine the characteristics and phylogenetic relationships of SIV isolates from respiratory disease outbreaks of swine, as well as to investigate the evolution of SIV within an endemically infected farm.

The efficiency of the outbreak surveillance must first be mentioned. In our hands, only about one half of the supposed influenza outbreaks could be confirmed by virological examinations. This fact points towards two different elements that are relevant to the development of surveillance programs for influenza in pigs. The first one relates to the actual ability of veterinarians to distinguish influenza from other respiratory diseases. On one hand, the presence of multiple respiratory pathogens in a farm may create confusion as regards establishing a clinical suspicion and, on the other hand, as shown

in two cases of the present study, influenza may only produce a mild disease without signs in adults. Besides this, it is worth noting that abortions were only seen associated to H3N2, a fact that would merit further field surveillance. Secondly, the short period in which SIV is thought to be shed (17, 18) may contribute to the failure in detecting true cases of SIV. In any case, a passive surveillance system for influenza in pigs based on the reporting of outbreaks does not seem to be very effective for the rapid detection of new strains.

In the present work, all SIV isolates from outbreaks corresponded to the expected subtypes and lineages known to circulate in pigs, namely H1N1, H3N2 and H1N2, with predominance of avian-like H1N1 and reassortant "human like" European H3N2 and H1N2. The pH1N1 virus was also found to circulate in pigs, demonstrating the potential for spread of this virus from humans to pigs, as others have shown (12, 19, 20) although, in our case, reassortment between pH1N1 and porcine SIV was not observed as others have reported (8, 9, 12) .

More interesting are the data regarding H1N2 isolates. In this case, H1N2 strains of the present study formed a cluster of their own and separate from other European H1N2, a fact that suggests the emergence of a locally generated variant in this area of NE Spain. Besides this, one H1N2 isolate harboured a *NA* that was common to American and Asian H1N2 and H3N2 strains. This observation suggests that reassortment between H1N2 or H1N2 and H3N2 strains occurred in the recent past. This H1N2 isolate was also close to some Italian SIV isolates that were reported to be related with human seasonal H3N2 viruses. A similar origin could be speculated for our H1N2/03.

It is also worth noting that 11/14 (79%) isolates retrieved from respiratory disease cases presented internal genes that clustered differently from *HA* and *NA*, further evidence indicating that probably most of the SIV circulating in pigs are reassortants or, in other words, that reassortment events are extremely frequent in SIV, probably much more than previously thought.

Genetic and antigenic drift can be seen as a process that develops relatively slowly and that would be favoured by sustained transmission along time in a susceptible population. Thus, an SIV endemic farm would be the ideal scenario to examine this drift. In the present study, 121 animals were followed from weaning to the end of their productive period. The analysis of the sequences of 22 isolates from that farm indicated that at least three different H1N1 reassortants circulated in that period, corresponding to two different *M* and *NP* genes, while the other six genome segments were constant. This observation opens up the question of the origin of such viruses. With the data currently available it is impossible to determine the sequence of events leading to the generation of those four reassortant viruses. However, the fact that M_b was already detected in the first sampling week (3-week-old piglets) suggest that if a reassortment with other influenza viruses occurred, this phenomenon should have taken place before the beginning of the study or just at the starting point. In any case, M_b viruses were not detected later on, indicating that probably they were not able to persist in the examined population. On the other hand, for *NP*, NP_b appeared suddenly at 7 weeks of age and then faded out. This would be consistent with either a reassortment with other undetected virus circulating in the farm at that time, or with a low level co-circulation of the NP_b virus in previous weeks that was only detected at 7^{th} week of age. Also, those

reassortants could have originated in any other part of the farm (for example sows) and be introduced in the fattening herd at a given time.

Further analysis of the other viral genes in SIV isolates of the farm, indicated the existence of two variants of the same strain that persisted over time. These two variants had less than 1% of dissimilarity and co-existed at almost all sampling times, indicating that they were probably equally efficient in terms of transmission and persistence within a herd. In any case, the biological implication of the coexistence of two viral variants within a farm should be studied in more depth.

Deep sequencing analysis of the *M* gene produced different results for M_a and M_b. While the analysis of M_a only provided evidence of a generation of quasi-species with a few nucleotide changes, the examination of M_b indicated that a small percentage of sequences corresponded to M_a. This was an unexpected result. A first hypothesis to explain it is that a contamination occurred either at sampling in the farm or in the laboratory. This cannot be ruled out, although blank samples produced the correct (negative) result. If the observed result reflects the real composition of the examined sample, then the alternative hypotheses to be considered are: a) the pig was indeed co-infected by two different viruses, a fact compatible with the generation of reassortants or, b) M_a and M_b are the product of mutation of a single virus, the latter being less likely given the relatively low similarity (94.7%) between M_a and M_b.

In conclusion, the present study shows that reassortment events are probably quite common in SIV, and also indicates that within a given farm infected endemically by SIV, different reassortants of the same subtype can co-circulate and even, different variants of the same strain can be maintained for months circulating within the herd. Thus, there is a need to gain further insight in the epidemiology of SIV in order to understand how new influenza strains may emerge.

Acknowledgements

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References

1. Olsen CW, Brown IH, Easterday BC, Van Reeth K: Swine influenza. Diseases of swine. Oxford: Blackwell Publishing. Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ, 9th Ed. 2006, 469-482.

2. de Jong JC, Smith DJ, Lapedes AS, Donatelli I, Campitelli L, Barigazzi G *et al.* Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. J Virol 2007, 81: 4315-22.

3. Noble S, McGregor MS, Wentworth DE, Hinshaw VS. Antigenic and genetic conservation of the haemagglutinin in H1N1 swine influenza viruses. J Gen Virol, 1993, 74: 1197-200

4. Campitelli L, Donatelli I, Foni E, Castrucci MR, Fabiani C, Kawaoka Y, *et al*. Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology, 1994, 232: 310-8.

5. Brown IH, Harris PA, McCauley JW, Alexander DJ. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J Gen Virol, 1998, 79: 2947-55.

6. Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology, 1993, 193:503-6.

7. de Jong JC, Claas ECJ, Osterhaus ADME, Webster RG, Lim WL. A pandemic warning. Nature, 1997, 389: 554

8. Tremblay D, Allard V, Doyon JF, Bellehumeur C, Spearman JG, Harel J, Gagnon CA. Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. J Clin Microbiol, 2011, 49: 4386-90

9. Moreno A, Di Trani L, Faccini S, Vaccari G, Nigrelli D, Boniotti MB, *et al.* Novel. H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. Vet Microbiol, 2011, 149: 472-7

10. World Organization for Animal Health (OIE): Swine influenza. Manual for diagnostic tests and vaccines for terrestrial animals. Paris 2008, 1128-1138.

11. Simon-Grifé M, Martín-Valls GE, Vilar MJ, Busquets N, Mora-Salvatierra M, Bestebroer TM, *et al.* Swine influenza virus infection dynamics in two pig farms; results of a longitudinal assessment. Vet Res, 2012 43: 24.

12. Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, *et al.* Long-term evolution and transmission dynamics of swine influenza A virus. Nature, 2011, 473: 519-22.

13. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, *et al.* Clustal W and Clustal X version 2.0", Bioinformatics, 2007, 23: 2947-48

14. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution, 2011, 28: 2731-39.

15. Guindon S., Dufayard J.F., Lefort V., Anisimova M., Hordijk W., Gascuel O. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0." Systematic Biology, 2010, 59: 307-21.

16. Igarashi M, Ito K, Yoshida R, Tomabechi D, Kida H, Takada A. Predicting the antigenic structure of the pandemic (H1N1) 2009 influenza virus hemagglutinin. PLoS One, 2010, 5(1):e8553

17. Landolt GA, Karasin AI, Phillips L, Olsen CW. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J Clin Microbiol, 2003, 41: 1936-41.

18. Van Reeth K, Gregory V, Hay A, Pensaert. Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. M. Vaccine, 2003, 21: 1375-81.

19. Moreno A, Di Trani L, Alborali L, Vaccari G, Barbieri I, Falcone E, *et al*. First Pandemic H1N1 Outbreak from a Pig Farm in Italy. Open Virol J, 2010, 4: 52-6.

20. Holyoake PK, Kirkland PD, Davis RJ, Arzey KE, Watson J, Lunt RA, Wang J, *et al.* The first identified case of pandemic H1N1 influenza in pigs in Australia. Aust Vet J, 2011, 89: 427-31.

Chapter 4. Study 2.

Antigenic characterization of swine influenza viruses isolated in Spanish pig farms in 2009-2011

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Abstract

Isolates obtained from respiratory outbreaks and longitudinal assessments in farrow-to-finish farms where analyzed antigenically by means of the HI by the use of mono specific sera obtained with immunized pigs and 100 serum samples obtained from seropositive and unvaccinated commercial farms. Based on these analyses, a high diversity was found when comparing the H1N1 viruses. In contrast, HN2 and H3N2 viruses circulating in Spanish swine seem to be more homologous regarding their cross-reactivity in the haemagglutination inhibition assay. When contrasted the amino acid sequences of the haemagglutinin of these isolates, the H1N1 viruses presented also a larger amount of changes than the other subtypes. The causes behind this different behavior depending on the subtype are unknown and probably reflect a different epidemiology.

Supplementary information (in Chapter 5): Table S1 shows the correspondences between the strains in Chapter 3 and Chapter 4. Table S4 shows the results obtained in the factorial analysis.

Introduction

H1N1, H1N2 and H3N2 are the three commonest subtypes of influenza A virus infecting pigs worldwide (Van Reeth *et al.,* 2012). Within each subtype different lineages that have a common geographical distribution have been reported and within each lineage there is also a considerable genetic diversity (Kuntz-Simon et al, 2009; Vijakrishna et al. 2011; Webby et al., 2004). Regarding the origin of the predominant strains within each subtype, both human and avian influenza genes are found in pig isolates (Brown *et al.* 1998, Karasin *et al.,* 2000a, b).

The adscription of a given swine influenza virus (SIV) isolate to a particular subtype and lineage can be done by sequencing and phylogenetic analysis of the haemagglutinin (HA) and neuraminidase (NA) genes (Vincent *et al.,* 2009; Moreno *et al.,* 2009 and 2012; Vijakrishna *et al.* 2011). However, the classification of an isolate as belonging to a given subtype and lineage is not enough prediction of neither the antigenic similarity between isolates. In fact, an amino acid substitution in one of several positions of the HA can result in a different antigenic clustering of phylogenetically related strains and may cause a different recognition by specific antibodies against HA (Strengell *et al.,* 2011). In H1 influenza A viruses, four main antigenic sites (Ca, Cb, Sa and Sb) have been described for the globular head of subunit HA1 while five sites are known for H3 viruses (A, B, C, D and E) (Caton *et al.,* 1981; Skehel *et al,* 1984; Webster, 1980).

Classically, the haemagglutination inhibition assay (HI) has been used as the reference serological technique for the analysis of cross reactivity between influenza A strains of the same subtype and to guess the degree of protection against infection afforded by a

given strain against another one (Van Reeth *et al.*, 2001; van Reeth *et al.,* 2004). Using the HI, it has been shown that porcine H1N1 and H1N2 influenza A viruses usually does not cross-react (Van Reeth *et al.,* 2004). Similarly, cross reactivity between SIV isolates belonging to the classical and avian H1N1 SIV lineages or of these with the pandemic H1N1 is limited although it has been reported that consecutive infections with different H1N1 may result in broader reactivity of anti-H1 antibodies (Kyriakis *et al.*, 2010).

At present, SIV vaccines marketed in Europe are made either of "old" (1970s-1990s) H1N1 and H3N2 or, for the newest products, of a combination of inactivated H1N1, H1N2 and H3N2 isolates circulating already almost one decade ago (2000 – 2003). The circulation in pigs of more recent SIV strains including human pandemic H1N1 reassortant viruses (Tremblay *et al.,* 2011; Moreno *et al.,* 2012), poses the question of the cross-reactivity of current strains with older ones or with vaccine-induced antibodies. The objectives of the present study were to analyze the antigenic clustering of recent field isolates from Spanish farms and to determine the cross reactivity of those isolates against monospecific antisera and field sera.

Materials and methods

Ethics statement

All the procedures involving animals in the present study were carried out according to an experimental protocol (code 5796) approved by the Ethics Committee from the

"Universitat Autònoma de Barcelona" and certified by the "Departament d'Agricultura, Ramaderia, Pesca i Medi Natural de la Generalitat de Catalunya" (reference: 1189).

Antigen preparation

Fourteen SIV isolates retrieved from pigs of Spanish farms between 2008 and 2011 were examined in the present study (Table 1). Thirteen of them have been isolated from respiratory disease outbreaks and the other two corresponded to endemic strains isolated in the course of longitudinal studies in 2009-2011. Additionally, one human pandemic H1N1 virus and one classical swine H1N1 obtained from the ATCC (ATCC number: VR-1683™) were included for comparative purposes. Viruses were grown in parallel in SPF embryonated chicken eggs (ECE) and MDCK cells (passage ≤3) according to previously described procedures (Simon-Grifé *et al.,* 2012). Chorio-allantoic fluids were collected at day 3 post-inoculation. In MDCK inoculated cells, after the development of cytophatic effect, cells were frozen at -80ºC, thawed and centrifuged at 550 g for 15 min. Cell culture supernatants or chorio-allantoic fluids were then examined by means of a real-time RT-PCR targeting the matrix gene segment of influenza A viruses (Simon-Grifé *et al.,* 2012) and titrated by means of the haemaglutination assay (OIE, 2008). For a given isolate, the highest titrating product in the haemagglutination assay (MDCK cell culture supernatants or chorio-allantoic fluids) was selected for inoculation of pigs. In case that the equal titres were obtained or that the isolate failed to haemagglutinate, the product yielding the higher number of genomic copies in the RT-PCR was selected.

Selected isolates were then inactivated with binary ethylenimine (BEI). With this purpose, 205 mg of 2-bromoethylenimine hidrobromide (Sigma-Aldrich, ref: 06670) were added to a 10 ml of a 0.175M NaOH solution. Then, BEI solution was incubated 1h at 37ºC and pH was measured (pH=9). Inactivation was performed by adding 10ml of virus in 351 µl of the BEI solution (28.5:1 v/v). The virus-BEI mixture was incubated at room temperature for 24 h. To inactivate BEI, a 1M solution of $Na_2S_2O_3$ was added in the virus-BEI mixture (1:10 v/V). BEI inactivation was confirmed by inoculation of BEI-treated viral suspensions in MDCK cells. The absence of cytopathic effect after three consecutive passages was considered as effective inactivation. The isolates were used for immunization at the highest possible haemagglutinating titre.

Immunization of pigs and selection of field samples

Eighteen groups (1 to 18) of two specific-pathogen-free (SPF) piglets of 28 days of age devoid of antibodies against SIV (as tested by Civtest influenza ELISA, HIPRA, Spain) were placed in separated pens of an experimental farm facility at *Institut de Recerca i Tecnologia Agroalimentaria* (IRTA). Groups 1 to 16 were vaccinated with the different inactivated SIV isolates (2 ml of a mixture containing 1ml of the virus and 1 ml of Diluvac Forte® as adjuvant). Group 17 received IM 2 ml of a mixture of 1ml of chorio-allantoic fluid from SPF ECE and 1 ml of Diluvac Forte®. Group 18 was administered IM a mixture of 1ml of MDCK cell culture supernatant and 1ml of Diluvac Forte® (Table 1). Pigs were immunized four times, at 42, 56, 70 and 84 days of age by IM injection of 1ml of the virus-adjuvant mixture in the neck and 1ml in the posterior limb. Blood samples were collected each time that pigs were inoculated and two weeks after the last inoculation when pigs were euthanized.

Also, 100 field sera were selected to be analysed by the HI against the 14 Spanish SIV isolates used in the present study. Sera were selected from a serum bank built in a previous study (Simón-Grifé, *et al.* 2011) through a random sampling of Spanish pig

Group	Strain	Origin/Year	Subtype	Phylogeny	HAU/ml	Production system
1	**S1**	Swine (outbreak)/2009	H1N1	Avian-like	1,280	ECE
2	**S2**	Swine (outbreak)/2010	H1N1	Avian-like	20,480	ECE
3	**S3**	Swine (outbreak)/2008	H1N1	Pandemic	80	MDCK
4	**S4**	Swine (outbreak)/2011	H1N1	Avian-like	1,280	ECE
5	**S5**	Swine (outbreak)/2011	H1N1	Reassortant HA-H1N2	0	MDCK
6	**S6**	Swine (endemic)/2009	H1N1	Avian-like	1,280	ECE
7	**S7**	Swine (outbreak)/2011	H1N2	euH1N2	1,280	ECE
8	**S8**	Swine (outbreak)/2011	H1N2	euH1N2	1,280	MDCK
9	**S9**	Swine (outbreak)/2011	H1N2	euH1N2	1,280	MDCK
10	**S10**	Swine (endemic)/2010	H1N2	Reassortant NA human H3N2	640	MDCK
11	**S11**	Swine (outbreak)/2009	H3N2	euH3N2	1,280	ECE
12	**S12**	Swine (outbreak)/2010	H3N2	euH3N2	20,480	ECE
13	**S13**	Swine (outbreak)/2010	H3N2	euH3N2	20,480	ECE
14	**S14**	Swine (outbreak)/2011	H3N2	euH3N2	1,280	MDCK
15	**phuH1N1**	Pandemic human 2009	H1N1	Pandemic	320	MDCK
16	**cSwH1N1**	ATCC : VR-1683™	H1N1	American	1,280	MDCK
17	**Egg/adjuvant**	SPF egg	N.A.		N.A.	N.A.
18	**MDCK/adjuvant**	MDCK supernatant	N.A.		N.A.	N.A.

Table 1. Distribution of groups and viruses used for immunization.

farms. Sera had been previously tested against H1N1, H1N2 and H3N2 SIV by HI using commercially available SIV isolates (GD Deventer, the Netherlands). The set of 100 samples was composed by five sera negative to all three subtypes and 95 positive sera of which 44 were positive against H1N1, 27 were positive against H1N2 and 56 were positive against H3N2. 14 samples were simultaneously positive to H1N1 and H1N2, 28 sera were positive to H1N1 and H3N2, 23 were positive to H1N2 and H3N2, and 13 were positive against the three subtypes. Range of titres for positive sera was 1:40 to 1:5,120.

Haemagglutination inhibition assay

All sera included in the study were analyzed by the HI assay using all the different SIV strains described above. The HI assay was performed according to the World Organization for Animal Health (OIE) protocol (OIE, 2008) using 4 haemagglutinating units (HAU) of each virus in a 25 µl volume. Each serum was analyzed in triplicate. In the case of the sera obtained from immunized pigs they were adjusted to a homologous HI of 1:320 for H1N2 and H3N2. In the case of H1N1 antisera, that produced much lower HI titers, they were adjusted to 1:160 or, when this was not possible, to the immediately lower feasible titer (1:80 or 1:40).

Analysis of the amino acid subtitutions in antigenic sites of HA1

To complement the analysis of the serological results, the amino acid sequence of the subunit HA1 of the HA from the isolates used in the present study was analyzed. For this reason, available sequences (Martin-Valls *et al.* 2012, in preparation) were aligned

using CLUSTAL W including as reference European SIV strains for which the complete HA sequence was available (H1N1: A/Swine/Spain/51915/2003; H1N2: A/Swine/Côtes d'Armor//2000; H3N2: A/Swine/Spain/54008/2004) and a consensus sequence was generated by subtype by using these sequences as well as the H1N1, H1N2 and H3N2 strains against which the field sera have been previously tested. Amino acid changes for each of the 14 Spanish field isolates included in the present study were recorded and compared by subtype to the consensus sequence. The HA1 from European SIV (156 H1N1 sequences, 88 H1N2 sequences and 51 H3N2 sequences available at the ISD database) was downloaded. Based on this dataset, positional entropy of the amino acid sequence of HA1 was calculated using Bio-Edit.

Statistical analysis

HI results obtained with the 100 field samples were analysed statistically in order to determine the influence of the use of each SIV strain and the agreement between assays.

Factorial analysis

To determine if HI titres could be explained only by subtypes or by lineages, a factorial analysis was carried out using R for Windows (R development team, 2008). For a proper comparison of the HI titres, these were re-categorized as 0 (negative), 1 (1:20), 2 (1:40), 3 (1:80), 4 (1:160), 5 (1:320), 6 (1:640), 7 (1:1280) and 8 (>1:1280).

Distribution of HI titers for field sera using each SIV isolate and comparison of results.

In order to figure out if the use of any of the studied isolates resulted in significantly higher prevalences or titres in the HI a statistical analysis was carried out. Comparison of prevalences for each isolate within a given subtype was performed by the McNemar

χ^2 (Fleiss, 1981) with binomial categorisation as positive (≥1:40) or negative. Also, a pairwise comparison of the average $\log_2$ (titre) produced by each strain was carried out within each subtype by means of the Kruskall-Wallis test for non-parametric data (Kruskal and Wallis, 1952). Significance level was set at $p<0.05$.

Agreement of results obtained with the different SIV isolates was calculated in two different ways: firstly, by a regression analysis using the categorisation of titres explained above for the factorial analysis and secondly, by means of the weighed Kappa values for ≥3 raters and categories (Fleiss, Nee and Landis, 1979). For the calculation of Kappa values, titres were categorized as negative (<1:40), low (1:40–1:80), moderate (1:160–1:640) and high (>1:640). StatsDirect software 2.7.9 was used for these calculations.

Results

Production and testing of monospecific antisera

Immunization with any of the isolates induced homologous HI titres ≥1:40 except for the H1N1 strain S5. In that case, the virus lacked HA activity in the haemagglutination assay and therefore, it was not possible to measure HI antibodies against it. However, sera of pigs inoculated with S5 reacted strongly with all H1N2 isolates included in this study indicating that the virus acted as an efficient immunogen. H1N1 pandemic strains (S3 and pH1N1) used in the present study induced low homologous HI titres in immunized pigs (1:80).

Analysis of the cross reactivity in HI using adjusted sera showed that H1N1 isolates had very low cross-reactivity among them (Table 2). In contrast, reactivity of anti-H1N2 or

anti-H3N2 antisera against isolates of the same subtype was much higher, and in the case of the H3N2 the use of one strain or another resulted in just two dilutions of difference.

HI analysis of the 100 field sera

Factorial analysis

Given the scarce cross-reactivity of H1N1 viruses in the previous HI analysis with monospecific sera, it was considered the possibility of distinguishing different groups of positive sera in field samples based on the reactivity against different H1N1 viruses. To clarify this, a factorial analysis was carried out. Results showed that positive sera grouped by subtype (H1N1, H1N2 and H3N2) but was not possible to distinguish pH1N1 or other H1N1 variant as an additional factor of grouping (Supplementary table S4).

Descriptive analysis: frequencies and mean comparison of the titers

In the case of H1N1 viruses, use of strain S4 in HI resulted in the higher percentage ($p < 0.05$) of positive samples (72%) and in higher average titres (7.28 $\log_2$) compared to the other strains of the same subtype (Figure 1). For H1N2, the use of one isolate or another did not produce significant differences except when titres against S10 were compared with titres against S7. For H3N2, differences were only seen regarding the percentage of positive samples obtained with each isolate.

		Antigen used in the hemagglutination inhibition test															
		H1N1								H1N2				H3N2			
	Monospecific Antisera	**pH1N1**	**cSwH1N1**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**	**S14**
H1N1	**pH1N1**	80	-	40	-	40	-	N.D.	-	-	-	-	-	N.A.	N.A.	N.A.	N.A.
	cSwH1N1	-	80	-	-	-	-	-	-	-	-	-	-	N.A.	N.A.	N.A.	N.A.
	S1	-	-	160	-	-	40	N.D.	-	-	-	-	-	N.A.	N.A.	N.A.	N.A.
	S2	-	-	-	160	-	-	N.D.	-	-	-	-	-	N.A.	N.A.	N.A.	N.A.
	S3	80	-	-	-	80	-	N.D.	-	-	-	-	40	N.A.	N.A.	N.A.	N.A.
	S4	-	-	-	-	-	40	N.D.	-	-	-	-	-	N.A.	N.A.	N.A.	N.A.
	S5	-	-	-	-	-	-	N.D.	-	160	160	320	160	N.A.	N.A.	N.A.	N.A.
	S6	-	-	-	-	-	-	N.D.	160	-	-	-	-	N.A.	N.A.	N.A.	N.A.
H1N2	**S7**	-	-	-	-	-	-	N.D.	-	320	160	80	80	N.A.	N.A.	N.A.	N.A.
	S8	-	-	-	-	-	-	N.D.	-	160	320	80	80	N.A.	N.A.	N.A.	N.A.
	S9	-	-	-	-	-	-	N.D.	-	80	80	320	80	N.A.	N.A.	N.A.	N.A.
	S10	-	-	-	-	-	-	N.D.	-	-	320	80	320	N.A.	N.A.	N.A.	N.A.
H3N2	**S11**	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	320	160	160	320
	S12	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	320	320	320	320
	S13	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	320	320	320	320
	S14	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.D.	N.A.	N.A.	N.A.	N.A.	N.A.	320	160	80	320

Table 2. Reactivity of antisera and antigens used in the study as determined in the haemagglutination inhibition (HI) assay. The table shows the reciprocal of the titer obtained in each assay (triplicates). For H1N1, antisera were adjusted to a homologous HI of 1:160 or the maximum possible (1:80 or 1:40) when this titer was not achieved. For H1N2 and H3N2, antisera were adjusted to produce a titre of 1:320 that was the common homologous titer.

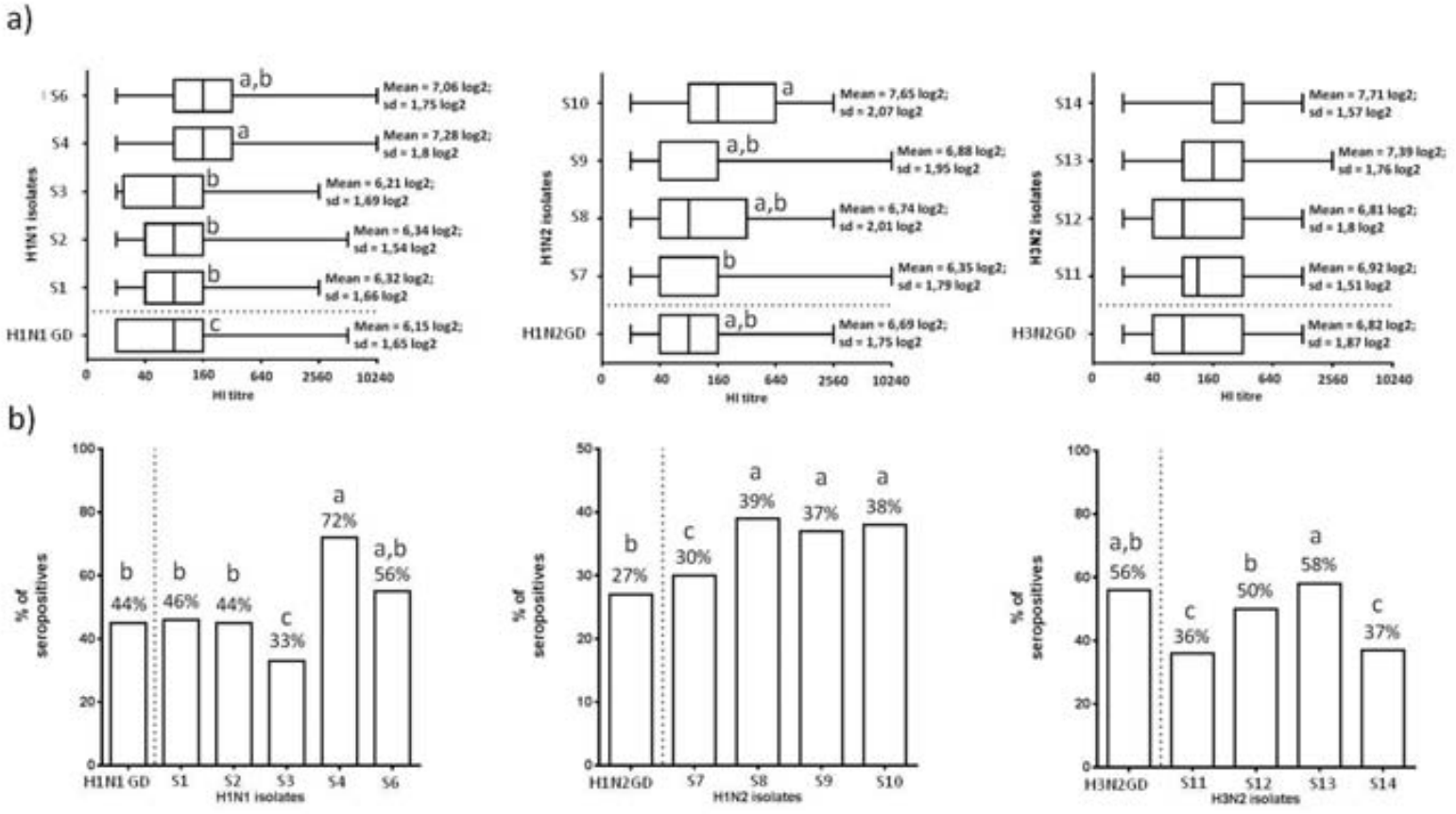

Figure 1. Titer means of the positive samples obtained by HI with the sera is represented in the a) graphs. Statistically significant differences were observed in H1N1 and H1N2 strains. Percentage of seropositive samples found by HI is represented in the b) histograms. Statistically significant differences were observed in all the subytpes

Correlation and Kappa values

Correlation of results for field sera and calculation of kappa values are shown in table 3. Inter-subtype comparison of results produced correlation coefficients <0.45 indicating a very low, if any, correspondence of HI results between different subtypes. In within subtype comparisons, correlation of HI results obtained with H1N1 isolates was in general lower (average correlation coefficient = 0.69) than that of H1N2 (average correlation coefficient = 0.79) and H3N2 (average correlation coefficient = 0.84).

Similarly, kappa values were higher for the comparison of HI results within H3N2 or H1N2 compared to H1N1 (Table 3).

Analysis of amino acid subtitutions in antigenic sites of HA1

Comparison of the HA1 sequence of Spanish isolates with the consensus sequence produced with the sequences of the isolates from the present study, revealed 36, 16 and 15 variable sites for H1N1, H1N2 and H3N2 isolates, respectively (Figure 2). It is worth to note that, individually, 3/4 avian-like H1N1 (S1, S2, S4 and S6) strains presented more than 10 amino acid changes; only 1/4 H1N2 had that number of substitutions and none of the H3N2 presented more than 6 changes. All the strains in the present study presented at least one change in an antigenic site, and in the case of the strain S2 six of the observed substitutions were located in Sa and Ca antigenic sites. The pandemic H1N1 strain presented 23 and 32 amino acid changes when compared with the H1N1 and H1N2 consensus sequences respectively (data not shown).

H1N1 strains

	Correlation						Kappa					
	H1N1 GD	**S1**	**S2**	**S3**	**S4**	**S6**	**H1N1 GD**	**S1**	**S2**	**S3**	**S4**	**S6**
H1N1 GD	1						1					
S1	0,75	1					0,59	1				
S2	0,69	0,72	1				0,58	0,56	1			
S3	0,62	0,68	0,69	1			0,44	0,52	0,30	1		
S4	0,63	0,66	0,67	0,52	1		0,37	0,38	0,36	0,23	1	
S6	0,69	0,73	0,57	0,67	0,68	1	0,49	0,56	0,42	0,49	0,56	1

H1N2 strains

	Correlation						Kappa					
	H1N2 GD	**S7**	**S8**	**S9**	**S10**	**S3**	**H1N2 GD**	**S7**	**S8**	**S9**	**S10**	**S3**
H1N2 GD	1						1					
S7	0,63	1					0,38	1				
S8	0,66	0,85	1				0,41	0,67	1			
S9	0,54	0,75	0,73	1			0,47	0,56	0,70	1		
S10	0,59	0,83	0,84	0,74	1		0,40	0,59	0,73	0,81	1	
S3	0,35	0,23	0,23	0,26	0,23	1	0,20	0,20	0,26	0,26	0,16	1

H3N2 strains

	Correlation					Kappa				
	H3N2 GD	**S11**	**S12**	**S13**	**S14**	**H3N2 GD**	**S11**	**S12**	**S13**	**S14**
H3N2 GD	1					1				
S11	0,71	1				0,48	1			
S12	0,79	0,86	1			0,61	0,68	1		
S13	0,78	0,78	0,97	1		0,59	0,53	0,83	1	
S14	0,66	0,90	0,80	0,74	1	0,51	0,83	0,72	0,65	1

Table 3. Pairwise correlation and weighed kappa values obtained with the 100 field sera. Results are shown by subtype. Pandemic strain S3 values are also shown against the H1N2 strains.

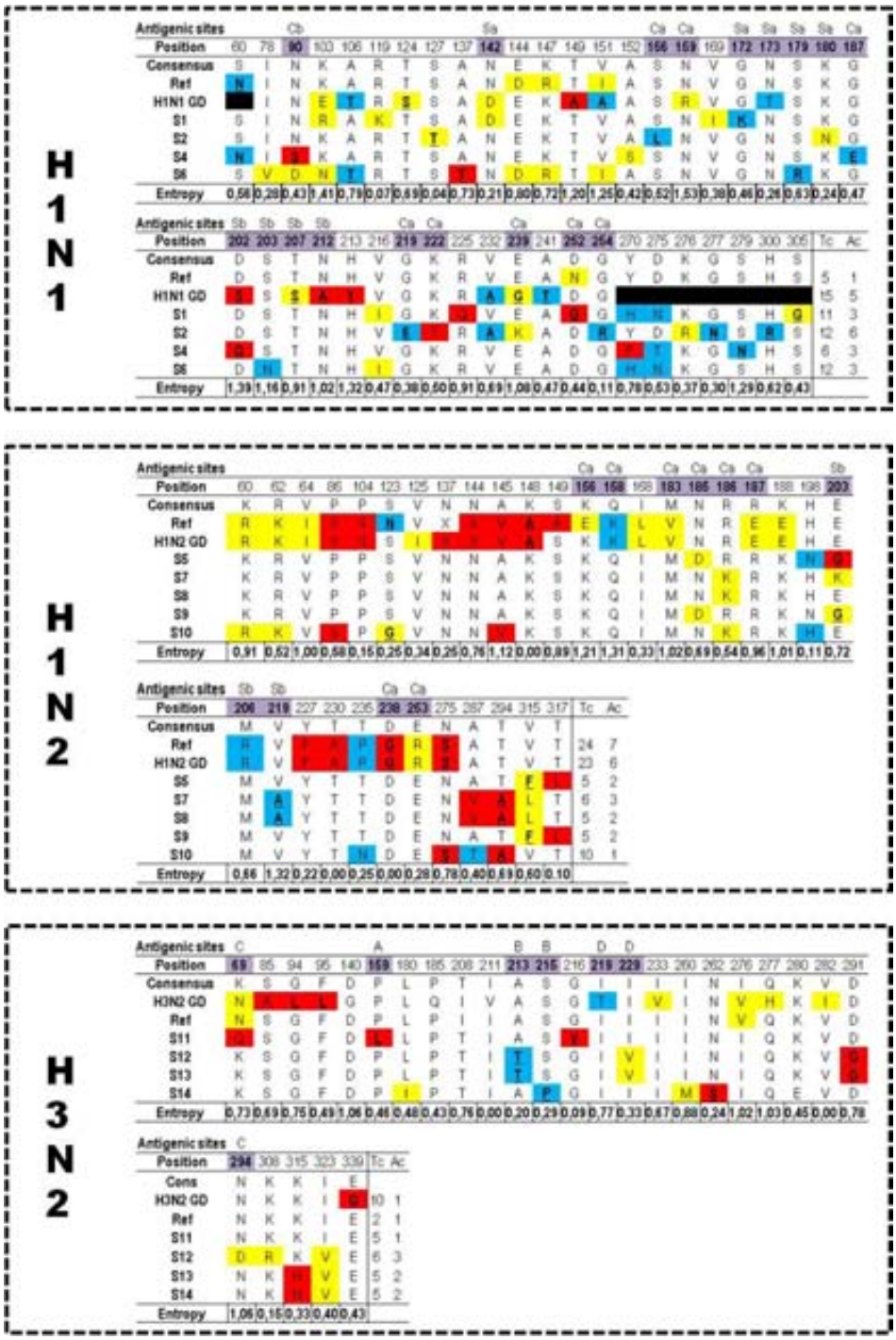

Figure 2. Amino acid changes in the HA1 subunit of the H1N1, H1N2 and H3N2 isolates. Antigenic sites are indicated as Ca, Cb, Sa, Sb for H1 viruses and A, B, C, D or E for H3 viruses. Consensus sequence (>50%) was produced based in the sequences of the isolates used in the present study. Hidrophobic, hidrophilic, neutral and size changes are marked in red, blue, yellow and bold-underlined, respectively. Positional entropy values obtained after the dataset of ISD sequences are shown at bottom row. First column show the strain for which results are presented.

Discussion

Pigs have been classically proposed as a mixing vessel for avian and mammalian influenza A viruses. However although birds can be a source of influenza viruses for pigs those avian viruses are not easily established in swine. One exception is the avian-like SIV H1N1 that is a wholly avian virus that was adapted and persisted in pigs (Kuntz-Simon *et al.,* 2008). In fact, the epidemiology of SIV is greatly influenced by the way in which pigs are produced. Thus, in countries were pig production is intensive three SIV subtypes, H1N1, H1N2 and H3N2, are almost the only ones found while when pigs are raised in more extensive conditions, particularly in Asia, viruses and antibodies against several different subtypes can be found, many of them of avian origin (Ninomiya *et al.,* 2002; Zhang *et al.,* 2011; Choi *et al.,* 2012; Kwon *et al.,* 2012).

In spite of the predominance of the three abovementioned subtypes, recent studies have shown that genetic diversity of SIV is greater than originally expected (Vincent *et al.,* 2009; Vijaykrishna *et al.,* 2011; Moreno *et al.,* 2012). In the present work, we aimed to analyze how the diversity of current SIV strains of a given country (Spain) resulted or not in an antigenic diversity.

The results obtained using monospecific antisera revealed that, in general, Spanish H1N1 SIV were diverse antigenically, presented smaller cross-reactivity between them and induced low HI titres when pigs were immunized with them. When field sera were analyzed using those same H1N1 viruses, it was noticed that apparently some cross-reactivity existed and it was not possible to clearly distinguish serological responses by the SIV isolate used in the HI. This apparent contradiction could be explained if

multiple different H1N1 strains were circulating in pig farms causing consecutive infections. As others have reported, consecutive infections with SIV result in a broader reactivity of sera in HI even against strains of other subtypes (Van Reeth *et al.,* 2004; Kyriakis *et al.,* 2010). However, compared to the other subtypes, HI results with H1N1 isolates resulted in the lower correlation coefficients, kappa values and the biggest differences on percentage of seropositive pigs compared to any other subtype, indicating that the observed antigenic diversity will probably have a serious impact on the measurement of antibodies against H1 and also poses the question of whether or not vaccines for SIV can provide effective immunity against this diversity of H1N1 variants.

It is interesting to note that field sera with relevant titres (>1:160) against isolate S3 (pandemic H1N1) were found. This result was somewhat surprising since sera reacting with the pandemic virus were collected before the emergence of pandemic H1N1 in Mexico in 2009. Two hypotheses could explain these results; a) a H1N1 strain related with the 2009 pandemic H1N1 strain was circulating in Spain before that date or b) cross-reactive antibodies against the pandemic virus were the result of repeated infections by other H1 viruses as mentioned above (Kyriakis *et al.,* 2010). The first hypothesis, although possible cannot be supported by experimental or published data. Since all the positive samples to S3 were positive to other "avian-like" H1N1 strains the second hypothesis seems to be the more plausible.

Isolate S5 was a H1N1 that induced antibodies cross-reactive with H1N2 viruses. Sequencing and phylogenetic analysis of the H1 of S5 (shown in chapter 2 of the

present thesis) showed that this strain is a reassortant including a H1 commonly found in H1N2 isolates of Europe. This type of reassortant strains has been detected in France (Kyriakis et al., 2011) but not in Spain or other European countries. However, we were unable to demonstrate haemagglutinating activity for S5. This phenomenon has been documented before for H1N2 viruses (Long *et al.*, 2004) that were unable to haemagglutinate chicken erythrocytes but haemagglutinated turkey or guinea pig red blood cells. In the present study only chicken red blood cells were used, and this could explain why S5 failed to haemagglutinate in spite of having adequate immunogenicity. Thus, double checking of SIV isolates with red blood cells of different origins could be convenient when lack of haemagglutinating activity occurs. Moreover, the results obtained with S5 point out that for swine H1 viruses precluding whether or not two isolates will cross-react based solely on the subtype can be inaccurate and thus, an adequate picture of the diversity of porcine H1 viruses will require serological and molecular characterizations.

When the amino acid sequence of the antigenic region HA1 was examined, H1N1 strains presented more amino acid changes than H1N2 or H3N2 isolates. These results could explain the lack of cross-reactivity observed between H1N1 strains and probably indicates different antigenic drift evolutionary lines.

Globally, the results obtained for H1N1 suggest that the introduction in the Spanish swine population of different H1N1 strains is very common or that the generation of antigenic variants by antigenic drift is higher than thought before; even both hypothesis can be true simultaneously. As a matter of fact, the phylogenetic analysis of the SIV

strains included in the present study supports the first hypothesis (chapter 2 of the present thesis) but also Simon-Grifé *et al.* (2012) have shown endemic circulation of H1N1 strains in a Spanish pig farm and deep sequencing of the variants circulating in that farm suggested the potential co-existence of several variants of the same strain (chapter 2 of the present thesis).

In contrast to H1N1, H1N2 and particularly H3N2 isolates seem to form a more compact group with very low antigenic diversity affecting HI. It is difficult to find an explanation for this evident difference with H1N1 isolates. It cannot be discarded that some bias have been inadvertently introduced in the selection of isolates because of the temporal or spatial frame of the sampling, however, after a careful review of the available data for the source outbreaks, such a bias could not be identified. A molecular and antigenic monitoring with a higher number of isolates could corroborate the observations of the present study.

In summary, Spanish H1N1 SIV present a high antigenic diversity while H1N2 and H3N2 isolates seem to be more closely related in HI. The results of the present study indicate that HI is not a suitable technique for the detection of anti-H1 antibodies except if a wide panel of variant viruses is used and also suggest the need for a continuous testing of available SIV vaccines against circulating H1N1 viruses. Moreover, more intensive epidemiological research is needed to gain understanding on the different diversity of H1N1 viruses compared to H1N2 and H3N2 ones.

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References

Caton, A.J., Brownlee, G.G., Yewdell, J.W., Gerhard, W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell, 2 :417-27.

Fleiss, J. L. 1981. Statistical methods for rates and proportions (2nd ed.). New York: John Wiley. p. 114. ISBN 0-471-06428-9.

Kruskal, W.H. and Wallis, W.A. 1952. Use of ranks in one-criterion variance analysis. Journal of the American Statistical Association, 47: 583–621

Kuntz-Simon, G., Madec, F. 2009. Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential. Zoonoses Public Health, 56: 310-25.

Kyriakis, C.S., Olsen, C.W., Carman, S., Brown, I.H., Brookes, S.M., Doorsselaere, J.V., Reeth, K.V. 2010. Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. Emerg Infect Dis, 16: 96-9.

Kyriakis, C.S., Brown, I.H., Foni, E., Kuntz-Simon, G., Maldonado, J., Madec, F., Essen, S., Chiapponi, C., Van Reeth, K. 2011. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. Zoonoses Public Health, 58: 93-101

Long, B.C., Goldberg, T.L., Swenson, S.L., Erickson, G., Scherba, G. 2004. Adaptation and limitations of established hemagglutination inhibition assays for the detection of porcine anti–swine influenza virus H1N2 antibodies. J Vet Diagn Invest, 162: 64–70

Moreno, A., Barbieri, I., Sozzi, E., Luppi, A., Lelli, D., Lombardi, G., Zanoni, M.G., Cordioli, P. 2009. Novel swine influenza virus subtype H3N1 in Italy.Vet Microbiol, 138: 361-7.

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: 472-7.

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, 156: 265-76.

Ninomiya, A., Takada, A., Okazaki, K., Shortridge, K.F., Kida, H. 2002. Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. Vet Microbiol, 88:107-14.

Olsen, B., Munster, V.J., Wallensten, A., Waldenström, J., Osterhaus, A.D., Fouchier, R.A. 2006. Global patterns of influenza a virus in wild birds. Science. 312: 384-8.

R Development Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Romagosa, A., Gramer, M., Joo, H.S., Torremorell, M. 2012. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza Other Respi Viruses, 6:110-8.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Douglas, A.R., Knossow, M. 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc Natl Acad Sci, 81: 1779–83

Simon-Grifé, M., Martín-Valls, G.E., Vilar, M.J., Busquets, N., Mora-Salvatierra, M., Bestebroer, T.M., Fouchier, R.A.M., Martín, M., Mateu, E., Casal, J. 2012. Swine influenza virus infection dynamics in two pig farms; results of a longitudinal assessment. Vet Res, 43: 24

Strengell, M., Ikonen, N., Ziegler, T., Julkunen, I. Minor changes in the hemagglutinin of influenza A(H1N1)2009 virus alter its antigenic properties. 2011. PLoS One. 6(10):e25848.

Tremblay, D., Allard, V., Doyon, J.F., Bellehumeur, C., Spearman, J.G., Harel, J., Gagnon, C.A. 2011. Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. J Clin Microbiol, 49: 4386-90.

Van Reeth, K., Brown, I., Essen, S., Pensaert, M. 2004. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res, 103: 115-24.

Van Reeth, K., Labarque, G., De Clercq, S., Pensaert, M. 2001. Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. Vaccine, 19: 4479-86.

Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, Riley S, Bahl J, Ma SK, Cheung CL, Perera RA, Chen H, Shortridge KF, Webby RJ, Webster RG, Guan Y, Peiris JS. 2011. Long-term evolution and transmission dynamics of swine influenza A virus. Nature, 473: 519-22.

Vincent, A.L., Ma, W., Lager, K.M., Gramer, M.R., Richt, J.A., Janke, B.H. 2009. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. Virus Genes, 39: 176-85.

Webby, R.J., Rossow, K., Erickson, G., Sims, Y., Webster, R. 2004. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res, 103: 67-73.

Webster, R.G., Laver, W.G. 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal

antibodies and the selection of variants with potential epidemiological significance. Virology, 104: 139–48

World Organization for Animal Health (OIE). 2008. Swine influenza. Manual for diagnostic tests and vaccines for terrestrial animals. Paris. pp. 1128–1138.

Zhang G, Kong W, Qi W, Long LP, Cao Z, Huang L, Qi H, Cao N, Wang W, Zhao F, Ning Z, Liao M, Wan XF. 2011. Identification of an H6N6 swine influenza virus in southern China. Infect Genet Evol, 11: 1174-7.

Chapter 5. Additional Data

Table S1. Correspondences of the nomenclature of the strains used in Chapter 3 and 4.

Chapter 3	Chapter 4
H1N1 01	S1
H1N1 02	S2
H1N1 03	Not used
H1N1 04	S3
H1N1 05	S4
H1N1 06	S5
Longitudinal strain 99.3	S6
H1N2 01	S8
H1N2 02	S9
H1N2 03	S7
H1N2 04	S10
H3N2 01	S11
H3N2 02	S12
H3N2 03	S13
H3N2 04	S14

Table S2. Accession numbers of the ISD dataset of whole genome viruses.

Whole genome strains		
Strain name	**Subtype**	**Accession numbers**
A/sw/Shizuoka/110/97	**H3N2**	AF225522.1; AF225542.1; AF225518.1; AF225526.1; AF225514.1; AF225534.1; AF225538.1; AF225530.1
A/sw/Shizuoka/115/97	**H3N2**	AF225523.1; AF225543.1; AF225531.1; AF225519.1; AF225527.1; AF225539.1; AF225535.1; AF225515.1
A/sw/Shizuoka/119/97	**H3N2**	AF225524.1; AF225544.1; AF225532.1; AF225520.1; AF225528.1; AF225540.1; AF225536.1; AF225516.1
A/swine/Alberta/14722/2005	**H3N2**	DQ469964.1; DQ469963.1; DQ469965.1; DQ469966.1; DQ469967.1; DQ469968.1; DQ469969.1; DQ469970.1
A/swine/Argentina/CIP051-A2/2008	**H3N2**	CY092328.1; CY092326.1; CY092324.1; CY092322.1; CY092327.1; CY092325.1; CY092323.1; CY092321.1
A/swine/Bakum/1832/2000	**H1N2**	EU053146.1; EU053148.1; EU053150.1; EU053149.1; EU053151.1; GQ161107.1; GQ161102.1; EU053147.1
A/swine/Bakum/IDT1769/2003	**H3N2**	GQ161134.1; EU478807.1; GQ161132.1; GQ161126.1; GQ161136.1; GQ161128.1; GQ161137.1; GQ161135.1
A/swine/Belgium/WVL1/1979	**H1N1**	CY037902.1; CY037900.1; CY037898.1; CY037896.1; CY037901.1; CY037899.1; CY037897.1
A/swine/Belgium/WVL2/1983	**H1N1**	CY037970.1; CY037968.1; CY037966.1; CY037971.1; CY037969.1; CY037967.1; CY037965.1
A/swine/Belgium/WVL5/1989	**H1N1**	CY037994.1; CY037992.1; CY037990.1; CY037988.1; CY037995.1; CY037993.1; CY037991.1; CY037989.1
A/swine/Binh Duong/03_06/2010	**H3N2**	AB598522.1; AB598516.1; AB598510.1; AB598504.1; AB598498.1; AB598492.1; AB598486.1; AB598480.1
A/swine/Binh Duong/03_08/2010	**H3N2**	AB598523.1; AB598517.1; AB598511.1; AB598505.1; AB598499.1; AB598493.1; AB598487.1; AB598481.1
A/swine/Binh Duong/03_10/2010	**H3N2**	AB598525.1; AB598519.1; AB598513.1; AB598507.1; AB598501.1; AB598495.1; AB598489.1; AB598483.1
A/swine/Binh Duong/03_13/2010	**H3N2**	AB598526.1; AB598520.1; AB598514.1; AB598508.1; AB598502.1; AB598496.1; AB598490.1; AB598484.1
A/swine/Binh Duong/03_14/2010	**H3N2**	AB598527.1; AB598521.1; AB598515.1; AB598509.1; AB598503.1; AB598497.1; AB598491.1; AB598485.1
A/swine/Bissendorf/IDT1864/2003	**H3N2**	GQ161173.1; GQ161168.1; GQ161167.1; GQ161170.1; GQ161169.; GQ161172.1; GQ161171.1
A/swine/Brabant/1984	**H3N2**	CY077946.1; CY077944.1; CY077942.1; CY077940.1; CY077945.1; CY077943.1; CY077941.1; CY077939.1
A/swine/British Columbia/28103/2005	**H3N2**	DQ469975.1; DQ469976.1; DQ469977.1; DQ469978.1; DQ469972.1; DQ469971.1; DQ469973.1; DQ469974.1
A/swine/Chachoengsao/2003	**H3N2**	AB434348.1; AB434346.1; AB434344.1; AB434342.1; AB434347.1; AB434345.1; AB434343.1; AB434341.1
A/swine/Cloppenburg/IDT4777/2005	**H1N2**	EU053145.1; EU053143.1; EU053141.1; EU053139.1; EU053144.1; EU053142.1; EU053140.1; EU053138.1
A/swine/Cote dArmor/3633/84	**H3N2**	AJ344025.1; AJ293933.1; AJ311508.1; AJ293943.1; AJ311458.1; AJ311464.1; AJ311461.1
A/swine/Denmark/WVL9/1993	**H1N1**	CY038026.1; CY038024.1; CY038022.1; CY038020.1; CY038027.1; CY038025.1; CY038023.1; CY038021.1
A/swine/Doetlingen/IDT4735/2005	**H1N2**	EU053136.1; EU053134.1; EU053132.1; EU053130.1; EU053137.1; EU053135.1; EU053133.1; EU053131.1
A/swine/England/1382/2010	**H1N2**	JF290394.1; JF290392.1; JF290390.1; JF290388.1; JF290395.1; JF290393.1; JF290391.1; JF290389.1
A/swine/England/WVL10/1993	**H1N1**	CY037910.1; CY037908.1; CY037906.1; CY037904.1; CY037909.1; CY037907.1; CY037905.1; CY037903.1
A/swine/England/WVL11/1994	**H1N1**	CY037918.1; CY037916.1; CY037914.1; CY037912.1; CY037917.1; CY037915.1; CY037913.1; CY037911.1
A/swine/England/WVL12/1995	**H1N1**	CY037924.1; CY037922.1; CY037920.1; CY037925.1; CY037923.1; CY037921.1; CY037919.1
A/swine/England/WVL14/1996	**H1N1**	CY037940.1; CY037938.1; CY037936.1; CY037934.1; CY037939.1; CY037937.1; CY037935.1
A/swine/England/WVL15/1997	**H1N1**	CY037948.1; CY037946.1; CY037944.1; CY037942.1; CY037947.1; CY037945.1; CY037943.1; CY037941.1
A/swine/England/WVL16/1998	**H1N1**	CY037956.1; CY037954.1; CY037952.1; CY037950.1; CY037955.1; CY037953.1; CY037951.1; CY037949.1
A/swine/England/WVL7/1992	**H1N1**	CY038010.1; CY038008.1; CY038006.1; CY038004.1; CY038011.1; CY038009.1; CY038007.1; CY038005.1
A/swine/Finistere/2899/1982	**H1N1**	EU417843.1; AJ344037.1; AJ316059.1; AJ344015.1; AJ311463.1; AJ311462.1; AJ311457.1
A/swine/France/WVL13/1995	**H1N1**	CY037932.1; CY037930.1; CY037928.1; CY037926.1; CY037933.1; CY037931.1; CY037929.1; CY037927.1
A/swine/France/WVL3/1984	**H1N1**	CY037978.1; CY037976.1; CY037974.1; CY037972.1; CY037979.1; CY037977.1; CY037975.1; CY037973.1

A/swine/France/WVL4/1985	**H1N1**	CY037986.1; CY037984.1; CY037982.1; CY037980.1; CY037987.1; CY037985.1; CY037983.1; CY037981.1
A/swine/France/WVL8/1992	**H1N1**	CY038018.1; CY038016.1; CY038014.1; CY038012.1; CY038019.1; CY038017.1; CY038015.1; CY038013.1
A/swine/Fujian/F2/2007	**H3N2**	JN105976.1; JN105974.1; JN105972.1; JN105970.1; JN105977.1; JN105975.1; JN105973.1; JN105971.1
A/swine/Granstedt/IDT3475/2004	**H1N2**	GQ161161.1; GQ161166.1; GQ161165.1; GQ161164.1; GQ161160.1; GQ161163.1; GQ161162.1
A/swine/Greven/IDT2889/2004	**H1N1**	GQ161159.1; GQ161154.1; GQ161153.1; GQ161156.1; GQ161155.1; GQ161158.1; GQ161157.1
A/swine/Guangdong/01/1998	**H3N2**	FJ830858.1; FJ830856.1; FJ830854.1; FJ830852.1; FJ830859.1; FJ830857.1; FJ830855.1; FJ830853.1
A/swine/Guangdong/01/2005	**H3N2**	EF455568.1; EF455566.1; EF455569.1; EF455567.1; EF455565.1; EF455564.1; EF455562.1; EF455563.1
A/swine/Guangdong/04/2005	**H3N2**	EU620746.1; EU620744.1; EU620742.1; EU620740.1; EU620745.1; EU620743.1; EU620741.1; EU620739.1
A/swine/Guangdong/102/2002	**H3N2**	GQ422426.1; GQ422420.1; GQ422414.1; GQ422408.1; GQ422402.1; GQ422396.1; GQ422390.1; GQ422437.1
A/swine/Guangdong/106/2002	**H3N2**	GQ422435.1; GQ422427.1; GQ422421.1; GQ422415.1; GQ422409.1; GQ422403.1; GQ422397.1; GQ422391.1
A/swine/Guangdong/107/2002	**H3N2**	GQ422432.1; GQ422422.1; GQ422416.1; GQ422410.1; GQ422404.1; GQ422431.1; GQ422401.1; GQ422395.1
A/swine/Guangdong/110/2002	**H3N2**	GQ422430.1; GQ422394.1; GQ422433.1; GQ422423.1; GQ422417.1; GQ422411.1; GQ422405.1; GQ422399.1
A/swine/Guangdong/111/2002	**H3N2**	GQ422436.1; GQ422428.1; GQ422412.1; GQ422406.1; GQ422398.1; GQ422392.1; GQ422425.1; GQ422419.1
A/swine/Guangdong/113/2002	**H3N2**	GQ422434.1; GQ422424.1; GQ422418.1; GQ422400.1; GQ422429.1; GQ422413.1; GQ422407.1; GQ422393.1
A/swine/Guangdong/164/06	**H3N2**	EU273779.1; EU273803.1; EU273799.1; EU273795.1; EU273791.1; EU273787.1; EU273783.1; EU273775.1
A/swine/Guangdong/165/06	**H3N2**	EU273780.1; EU273804.1; EU273800.1; EU273796.1; EU273792.1; EU273788.1; EU273784.1; EU273776.1
A/swine/Guangdong/166/06	**H3N2**	EU273781.1; EU273805.1; EU273801.1; EU273797.1; EU273793.1; EU273789.1; EU273785.1; EU273777.1
A/swine/Guangdong/223/2006	**H3N2**	GU086126.1; GU086128.1; GU086130.1; GU086132.1; GU086127.1; GU086129.1; GU086131.1; GU086133.1
A/swine/Guangdong/423/2006	**H3N2**	GU086135.1; GU086137.1; GU086139.1; GU086141.1; GU086134.1; GU086136.1; GU086138.1; GU086140.1
A/swine/Guangxi/1/2004	**H3N2**	FJ157993.1; FJ157991.1; FJ157989.1; FJ157987.1; FJ157992.1; FJ157990.1; FJ157988.1; FJ157986.1
A/swine/Haseluenne/IDT2617/2003	**H1N1**	EU478811.1; GQ161115.1; GQ161117.1; GQ161121.1; GQ161109.1; Q161124.1; GQ161118.1; GQ161120.1
A/swine/Heilongjiang/10/2007	**H3N2**	HM765435.1; HM765433.1; HM765431.1; HM765429.1; HM765436.1; HM765434.1; HM765432.1; HM765430.1
A/swine/Hong Kong/101/1979	**H1N1**	CY084885.1; CY084883.1; CY084881.1; CY084879.1; CY084884.1; CY084882.1; CY084880.1; CY084878.1
A/swine/Hong Kong/1111/2004	**H1N2**	CY085666.1; CY085664.1; CY085662.1; CY085660.1; CY085665.1; CY085663.1; CY085661.1; CY085659.1
A/swine/Hong Kong/1304/2003	**H1N2**	CY085594.1; CY085592.1; CY085590.1; CY085588.1; CY085593.1; CY085591.1; CY085589.1; CY085587.1
A/swine/Hong Kong/1435/2009	**H1N2**	CY061657.1; CY061655.1; CY061653.1; CY061651.1; CY061656.1; CY061654.1; CY061652.1; CY061650.1
A/swine/Hong Kong/1479/2009	**H1N2**	CY061665.1; CY061663.1; CY061661.1; CY061659.1; CY061664.1; CY061662.1; CY061660.1; CY061658.1
A/swine/Hong Kong/1559/2008	**H1N1**	CY085882.1; CY085880.1; CY085878.1; CY085876.1; CY085881.1; CY085879.1; CY085877.1; CY085875.1
A/swine/Hong Kong/1562/2005	**H1N2**	GQ229336.1; GQ229334.1; GQ229335.1; GQ229337.1; GQ229332.1; GQ229333.1; GQ229338.; GQ229331.1
A/swine/Hong Kong/1578/2003	**H1N2**	CY085610.1; CY085608.1; CY085606.1; CY085604.1; CY085609.1; CY085607.1; CY085605.1; CY085603.1
A/swine/Hong Kong/1669/2002	**H1N1**	CY085538.1; CY085536.1; CY085534.1; CY085532.1; CY085537.1; CY085535.1; CY085533.1; CY085531.1
A/swine/Hong Kong/189/2010	**H1N1**	CY061808.1; CY061806.1; CY061804.1; CY061802.1; CY061809.1; CY061807.1; CY061805.1; CY061803.1
A/swine/Hong Kong/201/2010	**H1N1**	CY061817.1; CY061815.1; CY061813.1; CY061811.1; CY061816.1; CY061814.1; CY061812.1; CY061810.1
A/swine/Hong Kong/2299/2009	**H1N1**	CY061736.1; CY061734.1; CY061732.1; CY061730.1; CY061737.1; CY061735.1; CY061733.1; CY061731.1
A/swine/Hong Kong/2314/2009	**H1N2**	CY061745.1; CY061743.1; CY061741.1; CY061739.1; CY061744.1; CY061742.1; CY061740.1; CY061738.1
A/swine/Hong Kong/2885/2009	**H1N1**	CY061768.1; CY061766.1; CY061764.1; CY061762.1; CY061769.1; CY061767.1; CY061765.1; CY061763.1
A/swine/Hong Kong/2886/2009	**H1N1**	CY061776.1; CY061774.1; CY061772.1; CY061770.1; CY061777.1; CY061775.1; CY061773.1; CY061771.1
A/swine/Hong Kong/2894/2009	**H1N1**	CY061784.1; CY061782.1; CY061780.1; CY061778.1; CY061785.1; CY061783.1; CY061781.1; CY061779.1

A/swine/Hong Kong/294/2009	**H1N2**	GQ229345.1; GQ229340.1; GQ229341.1; GQ229346.1; GQ229339.1; GQ229344.1; GQ229342.1; GQ229343.1
A/swine/Hong Kong/2974/2009	**H1N1**	CY061792.1; CY061790.1; CY061788.1; CY061786.1; CY061793.1; CY061791.1; CY061789.1; CY061787.1
A/swine/Hong Kong/2995/2009	**H1N1**	CY061800.1; CY061798.1; CY061796.1; CY061794.1; CY061801.1; CY061799.1; CY061797.1; CY061795.1
A/swine/Hong Kong/39/1977	**H1N1**	CY084597.1; CY084595.1; CY084593.1; CY084591.1; CY084596.1; CY084594.1; CY084592.1; CY084590.1
A/swine/Hong Kong/414/2009	**H1N1**	CY085994.1; CY085992.1; CY085990.1; CY085988.1; CY085993.1; CY085991.1; CY085989.1; CY085987.1
A/swine/Hong Kong/421/2006	**H1N1**	CY085770.1; CY085768.1; CY085766.1; CY085764.1; CY085769.1; CY085767.1; CY085765.1; CY085763.1
A/swine/Hong Kong/558/2007	**H1N1**	CY085842.1; CY085840.1; CY085838.1; CY085836.1; CY085841.1; CY085839.1; CY085837.1; CY085835.1
A/swine/Hong Kong/5609/1999	**H1N2**	CY087132.1; CY087130.1; CY087128.1; CY087126.1; CY087131.1; CY087129.1; CY087127.1; CY087125.1
A/swine/Hong Kong/59/1977	**H1N1**	CY084741.1; CY084739.1; CY084737.1; CY084735.1; CY084740.1; CY084738.1; CY084736.1; CY084734.1
A/swine/Hong Kong/638/2003	**H1N1**	CY085562.1; CY085560.1; CY085558.1; CY085556.1; CY085561.1; CY085559.1; CY085557.1; CY085555.1
A/swine/Hong Kong/676/2008	**H1N1**	CY085858.1; CY085856.1; CY085854.1; CY085852.1; CY085857.1; CY085855.1; CY085853.1; CY085851.1
A/swine/Hong Kong/69/2006	**H1N2**	CY085754.1; CY085752.1; CY085750.1; CY085748.1; CY085753.1; CY085751.1; CY085749.1; CY085747.1
A/swine/Hong Kong/70/1977	**H1N1**	CY084813.1; CY084811.1; CY084809.1; CY084807.1; CY084812.1; CY084810.1; CY084808.1; CY084806.1
A/swine/Hong Kong/715/2008	**H1N2**	CY085866.1; CY085864.1; CY085862.1; CY085860.1; CY085865.1; CY085863.1; CY085861.1; CY085859.1
A/swine/Hong Kong/72/2007	**H1N1**	CY085818.1; CY085816.1; CY085814.1; CY085812.1; CY085817.1; CY085815.1; CY085813.1; CY085811.1
A/swine/Hong Kong/78/1978	**H1N1**	CY084829.1; CY084827.1; CY084825.1; CY084823.1; CY084828.1; CY084826.1; CY084824.1; CY084822.1
A/swine/Hong Kong/78/2003	**H1N2**	GQ229313.1; GQ229308.1; GQ229309.1; GQ229314.1; GQ229307.1; GQ229312.1; GQ229310.1; GQ229311.1
A/swine/Hong Kong/8512/2001	**H1N1**	GQ229289.2; GQ229288.1; GQ229286.1; GQ229287.1; GQ229284.1; GQ229285.1; GQ229290.1; GQ229283.1
A/swine/Hong Kong/8944/2001	**H1N2**	CY085426.1; CY085424.1; CY085422.1; CY085420.1; CY085425.1; CY085423.1; CY085421.1; CY085419.1
A/swine/Hong Kong/915/2004	**H1N2**	GQ229273.1; GQ229272.1; GQ229270.1; GQ229271.1; GQ229268.1; GQ229269.1; GQ229274.1; GQ229267.1
A/swine/Hong Kong/95/1979)	**H1N1**	CY084933.1; CY084931.1; CY084929.1; CY084927.1; CY084932.1; CY084930.1; CY084928.1; CY084926.1
A/swine/Hong Kong/9656/2001	**H1N1**	GQ229361.1; GQ229356.1; GQ229357.1; GQ229362.1; GQ229355.1; GQ229360.1; GQ229358.1; GQ229359.1
A/swine/Hong Kong/NS129/2003	**H1N1**	CY085554.1; CY085552.1; CY085550.1; CY085548.1; CY085553.1; CY085551.1; CY085549.1; CY085547.1
A/swine/Hong Kong/NS152/2009	**H1N1**	CY085970.1; CY085968.1; CY085966.1; CY085964.1; CY085969.1; CY085967.1; CY085965.1; CY085963.1
A/swine/Hong Kong/NS157/2004	**H1N2**	CY085634.1; CY085632.1; CY085630.1; CY085628.1; CY085633.1; CY085631.1; CY085629.1; CY085627.1
A/swine/Hong Kong/NS1583/2009	**H1N1**	CY061728.1; CY061726.1; CY061724.1; CY061722.1; CY061729.1; CY061727.1; CY061725.1; CY061723.1
A/swine/Hong Kong/NS1809/2009	**H1N1**	CY061752.1; CY061750.1; CY061748.1; CY061746.1; CY061753.1; CY061751.1; CY061749.1; CY061747.1
A/swine/Hong Kong/NS1810/2009	**H1N1**	CY061760.1; CY061758.1; CY061756.1; CY061754.1; CY061761.1; CY061759.1; CY061757.1; CY061755.1
A/swine/Hong Kong/NS1890/2009	**H1N2**	CY061849.1; CY061847.1; CY061845.1; CY061843.1; CY061848.1; CY061846.1; CY061844.1; CY061842.1
A/swine/Hong Kong/NS1892/2009	**H1N2**	CY061857.1; CY061855.1; CY061853.1; CY061851.1; CY061856.1; CY061854.1; CY061852.1; CY061850.1
A/swine/Hong Kong/NS210/2005	**H1N1**	CY085722.1; CY085720.1; CY085718.1; CY085716.1; CY085721.1; CY085719.1; CY085717.1; CY085715.1
A/swine/Hong Kong/NS252/2009	**H1N2**	CY086002.1; CY086000.1; CY085998.1; CY085996.1; CY086001.1; CY085999.1; CY085997.1; CY085995.1
A/swine/Hong Kong/NS318/2004	**H1N1**	CY085658.1; CY085656.1; CY085654.1; CY085652.1; CY085657.1; CY085655.1; CY085653.1; CY085651.1
A/swine/Hong Kong/NS605/2003	**H1N2**	CY085578.1; CY085576.1; CY085574.1; CY085572.1; CY085577.1; CY085575.1; CY085573.1; CY085571.1
A/swine/Hong Kong/NS623/2002	**H1N2**	GQ229368.1; GQ229366.1; GQ229367.1; GQ229369.1; GQ229364.1; GQ229365.1; GQ229370.1; GQ229363.1
A/swine/Hong Kong/NS728/2002	**H1N2**	CY085522.1; CY085520.1; CY085518.1; CY085516.1; CY085521.1; CY085519.1; CY085517.1; CY085515.1
A/swine/Hong Kong/NS837/2001	**H1N1**	GQ229260.1; Q229261.1; GQ229266.1; GQ229259.1; GQ229265.1; GQ229264.1; GQ229262.1; GQ229263.1
A/swine/Hong Kong/NS857/2001	**H1N2**	GQ229351.1; GQ229349.1; GQ229350.1; GQ229352.1; GQ229354.1; GQ229348.1; GQ229353.1; GQ229347.1

A/swine/Hong_Kong/715/2008	**H1N2**	CY085866.1; CY085864.1; CY085862.1; CY085860.1; CY085865.1; CY085863.1; CY085861.1; CY085859.1
A/swine/Hungary/13509/2007	**H3N2**	FJ798775.1; FJ798773.1; FJ798771.1; FJ798769.1; FJ798776.1; FJ798774.1; FJ798772.1; FJ798770.1
A/swine/Hungary/19774/2006	**H1N1**	FJ798783.1; FJ798781.1; FJ798779.1; FJ798777.1; FJ798784.1; FJ798782.1; FJ798780.1; FJ798778.1
A/swine/Iowa/01700/2007	**H3N2**	FJ798783.1; FJ798781.1; FJ798779.1; FJ798777.1; FJ798784.1; FJ798782.1; FJ798780.1; FJ798778.1
A/Swine/Iowa/533/99	**H3N2**	AF251415.2; AF251417.1; AF251414.1; AF251411.1; AF251418.1; AF251416.1; AF251413.1; AF251412.3
A/Swine/Iowa/569/99	**H3N2**	AF251426.1; AF251424.1; AF251421.1; AF251419.1; AF251423.2; AF251425.1; AF251422.1; AF251420.1
A/swine/Iowa/A01049034/2010	**H3N2**	JN656961.1; JN656959.1; JN656963.1; JN656960.1; JF812322.1; JN656962.1; JF812299.1; JF812276.1
A/swine/Iowa/A01049035/2010	**H3N2**	JN656966.1; JN656964.1; JN656968.1; JN656965.1; JF812323.1; JN656967.1; JF812277.1; JF812300.1
A/swine/Iowa/H02AS8/2002	**H3N2**	GU135896.1; GU135901.1; GU135902.1; GU135897.1; GU135898.1; GU135899.1; GU135900.1; EU422987.1
A/swine/Iowa/H03BF5/2003	**H3N2**	GU135889.1; GU135890.1; GU135892.1; GU135893.1; GU135888.1; GU135891.1; GU135894.1; GU135895.1
A/swine/Iowa/H03HB4/2003	**H3N2**	GU135917.1; GU135918.1; GU135920.1; GU135921.1; GU135922.1; GU135915.1; GU135916.1; GU135919.1
A/swine/Iowa/H03HO7/2003	**H3N2**	GU135923.1; GU135924.1; GU135926.1; GU135925.1; GU135927.1; GU135928.1; GU135929.1; EU422988.1
A/swine/Iowa/H03LDH5/2003	**H3N2**	GU135938.1; GU135939.1; GU135941.1; GU135942.1; GU135940.1; GU135943.1; GU135944.1; GU135945.1
A/swine/Italy/116114/2010	**H1N2**	CY067661.1; CY067665.1; CY067664.1; CY067662.1; CY067659.1; CY067666.1; CY067663.1; CY067660.1
A/swine/Italy/1369-7/1994	**H1N1**	CY098504.1; CY098502.1; CY098500.1; CY098498.1; CY098503.1; CY098501.1; CY098499.1; CY098497.1
A/swine/Italy/18/2000	**H1N2**	HQ829648.1; HQ709207.1; HQ845031.1; HQ709203.1; HQ709204.1; HQ709216.1; HQ845022.1; HQ850074.1
A/swine/Italy/1850/1977	**H3N2**	HQ829648.1; HQ709207.1; HQ845031.1; HQ709203.1; HQ709204.1; HQ709216.1; HQ845022.1; HQ850074.1
A/swine/Italy/198260/2008	**H1N2**	HQ709211.1; HQ845037.1; HQ660247.1; HQ660248.1; HQ829649.1; HQ709218.1; HQ845026.1; HQ850075.1
A/swine/Italy/233139/2005	**H1N2**	HQ829650.1; HQ709209.1; HQ709217.1; HQ845024.1; HQ845032.1; HQ660251.1; HQ660252.1; HQ850076.1
A/swine/Italy/290271/2009	**H1N1**	CY053623.1; CY053621.1; CY053619.1; CY053617.1; CY053622.1; CY053620.1; CY053618.1; CY053616.1
A/swine/Italy/320546/2009	**H1N2**	HQ850077.1; HQ709197.1; HQ709198.1; HQ845027.1; JF432092.1; HQ709214.1; HQ709224.1; HQ845036.1
A/swine/Italy/3592/1999	**H1N2**	HQ845021.1; HQ658492.1; HQ709206.1; JF317564.1; HQ850078.1; HQ709220.1; HQ845030.1; HQ660233.1
A/swine/Italy/50568/2005	**H1N2**	HQ850079.1; HQ709221.1; HQ845023.1; HQ845033.1; HQ660235.1; HQ660236.1; HQ829651.1; HQ709208.1
A/swine/Italy/526/1985	**H3N2**	L05477.1; CY077917.1; CY077919.1; CY077921.1; CY077923.1; CY077918.1; CY077922.1; CY077920.1
A/swine/Italy/58769/2010	**H1N2**	HQ168025.1; HM771279.1; HM771277.1; HM771275.1; HQ168026.1; HM771278.1; HM771276.1; HM771274.1
A/swine/Italy/6/1981	**H3N2**	CY077930.1; CY077928.1; CY077926.1; CY077924.1; CY077931.1; CY077929.1; CY077927.1; CY077925.1
A/swine/Italy/62/1998	**H1N2**	JF317563.1; HQ709201.1; HQ709202.1; HQ709215.1; HQ845029.1; HQ709205.1; HQ850080.1; HQ845020.1
A/swine/Italy/626-2/2006	**H1N2**	HQ850081.1; HQ829652.1; HQ845025.1; HQ709210.1; HQ709222.1; HQ845038.1; HQ658489.1; HQ658490.1
A/swine/Italy/670/1987	**H1N1**	CY025259.1; CY025257.1; CY025255.1; CY025253.1; CY025260.1; CY025258.1; CY025256.1; CY025254.1
A/swine/Italy/671/1987	**H1N1**	CY022988.1; CY022986.1; CY022993.1; CY022991.1; CY022989.1; CY022987.1; M80963.1; CY022990.1
A/swine/Italy/70757/2009	**H1N2**	HQ709223.1; HQ845035.1; HQ660238.1; HQ850082.1; HQ829653.1; HQ709212.1; HQ845028.1; HQ660239.1
A/swine/Italy/81226/2009	**H1N2**	HQ850083.1; HQ829654.1; HQ709213.1; HQ709219.1; HQ845019.1; HQ660242.1; HQ845034.1; HQ660243.1
A/swine/Italy/85429/2009	**H1N1**	CY057081.1; Y057079.1; CY057077.1; CY057075.1; CY057082.1; CY057080.1; CY057078.1; CY057076.1
A/swine/Italy/85437/2009	**H1N1**	CY061550.1; CY061548.1; CY061546.1; CY061544.1; CY061551.1; CY061549.1; CY061547.1; CY061545.1
A/swine/Kansas/015252/2009	**H3N2**	CY045562.1; CY045560.1; CY045558.1; CY045556.1; CY045563.1; CY045561.1; CY045559.1; CY045557.1
A/swine/Kansas/10-91088/2010	**H3N2**	JN409388.1; JN409390.1; JN409392.1; JN409394.1; JN409389.1; JN409391.1; JN409393.1; JN409395.1
A/swine/Kansas/11-101926/2011	**H3N2**	JN409397.1; JN409399.1; JN409401.1; JN409403.1; JN409396.1; JN409398.1; JN409400.1; JN409402.1
A/swine/Kansas/11-104465/2011	**H3N2**	JN409397.1; JN409399.1; JN409401.1; JN409403.1; JN409396.1; JN409398.1; JN409400.1; JN409402.1

A/swine/Kansas/11-104467/2011	**H3N2**	JN409413.1; JN409415.1; JN409417.1; JN409419.1; JN409412.1; JN409414.1; JN409416.1; JN409418.1
A/swine/Kansas/11-107824/2011	**H3N2**	JN409420.1; JN409422.1; JN409424.1; JN409426.1; JN409421.1; JN409423.1; JN409425.1; JN409427.1
A/swine/Kansas/11-109700/2011	**H3N2**	JN409429.1; JN409431.1; JN409433.1; JN409435.1; JN409428.1; JN409430.1; JN409432.1; JN409434.1
A/swine/Kansas/11-110529/2011	**H3N2**	JN409436.1; JN409438.1; JN409440.1; JN409442.1; JN409437.1; JN409439.1; JN409441.1; JN409443.1
A/swine/Korea/CAN04/2005	**H3N2**	EU798930.1; EU798910.1; EU798890.1; EU798870.1; EU798850.1; EU798830.1; EU798810.1; EU798790.1
A/swine/Korea/CAS05/2004	**H3N2**	EU798909.1; EU798889.1; EU798869.1; EU798849.1; EU798829.1; EU798809.1; EU798789.1; EU798929.1
A/swine/Korea/CAS07/2005	**H3N2**	EU798911.1; EU798891.1; EU798871.1; EU798851.1; EU798831.1; EU798811.1; EU798791.1; EU798931.1
A/swine/Korea/CAS09/2006	**H3N2**	EU798932.1; EU798912.1; EU798892.1; EU798872.1; EU798852.1; EU798832.1; EU798812.1; EU798792.1
A/swine/Korea/CY04/2007	**H3N2**	EU798913.1; EU798893.1; EU798873.1; EU798853.1; EU798833.1; EU798813.1; EU798793.1; EU798933.1
A/swine/Korea/CY05/2007	**H3N2**	EU798934.1; EU798914.1; EU798894.1; EU798874.1; EU798854.1; EU798834.1; EU798814.1; EU798794.1
A/swine/Korea/CY07/2007	**H3N2**	EU798915.1; EU798895.1; EU798875.1; EU798855.1; EU798835.1; EU798815.1; EU798795.1; EU798935.1
A/swine/Korea/CY09/2007	**H3N2**	EU798936.1; EU798916.1; EU798896.1; EU798876.1; EU798856.1; EU798836.1; EU798816.1; EU798796.1
A/swine/Korea/CY10/2007	**H3N2**	EU798917.1; EU798897.1; EU798877.1; EU798857.1; EU798837.1; EU798817.1; EU798797.1; EU798937.1
A/swine/Manitoba/12707/2005	**H3N2**	DQ469980.1; DQ469979.1; DQ469981.1; DQ469982.1; DQ469983.1; DQ469984.1; DQ469985.1; DQ469986.1
A/swine/Minnesota/001332/2006	**H3N2**	CY099237.1; CY099233.1; CY099231.1; CY099238.1; CY099236.1; CY099232.1; CY099235.1; CY099234.1
A/swine/Minnesota/001444/2007	**H3N2**	CY099221.1; CY099219.1; CY099217.1; CY099222.1;CY099220.1; CY099218.1; CY099215.1; CY099216.1
A/swine/Minnesota/00484/2005	**H3N2**	CY099253.1; CY099251.1; CY099249.1; CY099254.1; CY099252.1; CY099250.1; CY099247.1; CY099248.1
A/swine/Minnesota/00611/2005	**H3N2**	CY099149.1; CY099150.1; CY099148.1; CY099145.1; CY099146.1; CY099143.1; CY099147.1; CY099144.1
A/swine/Minnesota/00709/2005	**H3N2**	CY099067.1; CY099065.1; CY099061.1; CY099068.1; CY099066.1; CY099063.1; CY099062.1; CY099064.1
A/swine/Minnesota/01146/2006	**H3N2**	CY099041.1; CY099038.1; CY099035.1; CY099042.1; CY099040.1; CY099037.1; CY099036.1; CY099039.1
A/swine/Minnesota/01862/2007	**H3N2**	CY099117.1; CY099118.1; CY099116.1; CY099113.1; CY099111.1; CY099115.1; CY099112.1; CY099114.1
A/swine/Minnesota/02782/2009	**H3N2**	CY099117.1; CY099118.1; CY099116.1; CY099113.1; CY099111.1; CY099115.1; CY099112.1; CY099114.1
A/swine/Minnesota/1300/2007	**H3N2**	FJ519965.1; JF346129.1; JF346136.1; JF346144.1; JF346152.1; JF411837.1; HQ315643.1; EU692894.1
A/swine/Minnesota/239105/2009	**H3N2**	CY086921.1; CY086924.1; CY086922.1; CY086919.1; CY086917.1; CY086923.1; CY086920.1; CY086918.1
A/swine/Minnesota/578/2007	**H3N2**	FJ519974.1; FJ519963.1; JF346127.1; JF346134.1; JF346142.1; JF346150.1; JF411835.1; EU692892.1
A/Swine/Minnesota/593/99	**H3N2**	AF251431.2; AF251433.1; AF251430.1; AF251434.1; AF251432.1; AF251429.1; AF251427.2; AF251428.2
A/swine/Minnesota/5947/2007	**H3N2**	FJ519966.1; HQ315644.1; JF346130.1; JF346137.1; JF346145.1; JF346153.1; JF411838.1; FJ519976.1
A/swine/Minnesota/65767/2006	**H3N2**	HQ315645.1; FJ519971.1; FJ519960.1; JF346131.1; JF346154.1; JF346139.1; JF346147.1; JF411840.1
A/swine/Minnesota/66853/2006	**H3N2**	FJ519972.1; FJ519961.1; JF346140.1; JF346148.1; JF411841.1; JF346132.1; JF346155.1; EU692890.1
A/swine/Minnesota/66960/2006	**H3N2**	FJ519973.1; FJ519962.1; JF346133.1; JF346156.1; JF346141.1; JF346149.1; JF411842.1; EU692891.1
A/swine/Minnesota/761/2007	**H3N2**	FJ519975.1; FJ519964.1; JF346128.1; JF346135.1; JF346143.1; JF346151.1; JF411836.1; EU692893.1
A/swine/Minnesota/7931/2007	**H3N2**	FJ519967.1; JF346138.1; JF346146.1; JF411839.1; JF346157.1; JF346158.1; FJ519977.1; EU692896.1
A/swine/Nakhon pathom/NIAH586-1/2005	**H3N2**	EU296613.1; EU296614.1; AB434364.1; AB434358.1; AB434363.1; AB434361.1; AB434359.1; AB434357.1
A/Swine/Nebraska/209/98	**H3N2**	AF251409.1; AF251406.1; AF251404.1; AF251407.2; AF251410.1; AF251408.1; AF251405.1; AF251403.1
A/swine/Nordkirchen/IDT1993/2003	**H3N2**	EU924274.1; EU924272.1; EU924270.1; EU924268.1; EU924273.1; EU924271.1; EU924269.1
A/swine/North Carolina/2003	**H3N2**	EF551057.1; EF551055.1; EF551053.1; EF551051.1; EF551056.1; EF551050.1; EF551052.1; EF551054.1
A/swine/North Carolina/R08-001877-D08-013371/2008	**H3N2**	CY041851.2; CY041847.2; CY041845.2; CY041849.2; CY041846.2; CY041844.2; CY041850.1; CY041848.1
A/swine/Norway/02_11342/2009	**H1N1**	JQ253796.1; JQ253794.1; JQ253792.1; JQ253790.1; JQ253797.1; JQ253795.1; JQ253793.1; JQ253791.1

A/swine/NY/A01104005/2011	**H3N2**	JN940426.1; JN940424.1; JN940422.1; JN940420.1; JN940425.1; JN940423.1; JN940421.1; JN940419.1
A/swine/Oedenrode/7c/1996	**H3N2**	CY077938.1; CY077936.1; CY077934.1; CY077932.1; CY077937.1; CY077935.1; CY077933.1
A/swine/Oklahoma/001142/2009	**H3N2**	CY045554.1; CY045552.1; CY045550.1; CY045548.1; CY045555.1; CY045553.1; CY045551.1; CY045549.1
A/swine/Oklahoma/008722/2007	**H3N2**	CY045570.1; CY045568.1; CY045566.1; CY045564.1; CY045571.1; CY045569.1; CY045567.1; CY045565.1
A/swine/Oklahoma/011506/2007	**H3N2**	CY045578.1; CY045576.1; CY045574.1; CY045572.1; CY045579.1; CY045577.1; CY045575.1; CY045573.1
A/swine/Ontario/00130/97	**H3N2**	AF251399.2; AF251402.1; AF251400.1; AF251397.1; AF251401.1; AF251398.1; AF251396.1; AF251395.2
A/swine/Ontario/33853/2005	**H3N2**	DQ469991.1; DQ469992.1; DQ469993.1; DQ469994.1; DQ469988.1; DQ469987.1; DQ469989.1; DQ469990.1
A/swine/Pennsylvania/057108-1/2010	**H3N2**	JF316647.1; JF316645.1; JF316643.1; JF316641.1; JF316646.1; JF316644.1; JF316642.1; JF316640.1
A/swine/Pennsylvania/62170-1/2010	**H3N2**	JF263535.1; HQ734207.1; HQ734208.1; HQ734209.1; HQ734210.1; HQ734212.1; HQ734211.1
A/swine/Pennsylvania/62170-3/2010	**H3N2**	JF263536.1; HQ734219.1; HQ734220.1; HQ734221.1; HQ734222.1; HQ734224.1; HQ734223.1
A/swine/QC/1685-5/2009	**H3N2**	HQ825207.1; HQ825209.1; HQ825210.1; HQ825211.1; HQ825213.1; HQ825214.1; HQ825208.1; HQ825212.1
A/swine/QC/1698-1/2009	**H3N2**	HQ825216.1; HQ825220.1; HQ825215.1; HQ825217.1; HQ825218.1; HQ825219.1; HQ825221.1; HQ825222.1
A/swine/QC/1840-2/2009	**H3N2**	HQ825232.1; HQ825234.1; HQ825236.1; HQ825237.1; HQ825239.1; HQ825233.1; HQ825235.1; HQ825238.1
A/swine/QC/2108-2/2009	**H3N2**	HQ825241.1; HQ825243.1; HQ825246.1; HQ825240.1; HQ825242.1; HQ825244.1; HQ825245.1; HQ825247.1
A/swine/QC/382/2009	**H3N2**	HQ825187.1; HQ825189.1; HQ825192.1; HQ825194.1; HQ825188.1; HQ825190.1; HQ825191.1; HQ825193.1
A/swine/QC/414/2009	**H3N2**	HQ825180.1; HQ825182.1; HQ825183.1; HQ825185.1; HQ825179.1; HQ825181.1; HQ825184.1; HQ825186.1
A/swine/QC/440-A/2009	**H3N2**	HQ825196.1; HQ825198.1; HQ825199.1; HQ825201.1; HQ825195.1; HQ825197.1; HQ825200.1; HQ825202.1
A/swine/Quebec/1265553/2010	**H3N2**	JF703684.1; JF682723.1; JF682721.1; JF682719.1; JF703685.1; JF682724.1; JF682722.1; JF682720.1
A/swine/Quebec/4001/2005	**H3N2**	EU826549.2; EU826547.2; EU826544.2; EU826550.2; EU826548.2; EU826545.2; EU826543.2; EU826546.1
A/swine/Ratchaburi/NIAH59/2004	**H3N2**	AB434372.1; AB434370.1; AB434368.1; AB434366.1; AB434371.1; AB434369.1; AB434367.1; AB434365.1
A/swine/Ratchaburi/NIAH874/2005	**H3N2**	EU296617.1; EU296618.1; AB434380.1; AB434374.1; AB434379.1; AB434377.1; AB434375.1; AB434373.1
A/swine/Scotland/WVL17/1999	**H1N1**	CY037964.1; CY037962.1; CY037960.1; CY037958.1; CY037963.1; CY037961.1; CY037959.1; CY037957.1
A/swine/Shandong/3/2005	**H3N2**	EU116038.1; EU116044.1; EU116042.1; EU116040.1; EU116037.1; EU116043.1; EU116041.1; EU116039.1
A/swine/Shizuoka/120/97	**H3N2**	AF225525.1; AF225529.1; AF225545.1; AF225533.1; AF225521.1; AF225537.1; AF225517.1; AF225541.1
A/swine/Sichuan/01/2006	**H3N2**	EU655695.1; EU655693.1; EU655691.1; EU655689.1; EU655696.1; EU655694.1; EU655692.1; EU655690.1
A/swine/Spain/33601/2001	**H3N2**	CY009379.1; CY009377.1; CY009375.1; CY009373.1; CY009378.1; CY009376.1; CY009374.1; CY009372.1
A/swine/Spain/39139/2002	**H3N2**	CY009387.1; CY009385.1; CY009383.1; CY009381.1; CY009386.1; CY009384.1; CY009382.1; CY009380.1
A/swine/Spain/42386/2002	**H3N2**	CY020507.1; CY020505.1; CY020503.1; CY020501.1; CY020508.1; CY020506.1; CY020504.1; CY020502.1
A/swine/Spain/50047/2003	**H1N1**	CY009899.1; CY009897.1; CY009895.1; CY009893.1; CY009898.1; CY009896.1; CY009894.1; CY009892.1
A/swine/Spain/51915/2003	**H1N1**	CY010579.1; CY010577.1; CY010575.1; CY010573.1; CY010578.1; CY010576.1; CY010574.1; CY010572.1
A/swine/Spain/53207/2004	**H1N1**	CY010587.1; CY010585.1; CY010583.1; CY010581.1; CY010586.1; CY010584.1; CY010582.1; CY010580.1
A/swine/Spain/54008/2004	**H3N2**	CY010571.1; CY010569.1; CY010567.1; CY010565.1; CY010570.1; CY010568.1; CY010566.1; CY010564.1
A/swine/Spain/WVL6/1991	**H1N1**	CY038002.1; CY038000.1; CY037998.1; CY037996.1; CY038003.1; CY038001.1; CY037999.1; CY037997.1
A/swine/Sweden/1021/2009	**H1N2**	GQ495136.1; GQ495134.1; GQ495132.1; GQ495130.1; GQ495135.1; GQ495133.1; GQ495131.1; GQ495129.1
A/swine/Sweden/9706/2010	**H1N2**	HM626485.1; HM626483.1; HM626481.1; HM626479.1; HM626486.1; HM626484.1; HM626482.1; HM626480.1
A/swine/Texas/4199-2/1998	**H3N2**	CY095679.1; CY095677.1; CY095676.1; CY095673.1; CY095672.1; CY095678.1; CY095675.1; CY095674.1
A/swine/Udon Thani/NIAH464/2004	**H3N2**	AB434356.1; AB434354.1; AB434352.1; AB434350.1; AB434355.1; AB434353.1; AB434351.1; AB434349.1
A/swine/Wisconsin/194/1980	**H3N2**	CY009315.1; CY009313.1; CY009311.1; CY009309.1; CY009314.1; CY009312.1; CY009310.1; CY009308.1

Table S3. Accession numbers of the added European strains for the determination of the closest relative strain for each gene segment.

Accession	Strain name	Subtype	Gene
AJ306849	A/swine/Italy/1521/98	H1N2	PB2
AJ306845	A/swine/Cotes d'Armor/790/97	H1N2	PB2
AJ306848	A/swine/Cotes d'Armor/1488/1999	H1N1	PB2
AJ306854	A/swine/Cotes d'Armor/2433/98	H1N2	PB2
AJ306842	A/swine/Ille et Vilaine/1455/1999	H1N1	PB2
AJ306843	A/swine/Italy/2064/99	H1N2	PB2
AJ306851	A/Swine/Italy/1513-1/98	H1N1	PB2
AJ306850	A/swine/Cotes d'Armor/604/99	H1N2	PB2
AJ311457	A/swine/Finistere/2899/1982	H1N1	PB2
AJ311459	A/Swine/Italy/1523/98	H3N2	PB2
JF290388	A/swine/England/1382/2010	H1N2	PB2
M55471	A/swine/Germany/2/1981	H1N1	PB2
AJ306866	A/swine/Ille et Vilaine/1455/1999	H1N1	PB1
AJ306856	A/swine/Cotes d'Armor/1488/1999	H1N1	PB1
AJ306864	A/swine/Cotes d'Armor/2433/98	H1N2	PB1
AJ306863	A/swine/Cotes d'Armor/604/99	H1N2	PB1
AJ306865	A/swine/Cotes d'Armor/790/97	H1N2	PB1
AJ306862	A/swine/Italy/1521/98	H1N2	PB1
AJ306861	A/Swine/Italy/1513-1/98	H1N1	PB1
JF432093	A/swine/Italy/321986/2009	H1N2	PB1
GU236515	A/swine/Skane/1321/1983	H1N1	PB1
DQ836168	A/swine/Jena/5/1996	H3N2	PB1
DQ836169	A/swine/Karrenzien/2/87	H3N2	PB1
DQ836170	A/swine/Bakum/8602/99	H3N2	PB1
DQ836171	A/swine/Potsdam/35/82	H3N2	PB1
DQ836172	A/swine/Lohne/1/97	H3N2	PB1
DQ836173	A/swine/Leipzig/1/97	H3N2	PB1
DQ836174	A/swine/Potsdam/15/81	H1N1	PB1
DQ836175	A/swine/Schwerin/103/89	H1N1	PB1
DQ836176	A/swine/Bakum/5/95	H1N1	PB1
DQ836177	A/swine/Belzig/2/2001	H1N1	PB1
AJ311462	A/swine/Finistere/2899/1982	H1N1	PB1
AJ311208	A/swine/Cotes d'Armor/1488/1999	H1N1	PA
AJ311207	A/swine/Cotes d'Armor/790/97	H1N2	PA
AJ311205	A/swine/Cotes d'Armor/2433/98	H1N2	PA
AJ312838	A/swine/Cotes d'Armor/604/99	H1N2	PA
AJ311210	A/swine/Italy/2064/99	H1N2	PA
AJ311209	A/swine/Italy/1081/00	H1N2	PA
AJ311206	A/swine/Italy/1521/98	H1N2	PA
AJ311463	A/swine/Finistere/2899/1982	H1N1	PA
AJ311465	A/Swine/Italy/1513-1/98	H1N1	PA
AJ307068	A/swine/Ille et Vilaine/1455/1999	H1N1	NP

AJ311510	A/swine/Italy/1509-6/97	H1N1	NP
AJ307066	A/swine/Cotes d'Armor/2433/98	H1N2	NP
U04855	A/swine/Northern Ireland/1938	H1N1	NP
U04856	A/swine/Cambridge/1939	H1N1	NP
AJ311510	A/swine/Italy/1509-6/97	H1N1	NP
AJ293942	A/Swine/Italy/1523/98	H3N2	NP
JN624324	A/swine/Italy/22530/2002	H1N2	NP
AJ307062	A/swine/Italy/2064/99	H1N2	NP
M63765	A/swine/Italy/2/1979	H1N1	NP
M63766	A/swine/Italy/141/1981	H1N1	NP
M60762	A/swine/Italy/147/1981	H1N1	NP
AJ307065	A/swine/Cotes d'Armor/790/97	H1N2	NP
FJ805965	A/swine/Belgium/1/1998	H1N1	NP
AJ307069	A/swine/Cotes d'Armor/1488/1999	H1N1	NP
AJ307074	A/swine/Cotes d'Armor/604/99	H1N2	NP
U85987	A/swine/England/191973/92	H1N7	NP
GU236518	A/swine/Skane/1321/1983	H1N1	NP
AM746619	A/wild boar/Germany/WS169/2006	H3N2	NP
AM920725	A/swine/Germany/Vi3161/95	H3N2	NP
AM920730	A/swine/Germany/Vi5698/1995	H1N1	NP
AM920735	A/swine/Germany/SEC27/97	H3N2	NP
AM920739	A/swine/Germany/SEk1178/2000	H1N2	NP
AM920744	A/swine/Germany/S043/2002	H3N2	NP
Z26856	A/swine/Germany/8533/1991	H1N1	NP
M63769	A/swine/Cambridge/1/1935	H1N1	NP
M30749	A/swine/Netherlands/12/1985	H1N1	NP
M22579	A/swine/Germany/2/1981	H1N1	NP
AJ293938	A/swine/Italy/1553-2/98	H3N2	M
AJ316052	A/swine/Italy/1521/98	H1N2	M
AJ316056	A/swine/Italy/2034/99	H1N1	M
AJ316058	A/swine/Italy/1566/98	H1N1	M
GQ404622	A/swine/Italy/v.45/1979	H1N1	M
EU478802	A/swine/England/17394/96	H1N2	M
U85985	A/swine/England/191973/92	H1N7	M
AM920726	A/swine/Germany/Vi3161/95	H3N2	M
AM920731	A/swine/Germany/Vi5698/1995	H1N1	M
AM920734	A/swine/Germany/SEC27/97	H3N2	M
AM920740	A/swine/Germany/SEk1178/2000	H1N2	M
AM920745	A/swine/Germany/S043/2002	H3N2	M
DQ186975	A/swine/Bakum/5/95	H1N1	M
DQ186984	A/swine/Bakum/8602/99	H3N2	M
EU478801	A/swine/Bakum/909/93	H3N2	M
EU478803	A/swine/Bakum/1362/98	H3N2	M
AJ311509	A/swine/Belgium/220/92	H3N2	M
DQ186976	A/swine/Belzig/2/2001	H1N1	M
EU478806	A/swine/Belzig/54/01	H3N2	M

EU478805	A/swine/Berlin/1578/00	H3N2	M
EU478846	A/swine/Bad Griesbach/IDT5604/06	H1N1	M
FJ805964	A/swine/Belgium/1/1998	H1N1	M
GQ161131	A/swine/Bakum/IDT1769/2003	H3N2	M
GQ404616	A/swine/Brno/1/1992	H1N1	M
AJ316047	A/swine/Cotes d'Armor/1482/1999	H1N1	M
AJ316049	A/swine/Cotes d'Armor/790/97	H1N2	M
AJ316051	A/swine/Cotes d'Armor/604/99	H1N2	M
AJ316061	A/swine/Ille et Vilaine/1455/1999	H1N1	M
DQ186974	A/swine/Schwerin/103/89	H1N1	M
DQ186979	A/swine/Potsdam/35/82	H3N2	M
DQ186980	A/swine/Karrenzien/2/87	H3N2	M
DQ186982	A/swine/Jena/5/1996	H3N2	M
DQ186983	A/swine/Lohne/1/97	H3N2	M
EU478796	A/swine/Potsdam/1/81	H1N1	M
EU478798	A/swine/Gent/1/84	H3N2	M
EU478799	A/swine/Gent/V230/1992	H1N1	M
EU478804	A/swine/Gent/7625/1999	H1N2	M
EU478809	A/swine/Ehren/IDT2570/03	H1N2	M
EU478815	A/swine/Nordwalde/IDT2197/03	H1N2	M
EU478817	A/swine/Vechta/2623/03	H1N1	M
EU478820	A/swine/Damme/IDT2890/04	H3N2	M
EU478821	A/swine/Geldern/IDT2888/2004	H1N1	M
EU478825	A/swine/Gudensberg/IDT2931/04	H1N2	M
EU478826	A/swine/Lohne/IDT3357/04	H3N2	M
EU478828	A/swine/Seesen/IDT3055/04	H3N2	M
EU478834	A/swine/Harkenblek/IDT4097/05	H3N2	M
EU478835	A/swine/Hertzen/IDT4317/05	H3N2	M
EU478837	A/swine/Laer/IDT3893/2005	H1N2	M
EU478838	A/swine/Laer/IDT4126/05	H3N2	M
EU478839	A/swine/Merzen/IDT4114/05	H3N2	M
EU478841	A/swine/Osterhofen/IDT4004/05	H3N2	M
EU478843	A/swine/Stadtlohn/IDT3853/05	H1N2	M
EU478845	A/swine/Wohlerst/IDT4093/05	H1N1	M
EU478847	A/swine/Herzlake/IDT5335/06	H3N2	M
GQ404581	A/swine/Czech Republic/1957	H1N1	M
GQ404585	A/swine/Minsk/1965	H1N1	M
GQ404591	A/swine/Czech Republic/2/1972	H3N2	M
GQ404594	A/swine/Czech Republic/1/1975	H3N2	M
GQ404607	A/swine/Czech Republic/1978	H3N2	M
GQ404620	A/swine/Schleswig-Holstein/1/1993	H1N1	M
GU236512	A/swine/Lidkoeping/1193/2002	H1N1	M
GU236520	A/swine/Skane/1321/1983	H1N1	M
M63525	A/swine/Netherlands/12/1985	H1N1	M
Z26861	A/swine/Germany/8533/1991	H1N1	M
Z26862	A/swine/Netherlands/25/1980	H1N1	M

EU091680	A/swine/Italy/66945/2006	H3N1	NS1
EU091704	A/swine/Italy/1484/2002	H3N2	NS1
AJ344040	A/swine/Cotes d'Armor/799/00	H1N2	NS1
AJ293940	A/swine/Italy/636/87	H3N2	NS1
AJ344041	A/swine/Cotes d'Armor/1121/2000	H1N1	NS1
EU091706	A/swine/Italy/10169/2001	H1N1	NS1
AJ344024	A/Swine/Italy/1523/98	H3N2	NS1
GU236521	A/swine/Skane/1321/1983	H1N1	NS1
AJ293939	A/swine/Italy/13962/95	H3N2	NS1
GU236513	A/swine/Lidkoeping/1193/2002	H1N1	NS1
Z26865	A/swine/Germany/8533/1991	H1N1	NS1
Z26866	A/swine/Netherlands/25/1980	H1N1	NS1
AJ344027	A/swine/Cotes d'Armor/1488/1999	H1N1	NS1
AJ344029	A/Swine/Italy/1513-1/98	H1N1	NS1
AJ344031	A/swine/Cotes d'Armor/2433/98	H1N2	NS1
AJ344033	A/swine/Ille et Vilaine/1455/1999	H1N1	NS1
AJ344035	A/swine/Italy/1654-1/99	H1N2	NS1
AJ344038	A/swine/Cotes d'Armor/1482/1999	H1N1	NS1
AJ519462	A/swine/Italy/3364/00	H1N1	NS1
AJ344026	A/swine/Cotes d'Armor/790/97	H1N2	NS1
AJ344032	A/swine/Italy/2064/99	H1N2	NS1
AJ344034	A/swine/Scotland/410440/94	H1N2	NS1
CY077923	A/swine/Italy/526/1985	H3N2	NS1
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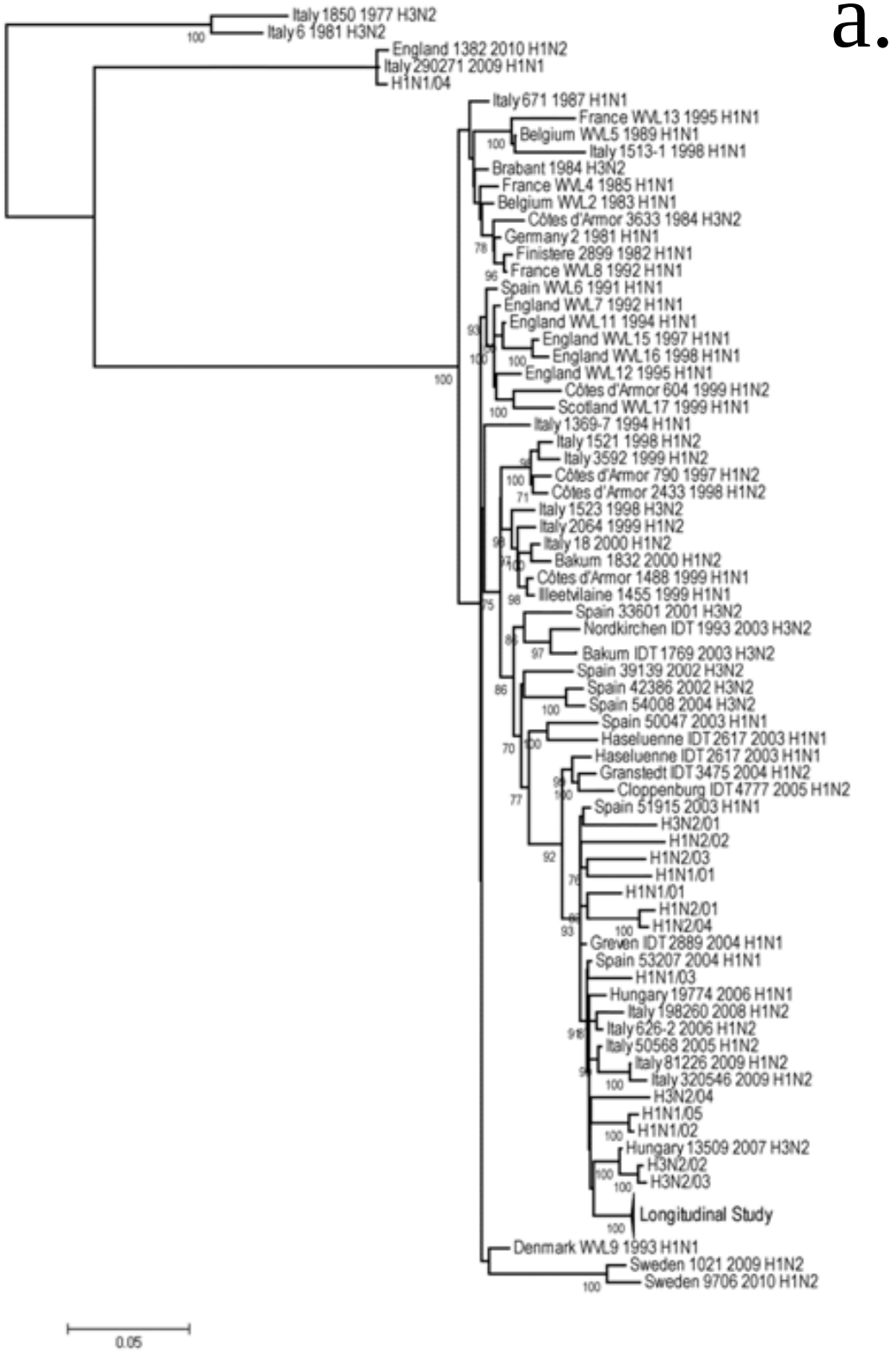
AM920741	A/swine/Germany/SEk1178/2000	H1N2	NS1
AM920746	A/swine/Germany/S043/2002	H3N2	NS1

Figures S1. Phylogenetic trees obtained with the European strains to determine the clustering of the isolates from the Chapter 3. Order as follows: a) PB2, b) PB1, c) PA, d) NP, e) M, f) NS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Belgium WVL5 1989 H1N1
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Brabant 1984 H3N2
France WVL4 1985 H1N1
Belgium WVL2 1983 H1N1
Côtes d'Armor 3633 1984 H3N2
Germany 2 1981 H1N1
Finistere 2899 1982 H1N1
France WVL8 1992 H1N1
Spain WVL6 1991 H1N1
England WVL7 1992 H1N1
England WVL11 1994 H1N1
England WVL15 1997 H1N1
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Scotland WVL17 1999 H1N1
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Spain 54008 2004 H3N2
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Haseluenne IDT 2617 2003 H1N1
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H3N2/01
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H1N2/03
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H1N2/04
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Sweden 1021 2009 H1N2
Sweden 9706 2010 H1N2
0.05


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Karrenzien 2 87 H3N2
Potsdam 15 81 H1N1
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England WVL15 1997 H1N1
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Lohne 1 1997 H3N2
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Oedenrode 7C 1996 H3N2
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H1N2/02
H1N1/03
H1N1/02
H1N1/06
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Longitudinal Study
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H1N1/06
H1N2/03
H1N2/04
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H1N1/05
H1N2/02
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England WVL10 1993 H1N1
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England WVL16 1998 H1N1
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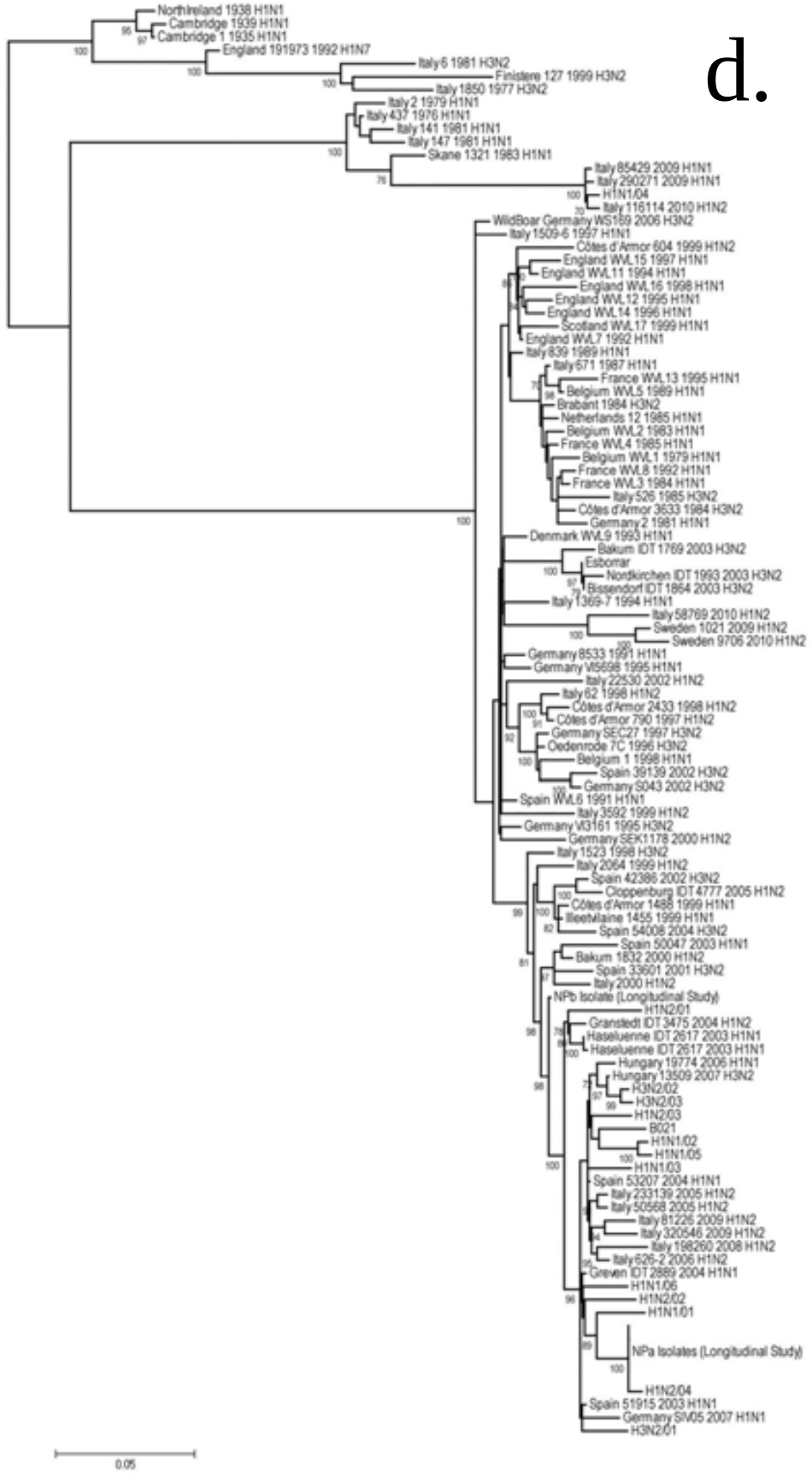
C.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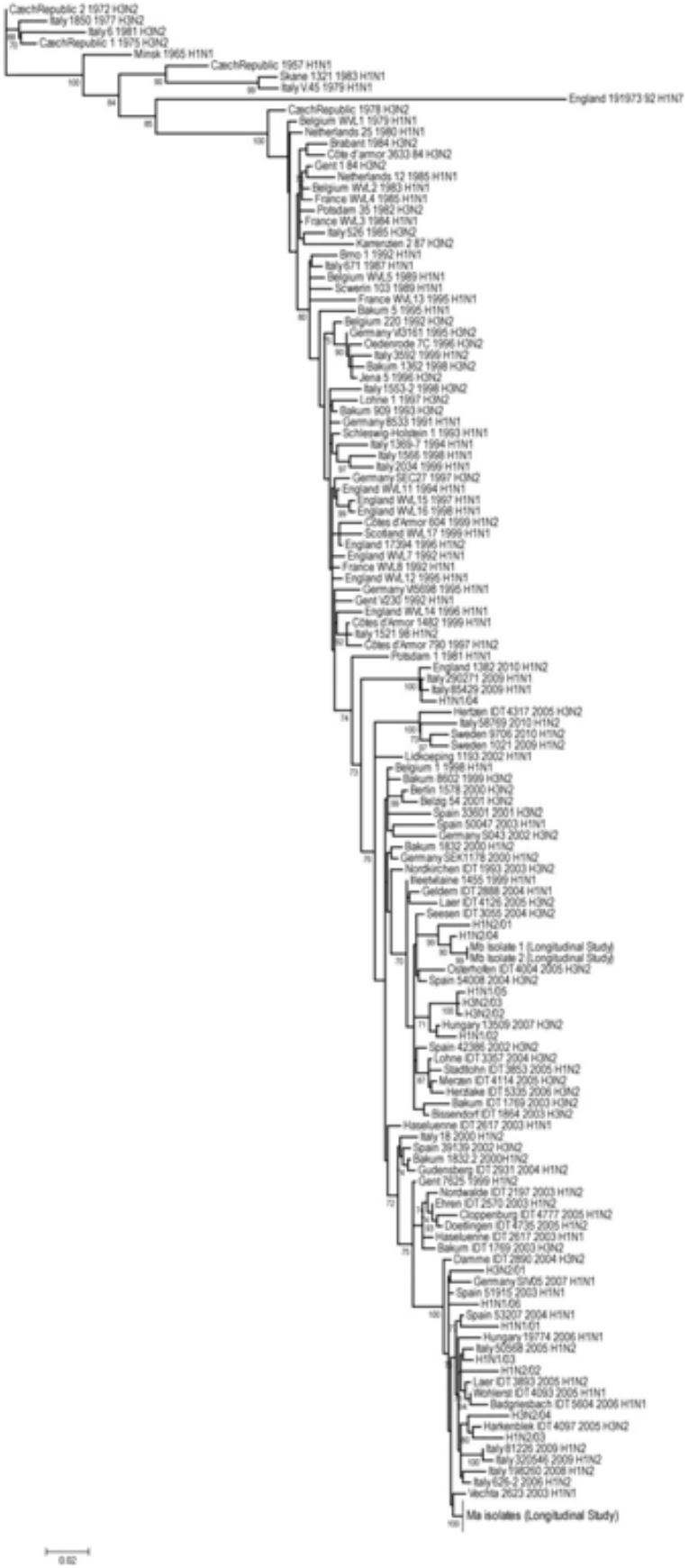
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e.

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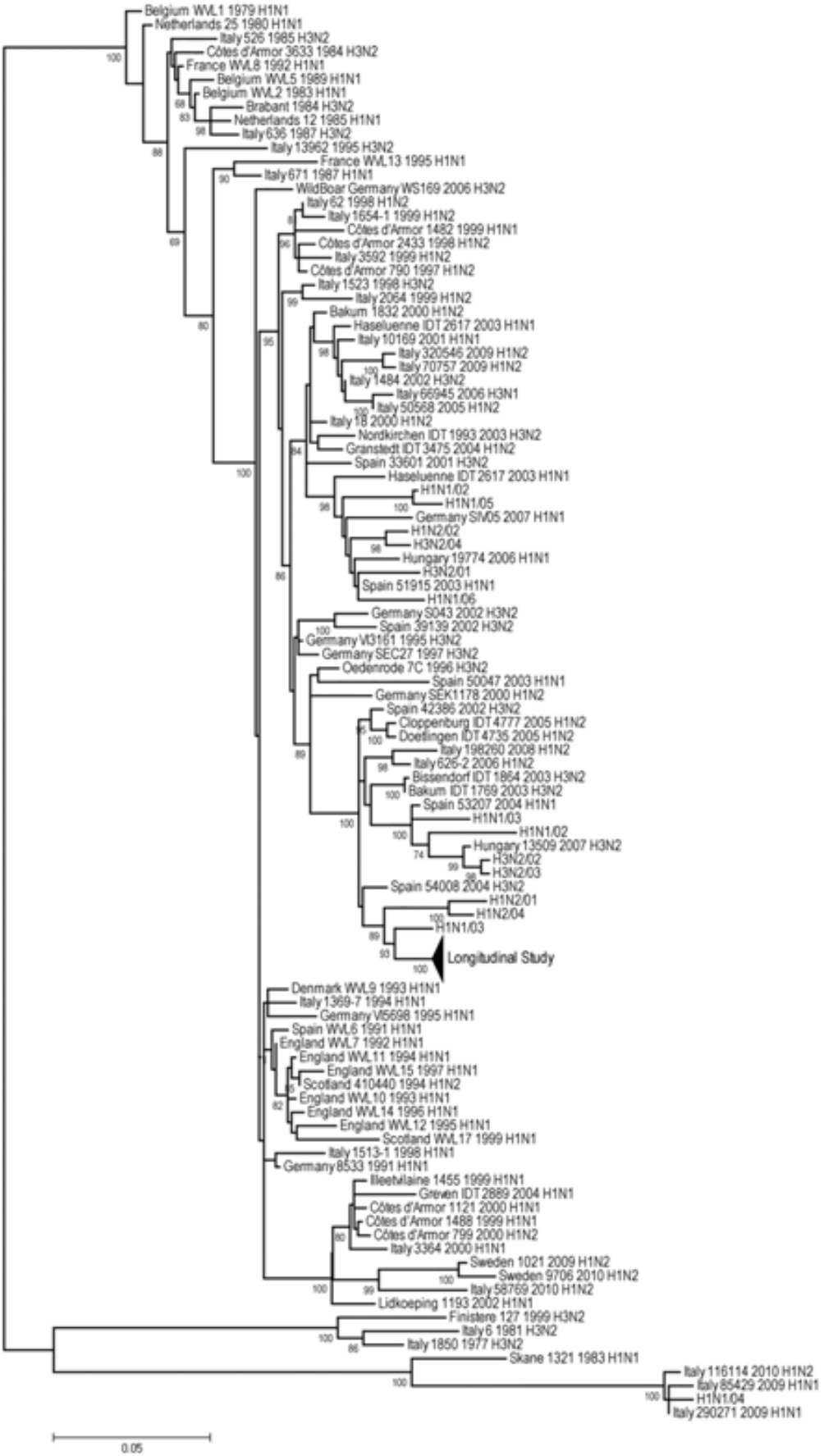
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H1N1/02
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Longitudinal Study
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0.05

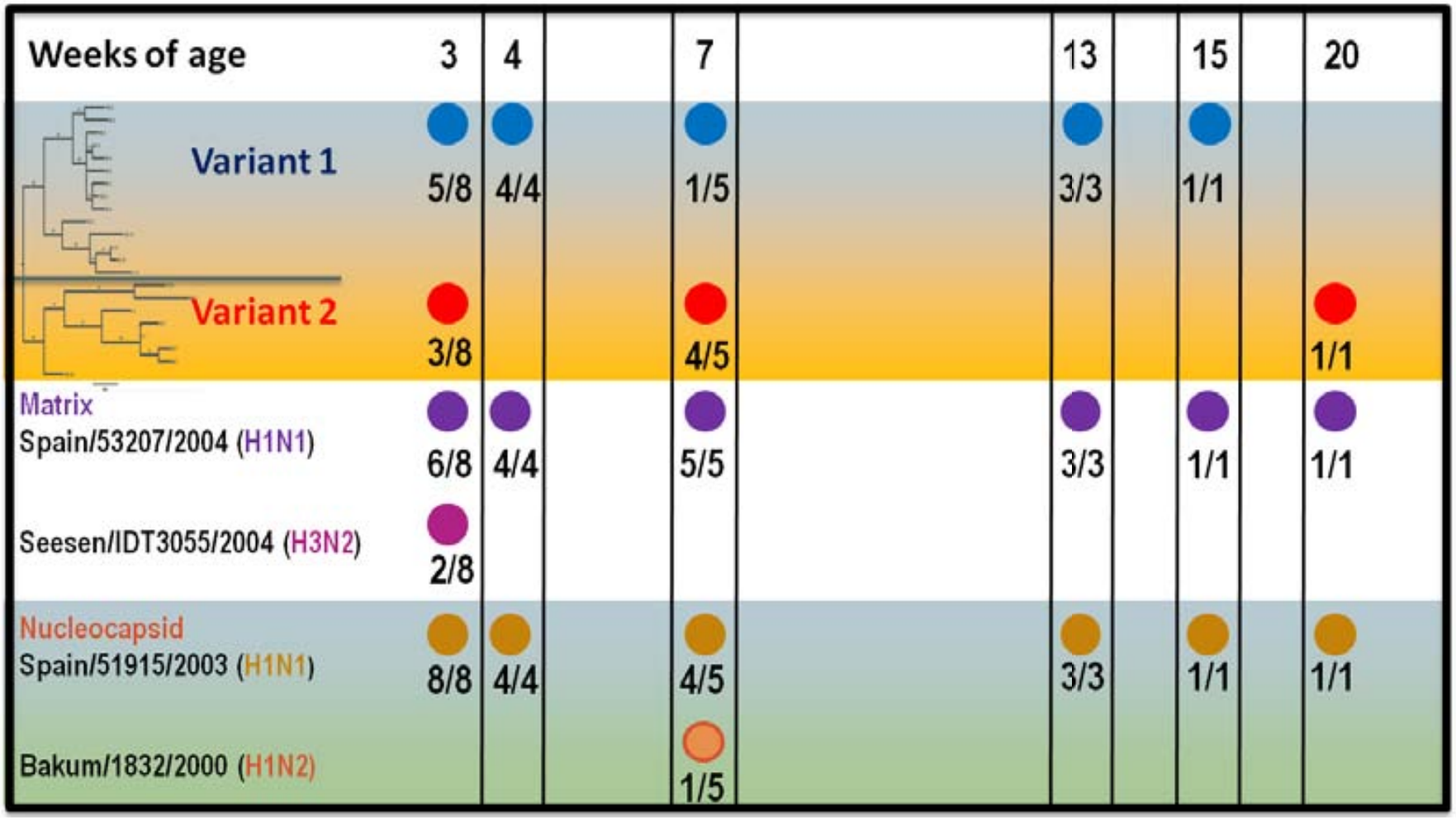

Figure S2. Summary of the results obtained in the longitudinal study. The types of isolates (by concatenated segments, type of Matrix and type of nucleocapsid gene segments) are represented by the circles in a given time of the sampling. Prevalence of each type per week are also shown.

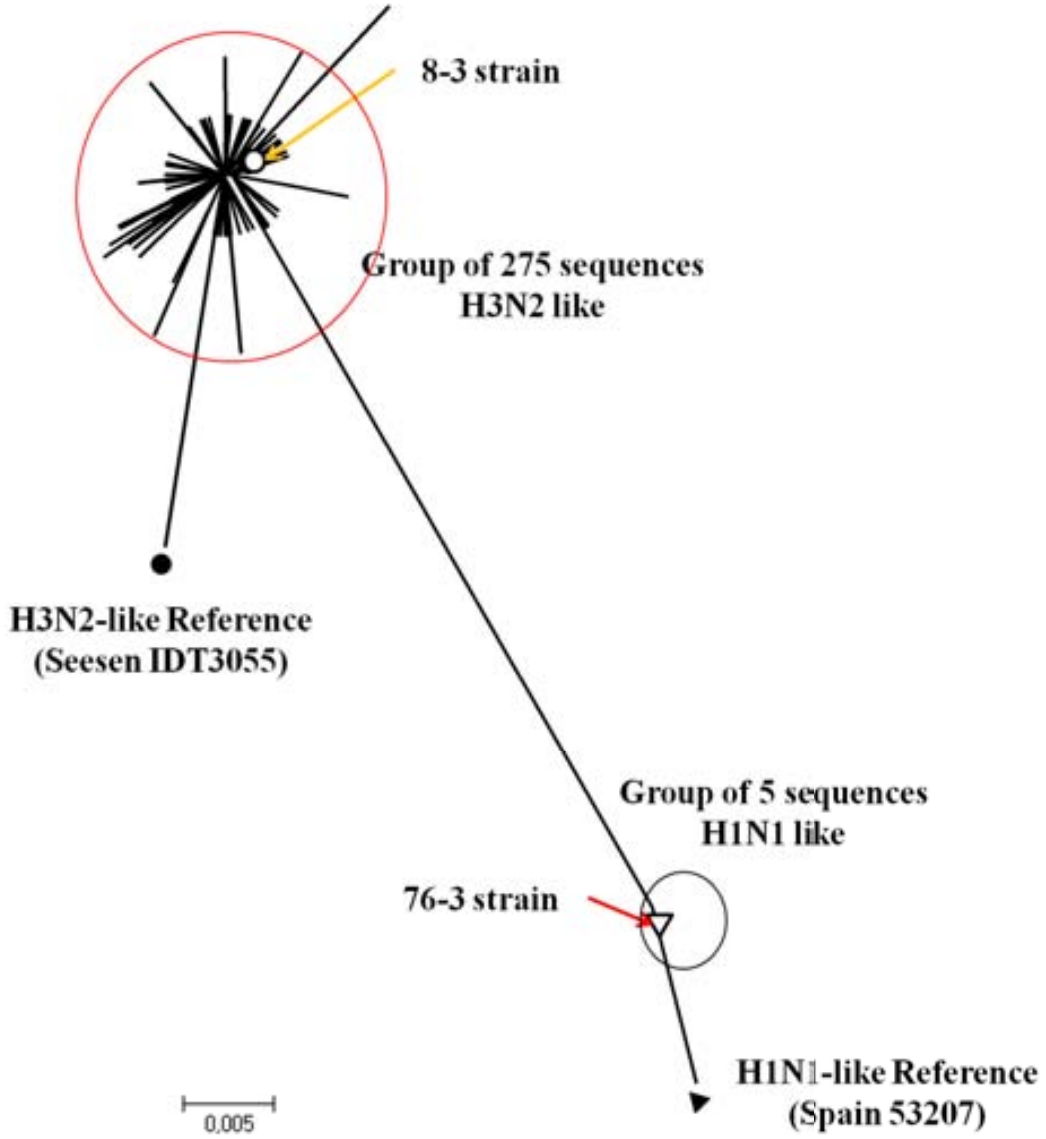

Figure S3. Maximum likelihood tree including 280 sequences obtained with the high-throughput sequencing from the animal 8.3. the consensus sequence obtained from the animals 8.3 and 76.3. and the closest relative strains for each one (reference strain). The segment used for the tree contained 357 nucleotides.

Table S4. Values of grouping obtained with the factorial analysis. Bold type indicates the values of the representative subtype for each factor

Strain used	Factor 1	Factor 2	Factor 3
GDH1N1	**0,806**	0,126	0,215
GDH1N2	0,111	0,174	**0,694**
GDH3N2	0,213	**0,679**	0,124
S1	**0,,896**	0,121	0,139
S2	**0,815**		
S3	**0,615**	0,236	0,294
S4	**0,681**	0,216	0,234
S6	**0,744**	0,195	0,179
S7	0,243		**0,754**
S8	0,155		**0,875**
S9	0,204		**0,833**
S10	0,178		**0,868**
S11		**0,772**	
S12	0,144	**0,96**	0,184
S13	0,176	**0,948**	0,146
S14	0,128	**0,739**	
SS Loadings	3,802	3,648	3,576
Proportion Var	0,238	0,228	0,224
Cumulative Var	0,238	0,466	0,689
Chi square statistic for 75 degrees of liberty			230,24
p-value			<0,01

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Chapter 6. General discussion

The historical importance of influenza is beyond any doubt and it still is one of the most important infectious diseases for humans and animals. Most of the scientific knowledge on influenza has been gathered in human and birds while other species of importance in the epidemiology of this infection such as swine have been less studied. However, since the last 2009 pandemic –that was caused by a strain closely related to the triple reassortant virus circulating in swine in the United States previously (Smith *et al.*, 2009b)- it has become increasingly evident that there is a need for a better understanding of the epidemiology of influenza in pigs. In particular it seems crucial to gain knowledge on how common is the emergence of new influenza variants in pigs. Also, since influenza is an important disease of pigs as well, the knowledge on the epidemiology of the infection would surely contribute to a better control and monitoring.

Previous research showed that SIV is a common pathogen of pigs in intensive swine producing areas where the prevalence of seropositive pigs can be very high (Simón-Grifé *et al.,* 2011) with all three subtypes, namely H1N1, H1N2 and H3N2 commonly circulating within and between herds both endemically or causing epidemic outbreaks (Madec *et al.,* 1985, Simon-Grifé *et al.,* 2012, Loeffen *et al.,* 2009). Knowledge on the molecular epidemiology of currently circulating SIV shows that genetic diversity is probably higher than supposed before (Karasin *et al.,* 2000a,b; Peiris *et al.,* 2001; Ma *et al.,* 2007; Lee *et al.,* 2009; Kyriakis *et al.,* 2011; Kwon *et al.,* 2011; Vijaykrishna *et al.,* 2011; Vincent *et al.,* 2011; Choi *et al.,* 2012; Moreno *et al.,* 2012). Unfortunately, there are still many gaps to be filled about the genetic and antigenic diversity of SIV, particularly in Europe where swine influenza has been largely neglected for many years. For example, it is almost unknown how frequent are subclinical infections compared to

clinical outbreaks; what role play endemic circulations of SIV within a herd for the generation of new SIV variants or the extent and importance of antigenic diversity within a given subtype. The present thesis was designed as a modest contribution for clarifying some of those issues regarding SIV circulating in Spanish farms.

1. Swine influenza virus infections in pigs

Contrarily of what happens in North America, in many European countries SIV has been considered as a second line respiratory pathogen and control strategies have been mostly used only in cases where the economic impact of influenza is beyond any doubt, especially when abortions may occur. In the present thesis, SIV was isolated in about one half of the respiratory disease outbreaks fulfilling a set of criteria of compatibility. This is evidence that SIV is clearly implicated in clinical respiratory outbreaks and is in accordance of what loeffen *et al.* (2009) reported. Taking into account that the shedding period for SIV is about one week after the onset of the signs (Van Reeth *et al.*, 2012), the chance for isolating SIV from infected pigs once an outbreak starts is relatively low and thus, it cannot be ruled out that some SIV-negative outbreaks were indeed influenza cases. Moreover, since multiple respiratory pathogens may circulate in a farm, masking of the clinical picture of influenza outbreaks is possible as well. In any case, the co-existence of subclinical infections makes monitoring of influenza in pigs a complex subject.

2. Genetic diversity of SIV and evolutionary patterns of swine influenza

For many years, it has been assumed that influenza viruses of swine were relatively less diverse than those of other species, particularly birds and human, and that swine viruses were relatively stable (Noble *et al.*, 1993; de Jong *et al.*, 2007). Results of the present thesis show that 11/14 isolates obtained in different Spanish farms resulted in a different phylogenetic grouping depending on the viral gene analysed. Fortunately, indication of new avian genes in porcine influenza viruses has not been found and, by now, reassortment events seem to be restricted to an exchange between swine and eventually human influenza genes. The most logical explanation for this observation is that reassortment of genes is easy and more frequent than previously thought. A very recent paper presented evidences in that direction (Lycett *et al.*, 2012).

In addition, when 22 SIV isolates retrieved from the same group of animals at different ages (in a longitudinal follow-up) were examined, it was shown that two variants of the same virus were co-circulating for about six months in the same batch of pigs. Each variant was isolated at different time points and presented common changes in 6/8 genes, indicating that genetic drift was dependent on each variant. Also, 3/22 strains presented evidences of reassortment in the M gene at 3 weeks of age and in the NP gene at seven weeks of age. Moreover, high throughput sequencing probably revealed the presence of two different SIV strains co-infecting a single animal, a perfect scenario for the generation of new SIV reassortants. Taken together, these results indicate that the frequency of reassortment events in SIV strains circulating in Spanish farms is very high. This observation leads to think that monitoring of potentially emergent SIV variants will be difficult since at some extent, most of swine isolates are reassortants in

one gene or the other. From a practical point of view, this means that surveillance should be exhaustive in terms of both sampling and sequencing of viral isolates. Moreover, the potential for co-infections with different influenza viruses within a farm also seem very high and this fact supports also the notion of the difficulty of carrying out a rationale and cost/effective surveillance in pigs.

3. Antigenic characterization of influenza A viruses isolated in swine

The H1N1 isolates from the present thesis were genetically diverse but the question about how that antigenic diversity will be reflected or not in an antigenic diversity remained open. The use of monospecific antisera showed that cross-reactivity between different H1N1 of swine was limited. Antigenic heterogeneity in avH1N1 has been also reported in the Netherlands (de Jong *et al.,* 2001) where different variants of avian-like H1N1 viruses were obtained from six different farms. Those viral variants were characteristic of the farm from where they were isolated, a fact that suggested that those H1N1 viruses circulated for a long time within the farm and did not spread to other farms in what could be considered an isolated evolution. A similar hypothesis could be suggested for the results obtained in the present study. In addition, a H1N1 virus reacted exclusively with anti-H1N2 sera indicating that this was a potential reassortant. This fact is an evidence that the mere determination of the subtype of an isolate cannot be used as an accurate predictor of the antigenic reactivity, not even in a relative homogoneus group such as H1N2.

Besides the above mentioned considerations, the high antigenic diversity indicates that for a good use of HI as an epidemiological or diagnostic tool, there is a need for a

update of the panel of viruses representing the most common variants circulating in a given region is given.

In contrast to what happened with H1N1 viruses, the results obtained with H1N2 or H3N2 isolates suggested that isolates of those subtypes are more closely related antigenically than H1N1 are, at least with respect to HA. The reasons lying behind the different patterns of genetic and antigenic diversity of H1N1 and H1N2 or H3N2 viruses are unknown and, in our opinion, deserve to be studied since most probably reflect a critical difference in the epidemiology of different influenza subtypes in pigs.

4. Prospects for future research

The data obtained in the present thesis suggest that SIV may result in either classical outbreaks or subclinical infection. Loeffen and co-workers (2009) indicated that SIV can infect pig farms endemically, but respiratory problems are not always present. In addition, the precise impact of SIV in the porcine respiratory complex is not well evaluated. From that point of view, one interesting research line that could be developed in the future would be the study of the factors leading to the development of clinical (classical outbreaks) or subclinical infections (virulence of different isolates? role of pre-existing homologous or heterologous immunity? role of colostral antibodies? etc…) and to the evaluation of the interactions between SIV and other respiratory pathogens in the context of the porcine respiratory disease complex.

Also, there is a lack of information about the endemic states of SIV in pig herds. How is the virus maintained in the farm? By means of a slow transmission rate, probably controlled by the partial immunity in the population and the presence of new susceptible animals (de Jong *et al.,* 2007, Romagosa *et al.,* 2012b) Or by the survival (weeks or months) in water (Stallknecht et al., 1999), in porous surfaces (Bean *et al.,* 1982; Boone *et al.,* 2005; Tiwari *et al.,* 2006) and in aerosols with low relative humidity and low temperature (Lowen *et al.,* 2007)? The evidence of infection in young seropositive animals (Simon-Grifé *et al.,* 2012) indicates that sow-to-piglet transmission probably occur in spite of colostral antibody transfer. This is very relevant for the understanding of the maintenance of SIV in endemic populations.

Finally, most of the studies based in the vaccination efficiency have been carried out under experimental conditions (Van Reeth *et al,* 2003; Kyriakis *et al.,* 2010) and there is a lack of information on the economic implication of the use of vaccines under field conditions. So, it would be interesting to study the efficiency of different vaccination methods together with different biosecurity measures for the control of influenza A viruses in pigs from the point of view of infection dynamics, production and economic impact.

Conclusions

1. Whole genome sequencing and phylogenetic analysis of currently circulating swine influenza virus of Spain showed that in 11/14 cases clustering of the isolates were different depending on the gene segment. These results suggest that reassortment events in Spanish swine influenza viruses are extremely common, probably more than previously expected.

2. Molecular monitoring of a H1N1 virus circulating endemically in a farrow-to-finish farm revealed that different drift variants or reassortants of the same virus can persist for months circulating within the herd. Moreover, high-throughput sequencing of the matrix gene indicated that a single pig can be co-infected by two different variants of a H1N1 virus. This is the perfect scenario for the generation of SIV reassortants and, consequently, the emergence of new SIV strains.

3. The evidence of reassortment events occurring between human and swine influenza viruses in isolates retrieved in respiratory outbreaks together with the short period of time needed for the appearance of variants or reassortants in endemic pig farms indicate that monitoring of influenza in pigs by a mere random sampling or sampling of clinical cases is of very limited usefulness for understanding the complex epidemiology of swine influenza.

4. The antigenic analysis of the examined isolates shows that H1N1 viruses currently circulating in Spanish pig farms are highly diverse and share limited cross-reactivity. Therefore, the use of the haemagglutination inhibition assay as an epidemiological or diagnostic tool requires the use of a constantly update panel of viruses representing at least the most common viral variants.

5. In contrast to H1N1, H1N2 and H3N2 viruses circulating in Spanish swine seem to be more homologous regarding their cross-reactivity in the haemagglutination inhibition assay. The causes behind this different behavior depending on the subtype are unknown and probably reflect a different epidemiology.

References (Introduction and General discussion)

Abed Y, Pizzorno A, Bouhy X, Boivin G. 2011. Role of permissive neuraminidase mutations in influenza A/Brisbane/59/2007-like (H1N1) viruses. PLoS Pathog, 7(12):e1002431.

Abusugra IA, Linné T, Klingeborn B. 1987. The provenance of the Swedish swine influenza H1N1 virus of 1983. Zentralbl Veterinarmed B, 34: 566-572

Adiego Sancho B, Omenaca Teres M, Martinez Cuenca S, Rodrigo Val P, Sanchez Villanueva P, Casas I, Pozo F, Perez Brena P. 2009. Human case of swine influenza A (H1N1), Aragon, Spain, November 2008. Euro Surveill, 14(7). pii: 19120.

Akey BL. 2003. Low-Pathogenicity H7N2 Avian Influenza Outbreak in Virginia During 2002. Avian Diseases, 47: 1099-1103.

Ali A, Khatri M, Wang L, Saif YM, Lee CW. 2012. Identification of swine H1N2/pandemic H1N1 reassortant influenza virus in pigs, United States. Vet Microbiol, 158: 60-68.

Alvarez AC, Brunck ME, Boyd V, Lai R, Virtue E, Chen W, Bletchly C, Heine HG, Barnard R. 2008. A broad spectrum, one-step reverse-transcription PCR amplification of the neuraminidase gene from multiple subtypes of influenza A virus. Virol J, 5:77.

Anthony SJ, St Leger JA, Pugliares K, Ip HS, Chan JM, Carpenter ZW, Navarrete-Macias I, Sanchez-Leon M, Saliki JT, Pedersen J, Karesh W, Daszak P,Rabadan R, Rowles T, Lipkin WI. 2012. Emergence of fatal avian influenza in new England harbor seals. MBio, 3(4). pii: e00166-12. doi: 10.1128/mBio.00166-12.

Aymard M, Brigaud M, Chastel MC, Fontaine M, Tillon JP, Vannier P. 1980. Comparison of influenza antibody serologic immunity in man and in pig. Comp Immunol Microbiol Infect Dis, 3: 111–119.

Bálint A, Metreveli G, Widén F, Zohari S, Berg M, Isaksson M, Renström LH, Wallgren P, Belák S, Segall T, Kiss I. 2009. The first Swedish H1N2 swine influenza virus isolate represents an uncommon reassortant. Virol J, 6:180.

Barbé F, Labarque G, Pensaert M, Van Reeth K. 2009. Performance of a commercial Swine influenza virus H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with European influenza viruses. J Vet Diagn Invest, 21: 88-96.

Barry JM. 2005. The Great Influenza. The story of the deadliest pandemic. Ed. Penguin Books, USA. ISBN-10: 0143034480.

Bean B, Moore BM, Sterner B, Peterson LR, Gerding DN, Balfour Jr HH. 1982. Survival of influenza viruses on environmental surfaces. J Infect Dis, 146: 47–51

Bikour MH, Cornaglia E, Weber JM, Elazhary Y. 1994. Antigenic characterization of an H3N2 swine influenza virus isolated from pigs with proliferative and necrotizing pneumonia in Quebec. Can J Vet Res, 58: 287-290.

Bikour MH, Frost EH, Deslandes S, Talbot B, Weber JM, Elazhary Y. 1995. Recent H3N2 swine influenza virus with haemagglutinin and nucleoprotein genes similar to 1975 human strains. J Gen Virol, 76: 697-703.

Bikour MH, Cornaglia E, Elazhary Y. 1996. Evaluation of a protective immunity induced by an inactivated influenza H3N2 vaccine after an intratracheal challenge of pigs. Can J Vet Res, 60: 312-314.

Blakemore F and Gledhill AW. 1941. Swine influenza in the British Isles. Proc R Soc Med, 34: 611.

Boone SA, Gerba CP. 2005. The occurrence of influenza A virus on household and day care center fomites. J Infect, 51: 103–109.

Böttcher J, Gangl A, Alex M, Niemeyer H. 2007. Endemic circulation of swine influenza virus (SIV) in the absence of disease. In: Markowska-Daniel, I. (ed.), 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 255.

Boulanger A, Ramirez OJ, Moscardi A. 2004. Serological evidence of swine influenza virus infection on Venezuela pigs farms. 18th International Pig Veterinary Society Congress; Hamburg, Germany.

Braucher DR, Henningson JN, Loving CL, Vincent AL, Kim E, Steitz J, Gambotto AA, Kehrli ME Jr. 2012. Intranasal vaccination with replication defective adenovirus-5 encoding influenza hemagglutinin elicits protective immunity to homologous challenge and partial protection to heterologous challenge in pigs. Clin Vaccine Immunol. Clin Vaccine Immunol., doi: 10.1128/CVI.00315-12 [Epub ahead of print] 29 Aug.

Brown IH, Done SH, Spencer YI, Cooley WA, Harris PA, Alexander DJ. 1993a. Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet Rec, 132: 598-602.

Brown IH, Manvell RJ, Alexander DJ, Chakraverty P, Hinshaw VS, Webster RG. 1993b. Swine influenza outbreaks in England due to a new H1N1 virus. Vet Rec, 132: 461-462.

Brown IH, Alexander DJ, Chakraverty P, Harris PA, Manvell RJ. 1994. Isolation of an influenza A virus of unusual subtype (H1N7) from pigs in England, and the subsequent experimental transmission from pig to pig. Vet Microbiol, 39: 125-34.

Brown IH, Chakraverty P, Harris PA, Alexander DJ. 1995. Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. Vet Rec, 136: 328-329.

Brown IH, Harris PA, McCauley JW, Alexander J. 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J Gen Virol, 79: 2947–2955.

Bryant NA, Rash AS, Russell CA, Ross J, Cooke A, Bowman S, MacRae S, Lewis, NS, Paillot R, Zanoni R, Meier H, Griffiths LA, Daly JM, Tiwari A, Chambers TM, Newton JR, Elton DM. 2009. Antigenic and genetic variations in European and

North American equine influenza virus strains (H3N8) isolated from 2006 to 2007. Vet Microbiol, 138: 41-52.

Callan RJ, Early G, Kida H, Hinshaw VS. 1995. The appearance of H3 influenza viruses in seals. J Gen Virol, 76: 199-203.

Cappuccio JA, Pena L, Dibárbora M, Rimondi A, Piñeyro P, Insarralde L, Quiroga MA, Machuca M, Craig MI, Olivera V, Chockalingam A, Perfumo CJ, Perez DR, Pereda A. 2011. Outbreak of swine influenza in Argentina reveals a non-contemporary human H3N2 virus highly transmissible among pigs. J Gen Virol, 92: 2871-2878.

Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG. 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology, 193: 503–506.

Castrucci MR, Campitelli L, Ruggieri A, Barigazzi G, Sidoli L, Daniels R, Oxford JS, Donatelli I. 1994. Antigenic and sequence analysis of H3 influenza virus haemagglutinins from pigs in Italy. J Gen Virol, 75: 371-379.

Chiapponi C, Moreno A, Barbieri I, Merenda M, Foni E. 2012. Multiplex RT-PCR assay for differentiating European swine influenza virus subtypes H1N1, H1N2 and H3N2. J Virol Methods, 184: 117-20.

Choi YK, Goyal SM, Farnham W, Joo HS. 2002. Phylogenetic analysis of H1N2 isolates of influenza A virus from pigs in the United States. Virus Res, 87: 173-179.

Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, Chaisingh A, Auewarakul P, Hanh NT, Ma SK, Hui PY, Guan Y, Peiris JS, Webster RG. 2004. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol, 79: 10821–10825.

Choi YK, Pascua PN, Song MS. 2012. Swine Influenza Viruses: An Asian Perspective. Curr Top Microbiol Immunol, [Epub ahead of print] 21 Jan.

Chutinimitkul S, Thippamom N, Damrongwatanapokin S, Payungporn S, Thanawongnuwech R, Amonsin A, Boonsuk P, Sreta D, Bunpong N, Tantilertcharoen R, Chamnanpood P, Parchariyanon S, Theamboonlers A, Poovorawan Y. 2008. Genetic characterization of H1N1, H1N2 and H3N2 swine influenza virus in Thailand. Arch Virol, 153: 1049-1056.

Cox NJ, Subbarao K. 2000. Global epidemiology of influenza: past and present. Annu Rev Med, 51: 407-421.

Damiani AM, Kalthoff D, Beer M, Müller E, Osterrieder N. 2012. Serological Survey in Dogs and Cats for Influenza A (H1N1) pdm09 in Germany. Zoonoses Public Health. doi: 10.1111/j.1863-2378.2012.01541.x. [Epub ahead of print] 16 Aug.

Deblanc C, Gorin S, Quéguiner S, Gautier-Bouchardon AV, Ferré S, Amenna N, Cariolet R, Simon G. 2012. Pre-infection of pigs with Mycoplasma hyopneumoniae modifies outcomes of infection with European swine **influenza** virus of H1N1, but not H1N2, subtype.. Vet Microbiol, 157: 96-105

Deng YM, Iannello P, Smith I, Watson J, Barr IG, Daniels P, Komadina N, Harrower B, Wong FY. 2012. Transmission of influenza A(H1N1) 2009 pandemic viruses in Australian swine. Influenza Other Respi Viruses, 6(3):e42-7. doi: 10.1111/j.1750-2659.2012.00337.x. [Epub ahead of print] 12 Feb.

Donis RO, Bean WJ, KawaokaY, Webster RG. 1989. Distinct lineages of influenza virus. H4 hemagglutinin genes in different regions of the world. Virology, 169: 408–417.

Drake, J; 1993. Rates of spontaneous mutation among RNA viruses. Proc Natl Acad Sci USA, 90: 4171–4175

Ducatez MF, Hause B, Stigger-Rosser E, Darnell D, Corzo C, Juleen K, Simonson R, Brockwell-Staats C, Rubrum A, Wang D, Webb A, Crumpton JC, Lowe J,Gramer M, Webby RJ. 2011. Multiple reassortment between pandemic (H1N1) 2009 and endemic influenza viruses in pigs, United States. Emerg Infect Dis, 17:1624-1629.

Ellström P, Latorre-Margalef N, Griekspoor P, Waldenström J, Olofsson J, Wahlgren J, Olsen B. 2008. Sampling for low-pathogenic avian influenza A virus in wild mallard ducks: oropharyngeal versus cloacal swabbing. Vaccine, 26: 4414–4416

Foni E, Chiapponi C, Fratta E, Garbarino C, Barigazzi G, Merenda M. 2003. Detection of swine influenza virus by RT-PCR and standard methods. 4th International Symposium on Emerging and Re-emerging Pig Diseases – Rome June 29th– July 2nd.

Forbes NE, Ping J, Dankar SK, Jia JJ, Selman M, Keleta L, Zhou Y, Brown EG. 2012. Multifunctional adaptive NS1 mutations are selected upon human influenza virus evolution in the mouse. PLoS One. 7(2):e31839.

Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA, 101: 1356-1361.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol, 79: 2814-2822.

Franck N, Queguiner S, Gorin S, Eveno E, Fablet C, Madec F, Kuntz-Simon G, 2007. Molecular epidemiology of swine influenza virus in France: identifications of novel H1N1 reassortants. 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, 24–27 June, p. 250.

Gall A, Hoffmann B, Harder T, Grund C, Beer M. 2008. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses. J Clin Microbiol, 46: 2561-2567.

Garten W, Klenk HD. 2008. Cleavage activation of the influenza virus hemagglutinin and its role in pathogenesis. In: Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza. Monogr Virol Basel Karger, 27: 156-167.

Gauger PC, Vincent AL, Loving CL, Lager KM, Janke BH, Kehrli ME, Roth JA. 2011. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. Vaccine, 29: 2712-2719.

Gibbs EP, Anderson TC. 2010. Equine and canine influenza: a review of current events. Anim Health Res Rev, 11: 43-51.

Gourreau JM, Kaiser C, Valette M, Douglas AR, Labie J, Aymard M. 1994. Isolation of two H1N2 influenza viruses from swine in France. Arch Virol, 135: 365–382.

Han JY, Park SJ, Kim HK, Rho S, Nguyen GV, Song D, Kang BK, Moon HJ, Yeom MJ, Park BK. 2012. Identification of reassortant pandemic H1N1 influenza virus in Korean pigs. J Microbiol Biotechnol, 5: 699-707.

Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science, 336: 1534-1541.

Hinshaw VS, Bean WJ Jr, Webster RG, Easterday BC. 1978. The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. Virology, 84: 51-62.

Hinshaw VS, Bean WJ, Geraci J, Fiorelli P, Early G, Webster RG. 1986. Characterization of two influenza A viruses from a pilot whale. J Virol, 58: 655-656.

Hiromoto Y, Parchariyanon S, Ketusing N, Netrabukkana P, Hayashi T, Kobayashi T, Takemae N, Saito T. 2012. Isolation of the Pandemic (H1N1) 2009 virus and its reassortant with an H3N2 swine influenza virus from healthy weaning pigs in Thailand in 2011. Virus Res, [Epub ahead of print] 10 Aug.

Hjulsager CK, Bragstad K., Botner A., Larsen LE. 2006. Isolation and genetic characterization of new reassortant H1N2 swine influenza virus from pigs in Denmark. 7th International Congress of Veterinary Society, Lisboa, Portugal, p. 133.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol, 146: 2275‑2289

Hofshagen M, Gjerset B, Er C, Tarpai A, Brun E, Dannevig B, Bruheim T, Fostad IG, Iversen B, Hungnes O, Lium B. 2009. Pandemic influenza A(H1N1)v: human to pig transmission in Norway? Euro Surveill, 14(45). pii: 19406.

Howden KJ, Brockhoff EJ, Caya FD, McLeod LJ, Lavoie M, Ing JD, Bystrom JM, Alexandersen S, Pasick JM, Berhane Y, Morrison ME, Keenliside JM, Laurendeau S, Rohonczy EB. 2009. An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can Vet J, 50: 1153–1161.

Iglesias I, Martínez M, Muñoz MJ, de la Torre A, Sánchez-Vizcaíno JM. 2009. First case of highly pathogenic avian influenza in poultry in Spain. Transbound Emerg Dis, 57: 282-285.

Imai M, Kawaoka Y. 2012. The role of receptor binding specificity in interspecies transmission of influenza viruses. Curr Opin Virol, 2: 160-167.

Isoda N, Sakoda Y, Kishida N, Bai GR, Matsuda K, Umemura T, Kida H. 2006. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. Arch Virol, 151: 1267– 1279.

Ito T, Couceiro JNSS, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol, 72: 7367-7373.

Johnson NPAS, Mueller J. 2002. Updating the accounts: global mortality of the 1918–1919 "Spanish" influenza pandemic. Bull Hist Med, 76: 105-115

de Jong JC, Heinen PP, Loeffen WL, van Nieuwstadt AP, Claas EC, Bestebroer TM, Bijlsma K, Verweij C, Osterhaus AD, Rimmelzwaan GF, Fouchier RA, Kimman TG. 2001. Antigenic and molecular heterogeneity in recent swine influenza A (H1N1) virus isolates with possible implications for vaccination policy. Vaccine, 19: 4452-4464.

de Jong JC, Smith DJ, Lapedes AS, Donatelli I, Campitelli L, Barigazzi G, Van Reeth K, Jones TC, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2007. Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. J Virol, 81: 4315-4322.

de Jong MC, Stegeman A, van der Goot J, Koch G. 2009. Intra- and interspecies transmission of H7N7 highly pathogenic avian influenza virus during the avian influenza epidemic in The Netherlands in 2003. Rev Sci Tech, 28: 333-340.

Karasin AI, Schutten MM, Cooper LA, Smith CB, Subbarao K, Anderson GA, Carman S, Olsen CW. 2000a. Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. Virus Res, 68: 71–85.

Karasin AI, Anderson GA, Olsen CW, 2000b. Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana. J Clin Microbiol, 38: 2453–2456.

Karasin AI, Brown IH, Carman S, Olsen CW. 2000c. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol, 74: 9322-9377.

Karasin AI, West K, Carman S, Olsen CW. 2004. Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol, 42: 4349-4354

Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka, Webster RG. 1994. Potential for transmission of avian influenza viruses to pigs. J Gen Virol, 75: 2183‑2188

Kitikoon P, Vincent AL, Gauger PC, Schlink SN, Bayles DO, Gramer MR, Darnell D, Webby RJ, Lager KM, Swenson SL, Klimov A. 2012. Pathogenicity and

transmission in pigs of the novel A(H3N2)v influenza virus isolated from humans and characterization of swine H3N2 viruses isolated in 2010-2011. J Virol, 86: 6804-6814.

Kilbourne ED. 2006. Influenza pandemics of the 20th century. Emerg Infect Dis, 12: 9-14.

Kim SH, Moon OK, Lee KK, Song YK, Yeo CI, Bae CW, Yoon H, Lee OS, Lee JH, Park CK. 2011. Outbreak of pandemic influenza (H1N1) 2009 in pigs in Korea. Vet Rec, 169: 155.

Koehler AV, Pearce JM, Flint PL, Franson JC, Ip HS. 2008. Genetic evidence of intercontinental movement of avian influenza in a migratory bird: the northern pintail (Anas acuta). Mol Ecol, 17: 4754-4762.

Koen, J.S., 1919. A practical method for field diagnosis of swine diseases. Am J Vet Med, 14: 468–470.

Kowalczyk A, Markowska-Daniel I. 2010. Phylogenetic analysis of swine influenza viruses isolated in Poland. Pol J Vet Sci, 13: 37-44.

Kuntz-Simon G, Madec F. 2009. Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential.Zoonoses Public Health, 56: 310-325.

Kumar SR, Deflube L, Biswas M, Shobana R, Elankumaran S. 2011. Genetic characterization of swine influenza viruses (H3N2) isolated from Minnesota in 2006-2007. Virus Genes, 43: 161-176.

Kwon TY, Lee SS, Kim CY, Shin JY, Sunwoo SY, Lyoo YS. 2011. Genetic characterization of H7N2 influenza virus isolated from pigs. Vet Microbiol, 153: 393-397.

Kyriakis CS, Gramer MR, Barbé F, Van Doorsselaere J, Van Reeth K. 2010. Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. Vet Microbiol, 144: 67-74.

Kyriakis CS, Brown IH, Foni E, Kuntz-Simon G, Maldonado J, Madec F, Essen SC, Chiapponi C, Van Reeth K. 2011. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. Zoonoses Public Health, 58: 93-101.

Lamont, HG. 1938. The problemns of the practitioner inconnection with the differential diagnosis and traetment ofdiseases of young pigs. Vet Rec, 50: 1377.

Larsen DL, Karasin A, Olsen CW. 2001. Immunization of pigs against influenza virus infection by DNA vaccine priming followed by killed-virus vaccine boosting. Vaccine, 19: 2842-2853.

Larsen DL, Olsen CW. 2002. Effects of DNA dose, route of vaccination, and coadministration of porcine interleukin-6 DNA on results of DNA vaccination against influenza virus infection in pigs. Am J Vet Res, 63: 653-659.

Lee JH, Gramer MR, Joo HS. 2007. Efficacy of swine influenza A virus vaccines against an H3N2 virus variant. Can J Vet Res, 71: 207–212.

Lee CS, Kang BK, Kim HK, Park SJ, Park BK, Jung K, Song DS. 2008. Phylogenetic analysis of swine influenza viruses recently isolated in Korea. Virus Genes, 37: 168-176

Lee C, Song D, Kang B, Kang D, Yoo J, Jung K, Na G, Lee K, Park B, Oh J. 2009. A serological survey of avian origin canine H3N2 influenza virus in dogs in Korea. Vet Microbiol, 137: 359-362.

Lekcharoensuk P, Lager KM, Vemulapalli R, Woodruff M, Vincent AL, Richt JA. 2006. Novel swine influenza virus subtype H3N1, United States. Emerg Infect Dis, 12: 787-794.

Liu JH, Okazaki K, Bai GR, Shi WM, Mweene A, Kida H. 2004. Interregional transmission of the internal protein genes of H2 influenza virus in migratory ducks from North America to Eurasia. Virus Genes, 29: 81-86.

Liu Q, Ma J, Liu H, Qi W, Anderson J, Henry SC, Hesse RA, Richt JA, Ma W. 2012. Emergence of novel reassortant H3N2 swine influenza viruses with the 2009 pandemic H1N1 genes in the United States. Arch Virol, 157: 555-562.

Loeffen WL, Hunneman WA, Quak J, Verheijden JH, Stegeman JA. 2009. Population dynamics of swine influenza virus in farrow-to-finish and specialised finishing herds in the Netherlands. Vet Microbiol, 137: 45-50.

Lorusso A, Vincent AL, Harland ML, Alt D, Bayles DO, Swenson SL, Gramer MR, Russell CA, Smith DJ, Lager KM, Lewis NS. 2011. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. J Gen Virol, 92: 919-930.

Louria DB, Blumenfeld HL, Ellis JT, Kilbourne ED, Rogers DE. 1959. Studies on influenza in the pandemic of 1957-1958. II. Pulmonary complications of influenza. J Clin Invest, 38: 213-265.

Lowen AC, Mubareka S, Steel J, Palese P. 2007. Influenza virus transmission is dependent on relative humidity and temperature. PLoS Pathog, 3: 1470-1476.

Lycett SJ, Baillie G, Coulter E, Bhatt S, Kellam P, McCauley JW, Wood JL, Brown IH, Pybus OG, Leigh Brown AJ, Cosi Consortium CS. 2012. Estimating reassortment rates in co-circulating Eurasian swine influenza viruses. J Gen Virol, [Epub ahead of print] 12 Sep.

Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, Gauger PC, Patnayak DP, Webby RJ, Richt JA. 2007. Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci USA, 104: 20949-20954.

Ma W, Brenner D, Wang Z, Dauber B, Ehrhardt C, Högner K, Herold S, Ludwig S, Wolff T, Yu K, Richt JA, Planz O, Pleschka S. 2010. The NS segment of an H5N1

highly pathogenic avian influenza virus (HPAIV) is sufficient to alter replication efficiency, cell tropism, and host range of an H7N1 HPAIV. J Virol, 84: 2122-2133.

MacCauley VW, Hongo S, Kavenin NV, Kochs G, Lamb RA, Mastrosovich MN, Pérez DR, Palese P, Presti RM, Rimstad E, Smith GSD. 2011. Family Orthomyxoviridae. In: King AMQ, Lefkowitz E, Adams MS, Canstens EB (Ed) Virus Taxonomy. 9th Report of the International Committee on taxonomy of viruses. Academic Press, London, 749-762.

Macklin MD, McCabe D, McGregor MW, Neumann V, Meyer T, Callan R, Hinshaw VS, Swain WF. 1998. Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. J Virol, 72: 1491-1496.

Madec F, Gourreau JM, Kaiser C, Le Dantec J, Vannier P, Aymard M. 1985. Étude de la persistance d'une activité du virus grippal H1N1 (swine) dans les élevages porcins en dehors des phases épidémiques. Comp Immunol Microbiol Infect Dis, 8: 247-258.

Maldonado J, Van Reeth K, Riera P, Sitjà M, Saubi N, Espuña E, Artigas C. 2006. Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J, 172: 377-381.

Maldonado J. 2007. Experiencias prácticas sobre el uso y las limitaciones de las analíticas de diagnóstico serológico para la Influenza porcina (IP). Proceedings of the XII Symposium of AVEDILA: 1-2 October, Bilbao.

Mancini DAP, Cunha EMS, Mendonça RMZ, Dias ALF, Castro AF, Pinto JR. 2006. Evidence of swine respiratory infection by influenza viruses in Brazil. Virus Reviews Research, 11: 39-43.

Mänz B, Brunotte L, Reuther P, Schwemmle M. 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. Nat Commun, 3: 802.

Masurel N, de Boer GF, Anker WJ, Huffels AD. 1983. Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. Comp Immunol Microbiol Infect Dis, 6: 141-149.

Miwa Y, Piao FZ, Goto H, Noro S. 1987. Isolation of human (H3N2) influenza virus and prevalence of the virus-antibody in swine. Nihon Juigaku Zasshi, 49: 1168-1170.

Moreno A, Barbieri I, Sozzi E, Luppi A, Lelli D, Lombardi G, Zanoni MG, Cordioli P. 2009. Novel swine influenza virus subtype H3N1 in Italy. Vet Microbiol, 138: 361-367.

Moreno A, Di Trani L, Alborali L, Vaccari G, Barbieri I, Falcone E, Sozzi E, Puzelli S, Ferri G, Cordioli P. 2010. First Pandemic H1N1 Outbreak from a Pig Farm in Italy. Open Virol J, 4: 52-56.

Moreno A, Chiapponi C, Boniotti MB, Sozzi E, Foni E, Barbieri I, Zanoni MG, Faccini S, Lelli D, Cordioli P. 2012. Genomic characterization of H1N2 swine influenza viruses in Italy. Vet Microbiol, 156: 265-276.

Morens DM, Taubenberger JK, Folkers GK, Fauci AS. 2010. Pandemic influenza's 500th anniversary. Clin Infect Dis, 51: 1442-1444.

Morens DM, Taubenberger JK, Folkers GK, Fauci AS. 2010. Pandemic influenza's 500th anniversary. Clin Infect Dis, 51: 1442-1444.

Muñoz-Sanz A. 2006. La gripe de Cristóbal Colón. Hipótesis sobre una catástrofe ecológica. Enferm Infecc Microbiol Clin, 24: 326-34.

Murphy FA, Gibbs JEP, Horzinek MC, Studdert MJ. 1999. Veterinary Virology, Academic press, Third Edition; ISBN: 0125113404 / 0-12-511340-4.

Nardelli L, Pascucci S, Gualandi GL, Loda P. 1978. Outbreaks of classical swine influenza in Italy in 1976. Zentralbl Veterinarmed B, 25: 853-857.

Nelson DL, Cox MM. 2010. Lehninger. Principles of Biochemistry. 5th Edition. WH Freeman. New York.

Nelson MI, Vincent AL, Kitikoon P, Holmes EC, Gramer MR. 2012. Evolution of Novel Reassortant A/H3N2 Influenza Viruses in North American Swine and Humans, 2009-2011. J Virol, 86: 8872-8872.

Neumeier E, Meier-Ewert H, Cox NJ. 1994. Genetic relatedness between influenza A (H1N1) viruses isolated from humans and pigs. J Gen Virol, 75: 2103-2107.

Neumann G and Kawaoka Y. 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis, 12: 881–886.

Ninomiya A, Takada A, Okazaki K, Shortridge KF, Kida H. 2002. Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. Vet Microbiol, 88: 107-114.

Noble S, McGregor MS, Wentworth DE, Hinshaw VS. 1993. Antigenic and genetic conservation of the haemagglutinin in H1N1 swine influenza viruses. J Gen Virol, 74: 1197-1200.

Nobusawa E, Sato K. 2006. Comparison of the mutation rates of human influenza A and B viruses. J Virol, 80: 3675-3678.

O'Donnell CD, Vogel L, Wright A, Das SR, Wrammert J, Li GM, McCausland M, Zheng NY, Yewdell JW, Ahmed R, Wilson PC, Subbarao K. 2012. Antibody pressure by a human monoclonal antibody targeting the 2009 pandemic H1N1 virus hemagglutinin drives the emergence of a virus with increased virulence in mice. MBio. 3(3). pii: e00120-12. doi: 10.1128/mBio.00120-12.

Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, Fouchier RA. 2006. Global patterns of influenza a virus in wild birds. Science, 312: 384-388.

Olsen CW. 2002. The emergence of novel swine influenza viruses in North America. Virus Research, 85: 199–210

Ottis, Bachmann PA, Webster RG, Kaplan MM. 1982. Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population. ArchVirol, 73: 103–108.

Park SJ, Moon HJ, Kang BK, Hong M, Na W, Kim JK, Poo H, Park BK, Song DS. 2012. Complete Genome Sequence of an Avian-Origin H3N2 Canine Influenza Virus Isolated from Dogs inSouth Korea. J Virol, 86: 9548-9549.

Pascua PN, Song MS, Lee JH, Choi HW, Han JH, Kim JH, Yoo GJ, Kim CJ, Choi YK. 2008. Seroprevalence and genetic evolutions of swine influenza viruses under vaccination pressure in Korean swine herds. Virus Res, 138: 43-49.

Patterson DK, Pyle GF. 1991. The geography and mortality of the 1918 influenza pandemic. Bull Hist Med, 65: 4-21

Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann, PA. 1981. Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bull. World Health Organ, 59: 75–78.

Pereda A, Cappuccio J, Quiroga MA, Baumeister E, Insarralde L, Ibar M, Sanguinetti R, Cannilla ML, Franzese D, Escobar Cabrera OE, Craig MI, Rimondi A, Machuca M, Debenedetti RT, Zenobi C, Barral L, Balzano R, Capalbo S, Risso A, Perfumo CJ. 2010. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis, 16: 304-307

Platt R, Vincent AL, Gauger PC, Loving CL, Zanella EL, Lager KM, Kehrli ME Jr, Kimura K, Roth JA. 2011. Comparison of humoral and cellular immune responses to inactivated swine influenza virus vaccine in weaned pigs. Vet Immunol Immunopathol, 142: 252-257.

Rajão DS, Alves F, Del Puerto HL, Braz GF, Oliveira FG, Ciacci-Zanella JR, Schaefer R, Dos Reis JK, Guedes RM, Lobato ZI, Leite RC. 2012. Serological evidence of swine influenza in Brazil. Influenza Other Respi Viruses, doi: 10.1111/j.1750-2659.2012.00366.x. [Epub ahead of print] 6 Apr.

Rapp-Gabrielson VJ, Gergen LR, Eddy BA, Junker DE, Weston CQ, Fleck R, Erickson G, Nemechek EC, Wasmoen TL. 2003. Protection of a swine influenza virus vaccine against an H3N2 virus antigenically and genetically distinct from the vaccine virus. In: Proceedings of the 4th International Symposium on Emerging and Re-emerging Pig Diseases, pp. 266-267.

Rivailler P, Perry IA, Jang Y, Davis CT, Chen LM, Dubovi EJ, Donis RO. 2010. Evolution of canine and equine influenza (H3N8) viruses cocirculating between 2005 an d 2008. Virology, 408: 71-79.

Romagosa A, Gramer M, Joo HS, Torremorell M. 2012a. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza Other Respi Viruses, 6: 110-118.

Romagosa A, Allerson M, Gramer M, Joo HS, Deen J, Detmer S, Torremorell M. 2012b. Vaccination of influenza a virus decreases transmission rates in pigs. Vet Res, 42:120.

Rhom C, Zhou N, Suss J, Mackenzie J, Webster RG. 1996. Characterization of a novel influenza hemagglutinin H15: criteria for determination of influenza A subtypes. Virology, 217: 508–516

Roberts, DH, Cartwright SF, Wibberley G. 1987. Outbreaks of classical swine influenza in pigs in England in 1986. Vet Rec, 121: 53–55.

Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin Virology, 127: 361‑373

Rosenbergova K, Lany P, Zajacova Z, Pospisil, Z. 2007. Characterization of swine influenza viruses in the Czech Republic. In: Markowska-Daniel, I. (ed.), 5th International Symposium on Emerging and Re-emerging Pig Diseases, Krakow, Poland, p. 260.

Saito T, Lim W, Suzuki T, Suzuki Y, Kida H, Nishimura SI, Tashiro M. 2001. Characterization of a human H9N2 influenza virus isolated in Hong Kong. Vaccine, 20: 125-33.

Scholtissek C. 1995. Molecular evolution of influenza viruses. Virus Genes, 11: 209-215.

Schultz U, Fitch WM, Ludwig S, Mandler J, Scholtissek C. 1991. Evolution of pig influenza viruses. Virology, 183: 61-73.

Seyer R, Hrincius ER, Ritzel D, Abt M, Mellmann A, Marjuki H, Kühn J, Wolff T, Ludwig S, Ehrhardt C. 2012. Synergistic adaptive mutations in the hemagglutinin and polymerase acidic protein lead to increased virulence of pandemic 2009 H1N1 influenza A virus in mice. J Infect Dis, 205: 262-71.

Shope RE. 1931. The etiology of swine influenza. Science, 73: 214-215

Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A,Webster RG. 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology, 252: 331–342.

Simon-Grifé M, Martín-Valls GE, Vilar MJ, García-Bocanegra I, Mora M, Martín M, Mateu E, Casal J. 2011. Seroprevalence and risk factors of swine influenza in Spain.Vet Microbiol, 149: 56-63.

Simon-Grifé M, Martín-Valls GE, Vilar MJ, Busquets N, Mora-Salvatierra M, Bestebroer TM, Fouchier RA, Martín M, Mateu E, Casal J. 2012. Swine influenza virus infection dynamics in two pig farms; results of a longitudinal assessment.Vet Res, 43: 24.

Shortridge KF, Stuart-Harris CH. 1982. An influenza epicentre? Lancet, 2: 812–813

Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, Chen H, Webster RG, Peiris JS, Guan Y. 2009a. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci USA, 106: 11709-11712.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009b. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature, 459: 1122-1125.

Song DS, Lee JY, Oh JS, Lyoo KS, Yoon KJ, Park YH, Park BK. 2003. Isolation of H3N2 swine influenza virus in South Korea. J Vet Diagn Invest, 15: 30-34.

Stallknecht DE, Brown JD. 2009. Tenacity of avian influenza viruses. Rev Sci Tech Off Int Epiz, 28: 59–67.

Starick E, Lange E, Grund C, Grosse Beilage E, Döhring S, Maas A, Noé T, Beer M, Harder TC. 2012. Reassortants of pandemic influenza A virus H1N1/2009 and endemic porcine HxN2 viruses emerge in swine populations in Germany. J Gen Virol, 93: 1658-1663.

Su S, Qi WB, Chen JD, Cao N, Zhu WJ, Yuan LG, Wang H, Zhang GH. 2012. Complete Genome Sequence of an Avian-Like H4N8 Swine Influenza Virus Discovered in Southern China. J Virol, 86: 9542.

Suarez DL. 2010. Avian influenza: our current understanding. Animal Health. Res Rev, 11: 18-33.

Subbarao K, Shaw MW. 2000. Molecular aspects of avian influenza (H5N1) viruses isolated from humans. Rev Med Virol, 10: 337-348.

Suzuki Y, Ito T, Suzuki T, Holland RE, Chambers TM, Kiso M, Ishida H, Kawaoka Y. 2000. Sialic acid species as a determinant of the host range of influenza A viruses. J Virol, 74: 11825-11831

Takemae N, Parchariyanon S, Damrongwatanapokin S, Uchida Y, Ruttanapumma R, Watanabe C, Yamaguchi S, Saito T. 2008. Genetic diversity of swine influenza viruses isolated from pigs during 2000 to 2005 in Thailand. Influenza Other Respi Viruses, 2: 181-189.

Tang Y, Wu P, Peng D, Wang X, Wan H, Zhang P, Long J, Zhang W, Li Y, Wang W, Zhang X, Liu X. 2009. Characterization of duck H5N1 influenza viruses with differing pathogenicity in mallard (Anas platyrhynchos) ducks. Avian Pathol, 38: 457-67.

Taubenberger JK, Reid AH, Janczewski TA, Fanning TG. 2001. Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. Philos Trans R Soc Lond B Biol Sci, 356: 1829-1839.

Taubenberger JK, Morens DM. 2006. 1918 Influenza: the mother of all pandemics. Emerg Infect Dis, 12: 15-22

Taubenberger JK, Morens DM. 2009. Pandemic influenza--including a risk assessment of H5N1. Rev Sci Tech, 28: 187-202.

Taubenberger JK, Morens DM. 2010. Influenza: the once and future pandemic. Public Health Rep, 125: 16-26.

Taubenberger JK, Kash JC. 2011. Insights on influenza pathogenesis from the grave.Virus Res, 162: 2-7.

Tiwari A, Patnayak DP, Chander Y, Parsad M, Goyal SM. 2006. Survival of two avian respiratory viruses on porous and nonporous surfaces. Avian Dis, 50: 284-287.

Torremorell M, Juarez A, Chavez E, Yescas J, Doporto JM, Gramer M. 2009. Procedures to eliminate H3N2 swine influenza virus from a pig herd. Vet Rec, 165: 74-77.

Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR,Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci USA, 109: 4269-4274.

Tremblay D, Allard V, Doyon JF, Bellehumeur C, Spearman JG, Harel J, Gagnon CA. 2011. Emergence of a new swine H3N2 and pandemic (H1N1) 2009 influenza A virus reassortant in two Canadian animal populations, mink and swine. J Clin Microbiol, 49: 4386-4390.

Van Reeth K, Labarque G, De Clercq S, Pensaert M. 2001. Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. Vaccine, 19: 4479-4486.

Van Reeth K, Labarque G, Pensaert M. 2006. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol, 19: 373-82.

Van Reeth K, Brown IH, Dürrwald R, Foni E, Labarque G, Lenihan P, Maldonado J, Markowska-Daniel I, Pensaert M, Pospisil Z, Koch G. 2008. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003. Influenza Other Respi Viruses, 2: 99-105.

Van Reeth K, Olsen CW, Brown IH, Easterday BC. 2012. Swine influenza, Diseases of Swine. Oxford: Blackwell Publishing. Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. 10th Edition. Pp xxx-xxx.

Vander Veen RL, Mogler MA, Harris DL, Kamrud KI. 2012. Analysis of the immunogenicity of an RNA particle (RP) swine influenza virus vaccine expressing the H3N2 cluster 4 HA gene at varying RP doses. 2012. Proceedings of the 22nd International Pig Veterinary Society congress (IPVS), Korea. Pp.

Vergara-Alert J, Argilaguet JM, Busquets N, Ballester M, Martín-Valls GE, Rivas R, López-Soria S, Solanes D, Majó N, Segalés J, Veljkovic V, Rodríguez F, Darji A. 2012. Conserved synthetic peptides from the hemagglutinin of influenza viruses induce broad humoral and T-cell responses in a pig model. PLoS One, 7(7):e40524.

Viboud C, Grais RF, Lafont BA, Miller MA, Simonsen L; Multinational Influenza Seasonal Mortality Study Group. 2005. Multinational impact of the 1968 Hong Kong influenza pandemic: evidence for a smoldering pandemic. J Infect Dis, 192: 233-248.

White DO, Fenner FJ. 1994. Orthomyxoviruses. Medical Virology, 4th edition. Academic Press. Chapter 31. Pp 489-499.

Vicente M, Soler A, Pérez MP, Nájera R. 1979. Estudios serológicos de influenza en porcinos de Chile. 4 Bol Of Sanit Pamm, 86.

Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, Riley S, Bahl J, Ma SK, Cheung CL, Perera RA, Chen H, Shortridge KF, Webby RJ, Webster RG, Guan Y, Peiris JS. 2011. Long-term evolution and transmission dynamics of swine influenza A virus.. Nature, 473: 519-522.

Vincent, A. L., K. M. Lager, B. H. Janke, M. R. Gramer, and J. A. Richt. 2008. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. Vet Microbiol, 126: 310-323.

Vincent AL, Ma W, Lager KM, Gramer MR, Richt JA, Janke BH. 2009. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. Virus Genes, 39: 176-185.

Vincent AL, Ma W, Lager KM, Richt JA, Janke BH, Sandbulte MR, Gauger PC, Loving CL, Webby RJ, García-Sastre A. 2012. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease. J Virol, 86: 10597-10605.

Wallensten A, Munster VJ, Elmberg J, Osterhaus AD, Fouchier RA, Olsen B. 2005. Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in Guillemot (Uria aalge). Arch Virol, 150: 1685-1692.

Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology, 84: 268–278

Webster RG, Hinshaw VS, Bean WJ, Van Wyke KL, Geraci JR, St Aubin DJ, Petursson G. 1981. Characterization of an influenza A virus from seals. Virology, 113: 712–724.

Webster RG, Laver WG, Air GM, Schild GC. 1982. Molecular mechanisms of variation in influenza viruses. Nature, 296: 115–121.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev, 56: 152-179.

Webster RG. 1993. Are equine influenza viruses still present in horses? Equine vet J, 25: 537-538

World Health Organization. 1980. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull World Health Organ. 58: 585–591.

World Health Organization. 2005. Avian influenza: assessing the pandemic threat. Geneva.

World Health Organization. 2009. Media centre; Inlfuenza Seasonal. Fact sheet nº11; http://www.who.int/mediacentre/factsheets/fs211/en/

World Health Organization; 2009, http://www.who.int/csr/resources/publications/swineflu/GenomePrimers0090512.pdf

World Organization for Animal Health (OIE). 2008. Swine influenza. Manual for diagnostic tests and vaccines for terrestrial animals. Paris. pp. 1128–1138.

Yoneyama S, Hayashi T, Kojima H, Usami Y, Kubo M, Takemae N, Uchida Y, Saito T. 2010. Occurrence of a pig respiratory disease associated with swine influenza A (H1N2) virus in Tochigi Prefecture, Japan. J Vet Med Sci, 72: 481-488.

Yassine HM, Khatri M, Zhang YJ, Lee CW, Byrum BA, O'Quin J, Smith KA, Saif YM. 2009. Characterization of triple reassortant H1N1 influenza A viruses from swine in Ohio H.M., Veterinary Microbiology, 139: 132–139

Yu H, Hua RH, Zhang Q, Liu TQ, Liu HL, Li GX, Tong GZ. 2008. Genetic evolution of swine influenza A (H3N2) viruses in China from 1970 to 2006. J Clin Microbiol, 46: 1067-1075.

Yu H, Zhang PC, Zhou YJ, Li GX, Pan J, Yan LP, Shi XX, Liu HL, Tong GZ. 2009. Isolation and genetic characterization of avian-like H1N1 and novel ressortant H1N2 influenza viruses from pigs in China. Biochem Biophys Res Commun, 386: 278-283.

Yu H, Zhou YJ, Li GX, Ma JH, Yan LP, Wang B, Yang FR, Huang M, Tong GZ. 2011. Genetic diversity of H9N2 influenza viruses from pigs in China: a potential threat to human health? Vet Microbiol, 149: 254-261

Yuen KY, Chan PK, Peiris M, Tsang DN, Que TL, Shortridge KF, Cheung PT, To WK, Ho ET, Sung R, Cheng AF. 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet, 351: 467-471.

Yus E, Sanjuan ML, Garcia F, Castro JM, Simarro I. 1992. Influenza A viruses: epidemiologic study in fatteners in Spain (1987–89). Zentralbl Veterinarmed B, 39: 113–118.

Zhang G, Kong W, Qi W, Long LP, Cao Z, Huang L, Qi H, Cao N, Wang W, Zhao F, Ning Z, Liao M, Wan XF. 2011. Identification of an H6N6 swine influenza virus in southern China. Infect Genet Evol, 11: 1174-1177.

Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow G, K, Liu L, Yoon KJ, Krauss S, Webster RG. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol, 73: 8851–8856.

Zhu W, Zhu Y, Qin K, Yu Z, Gao R, Yu H, Zhou J, Shu Y. 2012. Mutations in polymerase genes enhanced the virulence of 2009 pandemic H1N1 influenza virus in mice. PLoS One, 7(3):e33383.

Appendix

A. Other publications during the Ph.D studies:

1**. Busquets N, Segalés J, Córdoba L, Mussá T, Crisci E, Martín-Valls GE, Simon-Grifé M, Pérez-Simó M, Pérez-Maíllo M, Núñez JI, Abad FX, Fraile L, Pina S, Majó N, Bensaid A, Domingo M, Montoya M. 2010.** Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. Vet Res. 41(5):74.

2. **Simon-Grifé M, Martín-Valls GE, Vilar MJ, García-Bocanegra I, Mora M, Martín M, Mateu E, Casal J. 2011.** Seroprevalence and risk factors of swine influenza in Spain. Vet Microbiol. 149(1-2):56-63.

3. **Simon-Grifé M, Martín-Valls GE, Vilar MJ, Busquets N, Mora-Salvatierra M, Bestebroer TM, Fouchier RA, Martín M, Mateu E, Casal J. 2012.** Swine influenza virus infection dynamics in two pig farms; results of a longitudinal assessment. Vet Res. 2012 Mar 27;43(1):24.

4**. Vergara-Alert J, Argilaguet JM, Busquets N, Ballester M, Martín-Valls GE, Rivas R, López-Soria S, Solanes D, Majó N, Segalés J, Veljkovic V, Rodríguez F, Darji A. 2012.** Conserved synthetic peptides from the hemagglutinin of influenza viruses induce broad humoral and T-cell responses in a pig model. PLoS One.7 (7):e40524.

B. Oral comunications in international congresses

1. **Simon-Grife, M., Martin-Valls, G.E., Maria J. Vilar, M.J., Garcia-Bocanegra,I., Mora, M., Martin, M., Mateu, E., Casal, J.** 2010a. Seroprevalence and risk factors of swine influenza in Spain. Proceed. 21th IPVS Congress Vancouver (Canadà). 18-21 de juliol de 2010.

2. **Simon-Grife, M., Martín-Valls, G.E., Vilar, M.J. Mora, M., Martín, M., Busquets, N., Mateu, E., Casal, J.** 2010b. Longitudinal study of swine influenza virus infection in a farrow-to-finish farm. Proceed. 21th IPVS Congress Vancouver (Canadà). 18-21 de juliol de 2010.

3. **Simon-Grifé M, Martin-Valls GE, Vilar M, Busquets N, Mora M, Martin M, Mateu E, Casal J.2011.** Longitudinal study of swine influenza virus infection and

phylogenetic analysis of H1N1 isolated in a farrow to finish farm. 6th Emerging and Re-Emerging infectious diseases. Barcelona. 15 de Juny de 2011..

4. **Martin-Valls GE, Simon-Grifé M, Busquets, N, Bestebroer TM, Martin M, Casal J, Fouchier RAM, Mateu E.** 2012 a. Whole genome characterization of fourteen influenza viruses isolated from outbreaks in swine herds from Spain. Proceed. 22nd IPVS Congres Jeju (Korea). 11 Juny de 2012. Pp 163.

5. **Martin-Valls GE, Simon-Grifé M, Busquets, N, Bestebroer TM, Martin M, Casal J, Fouchier RAM, Mateu E. 2012b.** Evolution dynamics of H1N1 influenza viruses isolated in a farrow-to-finish farm Proceed. 22nd IPVS Congres Jeju (Korea). 12 Juny de 2012. p196. Presentació premiada amb el "Travel Award" en la categoria de "Virology & Viral Diseases".

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Original article

Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus

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Abstract – The recent pandemic caused by human influenza virus A(H1N1) 2009 contains ancestral gene segments from North American and Eurasian swine lineages as well as from avian and human influenza lineages. The emergence of this A(H1N1) 2009 poses a potential global threat for human health and the fact that it can infect other species, like pigs, favours a possible encounter with other influenza viruses circulating in swine herds. In Europe, H1N1, H1N2 and H3N2 subtypes of swine influenza virus currently have a high prevalence in commercial farms. To better assess the risk posed by the A(H1N1) 2009 in the actual situation of swine farms, we sought to analyze whether a previous infection with a circulating European avian-like swine A/Swine/Spain/53207/2004 (H1N1) influenza virus (hereafter referred to as SwH1N1) generated or not cross-protective immunity against a subsequent infection with the new human pandemic A/Catalonia/63/2009 (H1N1) influenza virus (hereafter referred to as pH1N1) 21 days apart. Pigs infected only with pH1N1 had mild to moderate pathological findings, consisting on broncho-interstitial pneumonia. However, pigs inoculated with SwH1N1 virus and subsequently infected with pH1N1 had very mild lung lesions, apparently attributed to the remaining lesions caused by SwH1N1 infection. These later pigs also exhibited boosted levels of specific antibodies. Finally, animals firstly infected with SwH1N1 virus and latter infected with pH1N1 exhibited undetectable viral RNA load in nasal swabs and lungs after challenge with pH1N1, indicating a cross-protective effect between both strains.

influenza virus / swine H1N1 / human A(H1N1) 2009 / cross-protection

1. INTRODUCTION

Influenza viruses belong to the *Orthomyxoviridae* family and are characterized by a segmented viral genome consisting of eight single stranded RNA fragments of negative polarity encoding 10 proteins [27]. In April 2009, a new A(H1N1) influenza virus was

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identified containing a distinct combination of gene segments from both North American and Eurasian swine influenza lineages as well as from avian and human lineages [8, 21], which rapidly reached pandemic proportions. Most human infections with this new swine-origin H1N1 influenza virus variant, A(H1N1) 2009, seem to be mild; however, there was a substantial number of hospitalized young individuals without previous underlying health problems, attesting to the pathogenic potential of A(H1N1) 2009 in humans.

Influenza viruses circulating in swine are closely related to the human H1N1 and H3N2 strains and reports of sporadic cross-species transfer of swine and avian influenza viruses to humans have been documented repeatedly during recent decades [18]. The mortality of swine influenza virus (SIV) infected pigs is usually low, although morbidity may approach 100% [14]. Swine influenza is characterized by sudden onset, coughing, respiratory distress, weight loss, fever, nasal discharge and rapid recovery [14]. Epithelial cells in the swine respiratory tract have receptors for both avian and mammalian influenza viruses [12]; thus, pigs could potentially serve as "mixing vessels" for the generation of new reassortant strains.

Currently, few studies have assessed the effect of the new pandemic A(H1N1) 2009 in pigs. No signs of disease were observed in miniature pigs infected with A(H1N1) 2009, although it replicated efficiently in the respiratory tract of these animals. According to this study, the asymptomatic infection, despite efficient virus replication, might explain why this new reassortant has never been found in swine before it was first identified in humans [13]. Another study analyzed replication dynamics, clinical symptoms and virus transmission in pigs infected with the novel A(H1N1) 2009. The inoculated pigs started nasal virus shedding from day 1 post-inoculation (PI) onwards and developed generally mild symptoms including fever, sneezing, nasal discharge, and diarrhoea. In that study, contact pigs became infected, shed virus and developed clinical symptoms similar to the inoculated animals [16].

The emergence of this novel human influenza virus A(H1N1) 2009 poses a potential global threat for human health. Since the new virus can infect other species, like pigs, a possible encounter with other influenza viruses circulating in swine herds may favour the possibility of generating new reassortants with higher virulence. To date, there are several reports confirming the diagnosis of A(H1N1) 2009 pandemic influenza virus in pig herds in all continents[1]. In all cases, the herds are believed to have been infected as a result of human-to-pig transmission. Therefore, the possibility that this novel human influenza virus A(H1N1) 2009 could affect a high percentage of swine herds has unknown consequences, not only for animal health but also for human health. A crucial question is whether previous immunity to circulating SIV protects pigs against pandemic A(H1N1) 2009 virus. Kyriakis et al. [17] have recently shown that pigs dually infected with some European SIV frequently exhibit cross-reactive hemagglutination inhibitory (HI) antibodies to pandemic A(H1N1) 2009 virus and related North American SIV, suggesting that pigs in Europe may have partial immunity to the pandemic A(H1N1) 2009 virus. The question of whether these antibodies would prevent an A(H1N1) 2009 infection remained unanswered. Thus, the objectives of the current study were (i) to experimentally investigate whether or not a previous infection with circulating H1N1 European avian-like swine influenza would confer protection to pigs which later encounter the novel pandemic human influenza virus A(H1N1) 2009 and (ii) to further explore the pathological and immunological parameters of this new A(H1N1) 2009 virus infection in pigs. The present study shows that a single exposure to a H1N1 European avian-like swine influenza protects pigs against a consecutive challenge with the pandemic A(H1N1) 2009 virus even in the absence of previous detectable cross-reactive HI antibodies.

[1] OIE, http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.08_SWINE_INFLUENZA.pdf..

2. MATERIALS AND METHODS

2.1. Viruses

Two Influenza A virus isolates were used in this study: the European avian-like swine A/Swine/Spain/53207/2004 (H1N1) isolated in 2004 (GenBank accession number CY010587) (hereafter referred to as SwH1N1) and the new human A/Catalonia/63/2009 (H1N1) influenza virus isolated in 2009 (GenBank accession numbers GQ464405-GQ464411 and GQ168897) (hereafter referred to as pH1N1). SwH1N1 was generously donated by Laboratorios HIPRA (Spain) and it was propagated following standard procedures by infecting Madin-Darby canine kidney (MDCK) cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (ref. 23 OIE). pH1N1 was isolated from a patient at the Hospital Clinic, Barcelona, Spain, and was propagated at 37.5 °C in the allantoic cavities of 11 day-old embryonated chicken eggs originating from a commercial specific-pathogen-free (SPF) flock (GDdeventer)[1]. Both viruses were titrated in MDCK cells, with the aid of trypsin in the post-infection media, and virus titre was calculated by the Reed and Muench method [19].

2.2. Animals

Twenty-two snatch-farrowed, colostrum-deprived Large White × Landrace piglets were obtained from a herd with a standard health status. Sows were seropositive to porcine reproductive and respiratory syndrome virus (PRRSV) and SIV, and seronegative to Aujeszky's disease virus. Piglets were obtained at the moment of delivery, immediately dried and umbilical cords clamped, cut and disinfected with an iodine solution. Piglets were fed *ad libitum* during the first two days with the milk substitute Patavie Porc (Oriane-Celtilait, Lesneven, France). Afterwards, animals received Startrite 100 (SCA Ibérica S.A., Mequinenza, Spain) mixed with milk or as dry meal from 10–15 days of age. Antibiotics administered in the feed included 205 000 UI of colistin (1 g of colimicine®, Laboratorios SP Veterinaria, in 1.5 L of milk) during the first week of age and 1.7 mg of enrofloxacin (Baytril oral solution 0.5%, Bayer Animal Health, Leverkusen, Germany) per kg body weight/day during the first two weeks of age. All piglets were housed in an experimental isolation room at the biosafety level 3 facilities of the Centre de Recerca en Sanitat Animal (CReSA, Barcelona, Spain). Animal care and procedures were in accordance with the guidelines of the Good Laboratory Practices (GLP) and under the supervision of the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona.

2.3. Experimental design

At the age of 40 days, pigs were randomly distributed into four groups, namely Mock/Mock ($n = 6$), SwH1N1/Mock ($n = 4$), Mock/pH1N1 ($n = 8$) and SwH1N1/pH1N1 ($n = 4$) balanced by sex and weight. Group Mock/Mock pigs were intranasally inoculated with 3.5 mL of MEM (half of the amount in each nostril) on days 0 and 21 of the experiment. Group SwH1N1/Mock animals were intranasally inoculated with 3.5 mL of a suspension containing $10^{7.04}$ tissue culture infectious doses 50% ($TCID_{50}$) per mL of SwH1N1 on day 0 and received the same volume of MEM on day 21. Pigs from group Mock/pH1N1 received the abovementioned amounts of MEM on day 0 and $10^{6.15}$ $TCID_{50}$ per mL of pH1N1 on day 21. Finally, pigs of group SwH1N1/pH1N1 were inoculated with both viruses, SwH1N1 on day 0 and pH1N1 on day 21, using the same route and dose mentioned for previous groups.

In order to study the events taking place at the early stages of infection with pH1N1, two Mock/Mock and two Mock/pH1N1 piglets were euthanized with an intravenous overdose of sodium pentobarbital on days 2 and 4 after pH1N1 inoculation (days 23 and 25 PI). All the remaining pigs in each group (2 animals of Mock/Mock group and 4 animals in each of the remaining groups) were euthanized on day 28 PI.

2.4. Clinical records and sampling procedures

Pigs were clinically monitored daily for the whole experimental period, with emphasis on potential respiratory disorders (coughing, sneezing and thumping) as well as systemic signs (depression, reluctance to move and fever). Specifically, rectal temperatures were taken on days 1, 2, 3, 5 and 7 after both viral inoculations, and also after 10 and 15 days post-SwH1N1 inoculation.

Nasal swabs were taken on days 1, 2, 4, 6, 10, 21, 22, 23, 25 and 28 PI, placed in 1 mL of PBS and frozen at −80 °C until further use. Complete necropsy was done on each animal, with special emphasis on the respiratory tract. Gross lung lesions were assessed for the presence or absence of pulmonary cranioventral multifocal consolidation and when present, extension was recorded. Right lung was used to perform a broncho-alveolar lavage (BAL) using 200 mL of PBS (animals from groups Mock/Mock and

Table I. Primers and probes used for TaqMan one-step qRT-PCR.

IAV	Primer/probe	Concentration (μM)	Sequence (5′-3′)	Reference
European	M+25	0.9	AGA TGA GTC TTC TAA CCG AGG TCG	
avian-like	M-124 E-A-L-S	1.8	TGC AAA RAC AYC TTC CAG TCT CTG	This study
swine	M+64 E-A-L-S	0.4	FAM[a]- TCR GGC CCC CTC AAA GCC GA-TAMRA[b]	This study
A(H1N1)	M+25	0.9	AGA TGA GTC TTC TAA CCG AGG TCG	[16]
2009	M-124 human09	0.9	TGC AAA GAC ACT TTC CAG TCT CTG	This study
	M+64	0.2	FAM- TCA GGC CCC CTC AAA GCC GA-TAMRA	[16]

[a] FAM, 6-carboxylfluorescein.
[b] TAMRA, 6-carboxyltetramethyl rhodamine.

Mock/pH1N1) and the left one sampled for histopathological and virological studies (animals from all experimental groups). Specifically, samples from lung (apical, middle and diaphragmatic lobes), nasal turbinate, nasal septae, trachea and tonsil were collected and fixed by immersion in 10% buffered formalin. Additional lung tissues (apical and cardiac lobes) were frozen at −80 °C until their use for viral RNA extraction.

2.5. Pathological procedures

Fixed tissue samples were dehydrated through graded alcohols, embedded in paraffin and stained with hematoxylin-eosin. Each tissue was microscopically studied for the presence of inflammatory lesions. In the lung, broncho-interstitial pneumonia (BIP) intensity was assessed by means a semi-quantitative scoring (0 to 3, indicating lack of, mild, moderate or severe pneumonia lesions, respectively).

2.6. Antibody detection

Anti-influenza A virus nucleoprotein (NP) antibody levels were studied in serum using the ID Screen® Influenza A Antibody Competition ELISA (ID-Vet, Montpellier, France) following manufacturer's instructions on days 0, 21 and 28 PI.

Detection of specific IgG and IgA in nasal swabs, serum and BAL was performed modifying the ID Screen® Influenza A Antibody Competition ELISA with a goat anti-porcine IgG (Fc):HRP (Serotec AAI41P) antibody at 1:100 000 dilution or a goat anti-porcine IgA:HRP (Serotec AAI40P) antibody at 1:10 000 dilution as secondary antibodies. Nasal swabs and BAL samples were used neat in the ELISA assay whereas serum samples were used at 1/100 dilution. Fifty μL of each sample or serum dilutions were used in the ELISA assay.

Hemagglutination-inhibition (HI) assay was used to measure antibody titres from serum samples at days 0, 21 and 28 PI. The HI test was performed according to standard procedures [25]. The test was standardized at 4 hemmagglutinin units (HAU). To remove non-specific inhibitors of hemagglutination and natural agglutinins of sera, samples were treated with receptor-destroying enzyme (RDE) from *Vibrio Cholerae* (Sigma, St. Louis, MO, USA) overnight, inactivated at 56 °C for 60 min and adsorbed into chicken red blood cells at 50% and 4 °C. The starting dilution was 1:20.

2.7. Hydrolysis probe and primer sets

The two influenza viruses used in this study and all the available European avian-like swine and the new human influenza virus A(H1N1) 2009 *M* gene sequences were downloaded, aligned from the Influenza Viruses Resource[2] and then compared to the *M* gene primer and probe sequences previously reported [22]. Three mismatches were identified for the European avian-like SIV sequences and four in the new human influenza virus A(H1N1) 2009 sequences in the target sequence of the primer M-124. Also, one mismatch was detected in the target sequence of the M+64 probe affecting the European avian-like SIV amplification. Taking all this information into account, primers and probes were modified to improve the European avian-like SIV detection

[2] http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html.

(Tab. I). Primers used for the new human influenza virus A(H1N1) 2009 detection in the present work were different from those of recent reports [28], although they were also based on previously reported *M* gene primer and probe sequences [22]. Primers and probes used in this study (Tab. I) were synthesized by Tib Molbiol (Berlin, Germany).

2.8 Quantitative RT-PCR (RT-qPCR)

SwH1N1 and pH1N1 viral loads in nasal swabs, lung tissue and BAL were assessed following a TaqMan one-step RT-qPCR in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). Viral RNA was extracted with QIAamp viral mini kit (Qiagen, Valencia, CA, USA) obtaining 60 µL of eluted viral RNA. The *M* fragment amplification was carried out using the primers and probe concentrations indicated in Table I and One-Step RT-PCR Master Mix Reagents (Applied Biosystems) following the manufacturer's instructions using 5 µL of eluted RNA in a total volume of 25 µL. The amplification conditions were as follows: reverse transcription at 48 °C for 30 min; initial denaturation reaction at 95 °C for 15 min and 40 PCR-cycles of 95 °C 15 s and 60 °C 1 min.

Standard curves and quantification were achieved by prior amplification of a 99 bp fragment of the *M* gene using both aforementioned strains as templates, primers described in Table I and the One-Step RT-PCR reagents (Qiagen) following the manufacturer's instructions, using 5 µL of eluted RNA in a total volume of 25 µL. The amplification conditions were the following: reverse transcription at 50 °C for 30 min; initial denaturation reaction at 95 °C for 15 min and 40 PCR-cycles of 94 °C 30 s, 55 °C 1 min and 72 °C 1 min. The obtained *M* gene fragment amplicon was cloned into pGEMT vector (Promega Madison, WI, USA) and transformed by heat shock in *Escherichia coli* competent cells (Invitrogen, Paisley, UK). The recombinant plasmid was purified using the QIA prep Spin kit (Qiagen) and spectrophotometrically quantified (Qubit, Invitrogen). The copy number of recombinant plasmids was calculated as previously described [7] following the formula: N (molecules per µL) = (C (DNA) µg/µL/K (fragment size in bp)) × 182.5×10^{23} (factor derived from the molecular mass per the Avogadro constant). Serial 10-fold dilutions of both plasmids of known concentration were made and the standard curves were generated using 1.83×10^2 to 1.83×10^6 copies of recombinant plasmid with the *M* gene fragment from the pH1N1 and 2.26×10^2 to 2.26×10^6 of recombinant plasmid with the *M* gene fragment from SwH1N1. The limit of detection (LoD) for pH1N1 was 11.65 plasmid copies per reaction, which corresponded to 0.05 $TCID_{50}$ per reaction. In the case of the SwH1N1 the LoD was 13.71 copies per reaction, which corresponded to 0.5 $TCID_{50}$ per reaction. The genome equivalent copies (GEC) of plasmid from the collected samples were determined based on these standard curves and taking into account their volumes. Thus, the LoD for pH1N1 was 2.82 $\log_{10}$ GEC and for SwH1N1 was 2.86 $\log_{10}$ GEC per nasal swab; for pH1N1 the LoD was 4.12 $\log_{10}$ GEC per gram of apical and middle lobes of lung and 2.82 $\log_{10}$ GEC per mL of BAL. Since the efficiency of retrotranscription was not directly determined, GEC numbers did not reflect exactly the number of viral RNA molecules. The RT-qPCR for SwH1N1 virus was used for samples taken between day 0 and day 21 PI in the experimental procedure, whereas RT-qPCR for pH1N1 virus was used for samples taken between days 21 to 28 PI.

2.9. Sequencing influenza virus

The complete amplification of the PB1, PB2, PA, HA, NP, MP and NS segments of pH1N1 of the inoculum (GenBank accession numbers GQ464405-GQ464411) before and after infection, were obtained using the genome primer set and protocol recommended by the World Health Organization (WHO)[3]. For SwH1N1, the complete NP segment and the 5′ end of the hemagglutinin (HA) segment were also sequenced before and after infection using the same set of primers. The amplified products were analyzed by agarose gel electrophoresis, stained with SYBR Gold® (Molecular Probes, Eugene, USA) and purified using the NucleoFast 96 PCR kit (MACHEREY-NAGEL GmbH & Co KG, Düren, Germany). Sequencing of both strands was performed using the BigDye technology (Applied Biosystems), with the primers M13F (5′-TGTAAAACGACGGC CAGT-3′) and M13R (5′-CAGGAAACAGCTATGA CC-3′). Sequence assembly was accomplished using the programs Phred [3, 4], Phrap and Consed [9], and Bioedit [10]. Homology and identity searches with respect to influenza genome sequences available at the GenBank were performed by using the BLAST utilities[4].

2.10. Statistical analysis

The Mann-Whitney test was used to compare ELISA titres in the different samples tested, and viral

[3] http://www.who.int/csr/resources/publications/swineflu/GenomePrimers_20090512.pdf.
[4] http://www.ncbi.nlm.nih.gov/BLAST.

loads in BAL, nasal swabs and lungs between experimental groups. All analyses were carried out with NCSS 2004 and PASS 2005 softwares (Kavysville, Utah, USA). The significance level was set at 0.05 with statistical tendencies reported when $p < 0.10$.

3. RESULTS

3.1. Clinical outcome

No relevant respiratory or systemic clinical signs were observed in any pig in any of the experimental groups. Moreover, no individual pig showed abnormal rectal temperatures ($\geq$ 40 °C) throughout the duration of the study.

3.2. Pathological studies

Significant gross lesions compatible with BIP were observed in 6 pigs, all of them corresponding to the Mock/pH1N1 group. Remarkably, 1 out of 2 pigs sacrificed at both 23 and 25 days PI (days 2 and 4 post-pH1N1-inoculation, respectively) had BIP-like lesions. All remaining Mock/pH1N1 pigs ($n = 4$) studied on day 28 PI also had BIP-compatible lesions (Fig. 1A). No gross lesions compatible with BIP were observed in any pig of the rest of the groups studied. Besides this, one control pig (Mock/Mock) euthanized on day 23 PI had serous arthritis of the left coxo-femoral joint, one double inoculated animal (SwH1N1/pH1N1) had fibrous/fibrinous polyserositis and another SwH1N1/pH1N1 pig had fibrous pleuritis and mild pulmonary cranio-ventral scars.

Microscopic pulmonary lesions were observed in all but the animals from Mock/Mock group (Figs. 1B and 1C). These lesions consisted of BIP, characterized by attenuation and loss of the bronchiolar epithelium, with mononuclear (lymphocyte and plasma cells) infiltration in the lamina propria of bronchi and bronchioli, as well as the presence of macrophages and lymphocytes within alveoli surrounding affected bronchi. Sometimes such inflammation also affected the lung interstitium. Bronchus-associated lymphoid tissue was occasionally hyperplastic. Such BIP lesions were present in all Mock/pH1N1 pigs, with mild ($n = 1$), moderate ($n = 4$) and severe ($n = 3$) intensity. Two pigs from each of the SwH1N1/Mock and SwH1N1/pH1N1 groups also showed BIP, although intensity was lower; one pig with mild and another with moderate lesions in group SwH1N1/Mock, and one animal with mild and another with mild-to-moderate lesions in group SwH1N1/pH1N1. Average histopathological BIP score is displayed in Figure 1D. No histological relevant findings were observed in the rest of studied tissues (nasal turbinates and septae, trachea and tonsil).

3.3. Antibody response against influenza viruses

Total anti-NP antibody levels, independent of their isotype, were measured with the competition ELISA kit in serum from all animals at the beginning of the assay (day 0), just before inoculation with pH1N1 (day 21 PI) and seven days after inoculation with pH1N1 (day 28 PI). Results represented in Figure 2A show that all animals were seronegative at the beginning of the experiment. At day 21 PI, only the animals in the two groups infected with SwH1N1 SIV (SwH1N1/Mock and SwH1N1/pH1N1) had seroconverted and had significantly higher antibody levels in serum ($p = 0.0001$) as compared with the four pH1N1 infected pigs in the Mock/pH1N1 group, which had total antibody levels equal or below 60% of inhibition, indicating that a primary immune response against pH1N1 was taking place.

No significant differences were observed when antibody levels from the four animals in group SwH1N1/pH1N1 were compared with those from group SwH1N1/Mock, although there was a clear statistical trend ($p = 0.11$) to suspect that pH1N1 induced a secondary response. The effect of pH1N1 infection was further evaluated comparing IgA delta values between day 21 and 28 between SwH1N1/Mock and SwH1N1/pH1N1 groups. The p-value was 0.06, indicating a strong tendency close to significance. Moreover, this tendency was also observed when serum samples were analyzed for specific IgA (Fig. 3A) and IgG (Fig. 3B) anti-NP antibodies in all the animals although to a lesser extent.

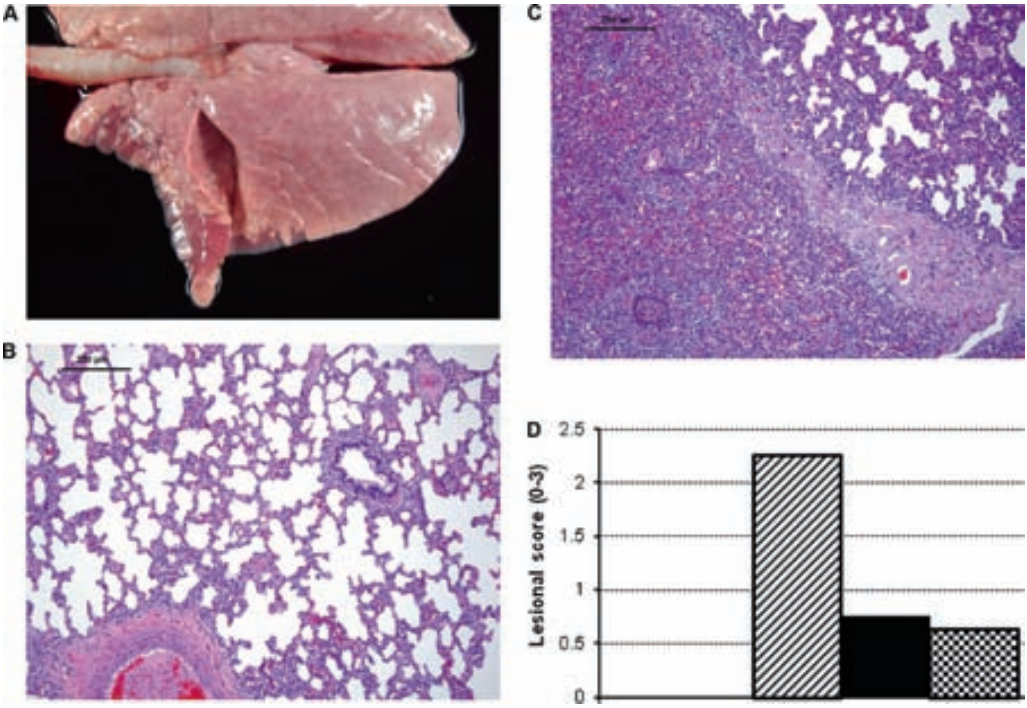

Figure 1. Pathological analysis at day 28 PI. (A) A representative picture of lung from group Mock/pH1N1. The lungs show moderate broncho-interstitial pneumonia (BIP)-compatible lesions. (B) Lung from a Mock/Mock control pig. Normal pulmonary parenchyma. Note the minimal thickness of interalveolar walls, characterised by pneumocytes and blood capillaries. Peribronchial lymphoid tissue is minimally developed. Haematoxylin and eosin stain. Bar = 200 μm. (C) Lung from a pH1N1 infected pig (day 7 post-pH1N1 inoculation). Marked presence of mononuclear inflammatory cells within alveoli, almost collapsing alveolar lumen. Lymphocyte perivascular cuffing together with moderate hyperplasia of the lymphoid tissue surrounding a bronchioli. Haematoxylin and eosin stain. Bar = 200 μm. (D) Pathological score for all the animals in the assay (Mock/Mock, Mock/pH1N1, SwH1N1/Mock, SwH1N1/pH1N1). (A color version of this figure is available at www.vetres.org.)

Humoral protection against influenza virus is predominantly mediated by antibodies against HA. Thus, serum samples were examined by HI assays against SwH1N1 and pH1N1 (Tab. II) at day 0, 21 and 28 PI. All animals in the group infected by SwH1N1 exhibited HI antibody titres at day 21 PI, which remained constant until day 28 PI. In the case of 3 out of 4 animals in group Mock/pH1N1, some low level of HI antibodies were present at day 28 PI (7 days after pH1N1 infection). However, with one exception, HI titres elicited by SwH1N1/pH1N1 animals exhibited an increment between day 21 and 28 PI when tested against SwH1N1. Also, HI antibody titres in the SwH1N1/pH1N1 group were higher than those observed in the animals from SwH1N1/Mock group (Tab. II). All sera collected at day 21 PI remained negative for HI when tested against pH1N1, with the exception of animal 152. However, at day 28 PI, HI titres against pH1N1 from animals of the SwH1N1/pH1N1 group were higher than for the Mock/pH1N1 group. Surprisingly, one animal (178) gave low serum responses in ELISA and HI tests, but exhibited high antibody OD ratios against the NP at the nasal cavity.

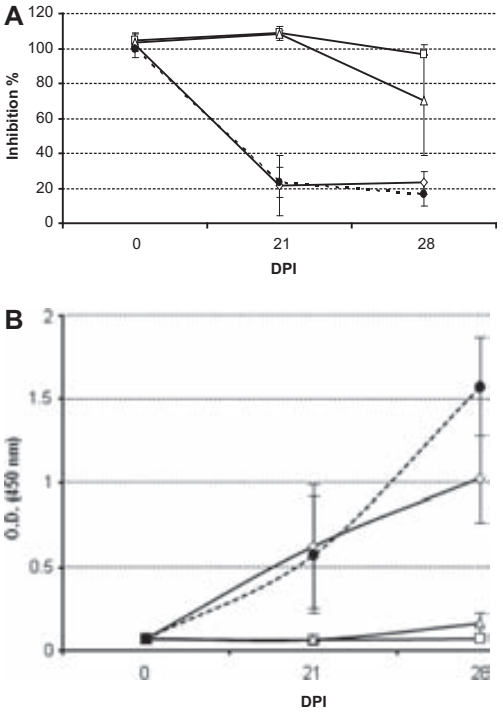

Figure 2. Antibodies in serum and nasal swabs. (A) Influenza total anti-NP antibodies in serum were analyzed by ELISA competition assay. (B) Influenza IgA anti-NP antibodies in nasal swabs were analyzed by ELISA at day 0, 21 and 28 PI. White squares, white rhomboids, white triangles and black circles represent average values from animals belonging to Mock/Mock (□), SwH1N1/Mock (◇), Mock/pH1N1 (Δ) and SwH1N1/pH1N1 (●) group, respectively. Error bars represent one SD above and below the mean.

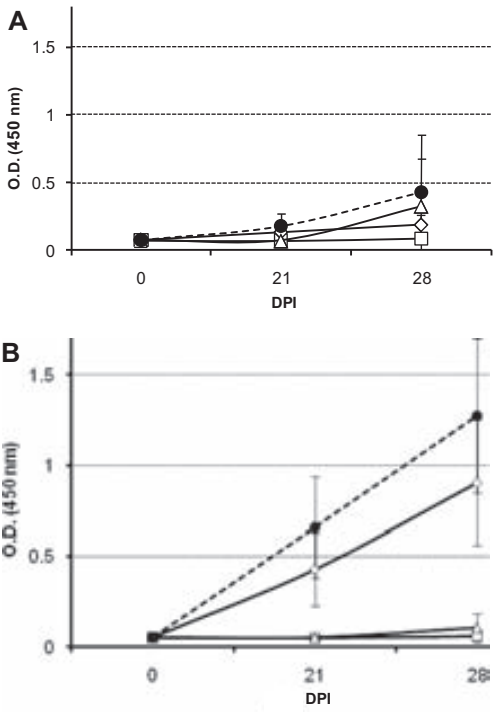

Figure 3. IgA and IgG antibodies in serum. (A) Influenza IgA and (B) IgG anti-NP antibodies in serum were analyzed by ELISA at day 0, 21 and 28 PI. White squares, white rhomboids, white triangles and black circles represent mean values from animals belonging to Mock/Mock (□), SwH1N1/Mock (◇), Mock/pH1N1 (Δ) and SwH1N1/pH1N1 (●) groups, respectively. Error bars represent one SD above and below the mean.

Specific IgA and IgG anti-NP antibodies were also detected in BAL of Mock/pH1N1 animals at tested times (23, 25 and 28 days PI, which were day 2, 4 and 7 PI for pH1N1 infection) (Fig. 4).

3.4. Viral RNA quantification

Viral RNA levels were investigated in respiratory tissues. SwH1N1 RNA was detected in nasal swabs of pig groups SwH1N1/Mock and SwH1N1/pH1N1 from day 1 to 7 PI, and had the maximum viral RNA load between days 2 and 4 PI (Figs. 5A and 5C). No viral RNA was detected on days 10 and 21 PI and in any day PI in the remaining groups (Mock/Mock and Mock/pH1N1). Similarly, pH1N1 RNA was detected between 1 and 7 days PI with this virus, with a peak load between days 2 and 4 PI (Fig. 5B). No pH1N1 viral RNA was detected in any of the samples in group SwH1N1/pH1N1 at any of the time-points analyzed after pH1N1 inoculation or in the rest of the animal groups (Mock/Mock and SwH1N1/

Table II. Individual hemagglutination inhibition titres from sera belonging to animals from different infection groups (Mock/Mock, SwH1N1/Mock, Mock/pH1N1 and SwH1N1/pH1N1). Samples were tested for hemagglutinin antigens from the viruses used in the experimental infection (SwH1N1 and pH1N1).

	Animal number	SwH1N1 (A/Swine/Spain/53207/2004)			pH1N1 (A/Catalonia/63/2009)		
		d0	d21	d28	d0	d21	d28
Mock/Mock	160	< 20	< 20	< 20	< 20	< 20	< 20
	186	< 20	< 20	< 20	< 20	< 20	< 20
SwH1N1/Mock	87	< 20	160	160	< 20	< 20	< 20
	152	< 20	160	160	< 20	20	< 20
	153	< 20	160	160	< 20	< 20	< 20
	159	< 20	160	160	< 20	< 20	< 20
Mock/pH1N1	82	< 20	< 20	< 20	< 20	< 20	40
	180	< 20	< 20	< 20	< 20	< 20	40
	188	< 20	< 20	< 20	< 20	< 20	20
	200	< 20	< 20	< 20	< 20	< 20	< 20
SwH1N1/pH1N1	84	< 20	160	320	< 20	< 20	80
	86	< 20	80	640	< 20	< 20	320
	168	< 20	80	320	< 20	< 20	80
	178	< 20	80	80	< 20	< 20	< 20

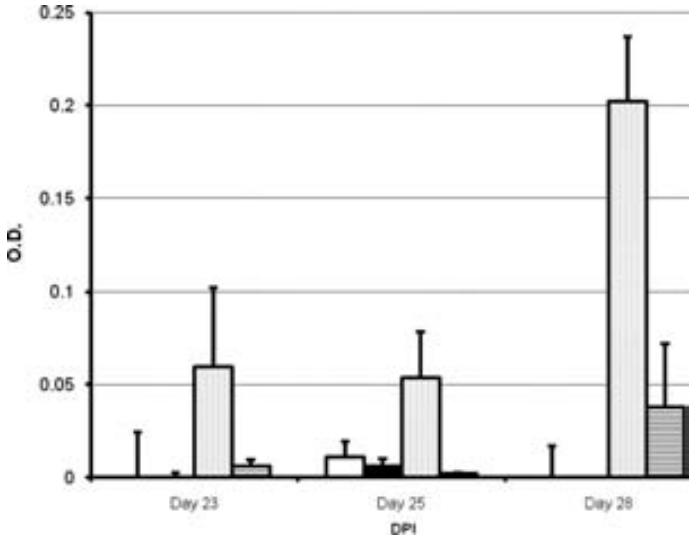

Figure 4. IgA and IgG antibodies in BAL. Influenza IgA and IgG anti-NP antibodies in BAL were analyzed by ELISA at day 23, 25 and 28 PI (corresponding to day 2, 4 and 7 after pH1N1 inoculation). White bars and dotted bars represent average IgA values of 2 and 4 animals belonging to Mock/Mock and Mock/pH1N1 groups, respectively. Black bars and lined bars represent average IgG values of 2 and 4 animals belonging to Mock/Mock and Mock/pH1N1 groups, respectively. Error bars represent one SD above and below the mean.

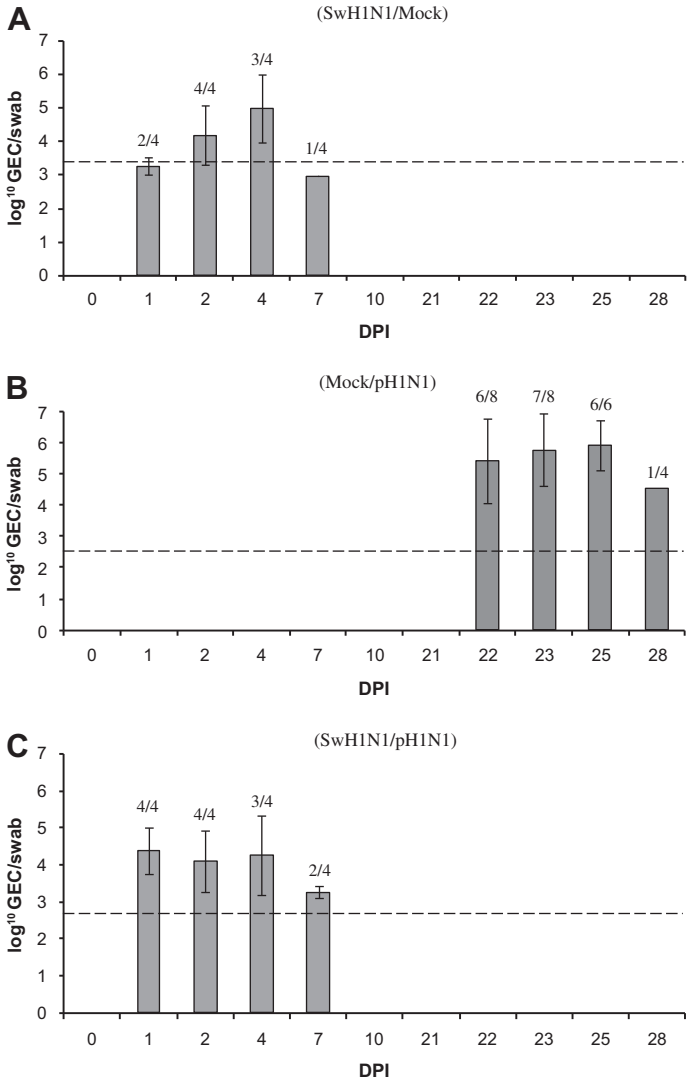

Figure 5. Influenza viral RNA load in nasal swabs. Quantification of influenza RNA was performed by RT-qPCR at the indicated days PI. Bars indicate average values of positive samples in genome equivalent copies (GEC) of plasmid per swab in group SwH1N1/Mock (A), Mock/pH1N1 (B) and SwH1N1/pH1N1 (C). The number of positive samples from the total number of animals was indicated above each bar. Samples from animals in group Mock/Mock were all below the limit of detection, which is indicated by the dotted line. Error bars represent one SD above and below the mean.

Mock). Differences between GEC values in animals from group Mock/pH1N1 compared with those from SwH1N1/pH1N1 animals on days 22, 23 and 25 of the experiment were statistically significant ($p = 0.02$; $p = 0.02$ and $p = 0.004$, respectively).

Influenza RNA load was also analyzed in the lungs (apical and cardiac lobes) of animals

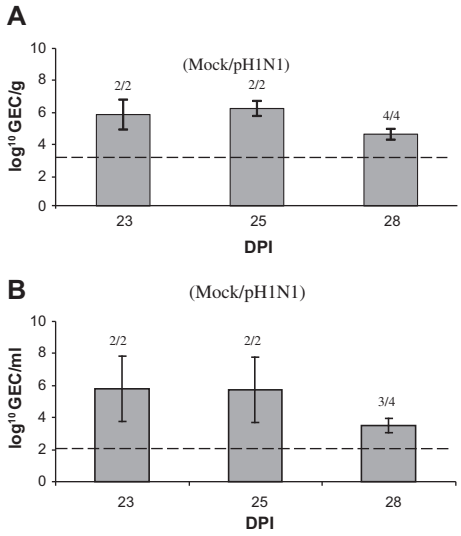

Figure 6. Influenza viral load in lung tissues and BAL. Quantitation of influenza RNA was performed by RT-qPCR at day 23, 25 and 28 PI (corresponding to day 2, 4 and 7 after pH1N1 inoculation). Bars indicate average values of positive samples in genome equivalent copies (GEC) of plasmid per swab in group Mock/pH1N1 for lung tissue (A) and BAL (B). The number of positive samples from the total number of animals was indicated above each bar. Samples from animals in groups Mock/Mock and SwH1N1/pH1N1 were all below the detection limit, indicated by the dotted line. Error bars represent one SD above and below the mean.

belonging to groups Mock/Mock, Mock/pH1N1 and SwH1N1/pH1N1 on days 23, 25 and 28 of the experiment (corresponding to day 2, 4 and 7 after pH1N1 infection) (Fig. 6A). No viral RNA was detected in the lungs of any animal belonging to the Mock/Mock group or SwH1N1/pH1N1 group in any lung tissue tested. Differences between viral RNA load in animals from group Mock/pH1N1 compared with values from lung from group SwH1N1/pH1N1 on days 22, 25 and 28 of the experiment were significantly different ($p = 0.03$). Influenza RNA was also detected in BAL from most of the animals in Mock/pH1N1 group (Fig. 6B) whereas no viral RNA was found in BAL from animals in Mock/Mock group.

3.5. Sequencing of SwH1N1 and pH1N1

The complete NP segment and the 5' end of HA sequences of SwH1N1 were analyzed and showed 100% identity to those reported previously (CY010583 and CY010580). Secondly, sequences of the complete PB2, PB1, PA, HA, NP, MP and NS genes of pH1N1 used for infection showed no variation when compared to other pandemic viruses. Furthermore, the sequences of pH1N1 isolated after pig infection were identical to those of the pH1N1 inoculum. Comparison of amino acid sequences from SwH1N1 with pH1N1 sequences resulted in an NP homology of 92% whereas in the case of HA this value was 78% and for NA it was 96%.

4. DISCUSSION

In the present study, the possible protective effect of an infection with a circulating European avian-like swine A/Swine/Spain/53207/2004 (H1N1) influenza virus (referred to as SwH1N1) in colostrum-deprived pigs was evaluated against a subsequent infection with the new human A/Catalonia/63/2009(H1N1) influenza virus (referred to as pH1N1). A prior exposure to the SwH1N1 strain induced immunity able to substantially reduce if not inhibit pH1N1 shedding and viral RNA load in respiratory tissues after a subsequent pH1N1 infection even in the absence of detectable cross-HI antibodies. It also protected pigs from lesion development. Additionally, previous results from studies of pigs infected with the new human A(H1N1) 2009 influenza virus were confirmed in the present work. The results presented here show that intranasal infection of pigs with $10^{6.15}$ $TCID_{50}$ of pH1N1 virus exhibited mild to moderate lung lesions, resulting in virus shedding and activation of a specific humoral immune response, as it has been previously shown [16].

The typical influenza-like symptoms were not observed in either group Mock/pH1N1 or SwH1N1 infected animals used in the present experiment. Such a situation should not be surprising, since clinical signs including fever

have only been reported when intratracheal inoculation was performed with a high virus dose ($\geq$ 7.5 $\log_{10}$ $TCID_{50}$) [23, 24]. Therefore, the experimental conditions used here induced a subclinical infection, a situation very often encountered on farms as most of SIV infections in field conditions cause subclinical infections [14].

After infection, virus RNA load in nasal swabs was detected very rapidly (at day 1 PI) not only for SwH1N1 but also for pH1N1. These results are in line with previous data showing that pigs are susceptible to the novel influenza virus A(H1N1) 2009 and they are able to infect contact pigs as soon as 3 days PI [16]. They also support data obtained from a naturally infected swine herd [28]. Thus, it is conceivable that this virus would probably spread quickly and efficiently if introduced into SIV H1N1 free farms. Reports from 19 countries world wide[1] about putative human-to-pig transmissions and also experimental studies of sequential passages of the virus in pigs [1] support this observation. However, the data presented in this work indicated that a previous infection with at least one circulating European avian-like H1N1 SIV strain would decrease the risk of a further infection with A(H1N1) 2009, and consequently, the potential of further reassorting.

Antiviral adaptive immune mechanisms against influenza virus involve neutralizing antibodies, including secretory IgA at mucosal surfaces, and CTL. Interestingly, primary influenza virus infection in pigs induced local antigen-specific lymphoproliferative responses and a long-lived increase of lung $CD8^+$ T cells which could play a role in the broad-spectrum immune protection to heterotypic virus strains [11]. However, humoral protection against influenza viruses is mainly mediated by antibody responses to HA [17]. In a previous study, pigs immunized as a result of intranasal inoculation with either H1N1 or H3N2 showed partial clinical protection against H1N2 challenge, and nasal and virus shedding was two days shorter than in naïve pigs [20]. Later, Heinen et al. [11] reported some heterotypic immunity in animals infected by aerosol firstly with H1N1 and later with H3N2. However, no cross-reactive HI antibodies from SIV H1N1-infected pigs were observed with H3N2 SIV or vice versa after primary infection. A recent report on serologic cross-reactivity with A (H1N1) 2009 in pigs serum showed that consecutive infection with two European SIV subtypes induced HI cross-reacting antibodies to A(H1N1) influenza virus, even though European viruses do not contain a classical swine H1 HA. These authors also described that HI antibodies induced by a single infection with European subtype H1N1 or H1N2 SIV did not cross-react with the pandemic (H1N1) 2009 virus [15]. The results presented in this work are in full agreement with all these previous data as no cross-reactive antibodies were detected between SwH1N1 and pH1N1 before pH1N1 infection. These results suggest that sequence divergence of around 20–30% in amino acids in proteins such as HA or NA do not interfere with the generation of memory cross-reactive B cells.

Furthermore, the sequences of pH1N1 inoculated and pH1N1 isolated from infected pigs were identical, suggesting that the virus did not evolve within the study period. This is in agreement with data obtained so far in humans where genetic variability of the virus is very limited. However, recent information from a naturally infected swine herd with A(H1N1) 2009 suggested that either a higher than normal mutation rate, strong positive selective pressure, or a combination of both might apply for this infection [28]. Further work will elucidate the genetic evolution of this new virus.

Davenport et al. [2] first described in 1953 the phenomenon of original antigenic sin. This observation was later expanded showing that the phenomenon of original antigenic sin responses to influenza viruses existed not just in humans but in other species as well [5, 6, 26]. In the context of sequential infection with two influenza viruses, the primary exposure induces proliferation of B cells that are either specific for the first virus only or cross-reactive with both viruses. Upon exposure to the second virus, memory B cells cross-reactive to both viruses outcompete naïve B cell clones just specific for novel epitopes from the second virus. Indeed, this theoretical explanation would fit with the results obtained in the present study, showing that original antigenic sin also takes

place for SIV infection in pigs. Cross-reactive memory B cells for SwH1N1 and pH1N1 might be generated upon infection with SwH1N1 by a single SwH1N1 exposure. The fact that cross-reacting antibodies were undetected before pH1N1 infection indicated that this cross-reactive memory B cell population was a minor population. These memory cells would be later activated by differentiating into plasma B cells when pH1N1 infection took place, explaining the rapid increase in pH1N1 specific HI antibodies 7 day after pH1N1 infection in animals from group SwH1N1/pH1N1 compared with HI antibodies titres in animals only infected with pH1N1 (primary infection) and the higher values of HI antibodies against SwH1N1 at day 28 (Tab. II).

In the present experiment, the boosting effect of pH1N1 infection in group SwH1N1/pH1N1 for IgA levels at mucosal sites and the HI cross-reactive antibodies generated after challenge would suggest that humoral responses with cross-neutralizing activity in respiratory tissues and sera played a major role in conferring protective immunity. Therefore, it seems that specific humoral immunity, particularly the one induced at the respiratory mucosa, correlated with protection in animals challenged with a subsequent infection with pH1N1.

To the authors' knowledge, this is the first study in which immunity generated against a circulating European avian-like swine (H1N1) influenza virus in pigs has been evaluated against a subsequent infection with a human (H1N1) influenza virus in pigs. In summary, a previous infection with one of the European circulating H1N1 SIV in the field was able to confer protective immunity to pigs against a challenge with the new variant A(H1N1) 2009. These data pave the way for understanding cross-protective immune responses generated between different influenza viral infections, within the same subtype or different subtypes. Further characterizations are required to understand the whole picture and all the fine mechanisms involved in cross-protection.

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REFERENCES

[1] Brookes S.M., Irvine R.M., Nunez A., Clifford D., Essen S., Brown I.H., et al., Influenza A (H1N1) infection in pigs, Vet. Rec. (2009) 164:760–761.

[2] Davenport F.M., Hennessy A.V., Francis T. Jr, Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus, J. Exp. Med. (1953) 98:641–656.

[3] Ewing B., Green P., Base-calling of automated sequencer traces using phred. II. Error probabilities, Genome Res. (1998) 8:186–194.

[4] Ewing B., Hillier L., Wendl M.C., Green P., Base-calling of automated sequencer traces using phred. I. Accuracy assessment, Genome Res. (1998) 8:175–185.

[5] Fazekas de St Groth S., Webster R.G., Disquisitions on original antigenic sin. II. Proof in lower creatures, J. Exp. Med. (1966) 124:347–361.

[6] Fazekas de St Groth S., Webster R.G., Disquisitions of original antigenic sin. I. Evidence in man, J. Exp. Med. (1966) 124:331–345.

[7] Fronhoffs S., Totzke G., Stier S., Wernert N., Rothe M., Bruning T., et al., A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction, Mol. Cell. Probes (2002) 16:99–110.

[8] Garten R.J., Davis C.T., Russell C.A., Shu B., Lindstrom S., Balish A., et al., Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans, Science (2009) 325:197–201.

[9] Gordon D., Abajian C., Green P., Consed: a graphical tool for sequence finishing, Genome Res. (1998) 8:195–202.

[10] Hall T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids Symp. (1999) 41:95–98.

[11] Heinen P.P., de Boer-Luijtze E.A., Bianchi A.T., Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection, J. Gen. Virol. (2001) 82:2697–2707.

[12] Ito T., Couceiro J.N., Kelm S., Baum L.G., Krauss S., Castrucci M.R., et al., Molecular basis for the generation in pigs of influenza A viruses with pandemic potential, J. Virol. (1998) 72:7367–7373.

[13] Itoh Y., Shinya K., Kiso M., Watanabe T., Sakoda Y., Hatta M., et al., In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses, Nature (2009) 460:1021–1025.

[14] Kothalawala H., Toussaint M.J., Gruys E., An overview of swine influenza, Vet. Q. (2006) 28:46–53.

[15] Kyriakis C.S., Olsen C.W., Carman S., Brown I.H., Brookes S.M., Doorsselaere J.V., Reeth K.V., Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe, Emerg. Infect. Dis. (2010) 16:96–99.

[16] Lange E., Kalthoff D., Blohm U., Teifke J.P., Breithaupt A., Maresch C., et al., Pathogenesis and transmission of the novel swine origin influenza virus A/H1N1 after experimental infection of pigs, J. Gen. Virol. (2009) 90:2119–2123.

[17] Murphy B.R., Clements M.L., The systemic and mucosal immune response of humans to influenza A virus, Curr. Top. Microbiol. Immunol. (1989) 146:107–116.

[18] Myers K.P., Olsen C.W., Gray G.C., Cases of swine influenza in humans: a review of the literature, Clin. Infect. Dis. (2007) 44:1084–1088.

[19] Reed L.J., Muench H., A simple method of estimating fifty per cent endpoint, Am. J. Hyg. (1938) 27:493–497.

[20] Reeth K.V., Brown I., Essen S., Pensaert M., Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs, Virus Res. (2004) 103:115–124.

[21] Smith G.J., Vijaykrishna D., Bahl J., Lycett S.J., Worobey M., Pybus O.G., et al., Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic, Nature (2009) 459:1122–1125.

[22] Spackman E., Senne D.A., Myers T.J., Bulaga L.L., Garber L.P., Perdue M.L., et al., Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes, J. Clin. Microbiol. (2002) 40:3256–3260.

[23] Sreta D., Kedkovid R., Tuamsang S., Kitikoon P., Thanawongnuwech R., Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial, Virol. J. (2009) 6:34.

[24] Van Reeth K., Labarque G., De Clercq S., Pensaert M., Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection, Vaccine (2001) 19:4479–4486.

[25] Webster R., Krauss S., World Health Organization Manual on Animal Influenza Diagnosis and Surveillance, in: W.H.O. (Ed.), Geneva, Switzerland, 2002.

[26] Webster R.G., Original antigenic sin in ferrets: the response to sequential infections with influenza viruses, J. Immunol. (1966) 97:177–183.

[27] Webster R.G., Bean W.J. Jr, Genetics of influenza virus, Annu. Rev. Genet. (1978) 12:415–431.

[28] Weingartl H.M., Berhane Y., Hisanaga T., Neufeld J., Kehler H., Emburry-Hyatt C., et al., Genetic and pathobiologic characterization of pandemic H1N1 2009 influenza viruses from a naturally infected swine herd, J. Virol. (2010) 84:2245–2256.

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Research article

Seroprevalence and risk factors of swine influenza in Spain

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ABSTRACT

Swine influenza is caused by type A influenza virus. Pigs can be infected by both avian and human influenza viruses; therefore, the influenza virus infection in pigs is considered an important public health concern. The aims of present study were to asses the seroprevalence of swine influenza subtypes in Spain and explore the risk factors associated with the spread of those infections. Serum samples from 2151 pigs of 98 randomly selected farms were analyzed by an indirect ELISA for detection of antibodies against nucleoprotein A of influenza viruses and by the hemagglutination inhibition (HI) using H1N1, H1N2 and H3N2 swine influenza viruses (SIV) as antigens. Data gathered in questionnaires filled for each farm were used to explore risk factors associated with swine influenza. For that purpose, data were analyzed using the generalized estimating equations method and, in parallel by means of a logistic regression. By ELISA, 92 farms (93.9%; $CI_{95\%}$: 89.1–98.7%) had at least one positive animal and, in total, 1340/2151 animals (62.3%; $CI_{95\%}$: 60.2–64.3%) were seropositive. A total of 1622 animals (75.4%; $CI_{95\%}$: 73.6–77.2%) were positive in at least one of the HI tests. Of the 98 farms, 91 (92.9%; $CI_{95\%}$: 87.7–98.1%) had H1N1 seropositive animals; 63 (64.3%; $CI_{95\%}$: 54.6–73.9%) had H1N2 seropositive pigs and 91 (92.9%; $CI_{95\%}$: 87.7–98.1%) were positive to H3N2. Mixed infections were detected in 88 farms (89.8; $CI_{95\%}$: 83.7–95.9%). Three risk factors were associated with seroprevalences of SIV: increased replacement rates in pregnancy units and, for fatteners, existence of open partitions between pens and uncontrolled entrance to the farm.

1. Introduction

Type A Influenza virus infections in swine are usually described as explosive outbreaks of acute respiratory disease similar in clinical course to human influenza (Olsen et al., 2006). A common assumption is that under certain circumstances related mostly to population dynamics, an epidemic outbreak of influenza in a pig farm may lead to the establishment of an endemic infection where SIV can circulate within the population without producing clearly noticeable outbreaks (Elbers et al., 1992)

The three commonest subtypes of SIV are H1N1, H1N2 and H3N2. In Europe, since 1979 the dominant H1N1 viruses have been 'avian-like' H1N1 viruses (Brown et al., 2000), while the most common H3N2 strains have been human-avian reassortants between hemagglutinin (HA) and neuraminidase (NA) genes from human-like swine H3N2 virus and the internal proteins from avian-like swine H1N1 virus (Castrucci et al., 1993; Campitelli et al., 1997). Finally, "triple reassortant" H1N2 contains HA from H1N1 human influenza virus, NA from swine H3N2 and internal proteins from avian-like swine H1N1 virus (Brown et al., 1998). However, the origin and nature of swine influenza

Abbreviation: ELISA, enzyme linked immunoassay.

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strains are different depending on their geographical location (Olsen et al., 2006). Thus, European H1N1, H3N2 and H1N2 SIV subtypes are genetically and antigenically different from those of North America (Kothalawala et al., 2006).

The HI, which is the classical serological test for detecting antibodies against SIV, is subtype-specific and is thought to be precise enough to discriminate between infections with different SIV subtypes provided that the viruses used as antigens in the test and the strains circulating in a region are antigenically close (Brown et al., 1998; Van Reeth et al., 2000, 2006).

The recent emergence of the new human pandemic A/H1N1 influenza virus, a triple human-swine-avian reassortant, was an example of the importance of pigs in the epidemiology of influenza. In Spain, as well as in other countries of Europe, non-random serological surveys revealed a high seroprevalence of H1N1, H1N2 and H3N2 in sows (Maldonado et al., 2006) and fattening pigs (Fraile et al., 2009) but knowledge on the risk factors for the introduction and spread of the infection in farms is scarce. The aims of the present study were: (i) to estimate the seroprevalence of H1N1, H1N2 and H3N2 subtypes in sows and fattening pigs and (ii) to evaluate the potential risk factors associated with seropositivity to the different SIV subtypes in pig farms from Spain.

2. Materials and methods

2.1. Study design

A cross-sectional survey (2008–2009) was designed to estimate the seroprevalence of different SIV subtypes. Sampling was restricted to Spanish pig census, which includes approximately 25,000,000 pigs located in about 94,000 herds. Approximately, 52% of the Spanish farms have an average stocking rate of more than 120 large animal units (Anonymous, 2010). Sampling was planned to be initially conducted in farms having more than 80 sows but finishing herds were excluded. With this restriction, 10 regions which accounted for 96.2% of the Spanish farms were included. Considering the previous data (Maldonado et al., 2006), an expected prevalence of 50% was assumed. Then, given the number of farms in Spain ($n > 10{,}000$), the precision (which was set at $\pm 10\%$) and the confidence level (which was set at 95%), the sample size (97 farms) was obtained.

For practical reasons, one hundred farms were considered, and the sampling was stratified by regions according to the proportion of farms in each one. Within each farm, 14 sows were randomly sampled – which was enough to detect antibodies against a given subtype if it was present in more than 20% of the sows – and, when available, samples from 10 finishing pigs were also obtained; enough to detect antibodies against a certain subtype if it affected more than 25% of fatteners. Ages of fattening pigs sampled ranged from 11 to 20 weeks, ensuring the absence of maternal antibodies and time enough for the exposure to influenza viruses. In total, 1400 sows and 849 fattening pigs were sampled. Unfortunately, sows from seven farms had been vaccinated against influenza in the previous 2 years, and as a consequence 98 sows samples were excluded from the analysis. In regions where the animal health authorities participated in the sampling (Andalusia, Castilla la Mancha, Catalonia, Extremadura, Galicia, and Navarra; representing 55% of the total Spanish census), farms were fully selected at random (random numbers applied to the registry reference number of the farm). In the other four regions, full random sampling was not always possible and, when needed, a convenience sampling, based on the availability of swine practitioners, was used to complete the selection of farms. At the end of the study a total of 85 farrow-to-finish and 13 farrow-to-weaning farms were surveyed. In total 1302 samples from sows collected in 93 pregnancy units (considering a unit as the whole group of sows of each farm), and 849 samples from fattening pigs collected in 85 fattening units (pigs between 11 and 20 weeks of age of each farm) were analysed. The geographical location of the farms is represented in Fig. 1.

2.2. Data collection: the questionnaire

Epidemiological data were gathered through an on-farm interview with the farmer. The questionnaire was designed using only "close-ended" questions to avoid ambiguities. Variables were grouped by topic: (a) general data: identification, location, herd size, presence of other domestic animal species (cats, dogs, birds or cattle), all-in/all-out (AIAO) management system and distance to the nearest farm; (b) production and health parameters: number of pigs per production phase (sows, weaners, fatteners and finishing pigs, boars), percentage of mortality in suckling pigs, weaners and fatteners, vaccination program and records of enteric and respiratory disease outbreaks during the last year; (c) facilities: floor type, floor material, presence of outdoor pens, type of waterers and feeders; (d) biosecurity: origin of replacement gilts

Fig. 1. Distribution of analysed farms (white dots) in Spain.

and boars, quarantine and adaptation periods for gilts, cleaning and disinfection methods and protocols, pest control programs (insects and rodents), use of bird-proof nets, presence and use of sanitary fords, sanitation of water, disposal of carcasses and presence of a perimeter fence.

The questionnaire was pre-tested in six farms that not were included in the present study. Pre-testing showed that the questionnaire took 30–45 min to complete, and resulted in minor changes in the questionnaire format. In all the farms included in the study, farmers agreed to answer the questionnaire.

2.3. Sample collection

Animals were bled using a sterile collection system (Vacutainer®, Becton-Dickinson, USA) and blood samples were transported to the laboratory under refrigeration (4 °C) within 24–48 h of sampling. Blood samples were centrifuged at $400 \times g$ for 15 min at 4 °C and sera were stored at −80 °C until further analysis.

2.4. Viruses

Three SIV strains were used: A/swine/Neth/Best/96 (avian-like H1N1), A/swine/Gent/7625/99 (H1N2) and A/swine/Neth/St-Oedenrode/96 (avian-like H3N2) (all of them provided by GD, Animal Service Center, Deventer, the Netherlands). Viral stocks were produced in Madin-Darby Canine Kidney (MDCK) cells cultured with added trypsin as usually reported for SIV (Dea et al., 1992; Herman et al., 2005). Cell culture supernatants were collected at approximately 75% of cytopathic effect, centrifuged and later titrated by hemagglutination using chicken erythrocytes. A single stock of each virus was used for all HI tests.

2.5. Diagnostic tests

Sera were examined by both an anti-nucleocapside type A indirect ELISA (Civtest Suis Influenza, Hipra Laboratories, Amer, Spain) and by hemagglutination inhibition tests for H1N1, H1N2 and H3N2. The ELISA was performed according to the manufacturer's instructions and samples were considered positive when sample-to-positive control ratio (S/P) was >0.2. The HI was performed according to standard procedures (OIE, 2008) and was standardized using four hemagglutinin units (HU) per well. To remove nonspecific inhibitors, sera were treated overnight (18 h) with receptor-destroying enzyme (RDE) from *Vibrio Cholerae* (Sigma–Aldrich, Madrid, Spain) and were then inactivated at 56 °C for 60 min. Subsequently, sera were adsorbed using a 50% chicken red blood cells (RBC) suspension at 4 °C. The starting dilution for testing sera was 1:20. Cut-off of HI was set to ≥1:20.

2.6. Statistical analysis

Prevalence of antibodies against SIV was estimated with the exact binomial confidence intervals of 95% (Martin et al., 1987). Agreement between ELISA and HI tests was tested by kappa statistics and discordance by McNemar's χ^2-test.

Two independent models were constructed to determine the risk factors associated with SIV, one for sows and another for fattening pigs, since variables that might be related to seroprevalence against SIV could differ according to the age group. Testing of association was performed in three steps. First, a general linear univariate analysis was performed for each virus subtype considering the farm as the experimental unit and seroprevalence of each subtype of SIV subtype as a dependent variable. All variables that yielded a p-value <0.25 in the analysis of covariance (ANCOVA) of the univariate analysis were considered as potential candidates for the multivariable model. This first analysis allowed to screening variables potentially associated with SIV seroprevalence, reducing thus the number of variables to be included in the multivariable model. With this strategy, manipulation of data included in the database was considerably reduced avoiding thus human errors. A subsequent analysis of collinearity (Spearman's correlation test) permitted the elimination of mutually linked variables. In such case ($p < 0.05$ and $R > 0.2$), only the variable more clearly linked (as decided bona fide) to swine influenza seropositivity was kept for further analysis (Table 1).

Finally, the effect of the remaining explanatory variables on the seroprevalence for a given subtype was introduced into an individual-level model, considering the individual as the experimental unit. A univariate model was performed considering the positivity/negativity status against each subtype as the dependent variable using generalized estimating equations (GEE), an extension of the logistic regression model for correlated responses (Liang and Zeger, 1986) that allows the control of biases produced by clustering of data (for example clustering in farms or regions). The number of seropositive animals was assumed to follow a binomial distribution and the farm and region to which each pig belonged were considered as random effects. The quasi-likelihood under independence model criterion (QIC) was used to determine the best model in terms of potential for explanation of the results (Pan, 2001). Once the best model was obtained, confounding was assessed. The model was evaluated by eliminating one variable (of those included in the final model) at a time until examining all potential combination of variables. Confounding was considered to occur when the removal of a variable modified the odds ratio (OR) of any of the remaining variables by 25% or more (Dohoo et al., 2003). Once the main-effects model was obtained, two-way interactions were generated and checked for statistical significance and biologic plausibility.

In parallel, a logistic regression model was used to analyze the relationship between potential explanatory variables and the presence of none or one *vs.* more than one SIV subtypes in the same farm. Regression analyses were performed independently for both sows and fatteners. Variables introduced in the initial regression were those selected after steps 1 and 2 as stated for GEE model. Logistic regression was performed using the backward stepwise method. SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Table 1
Variables included in the GEE model, and variables excluded due to collinearity.

Included variables	Variables removed due to collinearity
Sows model	
Presence of other livestock animals than pigs in the farm[a]	Number of nearby pig farms[b]
	Type of control system for ventilation in maternity[a]
	Type of control system for ventilation in pregnancy[a]
	Type of ventilation in maternity[a]
	Sow census[b]
Replacement rate value[b]	Controlled access to farm[a]
	Presence of pregnancy yards[a]
	Type of ventilation in maternity[a]
	Sow census[b]
Type of ventilation in pregnancy[a]	Type of control system for ventilation in maternity[a]
	Type of control system for ventilation in pregnancy[a]
	Type of ventilation in maternity[a]
	Sow census[b]
Fattening pigs model	
All in/all out performance in fatteners[a]	All in/all out performance in nursery[a]
Number of nearby pig farms[b]	
Controlled farm access[a]	
Partitions between pens in fattening units[a]	Partitions between pens in nursery[a]
Presence of bird-proof nets[a]	
Presence of other livestock animals than pigs in the farm[a]	
Type of control system for ventilation in fattening[a]	Type of ventilation in fattening[a]
	Type of control system in nursery[a]
	Type of control hitting in nursery[a]
Type of farm[a]	

[a] Corresponding to categorical variables.
[b] Corresponding to continuous variables.

3. Results

3.1. Seroprevalence of different SIV subtypes

Using ELISA, antibodies against SIV were found in animals of 92 out of 98 farms (93.9%; $CI_{95\%}$: 89.1–98.7%). The within-farm seroprevalence obtained for our sample ranged from 4.2% to 100% (median: 70.8%; lower quartile (Q1): 50%, upper quartile (Q3): 87.5%). In total, 1340/2151 pigs (62.3%; $CI_{95\%}$: 60.2–64.3%) were seropositive. Seroprevalence in sows was significantly higher ($p < 0.05$) than in fattening pigs (median = 92.9% *vs.* median = 40% respectively). Statistically significant differences were observed among regions ($p < 0.05$). Seroprevalence against SIV by regions ranged from 23.4% to 87.3%. Higher seroprevalences were detected in Catalonia (79.7%) and Aragon (77.7%), compared to Castilla y Leon (63.9%), Andalusia (58.6%), Castilla la Mancha (38.5%) and Extremadura (23.4%). Murcia, Valencia, Galicia and Navarra were excluded from this particular analysis because the number of farms sampled and the potential biases in the intentioned sampling.

Using the HI, all the farms had positive animals to at least one of the examined influenza subtypes. In 91/98 farms (92.9%; $CI_{95\%}$: 87.7–98.1%) H1N1 seropositive pigs were found; in 63/98 (64.3%; $CI_{95\%}$: 54.6–73.9%) pigs seropositive to H1N2 were detected and, 91 farms (92.9%; $CI_{95\%}$: 87.7–98.1%) had H3N2 seropositive pigs. Interestingly, positive animals to more than one subtype were found in 86/98 farms (87.8; $CI_{95\%}$: 81.2–94.4%) and serological evidence of co-circulation of all three SIV subtypes was found in 59/98 farms (60.0%; $CI_{95\%}$: 50.0–70.0%). For all subtypes, the proportion of seropositive sow units was significantly higher ($p < 0.05$) than that of fattening units for the same subtype. Within a given type of unit (sows or fattening), prevalence of positive units against H1N1 or H3N2 was higher ($p < 0.05$) than that of H1N2. The median within a farm seroprevalence, obtained in the sampled farms, for the different subtypes was: 58.3% for H1N1, 25.0% for H1N2, and 58.3% for H3N2. Of the 2151 sera tested, 1622 (75.4%; $CI_{95\%}$: 73.6–77.2%) were positive in at least one of the HI tests. The results are summarized in Tables 2 and 3.

Seroprevalence against the H3N2 subtype was significantly lower in gilts compared to older sows (parities 1–7 or more) ($p < 0.05$). This difference was not observed for the H1N1 or H1N2 subtypes. When HI titres for the different subtypes were compared, it was observed that the distribution of antibody titres of positive animals was different for the different subtypes. Thus, average anti-H3N2 titres were significantly higher ($p < 0.05$) (6.6 $\log_2$; $CI_{95\%}$: 6.5–6.7) than titres against either H1N1 or H1N2 (6.2 $\log_2$; $CI_{95\%}$: 6.2–6.3 and 6.2 $\log_2$; $CI_{95\%}$: 6.1–6.4, respectively). Likewise, H1N1 and H3N2 titres were significantly higher in sows (6.4 $\log_2$; $CI_{95\%}$: 6.3–6.5 and 6.8 $\log_2$; $CI_{95\%}$: 6.7–6.9, respectively) than in fattening pigs (5.7 $\log_2$; $CI_{95\%}$: 5.5–5.9; and 6.0 $\log_2$; $CI_{95\%}$: 5.8–6.2, respectively). There were no significant differences between those two age groups with regards to H1N2 subtype. Fig. 2 (sections a, b and c) shows the distribution of antibody titres against H1N1, H1N2 and H3N2.

According to the information provided by the owner, clinical disease compatible with swine influenza during the previous year was observed only in nine farms. Symptomatology was observed in fattening pigs in four of those farms and in the sows group in three of them. Only two farms presented clinical symptoms in both age groups.

Table 2
Number of herds ($n = 98$) with at least one sow and/or fattener with HI antibodies to one or more SIV subtypes.

Virus subtype(s)	Number and percentage of positive herds[a]					
	All herds		Pregnancy units		Fattening units	
	N	(%)	*N*	(%)	*N*	(%)
H1N1 (total)	91	92.9	88	94.6	51	60.0
H1N2 (total)	63	64.3	60	64.5	22	25.9
H3N2 (total)	91	92.9	82	88.2	51	60.0
H1N1only	5	5.1	6	6.5	12	14.1
H1N2only	0	0.0	1	1.1	1	1.2
H3N2only	5	5.1	4	4.3	14	16.5
H1N1 + H1N2	2	2.1	4	4.3	4	4.7
H1N1 + H3N2	25	25.5	23	24.7	20	23.5
H1N2 + H3N2	2	2.0	0	0.0	2	2.4
H1N1 + H1N2 + H3N2	59	60.2	55	59.1	15	17.6
Negative to the 3 subtypes	0	0.0	0	0.0	17	20.0

[a] Cut-off = 1:20.

3.1.1. Correlation between HI and ELISA

Of the 2151 examined pigs, 76.0% of the samples analyzed were classified either as positive or negative simultaneously by ELISA and at least one of the HI tests; 18.6% of the sera samples were positive by HI but negative by ELISA and 5.4% of the sera samples were negative by HI but positive by ELISA. Despite these differences, kappa statistics showed a fairly degree of agreement ($K = 0.45$; CI_{95}%: 0.41–0.49; $p < 0.05$). Forty-four farms had at least one animal negative to HI and positive to ELISA.

3.2. Risk factors associated with SIV seropositivity

3.2.1. GEE model

Separate statistical models were built for sows and fatteners. In the case of sows, three explanatory variables were selected for building up the GEE model: type of ventilation in pens for pregnant sows (natural *vs*. forced), presence of livestock animals of species other than pigs in the farm, and the replacement rate value. In the GEE model for fatteners eight explanatory variables were included: type of farm (farrow-to-finsh *vs*. multisite system) presence of livestock animals of species other than pigs in the farm, presence of bird-proof nets, type of control system for ventilation in fattening areas (manual *vs*. automatic), controlled farm access, type of separation between pens in fatteners (solid *vs*. open), number of nearby pig farms and AIAO management in fatteners (Table 1). No confounding factors or potentially relevant interactions between variables were observed in the finals models.

For sows, replacement rate was revealed as risk factor for H1N2 and H3N2 (OR = 1.02; $CI_{95\%}$: 1.01–1.04 and OR = 1.04; $CI_{95\%}$: 1.01–1.07, respectively). The OR obtained for the replacement rate is referred to each unit of increase on the replacement rate expressed in percentage units. On the other hand the presence of discontinuous partitions between pens was statistically related to seroprevalence against H1N2 in fattening units (OR = 5.31; $CI_{95\%}$: 1.59–17.70). Finally, an uncontrolled access to farm was resulted as a risk factor for seroprevalence against H1N1 (OR = 2.44; $CI_{95\%}$: 1.01–5.87). Other included variables in the model were not related to the seroprevalence of swine influenza. Table 4 shows the results of the GEE model in detail.

3.2.2. Logistic regression

Separate logistic regressions were built for sows and fattening pigs as well. For sows, the presence of antibodies against two or more SIV subtypes was significantly associated with higher replacement rates (OR = 1.07; $CI_{95\%}$: 1.02–1.12).

Table 3
Number of animals ($n = 2151$) and age groups (sows: $n = 1302$; fattening pigs: $n = 849$) with HI antibodies to one or more SIV subtypes.

Virus subtype(s)	Number and percentage of seropositive animals[a]					
	All animals		Sows		Fattening pigs	
	N	(%)	*N*	(%)	*N*	(%)
H1N1 (total)	1229	57.1	959	73.7	270	31.8
H1N2 (total)	444	20.6	352	27.0	92	10.8
H3N2 (total)	1170	54.4	874	67.1	296	34.9
H1N1only	338	15.7	220	16.9	118	13.9
H1N2only	31	1.5	22	1.7	9	1.1
H3N2only	293	13.6	139	10.7	154	18.1
H1N1 + H1N2	83	3.9	55	4.2	28	3.3
H1N1 + H3N2	547	25.4	460	35.3	87	10.2
H1N2 + H3N2	69	3.2	51	3.9	18	2.1
H1N1 + H1N2 + H3N2	261	12.1	224	17.2	37	4.4
Negative to the 3 subtypes	529	24.6	131	10.1	398	46.9

[a] Cut-off = 1:20.

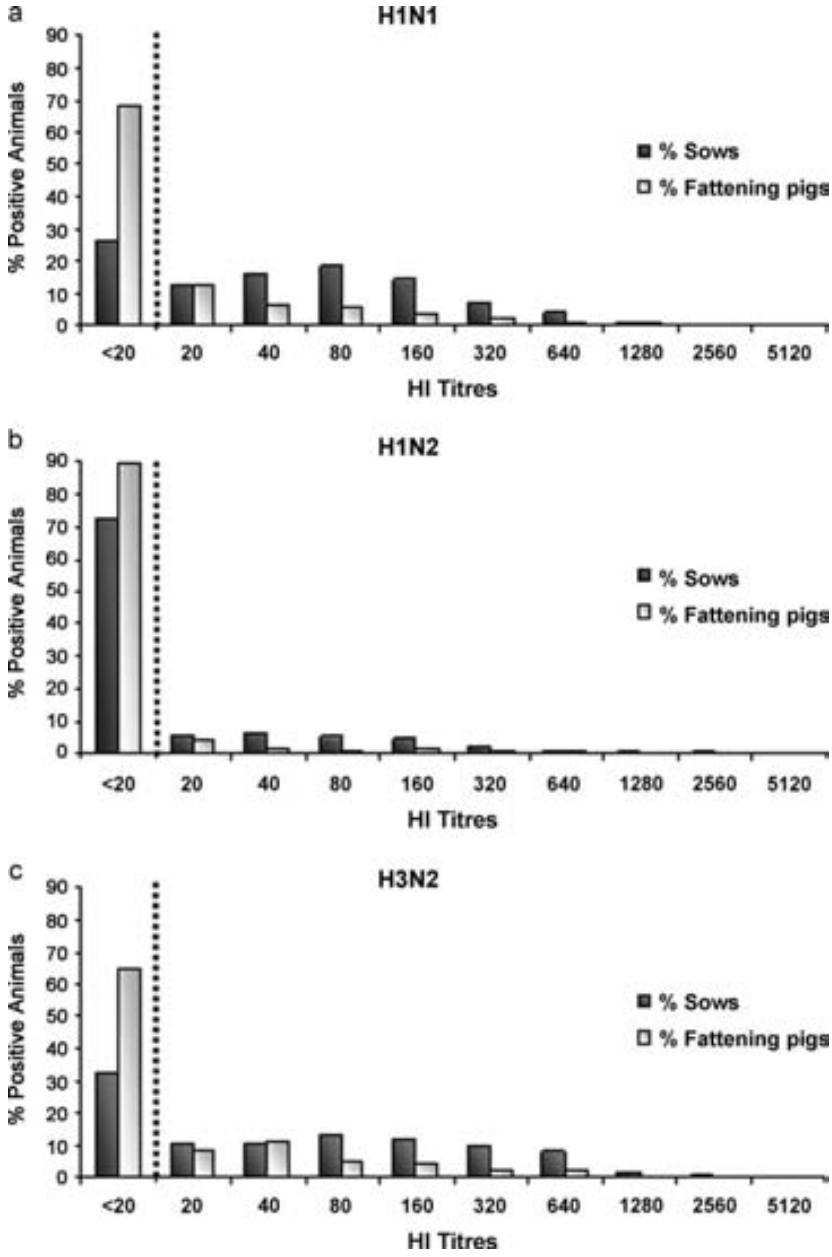

Fig. 2. Distribution of antibody titres against H1N1 (section a), H1N2 (section b) and H3N2 (section c). Dotted line represents the cut-off.

In the fattening pigs regression, two variables, absence of bird-proof nets (OR = 2.82; $CI_{95\%}$: 1.08–7.40) and uncontrolled access to farm (OR = 3.46; $CI_{95\%}$: 1.08–11.1), were significantly associated with the presence of antibodies against two or more SIV subtypes.

4. Discussion

Results of the present study show that swine influenza infection is widespread in pig farms of Spain. Seroprevalence in sows using HI (89.9%) was similar that reported by Van Reeth (2008) (79.2%) who analyzed sows from Spanish farms during the period 2002–2003. The seroprevalence obtained in our study are in the range of other European countries such as Germany (85.2%) or Belgium (94%) (Van Reeth et al., 2008) and indicate the ubiquitous nature of SIV in pig farms.

In the present study, the strains used for HI were from the Netherlands (H1N1 and H3N2) and Belgium (H1N2) and were at least 10 years old although belonged to the Eurasian clusters circulating in Europe. Unfortunately, the lack of Spanish strains or the lack of information on those available led us to that choice. The use of these strains and not the recent Spanish SIV strains may have resulted in an underestimation of the real seroprevalences. However, in a recent study (Martin-Valls et al., 2010), 94 sera samples

Table 4
Distribution of variables included in generalized estimating equations (GEE) model to determine the risk factors associated to swine influenza seroprevalence in pigs.

Variables	H1N1			H1N2			H3N2		
	OR	95% CI OR	*p*-Value	OR	95% CI OR	*p*-Value	OR	95% CI OR	*p*-Value
Sows									
Replacement rate value	0.99	0.98–1.01	0.563	1.02	1.01–1.04	0.007	1.04	1.01–1.07	0.005
Natural ventilation in pregnancy	0.64	0.35–1.16	0.144	0.96	0.52–1.78	0.898	0.81	0.43–1.51	0.505
Presence of livestock animals other than pigs	1.29	0.55–3.02	0.559	0.94	0.42–2.09	0.888	0.63	0.27–1.47	0.282
Fattening pigs									
Open partition between pens in fattening units	1.48	0.69–3.14	0.312	5.31	1.59–17.70	0.007	0.85	0.39–1.88	0.689
Uncontrolled access to farm	2.44	1.01–5.87	0.047	2.703	0.84–8.67	0.095	1.02	0.44–2.36	0.969
Absence of bird-proof nets	1.31	0.55–3.13	0.544	2.15	0.62–7.46	0.229	1.67	0.72–3.90	0.234
Manual control system of ventilation in fattening units	0.71	0.26–1.95	0.500	1.32	0.33–5.32	0.694	1.88	0.74–4.74	0.182
Multisite system	0.86	0.37–2.04	0.738	0.63	0.16–2.40	0.495	1.83	0.80–4.16	0.152
Not all in/all out performance in fatteners	1.56	0.72–3.38	0.257	0.81	0.24–2.69	0.729	0.74	0.35–1.59	0.443
Number of nearby pig farms	0.97	0.86–1.10	0.638	0.95	0.83–1.10	0.504	0.91	0.74–1.11	0.348
Presence of livestock animals other than pigs	2.28	0.89–5.84	0.086	1.74	0.40–7.50	0.458	0.78	0.29–2.12	0.630

CI, exact binomial confidence interval; OR, odds ratio.

were tested by HI using both a A/swine/Neth/Best/96 strain and a contemporary A/swine/Spain/53207/2004 H1N1 strain, resulting in 38.3% and 40.4% of positive sera, respectively. These data suggest that, at least for H1N1, results of the present study were not substantially affected by the use of Dutch or Belgian strains.

The fact that sows had higher seroprevalences than fattening pigs is in accordance with other studies (Jeong et al., 2007; Poljak et al., 2008) and agrees with the idea that the chances for infection with a ubiquitous agent increases with time. In this sense and, in agreement with other studies (Markowska-Daniel and Stankevicius, 2005; Poljak et al., 2008), H3N2 seropositivity also increased in higher-parity sows.

The introduction of the "triple reassortant" H1N2 virus into Spain is quite recent (1990s) in comparision with H1N1 and H3N2, which were already fully established (Brown, 2000). During these years, H1N2 subtype has had sufficient time to spread and become endemic in pigs of Spain. Interestingly, in this study the observed seroprevalence against H1N2 is significantly lower than that of H1N1 and H3N2. It could be speculated that these findings are difficult to interpret but the lower dissemination of H1N2 might be attributable to a lower ability of the H1N2 for being transmitted from pig to pig. Causes for such a lower spread should be investigated.

Eighty-eight farms were seropositive against more than one SIV subtype; at an individual level 44.6% of pigs also had antibodies against two or three subtypes. This is a relevant fact that indicates co-circulation of several subtypes. In the event of simultaneous circulation of different SIV viruses in the same farm, chances for the generation of reassortants increase. This is how H1N2 was probably originated (Brown et al., 1998).

Despite that all farms were positive to SIV, only 9% reported suffering or having suffered influenza-like disease during the previous year. With all caution that deserves clinical reports, this most probably reflects the subclinical nature of most SIV infections (Van Reeth, 2007; Simon-Grife et al., 2010).

When comparing ELISA and HI results by using the kappa value, agreement of qualitative results was only fair. In any case, if HI negative/ELISA positive results can be interpreted as the consequence of a lack of sensitivity of HI when strains used as a antigen in this test differs from currently circulating strains in a region or eventually, can also be attributed to other circulating subtypes different to avian-like H1N1, H1N2 or H3N2; ELISA negative/HI positive undoubtedly points to the lack of sensitivity of the ELISA. As a matter of fact, the study carried out by Maldonado (personal communication, 2007), demonstrates that H1N1-positive sera were detected better than H1N2- or H3N2-positive ones (S = 100%, 86.9% and 73.4%, respectively) when tested with the ELISA used in the present study.

Regarding factors associated with SIV presence and spread, in the present study, it was observed that increased replacement rates were associated with increased seropositivity of sows against H1N2 and H3N2, and with the proportion of farms seropositive against more than one SIV subtypes. These results suggest that the replacement rate could act both as a source of spread and as a source of introduction of influenzavirus on a farm through the introduction of replacement gilts that would act as a source of susceptible animals as well as a source of (subclinically) infected animals. In this sense, it has been postulated that SIV seroprevalence could decrease significantly and even that SIV could be eventually eliminated, if a temporary stop of the introduction of replacement animals and partial depopulation were performed (Torremorell et al., 2009).

Although AIAO management procedure is considered to provide a minimal disease exposure (Trapp et al., 2003) – including SIV – because of the segregation from non-contemporary animals, our results did not show any relationship between SIV seroprevalence and application of AIAO. This fact makes sense considering that SIV infection is presented as an acute process affecting the animals during a short period and not affecting a farm as a ubiquitous infectious agent.

Solid separations between pens are a measure frequently used to prevent pathogen transmission between pens. Dupont et al. (2009) showed that the dissemination of air-borne pathogens was facilitated by close contact between animals from different pens. In our case, solid pen separations also seemed to be related with lower prevalences against the subtype in fattening pigs.

In our study, uncontrolled access to the farm was associated with higher seroprevalence against H1N1 and with the proportion of farms seropositive against more than one subtype. As some studies have shown, people and vehicles can act as a source of introduction of influenza-virus in a herd (Alexander, 2007). For this reason, limiting visits is an important biosecurity measure in order to reduce the risk of infection.

Our results have revealed an association between lack of bird-proof nets and presence of antibodies against two or more subtypes of swine influenza in a farm. This result makes sense considering that birds, especially waterfowl, can transmit influenza virus to pigs either as virus shedding animals or as mechanical carrier (Alexander, 2007).

In summary, the present study indicated a widespread exposure to SIV in pig farms of Spain. Three risk factors were associated with high seroprevalences of SIV: increased replacement rates, open partitions between pens of fattening pigs and uncontrolled access to the farm. Therefore, advisable measures for reducing SIV prevalence in pig farms would be a careful check of replacement gilts for SIV shedding before entering them in the stock of sows, design pens with solid partitions to minimize contacts between animals from different pens and properly implemented biosecurity measures that minimize the contact of people, vehicles and wild animals with the farm animals.

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References

Alexander, D.J., 2007. An overview of the epidemiology of avian influenza. Vaccine 25, 5637–5644.

Anonymous, 2010. El sector de la carne de cerdos en cifras. Ministry of Environment and Rural and Marine Affairs. http://www.mapa.es/app/SCP/documentos/INDICADORES%20ECONOMICOS%20CARNE%20-DE%20CERDO%202009.pdf.

Brown, I.H., Harris, P.A., McCauley, J.W., Alexander, D.J., 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of a novel genotype. J. Gen. Virol. 79, 2947–2955.

Brown, I.H., 2000. The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. 74, 29–46.

Campitelli, L., Donatelli, I., Foni, E., Castrucci, M.R., Fabiani, C., Kawaoka, Y., Krauss, S., Webster, R.G., 1997. Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology 232, 310–318.

Castrucci, M.R., Donatelli, I., Sidoli, L., Barigazzi, G., Kawaoka, Y., Webster, R.G., 1993. Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology 193, 503–506.

Dea, S., Bilodeau, R., Sauvageau, R., Montpetit, C., Martineau, G.P., 1992. Antigenic variant of swine influenza virus causing proliferative and necrotizing pneumonia in pigs. J. Vet. Diagn. Invest. 4, 380–392.

Dohoo, I., Martin, W., Stryhn, H., 2003. Veterinary Epidemiology Research. AVC, Charlottetown (PEI), pp. 335–372.

Dupont, K., Hjulsager, C.K., Kristensen, C.S., Baekbo, P., Larsen, L.E., 2009. Transmission of different variants of PCV2 and viral dynamics in a research facility with pigs mingled from PMWS-affected herds and non-affected herds. Vet. Microbiol. 139, 219–226.

Elbers, A.R., Tielen, M.J., Cromwijk, W.A., Hunneman, W.A., 1992. Variation in seropositivity for some respiratory disease agents in finishing pigs: epidemiological studies on some health parameters and farm and management conditions in the herds. Vet. Quart. 14, 8–13.

Fraile, L., Alegre, A., López-Jiménez, R., Nofrarías, M., Segalés, J., 2009. Risk factors associated with pleuritis and cranio-ventral pulmonary consolidation in slaughter-aged pigs. Vet. J., doi:10.1016/j.tvjl.2009.03.029.

Herman, M., Haugerud, S., Malik, Y.S., Goyal, S.M., 2005. Improved in vitro cultivation of swine influenza virus. Int. J. Appl. Res. Vet. M. 3, 124–128.

Jeong, K., Park, Y., Jin, W., Hee Han, J., 2007. Seroprevalence of swine influenza and porcine and reproductive and respiratory syndrome in Korea. Korean J. Vet. Serv. 30, 197–203.

Kothalawala, H., Toussaint, M.J.M., Gruys, E., 2006. An overview of swine influenza. Vet. Quart. 28, 45–53.

Liang, K.Y., Zeger, S.L., 1986. Longitudinal data analysis using generalized linear models. Biometrics 73, 13–22.

Maldonado, J., Van Reeth, K., Riera, P., Sitjà, M., Saubi, N., Espuña, E., Artigas, C., 2006. Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet. J. 172, 377–381.

Markowska-Daniel, I., Stankevicius, A., 2005. Seroprevalence of antibodies against swine influenza virus in pigs of different age. Bull. Vet. I. Pulawy 49, 3–7.

Martin, S., Meek, A., Willeberg, P., 1987. Veterinary Epidemiology: Principles and Methods. Iowa State University Press, Ames, IA.

Martin-Valls, G.E., Simon-Grife, M., Nuria Busquets, N., Ivan Diaz, I., Martin, M., Casal, J., Mateu, E., 2010. Analysis of cross reactivity of porcine sera in the haemagglutination inhibition assay using swine H1N1 or the new pandemic H1N1 influenza virus. In: Proceedings of 21st International Pig Veterinary Society (IPVS). pp. 590.

OIE (World Organization for Animal Health), 2008. Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris, France, pp. 1130–1131.

Olsen, C.W., Brown, I.H., Easterday, B.C., Van Reeth, K., 2006. Swine influenza. In: Straw, B.E., Zimmerman, J.J., d'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine. Blackwell Publishing, Oxford, UK, pp. 469–482.

Pan, W., 2001. Akaike's information criterion in generalized estimating equations. Biometrics 57, 120–125.

Poljak, Z., Dewey, C.E., Martin, S.W., Christensen, J., Carman, S., Friendship, R.M., 2008. Prevalence of and risk factors for influenza in southern Ontario swine herds in 2001 and 2003. Can. J. Vet. Res. 72, 7–17.

Simon-Grife, M., Martin-Valls, G.E., Vilar, M.J., Mora, M., Martin, M., Busquets, N., Mateu, E., Casal, J., 2010. Longitudinal study of swine influenza virus infection in a farrow-to-finish farm. In: Proceedings of 21st International Pig Veterinary Society (IPVS). pp. 77.

Torremorell, M., Juarez, A., Chavez, E., Yescas, J., Doporto, J.M., Gramer, M., 2009. Procedures to eliminate H3N2 swine influenza virus from a pig herd. Vet. Rec. 165, 74–77.

Trapp, S.A., Hill, B.E., Hankins, S.L., Schinckel, A.P., Richert, B.T., 2003. The Effect of Rearing Environment on Two Ractopamine Use Programs. Swine Research Report, Purdue University. pp. 5–17.

Van Reeth, K., Brown, I.H., Pensaert, M., 2000. Isolations of H1N2 influenza A virus from pigs in Belgium. Vet. Rec. 146, 588–589.

Van Reeth, K., Labarque, G., Pensaert, M., 2006. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 Swine influenza viruses. Viral Immunol. 19, 373–382.

Van Reeth, K., 2007. Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet. Res. 38, 243–260.

Van Reeth, K., Brown, I.H., Dürrwald, R., Foni, E., Labarque, G., Lenihan, P., Maldonado, J., Markowska-Daniel, I., Pensaert, M., Pospisil, Z., Koch, G., 2008. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002–2003. Influenza Other. Respi. Viruses 2, 99–105.

VETERINARY RESEARCH

RESEARCH Open Access

Swine influenza virus infection dynamics in two pig farms; results of a longitudinal assessment

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Abstract

In order to assess the dynamics of influenza virus infection in pigs, serological and virological follow-ups were conducted in two whole batches of pigs from two different farms (F1 and F2), from 3 weeks of age until market age. Anti-swine influenza virus (SIV) antibodies (measured by ELISA and hemagglutination inhibition) and nasal virus shedding (measured by RRT-PCR and isolation in embryonated chicken eggs and MDCK cells) were carried out periodically. SIV isolates were subtyped and hemagglutinin and neuraminidase genes were partially sequenced and analyzed phylogenetically. In F1, four waves of viral circulation were detected, and globally, 62/121 pigs (51.2%) were positive by RRT-PCR at least once. All F1 isolates corresponded to H1N1 subtype although hemagglutination inhibition results also revealed the presence of antibodies against H3N2. The first viral wave took place in the presence of colostral-derived antibodies. Nine pigs were positive in two non-consecutive sampling weeks, with two of the animals being positive with the same isolate. Phylogenetic analyses showed that different H1N1 variants circulated in that farm. In F2, only one isolate, H1N2, was detected and all infections were concentrated in a very short period of time, as assumed for a classic influenza outbreak. These findings led us to propose that influenza virus infection in pigs might present different patterns, from an epidemic outbreak to an endemic form with different waves of infections with a lower incidence.

Introduction

Swine influenza (SI) is caused by *Influenzavirus* type A. In pigs, the disease is reported to be very similar to human influenza: high fever (40.5-41.7°C), lethargy, coughing and laboured breathing, anorexia and weight loss [1,2]. Sneezing, conjunctivitis, nasal discharge and abortions may also be observed [2]. SI-associated gross lung lesions observed in pigs are mainly those of a viral pneumonia, and are characterized by a broncho-intersticial pneumonia (BIP) [3].

Pigs can be infected with avian, swine and human influenza A viruses, and for that reason, swine has been classically proposed to be the mixing vessel where reassortant influenza strains can arise [4,5]. Although this "mixing vessel" concept is now narrower than some years ago, the recent emergence of a human pandemic influenza A virus harbouring genes thought to be originally of swine origin stressed again the interest in the epidemiology of influenza in pigs [6].

Traditionally, the entry of a new influenza virus in a herd was considered to cause the appearance of the clinical signs in a high percentage of animals [3]. However, Swine Influenza Virus (SIV) seems to be more widespread in pigs than previously thought [7]. Besides, the fact that the incidence of confirmed clinical outbreaks of influenza in pigs is relatively low suggests that in most cases, infections are of a subclinical nature [8-10]. On the other hand, although the persistence of SIV activity after an acute outbreak has been described [11], and the existence of endemically infected herds has been postulated [3,7], the establishment of endemic infections in swine herds has never been demonstrated. Beyond the picture of a classic epidemic outbreak, there is very little knowledge about the dynamics of SIV within pig farms.

The aim of the present study was to assess the dynamics of influenza virus infection in pig farms, through serological and virological follow-ups of two whole batches of pigs from two commercial farrow-to-finish pig farms.

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Materials and methods

Ethics statement

This study was carried out in strict accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union. All experimental procedures were conducted in accordance with the recommendations approved by the Animal and Human Ethics experimentation Committee (CEEAH) of the Universitat Autònoma de Barcelona, that ensures the protection and welfare of the animals used in research, in agreement with the current European Union Legislation.

Selection of herds

Selection criteria were: a previous knowledge of the serological status of the farm; absence of SIV vaccination and, the willingness of the owner to cooperate in such a long-term survey. In a previous study conducted between 2008 and 2009 [10], SIV seroprevalence in sows and fattening pigs was assessed in 98 Spanish farms, of which two farrow-to-finish farms located in Catalonia (NE Spain) were selected for this study. Farm 1 (F1) was a 300-sows farrow to finish swine farm located in a high pig density area, while Farm 2 (F2) was a farrow-to-finish operation of 90 sows located in a region of low pig density.

Before the start of the present study, 10 gilts, 20 sows and 20 pigs of each age (3, 6, 9, 12, 15 and 20 weeks) were tested serologically (ELISA, CIVTEST-Suis, *Laboratorios* Hipra SA, Amer, Spain) to re-confirm the SIV status of the two farms.

Farm facilities and biosecurity practices

Farm 1 (F1)

In F1, dry and pregnant sows were housed in stalls. Piglets remained with the sows until the 4th week of age, when they were moved to nursery facilities. In nurseries, pigs were housed in three separated and independent outdoor modules, with no temperature or ventilation control systems. At 10 weeks of age, pigs were transferred into two independent buildings for fatteners. Finally, at 16 weeks of age, pigs were moved to finishing facilities, where they remained until sent to the slaughterhouse at 24 weeks of age. Fattening and finishing facilities had natural ventilation and open separations between pens.

The management practices in this farm included the use of all in/all out (AIAO) production in the nursery, but not in the growing-finishing facilities. The main biosecurity measures included the application of quarantine to the replacement stock, presence of a perimetral fence around the farm and the application of a rodent control. However, it is worth noting that biosecurity measures such as presence of bird-proof nets in windows or a changing room with showers were not present in F1.

Farm 2 (F2)

In F2, sows were housed in individual stalls during gestation. Piglets were transferred to nurseries at 4 weeks of age, where they remained until the 11th week of age. Then, pigs were transferred to pens for fatteners where they were housed until sent to the slaughterhouse. In this farm, pigs were sent to the slaughterhouse in two sittings, at 21 weeks of age (18 pigs) and at 22 weeks of age (57 pigs) depending on their weight. Nurseries were equipped with a forced ventilation system, while fattening units had natural ventilation; both facilities had open separations between pens. Animals were managed on an AIAO basis until reaching market weight.

In this farm quarantine practices were not applied before introduction of replacement stock. Biosecurity measures applied in F2 included the presence of a perimeter fence around the farm, as well as a control program for rodents. Most of the biosecurity measures aimed at reducing disease introduction from people, such as presence of changing room with showers or clothes and boots provided by the farm, were not applied in F2. It is important to note, however, that only the owner and the veterinarian had direct contact with pigs from this farm. Bird-proof nets in windows were not present in F2.

It is noteworthy that in both herds, the distribution of pigs in the different pens was at random and, in consequence, pigs from different litters or previous pens could be mixed.

Sampling and data collection

Every time the farm was visited, pigs were clinically inspected and the distribution of pigs per pen was recorded. Between visits, farmers were asked to record any abnormal event or presence of clinical signs. In F1, the follow-up started in July 2009 and ended in December 2009, while in F2 animals were followed between January 2010 and June 2010.

In each herd, a whole batch of 3-weeks-old piglets (all piglets of that age present at the farm) was selected for the study, and animals were identified (ear-tagged) individually. In total, 121 pigs (11 litters) and 79 pigs (8 litters) were sampled in F1 and F2, respectively. Sera from sows were also collected.

Pigs were followed from 3 weeks of age until sent to the slaughterhouse. Nasal swabs of sterile cotton (ref. 300251, Deltalab, Barcelona, Spain) and serum (jugular venipuncture) were taken periodically. After collection, nasal swabs were placed with vigorous shaking in 1 mL of phosphate-buffered saline plus 10% glycerol and antibiotics (1000 units/mL penicillin and 1000 units/mL of streptomycin) immediately after collection and stored at -80°C until tested.

Initially sampling was planned to be carried out weekly between the 3rd and the 13th week of age, and afterwards, at 14 weeks (only nasal swabs that week), 15, 17, 20 and 24 weeks of age. However, F2 was sampled weekly between the 3rd and 21st or 22nd week of age because of the failure to detect SIV during the first weeks of sampling.

Serology

Sera were examined initially by a commercial ELISA directed to detect antibodies against type A influenza nucleocapside (ELISA, CIVTEST-Suis, *Laboratorios* Hipra SA, Amer, Spain). Also, presence of anti-influenza antibodies in nasal swab suspensions of 3-week-old piglets was assessed by means of a competition ELISA nucleoprotein (NP) using the (ID Screen® Influenza A Antibody Competition, ID VET, Montpellier, France). In this case, nasal swab suspensions were examined at a 1/2 dilution, and known positive and negative samples were used as test controls [12].

Sera collected from sows and finishers (17, 20 and 24 weeks of age in F1 and 17 and 20 weeks of age in F2) were analyzed by the hemagglutination inhibition (HI) assay performed according to standard procedures [2] with 4 hemagglutinin units (HU) per well. Cut-off of HI was set to ≥ 1:20 as reported before [9,13]. Three SIV strains that belonged to Eurasian clusters circulating in Europe were used for HI: A/swine/Neth/Best/96 (avian-like H1N1), A/swine/Gent/7625/99 (triple reassortant H1N2), A/swine/Neth/St Oedenrode/96 (avian-like H3N2) (all of them provided by GD, Animal Service Center, Deventer, The Netherlands). Viral stocks were produced in MDCK cells and a single viral batch was used for all HI tests. For those pigs found to be viral shedders more than once, sera were also examined in HI using the isolate previously retrieved from those pigs.

Nasal shedding of SIV

Detection of SIV in nasal swabs was assessed by means of a Taq-Man real time reverse transcriptase/polymerase chain reaction (RRT-PCR) directed to the detection of the *M* gene of influenza A viruses [12] performed in a Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). Viral RNA was extracted with QIAamp viral kit (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer.

All SIV RRT-PCR positive samples were inoculated into specific pathogen free (SPF) embryonated chicken eggs (ECE) in order to attempt SIV isolation [2]. Briefly, nasal swab suspensions were centrifuged, and 100 μL of the supernatant were inoculated into the allantoic cavity of 9-11-day-old ECE. Allantoic fluid was harvested 3 days after inoculation, and SIV presence was detected by hemagglutination. Non hemagglutinating allantoic fluids in the first passage were inoculated again in ECE. If the allantoic fluid was negative after the second passage, nasal swab suspensions were inoculated in Madin-Darby Canine Kidney (MDCK) cells cultured with added trypsin (5 μg/mL). Cell culture supernatants were collected at approximately 75% of cytopathic effect, centrifuged and later tested by RRTPCR. Samples that did not produce cytophatic effect were subjected to a second passage in MDCK cells. Samples were discarded if negative after the second passage.

Subtyping and phylogenetic analysis

Viral isolates were subtyped by multiplex RT-PCR described by Chiapponi et al. [14] for the detection of H1, H3, N1 and N2 genes and sequenced using Big Dye Terminator v3.1 cycle sequencing Kit (Applied Biosystems, Madrid, Spain) and the ABI Prism 3100 sequence analyser (PerkinElmer, Madrid, Spain). The isolates that could not be amplified and sequenced using the methodology cited were analyzed with different primers as an alternative to subtype these strains. Moreover, these primers were also used to sequencing a long fragment of the HA (1676 bp) and NA (1349 bp) genes of 26 isolates randomly selected from the isolates obtained in the different weeks of sampling. Finally, the internal genes from one isolate of 3, 7 and 13 weeks of age were partially sequenced. The sequences of the primer set used to amplify each segment are shown in Additional file 1: Table S1.

Comparison with published sequences (available at NCBI) was carried out using CLUSTAL W, and the unrooted phylogenetic trees were generated by the distance-based neighbor-joining method (1000 iterations) using MEGA 4.1. Relevant and not redundant HA and NA sequences from different countries, species and years were included in the phylogenetic analysis. GenBank accession numbers for all sequences used in this study are listed in Additional file 2: Table S2.

Statistical analysis

SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

Farm 1

Antibodies against influenza A viruses

Figure 1 summarizes the evolution of seroprevalence. At 3 weeks of age, seroprevalence by ELISA was 56.2% (68/121), and declined to a 10.3% (12/116) at 6 weeks of age. Afterwards and until the 15th week of age, seroprevalence varied between 18.3 and 44.9%. Almost all the 15-week-old seronegative animals seroconverted afterwards. Anti-NP antibodies were not detected in nasal swabs from animals of 3 weeks of age.

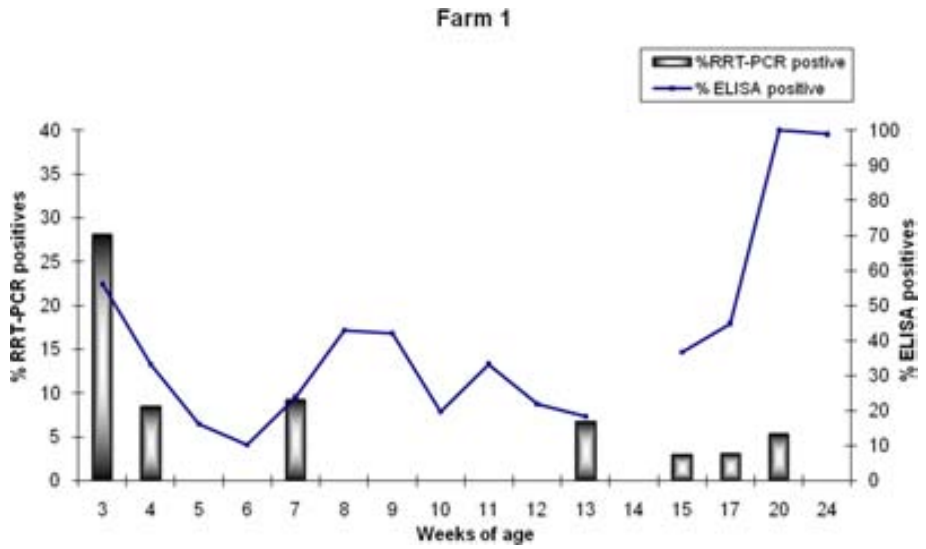

Figure 1 Seroprevalence and incidence of SIV in Farm 1. Antibodies against SIV were analyzed by ELISA (line) and nasal shedders were determined by RRT-PCR (bars) at each sampling time.

Table 1 shows the proportion of pigs positive by the hemagglutination inhibition test (≥ 1:20) to H1N1, H1N2 and H3N2 subtypes at 17, 20 and 24 weeks of age. In all cases H1N1 and H3N2 seropositive animals were detected, but no antibodies against H1N2 were found. When the sera from pigs that were positive by RRT-PCR more than once were analyzed by HI test using the strain previously isolated from them as antigen, only 4/9 showed titres ≥ 1:20 at the time of the second detection. These sera with antibodies against the strain isolated in the farm belonged to animals of 7 weeks of age (1/9), 13 weeks of age (2/9) and 15 weeks of age (1/9), while sera without antibodies were from pigs of 7 weeks of age (2/9), 13 weeks of age (2/9) and 24 weeks of age (1/9).

Regarding sows, all were seropositive for H3N2 and 9/11 had antibodies against H1N1.

Viral shedding

Using RRT-PCR, 62 animals (51.2%) were positive at least once. As shown in Figure 1, four waves of viral circulation were observed: in farrowing units (at 3 and 4 weeks of age), in nurseries (at 7 weeks of age), in fattening units (at 13 weeks of age), and in finishing units (at 15, 17, and 20 weeks of age), with incidences ranging from 3.0 to 28.1%. Interestingly, nine animals (7.4%) were positive in at least two different occasions.

S IV was isolated either in ECE or MDCK in at least one sample from all weeks, in exception of the 17 weeks of age, that had RRT-PCR positive nasal swabs; namely 42 isolates (58.3% of the positive samples). Isolations were obtained from animals with ages of 3 weeks (19 isolates), 4 (8 isolates), 7 (8 isolates), 13 (4 isolates), 15 (2 isolates), and 20 (1 isolates). Of the 42 isolates, 40 were partially sequenced for hemagglutinin (HA), 34 for neuraminidase (NA) and 34 were subtyped for both HA and NA. Two isolates could not be amplified and sequenced neither HA nor NA.

The similarity of the complete nucleotide sequences of the HA (1676 bp) and NA (1349 bp) from the 26 isolates analyzed ranged from 99.2% to 100% and from 99.4% to 100% for HA and NA genes, respectively. On the other hand, analysis of the nucleotide sequences of the internal genes of three isolates showed a high similarity; from 99.7% to 99.8% for polymerase gene 2 (PB2) and polymerase gene 1 (PB1), from 99.6 to 99.9 for polymerase gene A

Table 1 Seroprevalence of antibodies against H1N1, H1N2 and H3N2 in Farm 1 obtained by HI test

	Percentage of seropositive animals*					
Age (weeks)	H1N1		H1N2		H3N2	
	Seroprevalence (%)	95% CI	Seroprevalence (%)	95% CI	Seroprevalence (%)	95% CI
17	83.7	74.5-90.1	0	0-4.7	80.6	71.1-87.6
20	51.6	41.2-61.9	0	0-4.8	96.8	90.5-99.1
24	53.2	42.7-63.5	0	0-4.9	77.7	67.7-85.3

* Cut-off = ≥ 1:20

CI, exact binomial confidence interval

(PA), from 99.8 to 100% for nucleoprotein gene (NP), of 100% for matrix gene (MA) and from 99.2 to 99.6 for non-structural gene (NS).

The phylogenetic analysis of the HA gene showed two distinct clusters designated as I and II (Figure 2). Cluster I was made up of isolates belonging mainly to farrowing area (3 and 4 weeks of age) and to fattening area (13 and 15 weeks of age). In contrast, cluster II was composed of isolates belonging to farrowing area (3 and 4 weeks of age), weaning area (7 weeks of age) and finishing area (20 weeks of age). The NA phylogenetic analysis showed at least 4 different clusters designated as III, IV, V, VI (Figure 2). Cluster III and V included isolates belonging mainly to farrowing area and to fattening area. Cluster IV was made up of isolates from pigs of 3 and 4 weeks of age. Finally, cluster VI was composed of isolates belonging to farrowing area, weaning area and finishing area.

Interestingly, nine animals were found to be positive by RRT-PCR at two sampling times. The SIV could be isolated at the two sampling points only from three out of the nine positive animals (designed as 8, 103 and 109). SIV isolated from the animals 8 and 103 were grouped in cluster II and in cluster I, respectively. In contrast, the distinct isolates obtained from pig 109 were classified in cluster I (isolate obtained at 4 weeks of age) and in cluster II (isolate obtained at 20 weeks of age).

All the isolates from this farm were subtyped as H1N1 and grouped with other European H1N1 SIV of an avian-like clade (Figure 3).

When the distribution of RRT-PCR positive pigs was examined, it was shown that in farrowing units 10/11 litters had at least one positive piglet at 3 weeks of age, but this proportion decreased to 4/11 one week later. In nurseries, all positive pigs of 7 weeks of age were housed in the same pen. In the other two pens viral shedders were not found throughout the whole 6 week period for which they were allocated there. Virus positive animals at 13th and 15th weeks of age were detected in two pens (4 and 6). Finally, for finishers 6/8 pens had at least one positive animal at 17 or 20 weeks of age. The distribution of positive animals throughout the study is represented in Figure 4.

Clinical signs and gross lesions

Only a low percentage of pigs (≤ 4%) showed mild influenza-like signs throughout the study, but mortality rates reached 20.3%. When possible, the necropsy of these

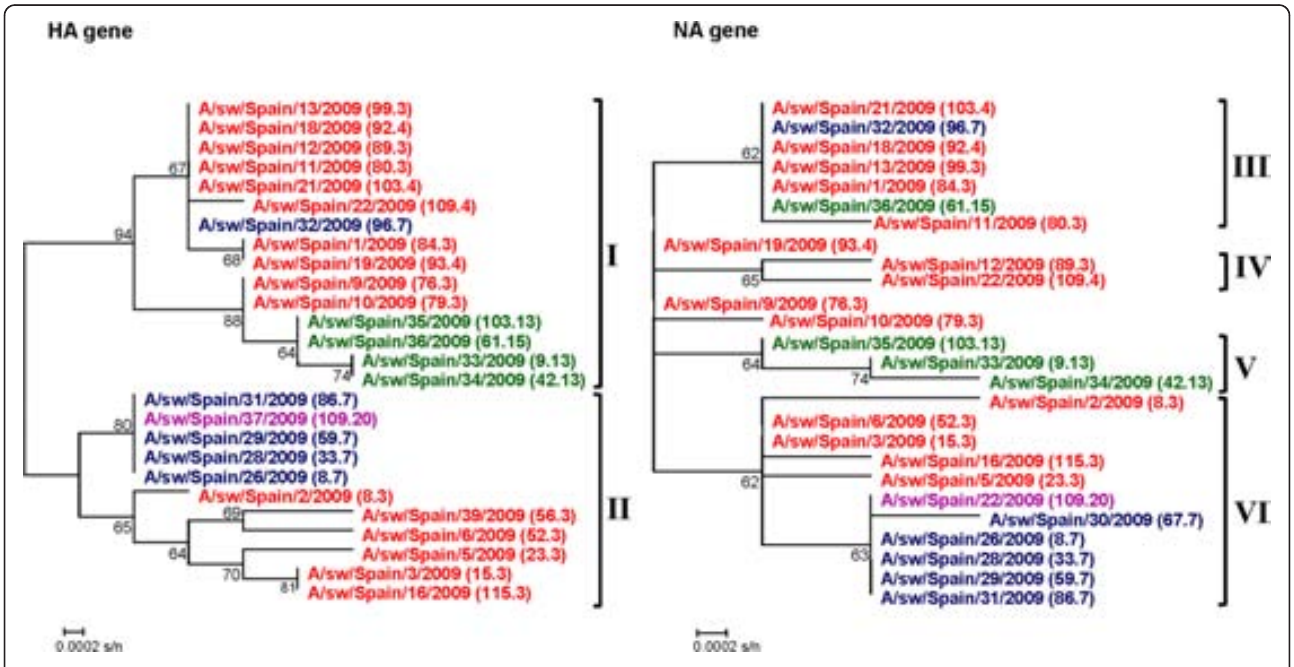

Figure 2 Phylogenetic tree of the HA1 and NA1 genes of SIV isolates from Farm 1. The accession numbers of sequence data of influenza virus were deposited in GenBank under the accession numbers [GenBank: JF960169, JF960172 - JF960174, JF960176, JF960177, JF960180 - JF960184, JF960187, JF960189, JF960190, JF960192, JF960193, JF960197, JF960199 - JF960208, JQ301920 - JQ301944]. The strains are indicated by the isolate name and between brackets by the animal number following by the age of animals in which the virus was isolated (in weeks). Strains given in red correspond to available isolates from pigs of 3 and 4 weeks of age. Strains given in blue correspond to available isolates from pigs of 7 weeks of age. Strains given in green correspond to available isolates from pigs of 13 and 15 weeks of age. Strains given in purple correspond to available isolates from pigs of 20 weeks of age. Abbreviations: cluster I, I; cluster II, II; cluster III, III; cluster IV, IV; cluster V, V; and cluster VI, VI.

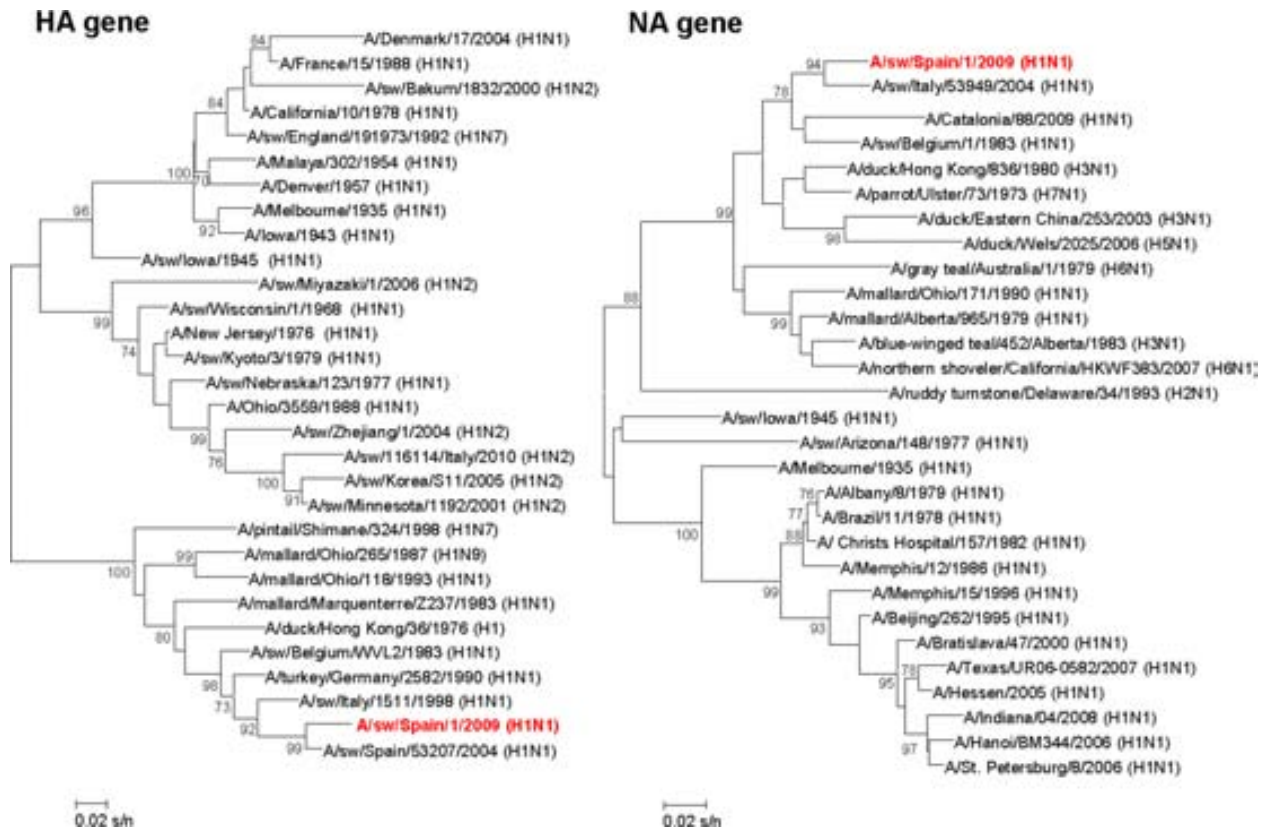

Figure 3 Phylogenetic analysis for HA and NA of SIV isolates retrieved in Farm 1. The strains isolated in our study are highlighted in red. Unrooted bootstrapped neighbour-joining trees of nucleotide sequences of hemagglutinin and neuraminidase. Bootstrap values, calculated on 1000 replicated trees, are shown if ≥ 70 percent. Scale bars indicate substitutions per site. The accession numbers of sequence data are provided in Additional file 2: Table S2.

animals was performed but only two of the nine necropsied pigs presented lesions compatible with BIP. Besides this, two pigs had fibrous/fibrinous polyserositis, and another pig had pulmonary haemorrhage and necrosis compatible with *Actinobacillus pleuropneumoniae*. Taken together, the lesions observed seem to indicate that this farm was affected by a porcine respiratory disease complex.

No viral RNA was detected by RRT-PCR in the lungs of any of the necropsied pigs.

Farm 2

Antibodies against influenza A viruses

In the first sampling (3 weeks of age) seroprevalence by ELISA was 93.7% (74/79). Then, seroprevalence decreased, and by 11 weeks of age all pigs were seronegative and remained so until 17 weeks of age. Seroconversions started at 18 weeks of age and in the last sampling (22 weeks of age) 84.2% (48/57) of animals were seropositive. Figure 5 summarizes these results. Anti-NP antibodies were not detected in nasal swabs from animals of 3 weeks of age.

Using HI, at 20 weeks of age, 92% (69/75) of the pigs were seropositive against H1N2, but no antibodies against H1N1 or H3N2 were found. Regarding sows, 4/8 were positive for H1N2 and 7/8 and 8/8 were seropositive to H1N1 and H3N2, respectively.

Viral shedding

Fifty-seven pigs (72.2%) were positive by RRT-PCR; of them, one was positive at 6 weeks of age and all the others at 17 weeks of age.

No viral isolation was obtained from nasal swabs in ECE; while, 53 isolates were obtained in MDCK cells, representing 92.9% of RRT-PCR positive samples. Unfortunately, SIV could not be isolated from the only RRT-PCR positive nasal swab at 6 weeks. For further characterization seven isolates were randomly selected, and their HA and NA genes were partially sequenced, corresponding to H1N2 SIV. Analysis of the nucleotide sequences of the HA (382 bp) and NA (702 bp) showed a similarity ranging from 99.4% to 100% and from 99.8 to 100%, for HA and NA genes respectively, indicating the presence of just one viral strain. The H1 sequences were phylogenetically related

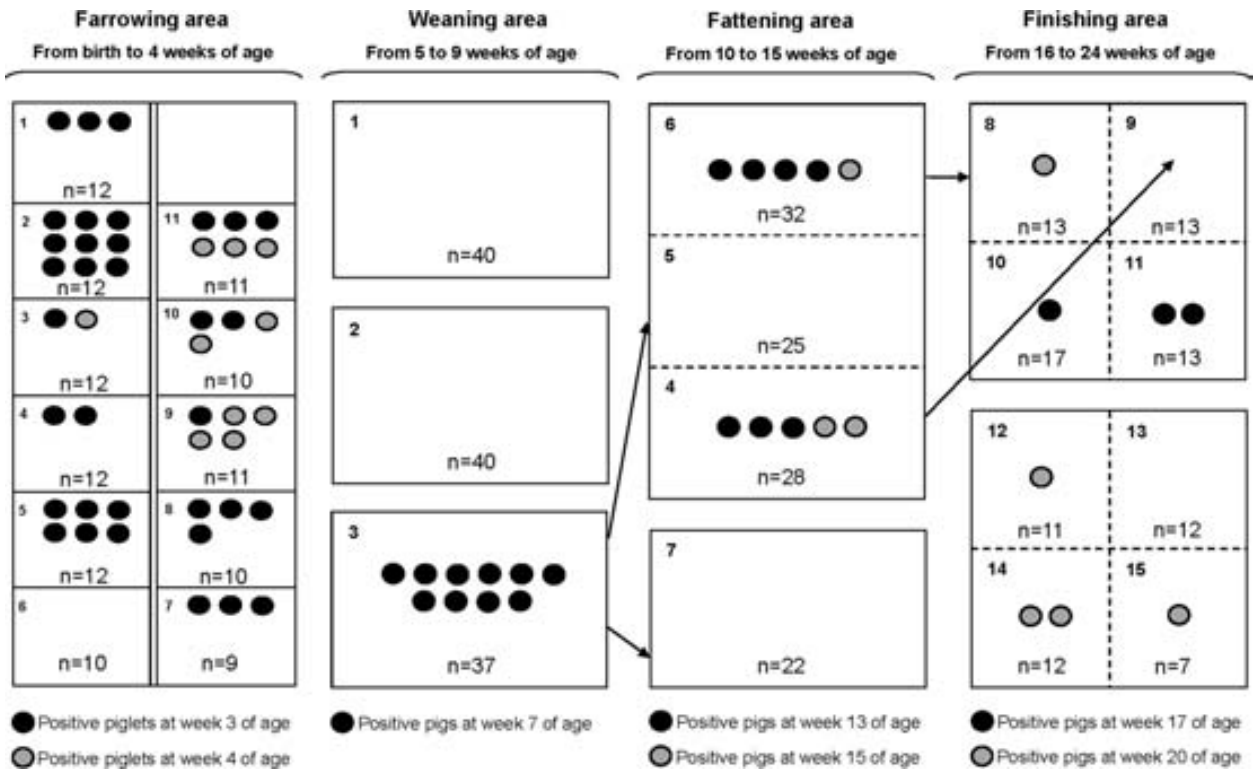

Figure 4 Distribution of viral shedders according to RRT-PCR results in Farm 1. Open separations between pens are represented as dashed lines; closed separations between pens are shown with solid lines. Arrows show movements of RRT-PCR positive pigs.

to one SIV isolated in 2000 in Germany (A/swine/Bakum/1832/2000 (H1N2)), and could be classified in a cluster where human and swine influenza viruses are included. In contrast, the N2 sequences were grouped with North-American SIV corresponding to H3N2 subtype (Figure 6). Positive animals detected in week 17th were distributed among all the pens that housed fatteners.

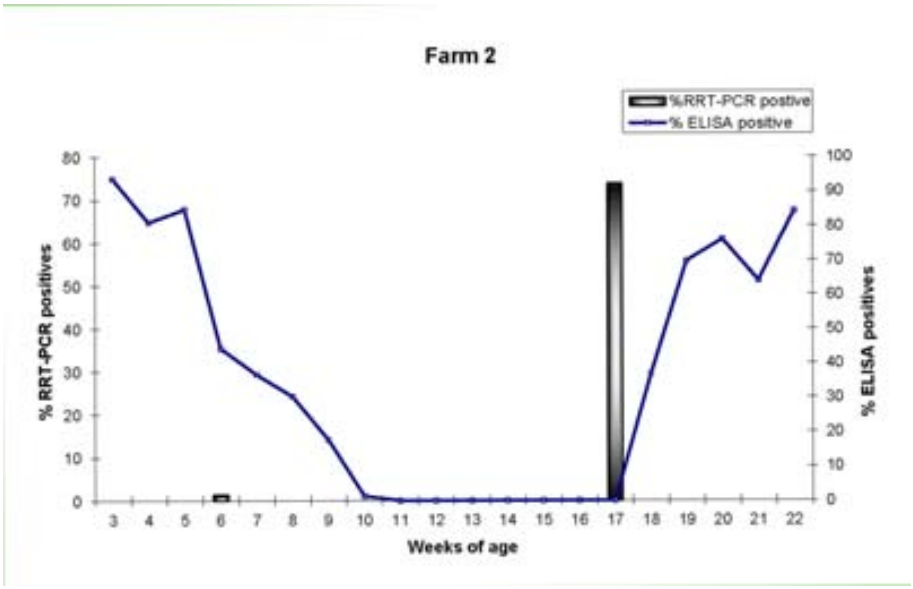

Figure 5 Seroprevalence and incidence of SIV in Farm 2. The techniques and symbols used are the same as those used in Figure 1.

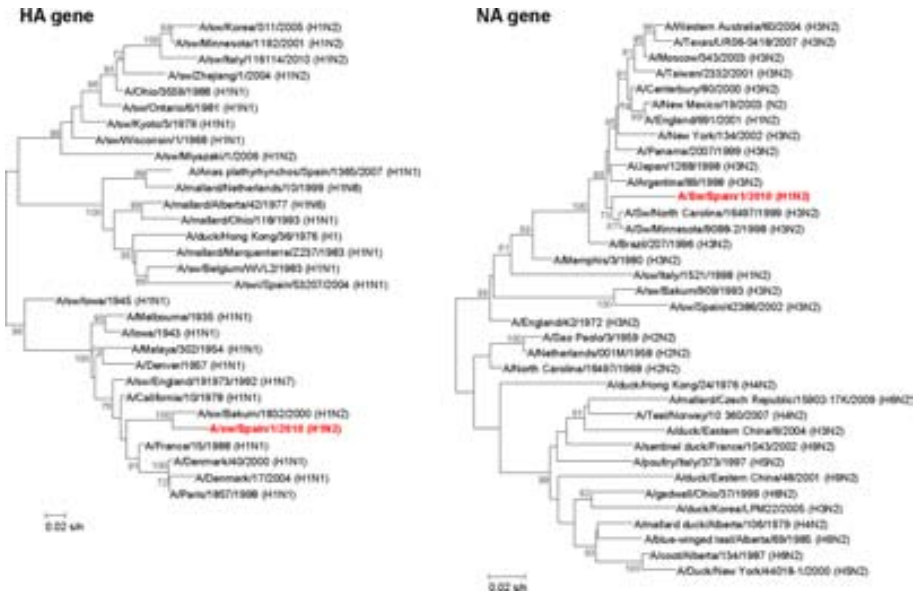

Figure 6 Phylogenetic analysis for HA and NA of SIV isolates retrieved in Farm 2. Color scheme, rooting, and scale are the same as those used in Figure 3. The accession numbers of sequence data are provided in Additional file 2: Table S2.

Clinical signs and gross lesions

Mild clinical signs compatible with influenza were detected at 17th and 18th weeks of age, but only affected a low percentage of pigs (6.6% and 1.3%, respectively). The mortality rate in this batch during the study was 5.1%.

Discussion

Results of the present study illustrate the existence of epidemic and endemic influenza infections in pig farms. While the presentation of the infection in F2 agrees with the traditional picture of an epidemic form of influenza, although of low virulence, the pattern of F1 depicts an endemic situation with an insidious spread, no overt signs but high mortality, and with the co-circulation of different H1N1 variants and probably an additional H3N2, as shown by HI. This opens several questions about the epidemiology, the clinical significance and even about the protection against infection with similar strains of SIV.

In F1 four viral waves were detected followed by seroconversion of a number of pigs. In this farm we identified shedder piglets at 3 and 4 weeks of age, while they were still with the sows. This finding contrasts with previous data suggesting that most SIV infections take place after the decay of maternal antibodies which occurs after 10 weeks of age [15,16]. On the other hand, mucosal IgA is considered to be a correlate of protection against virus replicating in the upper airways [17,18]. In the present case, piglets with maternal-derived antibodies against SIV were found to be positive by RRT-PCR, reinforcing the idea that the measurement of maternal antibodies does not correlate with protection against SIV at a mucosal level [19-23]. All the SIV positive piglets of 3-4 weeks of age showed a lack of specific IgA anti- SIV in nasal level. Beyond a potential lack of sensitivity of the test for the detection of antibodies in nasal mucus, this result would explain the finding of seropositive but infected piglets.

Interestingly, nine pigs were detected as positive by RRT-PCR at two sampling times separated at least four weeks. Although it may be possible that these pigs were infected continuously, but positivity was not detected at some of the sampling times, it seems unlikely since such a long duration of SIV nasal viral shedding has never been reported [24].

Humoral protection against influenza viruses is mainly mediated by antibody responses to HA [25]. In this sense, we also identified two pigs infected in different weeks by SIV strains belonging to the same HA clade in spite of having HI titres > 1:20 against the infecting strain. This observation can be either the result of a true infection, in which case the predictive value of HI antibodies for determining protection could be questioned, or the consequence of an external contamination of the sample, produced, for example by a recent contact of the pig with a shedder pen-mate.

The presence of infected piglets in farrowing units also raises the question of the potential sources of infection. The most obvious source of virus for the piglets could be the sows, although most of them were seropositive for H1N1. Unfortunately we did not test them virologically, and this point cannot be clarified. In any case, the role of

sows for maintaining viral circulation in SIV endemic farms is unknown and would deserve more in-depth studies.

One of the most interesting findings of the study was the detection of different H1N1 variants in the same batch of pigs from F1 accordingly the phylogenetic analysis of HA and NA instead the internal genes from the three isolates analyzed showed a high similarity between them. Moreover, the isolates seem to be grouped in the different clusters according to the weeks of age of the animals. Taken together, these results suggest that drift processes have occurred in F1 and as a consequence drift variants have been generated during the sampling frame. To our knowledge this is the first report of some close related H1N1 variants co-circulating endemically in a herd. Besides this, the existence of variants belonging to the H1N1 subtype with small genetic divergence suggests that this virus have been circulating in the herd for a long time. The endemic circulation of distinct H1N1 strains in F1 emphasizes the potential for the emergence of reassortant viruses in pig farms. However, the evidence of simultaneous infection of the same pigs is still lacking. Interestingly, recent studies have shown that intra-subtype reassortment events have played an important role in the evolutionary history of A/H1N1; for example, in the genesis of strains associated with influenza epidemics in humans caused by A/H1N1 viruses in 1947 and 1951 [26]. Furthermore, the presence of drift variants in the same batch of pigs may explain the detection of positive pigs by RRT-PCR more than once sampling time since antigenic drift may facilitate viral escape from antibody neutralization [27]. These facts can be also explained by the development of a weak immunity against the homologous or the heterologous strain, suggesting a partial protection unable to prevent the second infection. However, these finding should be thoroughly investigated by means of transmission-by-contact models.

Another point of interest is the source of SIV infection in the studied farms. The introduction of asymptomatic carrier pigs as well as the transmission from humans could explain the introduction of the virus in these farms. Furthermore, the dissemination of the virus from a neighbouring farm, by aerial transmission could be another potential mechanism of SIV introduction [3]. In this sense, F1 was located in a region of higher pig density areas compared to where F2 was located, and since pig density in a region has been related with SIV prevalence [28] it may seem that F1 was at a higher risk of SIV introduction compared to F2. Finally, other possible means of SIV introduction in these farms could be via fomites or birds.

Influenza viruses are usually classified into Eurasian and north American lineages. The phylogenetic analysis of strains isolated in F2 revealed that the NA was more related to those of swine and human H3N2 virus from North-American lineage. These findings are in agreement with an earlier study by Liu et al. [29], who proposed that the classification of influenza viruses should be more complicated than these two lineages. Moreover, these results highlight the potential intercontinental virus exchange, gene flow and reassortant between Eurasian and North American lineages.

Closed separations between pens which do not allow direct physical contact between pigs from different pens are often considered as a preventive measure against dissemination of airborne pathogens, including SIV [10,30]. In our study, the lack of transmission among pigs housed in different pens with closed separations indicates that it would be advisable to design farm facilities with closed partitions between pens in order to minimize spread of SIV infection.

From a methodological point of view, it is worth to note that only H1N1 and H1N2 viruses could be isolated in spite of evidences for H3N2 being present in F1. Multiples reasons could explain the unsuccessful isolation of this subtype, among them the inactivation of the virus during transport or failure to replicate in eggs [31] or in MDCK cells cultures [32,33] due to a low HA receptor-binding activity. The inactivation hypothesis seems unlikely because of the considerable rate of isolation for other samples treated exactly the same than those containing H3N2. Furthermore, some of the isolates could not be sequenced because RT-PCR failed to amplify the HA or NA genes. In any case, these results can be interpreted as the consequence of a high variability of HA sequences or eventually, can also be attributed to other circulating HA and NA different to H1, H3, N1 or N2. Regarding HI test, it is important to consider that the strains used as antigen were from The Netherlands and Belgium, and all of them were at least 12 years old. The use of these strains can result in an underestimation of the true percentage of seropositive animals. However, in a recent study [34] on cross reactivity between A/swine/Neth/Best/96 and A/swine/Spain/53207/2004, both strains produced fairly similar titres. These data suggest that, at least for H1N1, the results of the present study were not substantially affected by the use of Dutch or Belgian strains. Furthermore, it is important to note that despite the high specificity (100%) of the indirect ELISA, the sensitivity (Se) seems be better for H1N1 subtype than for H1N2 and H3N2 subtypes (Se = 100%, 86.9% and 73.4%, respectively) [35], and this could also result in an underestimation of the seroprevalences obtained.

In conclusion, we report that influenza infection in pigs from commercial herds can occur with different patterns, from an acute outbreak with epidemic spread to an endemic situation. This work also shows that SIV infection

can occur in piglets in presence of colostral-derived antibodies against the subtypes circulating in the farm. Moreover, evidences suggest that homologous protection after infection with one strain could not fully prevent a second infection with the same strain or a closely related one. Also, in an endemic farm, several SIV may co-circulate for extended periods of time. A better knowledge of the SIV epidemiology may contribute to improve the understanding of the arising of pandemic viruses.

Additional material

Additional file 1: Table S1 Primer set used to amplify each segment of the SIV. Primer set used to amplify each segment of the SIV and information about the begin and end positions of each one.

Additional file 2: Table S2 GenBank accession numbers of HA and NA sequences used in phylogenetic analysis. GenBank accession numbers and background information for sequences of influenza A virus used in the phylogenetic analysis.

Abbreviations

ELISA: Enzyme linked-immunosorbent assay; MDCK: Madin-Darby canine Kidney.

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Authors' contributions

MSG participated in the design of the study, carried out the sample collection, performed the experiments and the statistical analysis and wrote the paper. GEMV participated in the design of the study, performed the experiments and helped in the sample collection. MJV participated in the sample collection and immunoassays NB participated in molecular studies and helped to draft the manuscript. RAMF participated in molecular studies and helped to draft the manuscript. TMB helped in molecular studies. MMS helped in the sample collection and participated in molecular studies and immunoassays. MM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. EM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. JC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Van Reeth K: **Avian and swine influenza viruses: our current understanding of the zoonotic risk.** *Vet Res* 2007, **38**:243-260.
2. World Organization for Animal Health (OIE): **Swine influenza.** *Manual for diagnostic tests and vaccines for terrestrial animals. Paris* 2008, 1128-1138.
3. Olsen CW, Brown IH, Easterday BC, Van Reeth K: **Swine influenza.** In *Diseases of swine. 9th edition.* Edited by: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Iowa: Blackwell Publishing Professional; 2006:469-482.
4. Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG: **Genetic reassortment between avian and human influenza A viruses in Italian pigs.** *Virology* 1993, **193**:503-506.
5. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y: **Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.** *J Virol* 1998, **72**:7367-7373.
6. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JSM, Guan Y, Rambaut A: **Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.** *Nature* 2009, **459**:1122-1125.
7. Brown IH: **The epidemiology and evolution of influenza viruses in pigs.** *Vet Microbiol* 2000, **74**:29-46.
8. Maldonado J, Van Reeth K, Riera P, Sitjà M, Saubi N, Espuña E, Artigas C: **Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain.** *Vet J* 2006, **172**:377-381.
9. Van Reeth K, Brown IH, Dürrwald R, Foni E, Labarque G, Lenihan P, Maldonado J, Markowska-Daniel I, Pensaert M, Pospisil Z, Koch G: **Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003.** *Influenza Other Respi Viruses* 2008, **2**:99-105.
10. Simon-Grifé M, Martín-Valls GE, Vilar MJ, García-Bocanegra I, Mora M, Martin M, Mateu E, Casal J: **Seroprevalence and risk factors of swine influenza in Spain.** *Vet Microbiol* 2011, **149**:56-63.
11. Madec F, Gourreau JM, Kaiser C, Le Dantec J, Vannier P, Aymard M: **Etude de la persistance d'une activité du virus grippal H1N1 (swine) dans les élevages porcins en dehors des phases épidémiques.** *Comp Immunol Microbiol Infect Dis* 1985, **8**:247-258, (in French).
12. Busquets N, Segalés J, Córdoba L, Mussá T, Crisci E, Martín-Valls GE, Simon-Grifé M, Pérez-Simo M, Pérez-Maíllo M, Núnez JI, Abad FX, Fraile L, Pina S, Majó N, Bensaid A, Domingo M, Montoya M: **Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus.** *Vet Res* 2010, **41**:74.
13. Kyriakis CS, Brown IH, Foni E, Kuntz-Simon G, Maldonado J, Madec F, Essen SC, Chiapponi C, Van Reeth K: **Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008.** *Zoonoses Public Health* 2009, **58**:93-101.
14. Chiapponi C, Fallacara F, Foni E: **Subtyping of H1N1, H1N2 and H3N2 swine influenza virus by two multiplex RT-PCR.** *Proceedings of the 4th International Symposium on Emerging and Re-emerging Pig Diseases: 29 June - 2 July 2003; Rome* 2003, 257-258.
15. Loeffen WLA, Nodelijk G, Heinen PP, van Leengoed LAMG, Hunneman WA, Verheijden JHM: **Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study.** *Vet Microbiol* 2003, **91**:295-308.
16. Loeffen WLA, Hunneman WA, Quak J, Verheijden JHM, Stegeman JA: **Population dynamics of swine influenza virus in farrow-to-finish and specialised finishing herds in the Netherlands.** *Vet Microbiol* 2009, **137**:45-50.
17. Tomoda T, Morita H, Kurashige T, Maassab HF: **Prevention of influenza by the intranasal administration of cold-recombinant, live-attenuated influenza virus vaccine: importance of interferon-y production and local IgA response.** *Vaccine* 1995, **13**:185-190.
18. Renegar KB, Small PA, Boykins LG, Wright PF: **Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract.** *J Immunol* 2004, **173**:1978-1986.
19. Easterday BC: **Immunological considerations in swine influenza.** *J Am Vet Med Assoc* 1972, **160**:645-648.

20. Macklin MD, McCabe D, McGregor MW, Neumann V: **Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus.** *J Virol* 1998, **72**:1491-1496.
21. Loeffen WLA, Heinen PP, Bianchi ATJ, Hunneman WA, Verheijden JHM: **Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus.** *Vet Immunol Immunopathol* 2003, **92**:23-35.
22. Choi YK, Goyal SM, Joo HS: **Evaluation of transmission of swine influenza type A subtype H1N2 virus in seropositive pigs.** *Am J Vet Res* 2004, **3**:303-306.
23. Kitikoon P, Nilubol D, Erickson BJ, Janke BH, Hoover TC, Sornsen SA, Thacker EL: **The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination.** *Vet Immunol Immunopathol* 2006, **112**:117-128.
24. Brookes SM, Nuñez A, Choudhury B, Matrosovich M, Essen SC, Clifford D, Marek J, Slomka MJ, Kuntz-Simon G, Garcon F, Nash B, Hanna A, Heegaard PMH, Quéguiner S, Chiapponi C, Bublot M, Maldonado-Garcia J, Gardner R, Foni E, Loeffen W, Larsen L, Van Reeth K, Banks J, Irvine RM, Brown IH: **Replication, pathogenesis and transmission of pandemic (H1N1) 2009 virus in non-immune pigs.** *PLoS One* 2010, **5**:e9068.
25. Murphy BR, Clements ML: **The systemic and mucosal immune response of humans to influenza A virus.** *Curr Top Microbiol Immunol* 1989, **146**:107-116.
26. Nelson MI, Viboud C, Simonsen L, Ryan T, Bennett RT, Griesemer SB, George KS, Taylor J, Spiro DJ, Sengamalay NA, Ghedin E, Taubenberger JK, Holmes EC: **Multiple reassortment events in the evolutionary history of H1N1 Influenza A virus since 1918.** *PLoS Pathog* 2008, **4**:e1000012.
27. Palese P: **Influenza: old and new threats.** *Nat Med* 2004, **10**:S82-S87.
28. Maes D, Deluyker H, Verdonck M, Castryck F, Miry C, Vrijens B, de Kruif A: **Herd factors associated with the seroprevalences of four major respiratory pathogens in slaughter pigs from farrow-to-finish pig herds.** *Vet Res* 2000, **31**:313-327.
29. Liu S, Ji K, Chen J, Tai D, Jiang W, Hou G, Chen J, Li J, Huang B: **Panorama phylogenetic diversity and distribution of type A influenza virus.** *PLoS One* 2009, **4**:e5022.
30. Dupont K, Hjulsager CK, Kristensen CS, Baekbo P, Larsen LE: **Transmission of different variants of PCV2 and viral dynamics in a research facility with pigs mingled from PMWS-affected herds and non-affected herds.** *Vet Microbiol* 2009, **139**:219-226.
31. Lu B, Zhou H, Ye D, Kemble G, Jin H: **Improvement of influenza A/Fujian/411/02 (H3N2) virus growth in embryonated chicken eggs by balancing the hemagglutinin and neuraminidase activities, using reverse genetics.** *J Virol* 2005, **79**:6763-6771.
32. Price GE, Smith H, Sweet C: **Differential induction of cytotoxicity and apoptosis by influenza virus strains of differing virulence.** *J Gen Virol* 1997, **78**:2821-2829.
33. Schultz-Cherry S, Dybdahl-Sissoko N, McGregor M, Hinshaw VS: **Mink lung epithelial cells: unique cell line that supports influenza A and B virus replication.** *J Clin Microbiol* 1998, **36**:3718-3720.
34. Martín-Valls GE, Simon-Grife M, Busquets N, Diaz I, Martin M, Casal J, Mateu E: **Analysis of cross reactivity of porcine sera in the haemagglutination inhibition assay using swine H1N1 or the new pandemic H1N1 influenza virus.** *Proceedings of 21st International Pig Veterinary Society: 18-21 July 2010; Vancouver* 2010, 590.
35. Maldonado J: **Experiencias prácticas sobre el uso y las limitaciones de las analíticas de diagnóstico serológico para la Influenza porcina (IP).** *Proceedings of the XII Symposium of AVEDILA: 1-2 October 2007; Bilbao* 2007.

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PLoS one

Conserved Synthetic Peptides from the Hemagglutinin of Influenza Viruses Induce Broad Humoral and T-Cell Responses in a Pig Model

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Abstract

Outbreaks involving either H5N1 or H1N1 influenza viruses (IV) have recently become an increasing threat to cause potential pandemics. Pigs have an important role in this aspect. As reflected in the 2009 human H1N1 pandemia, they may act as a vehicle for mixing and generating new assortments of viruses potentially pathogenic to animals and humans. Lack of universal vaccines against the highly variable influenza virus forces scientists to continuously design vaccines *à la carte*, which is an expensive and risky practice overall when dealing with virulent strains. Therefore, we focused our efforts on developing a broadly protective influenza vaccine based on the Informational Spectrum Method (ISM). This theoretical prediction allows the selection of highly conserved peptide sequences from within the hemagglutinin subunit 1 protein (HA1) from either H5 or H1 viruses which are located in the flanking region of the HA binding site and with the potential to elicit broader immune responses than conventional vaccines. Confirming the theoretical predictions, immunization of conventional farm pigs with the synthetic peptides induced humoral responses in every single pig. The fact that the induced antibodies were able to recognize *in vitro* heterologous influenza viruses such as the pandemic H1N1 virus (pH1N1), two swine influenza field isolates (SwH1N1 and SwH3N2) and a H5N1 highly pathogenic avian virus, confirm the broad recognition of the antibodies induced. Unexpectedly, all pigs also showed T-cell responses that not only recognized the specific peptides, but also the pH1N1 virus. Finally, a partial effect on the kinetics of virus clearance was observed after the intranasal infection with the pH1N1 virus, setting forth the groundwork for the design of peptide-based vaccines against influenza viruses. Further insights into the understanding of the mechanisms involved in the protection afforded will be necessary to optimize future vaccine formulations.

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Introduction

In the last decades, several cases of human infection with the highly pathogenic avian influenza virus (HPAIV) H5N1 have been reported by the World Health Organization http://www.who.int/influenza/human_animal_interface/avian_influenza/en/). It is a common assumption that the pig may act as mixing vessel to generate new reassortant influenza viruses due to the presence of receptors for both avian and mammalian influenza viruses in the epithelial cells of their respiratory tract [1]. A recent example of the latter caused the first pandemia of the 21st century, starting in 2009 as a consequence of the global spread of a swine-origin influenza virus A H1N1 (pH1N1). This was a virus that contained genes from avian, pig and human origin [2]. Although the virus was not as pathogenic to humans as expected, severe disease cases associated with pH1N1 have been more recently reported in England (http://www.who.int/influenza/surveillance_monitoring/updates/2010_12_30_GIP_surveillance/en/). The future evolution of this or any emergent influenza virus (IV) is uncertain. This is a distressing matter particularly because available vaccines and therapies are strictly restricted to phylogenetically closely related circulating viruses. Therefore, finding universal and effective vaccines and therapeutic measures to fight against future IV is a must for public health.

IV hemagglutinin (HA) is a viral surface polypeptide that mediates both, the binding of IV to the host cell surface and the fusion of viral and endosomal membranes [3]. HA is formed by subunit 1 (HA1) and subunit 2 (HA2) and both the N- and C-terminal parts of HA1 together with HA2 comprise the stalk of the molecule [4]. Vaccines designed to elicit antibodies against the stalk of HA are reported to confer protection against IV infection in mice [5]. HA1, although highly variable, encodes specific and highly conserved domains which may be involved in determining the recognition and targeting (RTD) of influenza viruses to their receptor as revealed by the Informational Spectrum Method (ISM) [6]. This includes the VIN1 domain, located within the site E in the N-terminus of HA1 [7]. In contrast with the high variability suffered by the globular part of the HA1 molecule, which is directly responsible for the receptor tropism, the site E remains relatively highly conserved [8]. Thus, representing potential targets to develop broad array of protective therapies and vaccines against IV infection.

Due to the already mentioned recent **cases** related to H5N1 and H1N1 IV subtypes, and because their potential to cause future outbreaks among the population, we focused our efforts on designing a vaccine capable of confering protection against both viral subtypes. As previously reported, RTD of HA1 from different H1N1 strains and HA1 from the recently emerged in Egypt H5N1 IV encode the same information. However, HA1 from H3N2 and all other H5N1 viruses encode different RT information [6,7]. Thus, aiming to increase the vaccine coverage, one HA1-peptide from the VIN1 domain of H1N1 and three HA1-peptides from two different H5N1 IV strains were designed and selected based on ISM.

In order to test the immunogenicity of our experimental vaccine, we decided to immunize conventional pigs with the combination of the synthesized peptides. Pigs allow the evaluation of the protective efficacy of experimental vaccines against several viral strains, including the recently pandemic H1N1 virus, pH1N1 [11]. Confirming the rationale behind their use as a pre-clinical animal model, immunization of conventional pigs with the VIN1-peptide cocktail allow us to demonstrate the induction of peptide-specific antibody and T-cell responses in every single animal, independently of their swine leukocyte antigen (SLA)-haplotype. Specific B and T-cell responses were induced against each one of the H1 and H5-peptides used, confirming their immunogenicity *in vivo*. Interestingly, the elicited antibodies also recognized several heterologous viruses *in vitro*, including the pH1N1, two swine influenza field isolates (SwH1N1 and SwH3N2) and a H5N1 highly pathogenic avian virus. This, together with the fact that the specific T-cell responses induced were also able to recognize the inactivated pH1N1, encouraged us to challenge all pigs with the pH1N1 influenza virus. Albeit preliminary, our results demonstrate that VIN1-vaccination was able to confer a partial protection against intranasal challenge with pH1N1, as demonstrated with the partial and total viral clearance from the lung lavages in two out of four immunized pigs. We believe that our results could contribute to the obtainment of a broader array of protective vaccines against future influenza outbreaks or even pandemics.

Results

VIN1 as a Synthetic Peptide-vaccine

The highly conserved VIN1 domain, located within the E site in the N-terminus of the HA1 molecule, plays an important role in the recognition and targeting (RT) between virus and receptor, therefore representing an ideal target for an antibody-mediated therapy against influenza infection [7]. Informational spectral analysis revealed that the RT domains of HA1 from H1N1/1918, pH1N1/2009, seasonal H1N1 and H5N1 emerged in Egypt encode the same information despite differences in their primary structures. Thus, based on ISM and using information available on the properties of HA and its receptors, a single 34-mer peptide (NF-34) from the H1N1 subtype was selected from within the VIN1 region. NF-34 corresponds to positions 87–120 from the A/South Carolina/1/18 (H1N1) virus (Table 1). Additionally, a peptide (ES-34) from the VIN1 domain from the A/Egypt/0636-NAMRU3/2007 (E; H5N1) was also selected and included in the vaccine. In previous studies, we also showed that HA1 from H3N2 and all other H5N1 encode different RT information [6,7]. Aiming to increase the vaccine coverage, two additional peptides (LE-35.1 and LE-35.2) were selected from the A/Hong Kong/213/03 (HK; H5N1) IV and both peptides were added to NF-34 and ES-34. LE-35.1 and LE-35.2 differ only in positions 43 and 48 (Table 1), representing "hot spots" of variability within this H5N1 sequence.

Amino acid sequences from pH1N1 virus and VIN1-peptides are given in Table 1. The identity between pH1N1 virus and NF-34 (H1-peptide) is 92%. The similarity between all the H5-peptides and the pH1N1 virus is less than 75% with even lower identities, being less than 40% when comparing pH1N1 and ES-34. There was no homology between the HK-derived peptides (LE-35.1 and LE-35.2) and the E-derived peptide (ES-34). The two amino acid differences between LE-35.1 and LE-35.2 represent a 95% of identity between them.

Immunogenicity of VIN1 Peptides in a Pig Model

The pig is a good model not only to study influenza pathogenesis and therapy [9], but also for developmental immunology [10]. Thus, to confirm the bioinformatic predictions related to the capacity of VIN1-peptides to elicit humoral responses, four of the influenza-seronegative pigs were immunized three times with the VIN1-peptide mixture with two week intervals between immunizations. Four extra-pigs (also negative for IV antibodies) were inoculated with saline solution and remained as negative controls during the assay. To evaluate the ability of the VIN1-peptide cocktail to elicit antibodies, sera obtained 15 days after each immunization were tested against the peptides. Results obtained by ELISA showed that the immunization was efficient since every single immunized pig produced specific antibodies against the VIN1-peptide pool, detectable after the second immunization (Fig. 1A). VIN1-peptides also elicited high antibody titres against each one of the single peptides included in the vaccine (Fig. 1B). In correlation with the high specificity shown in the peptide-ELISA, sera from peptide-immunized pigs specifically reacted against the purified hemagglutinin protein of H5- and H1-subtypes, with only one serum from the V1N1 group showing background OD values in the H1-ELISA (Fig. 1C).

Finally, we were intrigued to find that VIN1-peptides also had the ability to induce T-cell responses. PBMC isolated from VIN1-immunized pigs specifically secreted IFN-γ in response to *in vitro* stimulation with VIN1-peptide cocktail (Fig. 2). First, we noted that VIN1-PBMC specifically secreted IFN-γ two weeks after the first immunization. Second, a homogeneous T-cell activity against the V1N1-peptide cocktail was detected between animals after the third immunization (Fig. 2*A*). And third, that all peptides were recognized by the specific T-cell induced (Fig. 2B). These results demonstrated that VIN1-peptides could act as productive immunogens in pigs.

Table 1. Amino acid sequences from the peptides used for immunization compared to the homologue sequence of the HA receptor recognition domain of the challenging strain (pH1N1) and the HA purified proteins used for the serologic tests.

Strain	Short name	Residues	Sequence
Challenge			
A/Catalonia/63/2009 (H1N1)	pH1N1	59–92	**S**S**D**NGTCYPGDFIDYEELREQLSSVSSFE**R**FEIF
Immunization			
A/South Carolina/1/18 (H1N1)	NF-34	87–120	**N**S**E**NGTCYPGDFIDYEELREQLSSVSSFE**K**FEIF
A/Egypt/0636-NAMRU3/2007 (H5N1)	ES-34	99–132	EELKHLLSRINHFEKIQIIPK**N**SWS**D**HEA**S**GVSS
A/Hong Kong/213/03 (H5N1)	LE-35.1	41–75	LC**D**LDGV**H**PLILRDCSVAGWLLGNPMCDEFINVPE
A/Hong Kong/213/03 (H5N1)	LE-35.2	41–75	LC**N**LDGV**K**PLILRDCSVAGWLLGNPMCDEFINVPE
HA purified proteins			
A/VietNam/1203/04 (H5)	VN04	115–149 57–91	EELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSS LCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPE
A/New Caledonia/20/99 (H1)	NCD99	101–134	NPENGTCYPGYFADYEELREQLSSVSSFERFEIF

In bold type, the amionacids differences between sequences are represented. Differences between the pH1N1 virus and the H1-peptide (NF-34) in homologous positions within the HA receptor recognition domain are marked. Aminoacid differences in the two H5-HK derived peptides (LE-35.1/2) are also represented.
doi:10.1371/journal.pone.0040524.t001

VIN1 Peptide Immunization Partially Prevent pH1N1 Virus Replication in BAL

As previously reported, the pig can be used to evaluate the protection of experimental pH1N1 influenza vaccines since they are natural receptive hosts for this virus subtype [11]. Aiming to evaluate the protective potential of our vaccine prototype, VIN1 peptide-vaccinated and control pigs were subjected to intranasal challenge with 10^6 $TCID_{50}$ of pH1N1 IV. The pH1N1 virus differs in three amino acids from the H1-peptide (NF-34) used in the vaccine (Table 1).

Intranasal infection of control pigs caused a subclinical infection and minor hystopathological changes. Moreover, mild to moderate BIP was recorded at necropsy (6 dpi), albeit virus was recovered from BAL at this time-point (Fig. 3). These results are in concordance with previously reported data obtained using colostrums-deprived pigs [11]; therefore, validating the use of seronegative conventional animals for vaccine testing. We did not detect differences in the severity of the lesions in lungs of vaccinated and non vaccinated animals. However, in contrast with control pigs, 2 out of 4 VIN1-peptide vaccinated pigs showed no or less viral RNA in their BAL (Fig. 3), which demonstrates a partially protective effect of our experimental vaccine.

VIN1 Peptides Induce Antibodies and T-cells that Specifically Recognize the pH1N1 Virus

In an attempt to correlate the protection provided from the immunological outcome induced by our vaccine, sera from immunized and control pigs were used to evaluate their capability to *in vitro* recognize the pH1N1. Sera obtained before the challenge from pigs vaccinated with VIN1-peptides, specifically detected pH1N1 infected-MDCK cells, as shown by indirect IF (Fig. 4 panel A). As expected, sera from control animals showed no reaction (Fig. 4 panel B); thus, demonstrating the ability of the peptide-induced antibodies to specifically identify the virus. Importantly, every single cell infected by pH1N1 was also recognized by the specific NS1-monoclonal antibody (Fig. 4 panel C) confirming the specificity of the reactions. Furthermore, sera from 2 of the pigs immunized with VIN1-peptides showed detectable HI activity, albeit at low titre and only those obtained at 6 dpi (Fig. 5A). As expected, sera from the control pigs did not show any specific response even at 6 dpi, which confirms the efficacy of our experimental vaccine to prime for viral-specific antibody responses. Regarding the presence of SNT antibodies, no significant differences were observed between the animal groups, at least at day 6pi (Fig. 5A).

As occurred for the antibodies, the induced T-cell responses measured by IFN-γ ELISPOT, not only specifically recognized the synthetic peptides, but also the pH1N1 virus. Thus, before the challenge only one out of four of the VIN1-vaccinated pigs showed detectable T-cell responses in response to *in vitro* stimulation with the inactivated pH1N1, while all vaccinated pigs responded at 6 dpi (Fig. 5B).

VIN1 Peptides Induce Antibodies that Recognize Distinct Viral Subtypes

Current influenza vaccines protect mostly against homologous virus strains. The presented VIN1-peptide cocktail did not confer efficient neutralizing antibodies and only one pig did not show viral RNA in BAL 6 dpi. However, an IF was performed to demonstrate that vaccination elicits antibodies that recognize different viruses. VIN1-sera obtained after three immunizations specifically detected SwH1N1 and H5N1 infected-MDCK cells, as shown by IF (Fig. 6). Furthermore, antibodies elicited after VIN1-immunization specifically detected SwH3N2 (Fig. 6).

To further investigate the antibody response, an HI assay was performed against the same virus subtypes: H5N1 HPAIV, SwH1N1 IV and SwH3N2 IV. No inhibition activity was recorded against any of the mentioned virus for any sera.

Discussion

The search for universal vaccines against influenza viruses is a must. Most efforts have been focussed on driving the immune response against well conserved epitopes or proteins of IV, such as the **influenza ion channel M2 protein**, and conserved epitopes from the influenza NP and **matrix 1 (M1)** [12,13,14]. More recently, the potential use of highly conserved synthetic peptides from HA2 as an efficient vaccine in mice has also been demonstrated [15]. In this report, we show evidence of the potential use of conserved HA1 peptides in future vaccine formulations using conventional pigs.

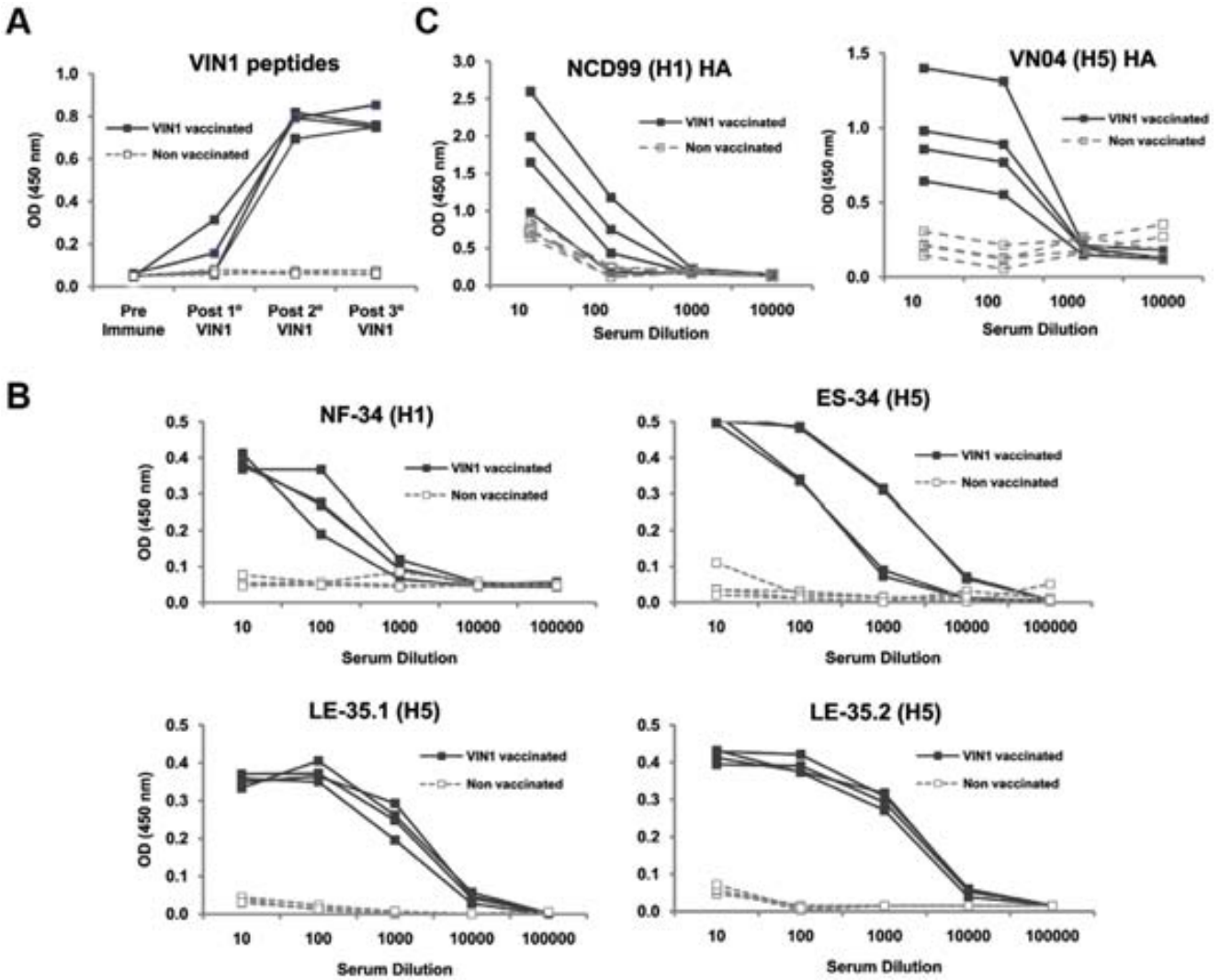

Figure 1. VIN1-peptide cocktail acts as a potent immunogen and the elicited sera reacts with different hemagglutinin subtypes and against VIN1-peptides. (A) Sera from individuals were obtained 15 days after each immunization and were tested for binding to a mixture of the VIN1-peptides (serum dilution 1:100) by ELISA. (B) Sera from individual pigs were obtained 15 days after the third immunization and were serially diluted and tested for binding to each single peptide by ELISA and (C) Sera described in B) were tested for binding to H5- or H1- recombinant hemagglutinin by ELISA.
doi:10.1371/journal.pone.0040524.g001

Peptides derived from the HA1-VIN1 domain were selected by ISM [6,7] and were used for the immunization carried out in the present study. As predicted, immunization of pigs with VIN1-peptides induced specific anti-VIN1 peptides antibodies that recognized the VIN1-peptides (Fig. 1A and 1B), the H1 and H5 recombinant proteins (Fig. 1C) and also the heterologous pH1N1 IV (Fig. 4). Even though it was not predicted, VIN1-peptide immunization was also able to induce T-cell responses in every single conventional pig that, again, not only recognized the specific peptides but also the heterologous pH1N1 IV. Interestingly enough, not all peptides seemed to be equally recognized, with both the NF-34 and ES-34 from the H1 and H5 hemagglutinin, respectively, being optimally recognized. The fact that these two epitopes are located in equivalent regions within the primary structure of the HA1 subunit, validate even more the ISM predictions.

An **ideal vaccine should elicit** both humoral and cellular responses in the context of highly variable Major Histocompatibility Complex (MHC), which is what we found with our vaccine. The fact that swine and human MHC complexes are remarkably similar [16], opens avenues for the extrapolation of these and future results for human medicine.

We observed an increase in virus clearance after the challenge with pH1N1 virus, which differs in 3 amino acids from NF-34 (the H1-peptide used in the VIN1-vaccine) (Table 1), in 2 out of 4 of the immunized pigs. This also opens new expectations for the use of VIN1-modified peptides in future vaccine formulations. Apart from sequence diversity, there is a clear lack of correlation between the protection observed and the immune responses detected at the individual level. Intriguingly, as soon as at day six post infection, only 2 pigs (pigs 1 and 2 from the V1N1 peptide-immunized group) showed concomitant detection of neutralizing and HI activity that did not totally correlate with protection. While pig 2 showed a clear reduction in viral load, pig 1 showed virus titres indistinguishable from those found in the control group. Although disappointing, our results seem to point towards the very

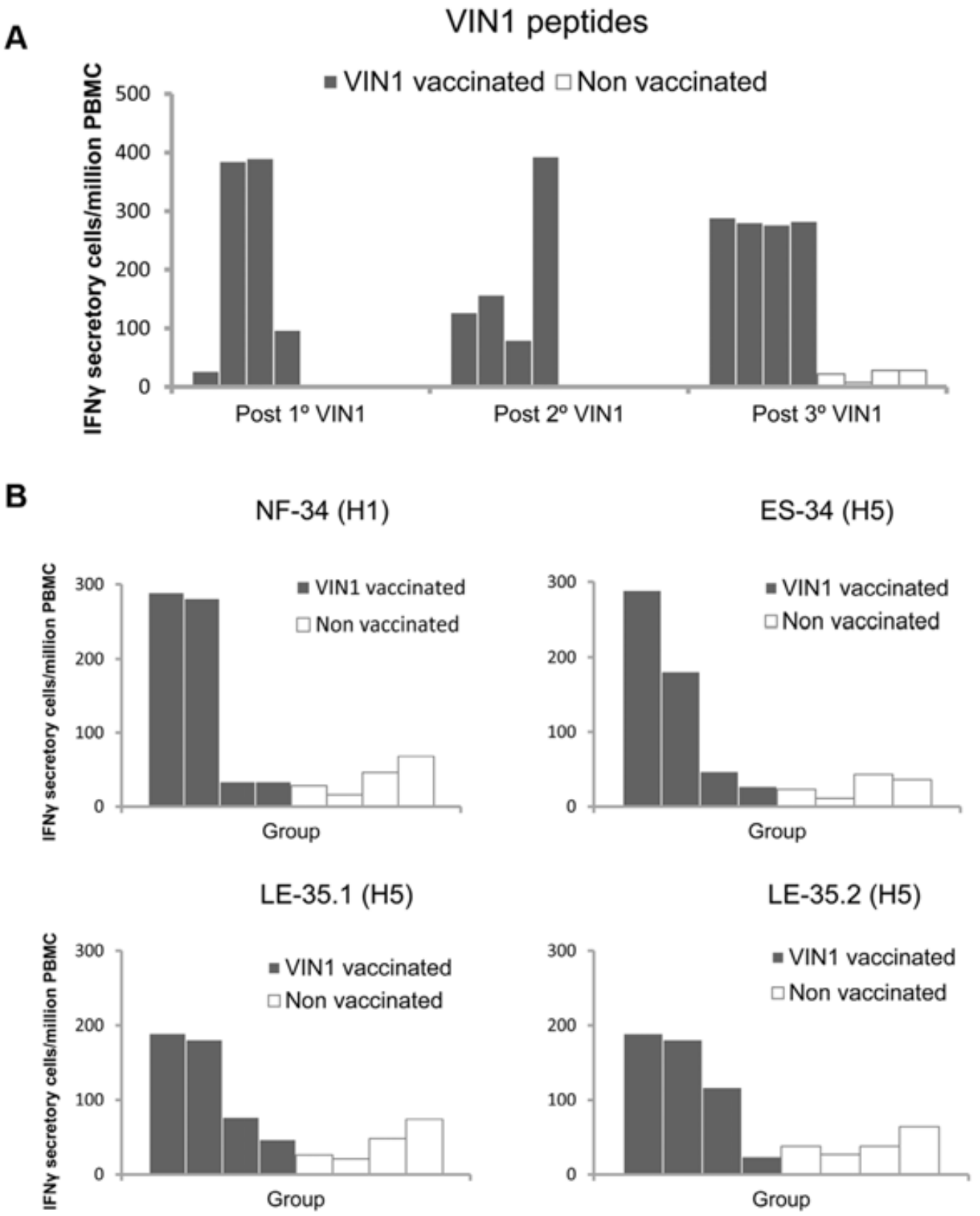

Figure 2. VIN1-HA1 derived peptides immunization induces strong T-cell responses in pigs. (A) Kinetics of the VIN1 peptide-specific T-cell responses induced 15 days after all immunizations measured by IFNγ-ELISPOT. (B) Specific T-cell responses induced 4 weeks after the third immunization were tested for each single peptide by IFNγ-ELISPOT.
doi:10.1371/journal.pone.0040524.g002

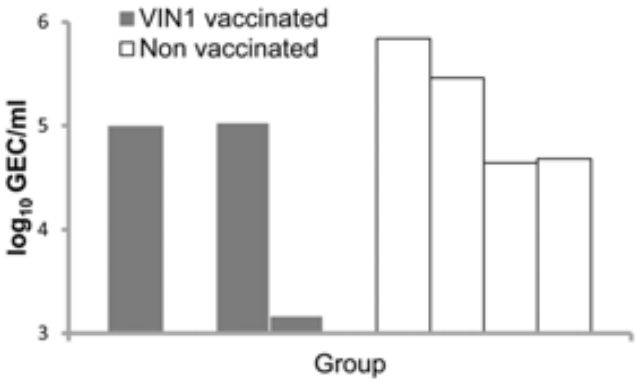

Figure 3. Immunization with VIN1-HA1 partially protects pigs *in vivo* against heterologous challenge with pH1N1. Influenza viral RNA quantification in BAL was performed by RT-qPCR at 6 dpi, corresponding to necropsy day. Bars indicated positive samples in genome equivalent copies (GEC) per ml of BAL. The detection limit in the assay was 3 $\log_{10}$ GEC/ml.
doi:10.1371/journal.pone.0040524.g003

important role of T-cells in the protection afforded which could be an important tool for developing more efficient vaccines for the future. Thus, the partial protection observed might correspond with the induction of non-detectable specific cytotoxic T-cell activity (CTL), as has been reported before for influenza [17,18] or with any other kind of T-cell activity independent from the induction of IFN-γ that might be involved in cross-protection [19]. We are currently addressing these issues, including the identification of shorter specific CTL-peptides.

The length of the peptides used, as well as the fact that the T-cells specifically secreted IFN-γ in response to *in vitro* stimulation with both the NF-34 peptide and the pH1N1 IV, point towards the induction of specific CD4+-T cells in every single vaccinated farm pig and independent of its SLA II haplotype. This, together with the fact that the specific antibodies induced are also able to recognize the pH1N1 virus, seem to validate the use ISM to optimize the prediction of highly conserved epitopes with better protective ability and to design future vaccine formulations, capable of inducing concomitantly, universal B and T-cell responses against H1N1 influenza viruses [20].

Unexpectedly, the reduction in the viral loads shown by pigs 2 and 4 did not correlate with less severity in the lung lesions. All pigs from either control or immunized groups show indistinguishable minor hystopathological changes. Despite the fact that these results could reflect a limitation of our T cell-centric vaccines to reduce disease, pigs might not be ideal models to test so, mainly because of the mild disease found after pH1N1 infection. For that reason, our hypothesis are also being tested in mice and chickens, which are ideal models for the characterization of the protective capability of experimental vaccines against an infection with highly pathogenic H5N1 IV; most probably, the responsible of future pandemic episodes [21].

Materials and Methods

Ethics Statement

All experiments with the pH1N1 IV were performed at the Biosafety Level 3 facilities of the *Centre de Recerca en Sanitat Animal* (CReSA-Barcelona). Sample from the patient infected by pH1N1 IV was coded prior to isolating the virus to ensure anonymity. For this reason, the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona* (UAB) exempted this study from the requirement to have the consent of the patient, who was infected with pH1N1 IV. The present study was performed in accordance with the Guidelines of the Good Experimental Practices and under the supervision and approvement of the Ethical and Animal Welfare Committee of the UAB (*Permit Number:* DMAH-5796).

Animal Experimental Design

A total of eight 8-wk-old conventional crossbreed pigs from a three-way cross (Duroc x Landrace hybrids paired with Pietrain boars) seronegative against influenza A virus were immunized three times two weeks apart. We immunized the pigs with either 15 μg of the VIN1-peptide cocktail (3.5–4 μg of each peptide) or saline solution in complete Freund's adjuvant (first immunization), incomplete Freund's adjuvant (second dose) and without adjuvant (last dose), by i.m. administration. Four weeks after the second boost, the pigs were intranasally inoculated with 10^6 $TCID_{50}$ of the pH1N1 virus. Animals were monitored daily for flu-like clinical signs. Sera and peripheral blood mononuclear cells (PBMC) obtained before each immunization, before the challenge and at 6 days post-infection (dpi), were used to detect specific humoral and cellular responses, respectively. Animals were euthanized at 6 dpi and a complete necropsy was carried out for each animal. Bronchoalveolar lavages (BAL) from the right lung of each pig were performed in 200 ml of PBS 1× immediately after post-mortem examination. BAL were frozen at −80°C until their use for viral RNA extraction and quantification. For histopathological analysis, samples from lung (apical, middle and diaphragmatic lobes), nasal turbinate and trachea were collected and fixed by immersion in 10% neutral buffered formalin. In the lung, broncho-interstitial pneumonia (BIP) intensity was assessed by means a semi-quantitative scoring (0 to 3, indicating lack of, mild, moderate or severe pneumonia lesions, respectively), as previously described [11].

Virus and Purified Hemagglutinins

Viruses used were pH1N1 virus (the pandemic swine-origin A/Catalonia/63/2009 H1N1 IV) [GenBank GQ464405-GQ464411 and GQ168897], SwH1N1 virus (A/Swine/Spain/003/2010 H1N1 IV) [GenBank JQ319725 and JQ319727], SwH3N2 virus (A/Swine/Spain/001/2010 H3N2 IV) [GenBank JQ319724 and JQ319726] and H5N1 HPAI virus (A/great crested grebe/Basque Country/06.03249/2006 H5N1 HPAIV) [GenBank EU636810 and EU636811]. After propagation at 37°C in the allantoic fluid of 11-day-old embryonated chicken eggs from a specific-pathogen-free flock, the infectious virus titre was determined in Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells and measured as tissue culture infectious doses 50% ($TCID_{50}$) by following the Reed and Muench method [22]. Purified hemagglutinin for A/VietNam/1203/04 (H5) and A/New Caledonia/20/99 (H1) were purchased from Abcam.

Peptide Synthesis

Four peptides were designed based on ISM predictions [6,7] and were mixed and used to immunize conventional pigs. The selected peptides were highly conserved and mapped to the flanking region of the HA1 within the VIN1 domain. Two peptides (LE-35.1 and LE-35.2) were derived from A/Hong Kong/213/03 (H5N1) [GenBank AB212056] and one (ES-34) from A/Egypt/0636-NAMRU3/2007 (H5N1) [GenBank EF382359]. The fourth peptide (NF-34) was derived from the HA1 of the human A/South Carolina/1/18 (H1N1) strain [GenBank AF117241]. The peptides were produced by GL Biochem (Shanghai) Ltd. Sequences from the synthetic peptides (thereafter referred as VIN1-peptides) are shown in Table 1.

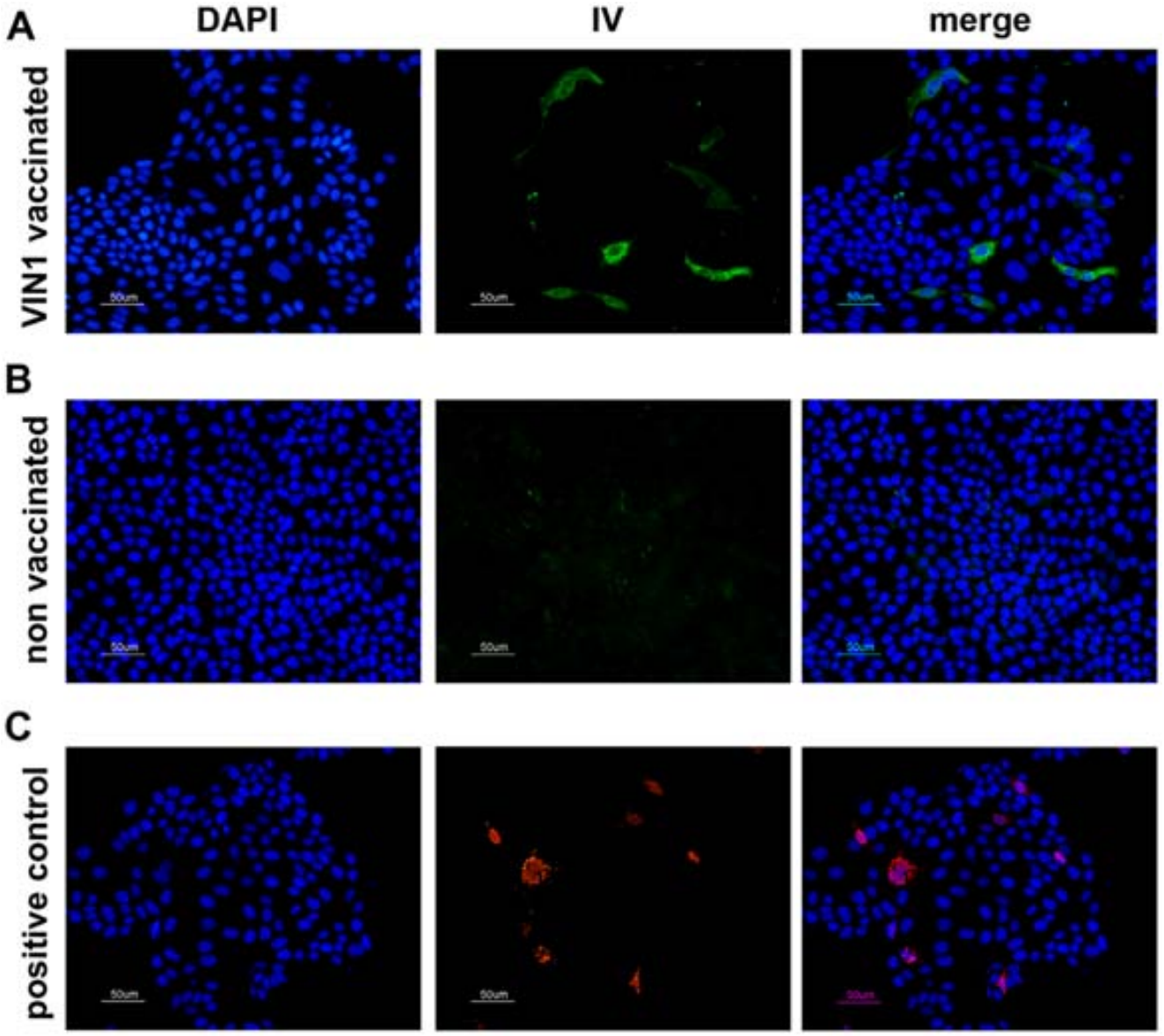

Figure 4. VIN1-sera recognize pH1N1 virus *in vitro.* Indirect immunofluorescence of pH1N1-infected MDCK cells at 16 hpi using as primary antibody: (A) the serum from one pig (representative of the group), immunized three times with VIN1-peptides; (B) the serum from one negative control pig (representative of the group), immunized three times with PBS; and (C) A monoclonal antibody against the NS-1 protein was used as control for the infection (right panel).
doi:10.1371/journal.pone.0040524.g004

Quantitative Real Time RT-PCR (RT-qPCR)

Viral RNA quantification using TaqMan RT-qPCR was performed in BAL. Viral RNA was extracted with QIAamp Viral Mini kit (Qiagen, Inc.). Amplification of a matrix (*M*) gene fragment was carried out using primers, probe, one-Step RT-PCR Master Mix Reagents (Applied Biosystems) and amplification conditions as described previously by Busquets *et al.* 2010 [11] in Fast7500 equipment (Applied Biosystems).

Influenza Nucleoprotein (NP)-specific ELISA

Sera from animals before starting the experiment were examined for the presence of specific antibodies against influenza NP using the ID Screen® Influenza A Antibody Competition ELISA (ID VET, France), following manufacturer's instructions. Pig serum samples were used at 1:100 dilution. Known positive and negative sera were used as test controls.

Peptide-specific ELISA

A peptide-based ELISA method was developed for the evaluation of the presence of specific antibodies in serum samples. Briefly, 96 well plates (Costar, Corning Incorporated) were coated with 1 μg/ml of each peptide individually, the VIN1-peptides cocktail or H5−/H1- purified hemagglutinin in coating buffer (sodium bicarbonate 0.1 M) overnight at 4°C. After blocking with 1% casein/PBS 1× for 1-h at 37°C, serum from individuals were added to the coated plate diluted at 1:100 or titrated with 10-fold dilutions (starting from 1:10), followed by 2-h incubation at 37°C. Plates were washed four times with PBS 1×/0.1% Tween20 and anti-pig IgG (whole molecule)-Peroxidase (Sigma) diluted 1:20,000

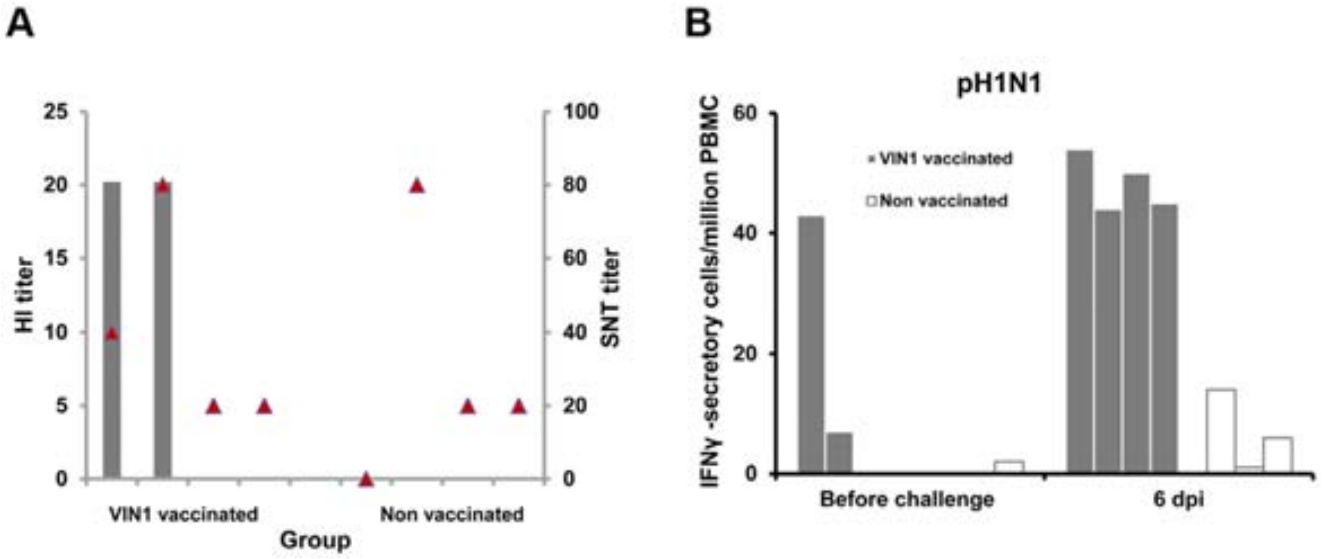

Figure 5. Immunization with the VIN1-HA1 peptide induces specific antibodies and T-cells against the heterologous pH1N1 virus. (A) HI and SNT titers obtained with sera from pigs immunized either with the VIN1-peptides or with saline solution (control), at 6 dpi with the pH1N1 virus. Grey bars represent HI titres and red triangles show SNT. (B) IFNγ-ELISPOT using pH1N1 virus as stimulus and PBMCs from pigs immunized either with the VIN1-peptides or with saline solution (control). The assay was done using PBMCs isolated either before the infection with the pandemic H1N1 virus or at 6 dpi.
doi:10.1371/journal.pone.0040524.g005

was added to wells followed by 45 min incubation at 37°C. After washing the plates four times (PBS 1×/0.1% Tween20), fifty µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution were added to the wells and allowed to develop for 8–10 min at room temperature (RT) protected from light. Optical density (OD) was measured at 450 nm.

Haemagglutination Inhibition (HI) Assay

An HI assay was performed following the standard procedures [23] using chicken red blood cells (RBC) and 4 haemagglutination units of either pH1N1 IV, SwH1N1 IV, SwH3N2 IV or H5N1 HPAIV. To avoid unspecific inhibitions, sera from individuals were treated prior to use. Briefly, one volume of serum samples was treated overnight at 37°C with four volumes of Receptor Destroying Enzyme (Sigma) solution (100 U/ml). Next day, serum samples were incubated for 30 min at 56°C after the addition of five volumes 1.5% sodium citrate. Finally, one volume of a 50% suspension of RBC was added and incubated for 1-h at 4°C. Known positive and negative sera were used as controls. HI titres of ≥20 were considered positive.

Seroneutralization (SNT) Assay

A SNT assay was done following the protocol described by Sirskyi and collaborators (2010) [24], with some modifications. Serum samples were diluted serially and incubated with 100 $TCID_{50}$ of pH1N1 virus for 2-h at 37°C. The mixture was then added to 10^5 MDCK cells/well and incubated overnight. After two washes with PBS 1×, the cells were fixed with cold 80% acetone for 10 min. Cells were air-dried, washed five times with PBS 1×/0.05% Tween-20 and incubated at RT for 1-h and a half with biotinylated influenza A anti-NP primary antibody (CAT # MAB8252B, Millipore, CA) diluted 1/2,000 in 5%FBS/PBS 1×. Plates were then washed five times with PBS 1×/0.05% Tween-20 and incubated 30 min in the dark with HRP-conjugate streptavidin (Millipore, CA) diluted 1/10,000 in 5%FBS/PBS 1×. Finally, after five washes with PBS 1×/0.05% Tween-20, TMB substrate (Sigma) was added to develop the reaction and stopped with Stop-solution H_2SO_4 (1N). Plates were then read at 450 nm.

IFN-γ ELISPOT Assay

An IFN-γ ELISPOT was performed as previously described [25], with some modifications. Briefly, PBMC were isolated from whole blood by Histopaque-1077 gradient (Sigma). Ninety-six-well plates (Costar, Corning Incorporated) were coated overnight with IFNγ-capture antibody (P2G10 clon, BD Pharmingen) diluted 1:100. After blocking the plates 1-h at 37°C, 500,000 PBMC/well were seeded and stimulated with either 2 µg/ml of VIN1-peptide cocktail or individually, or with 10^5 $TCID_{50}$ of inactivated pH1N1 IV per well for 20-h. Cells were removed and a biotin mouse anti-pig IFN-γ detection antibody (BD Pharmingen), diluted 1:1,000, was used followed by streptavidin-peroxidase (0.5 µg/ml). Insoluble TMB blue (Calbiochem) was added as final substrate. Positive spots were counted using a microscope.

Immunofluorescence Microscopy

MDCK cells (300,000 cells/well) were either mock infected or infected with pH1N1 IV, SwH1N1 IV, SwH3N2 IV or H5N1 HPAIV for 16-h at a MOI of 0.01. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then blocked with 3%BSA/PBS 1× for 1-h and incubated with the sera from the pigs (1:100) for 1-h in the blocking solution at RT. After three washes with PBS 1×, the cells were incubated with anti-IgG pig:FITC antibody (1:300) (Jackson Immunoresearch Europe LTd) for 1-h in blocking solution at RT. Finally, nuclei were counterstained with DAPI (1 µg/ml) and coverslips were mounted with Vectaschield. Protocol was modified from the previously described by Ballester *et al.* 2011 [26]. Fluorescence images were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). The images were processed by using the Image J v1.45l software (http://rsb.info.nih.gov/ij).

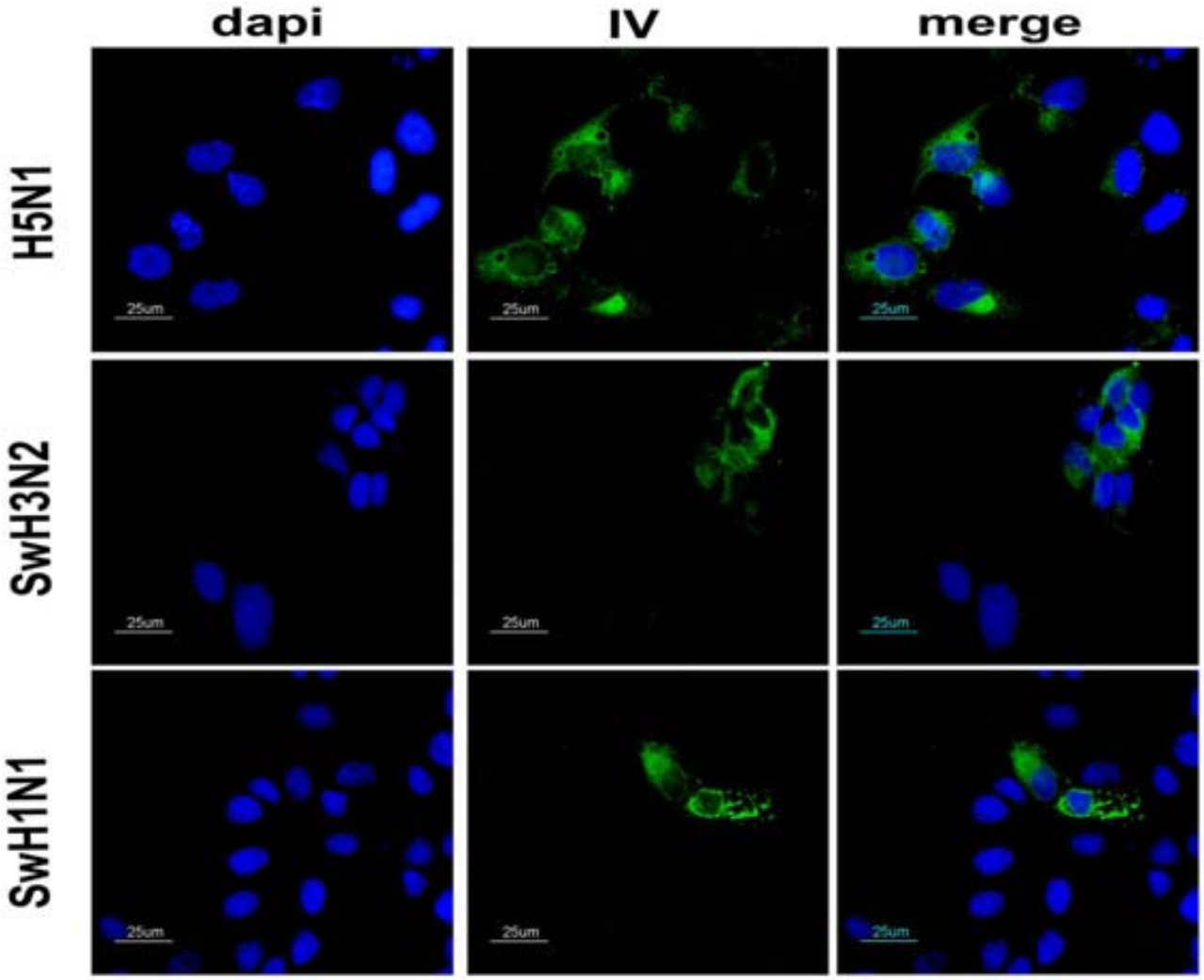

Figure 6. VIN1-sera recognize distinct viral subtypes. Indirect immunofluorescence of either H5N1, SwH3N2 or SwH1N1-infected MDCKs cells at 16 hpi using as primary antibody the serum from one pig (representative of the group), immunized three times with VIN1 peptides.
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The manuscript was edited by both Dr. Maureen V. Foster and Dr. Kevin Dalton.

Author Contributions

Conceived and designed the experiments: JVA FR AD. Performed the experiments: JVA JMA NB MB GEMV RR SLS DS NM JS. Analyzed the data: JVA JMA NB FR AD. Contributed reagents/materials/analysis tools: VV. Wrote the paper: JVA FR AD.

References

1. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, et al. (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72: 7367–7373.
2. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA (2008) Swine influenza viruses a North American perspective. Adv Virus Res 72: 127–154.
3. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931–939.
4. Wilson IA, Skehel JJ, Wiley DC (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature 289: 366–373.
5. Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, et al. (2010) Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 1.
6. Veljkovic V, Niman HL, Glisic S, Veljkovic N, Perovic V, et al. (2009) Identification of hemagglutinin structural domain and polymorphisms which may modulate swine H1N1 interactions with human receptor. BMC Struct Biol 9: 62.
7. Veljkovic V, Veljkovic N, Muller CP, Muller S, Glisic S, et al. (2009) Characterization of conserved properties of hemagglutinin of H5N1 and human influenza viruses: possible consequences for therapy and infection control. BMC Struct Biol 9: 21.
8. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, et al. (2000) Early alterations of the receptor-binding properties of H1, H2, and H3 avian i nfluenza virus hemagglutinins after their introduction into mammals. J Virol 74: 8502–8512.
9. Barnard DL (2009) Animal models for the study of influenza pathogenesis and therapy. Antiviral Res 82: A110–122.
10. Rothkotter HJ, Sowa E, Pabst R (2002) The pig as a model of developmental immunology. Hum Exp Toxicol 21: 533–536.
11. Busquets N, Segales J, Cordoba L, Mussa T, Crisci E, et al. (2010) Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. Vet Res 41: 74.

12. El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, et al. (2011) Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 186: 1022–1031.
13. Kitikoon P, Vincent AL, Janke BH, Erickson B, Strait EL, et al. (2009) Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates. Vaccine 28: 523–531.
14. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, et al. (2007) Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including Subtype H5N1. Emerg Infec Dis 13: 426–435.
15. Wang TT, Tan GS, Hai R, Pica N, Ngai L, et al. (2010) Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci U S A 107: 18979–18984.
16. Molder T, Adojaan M, Kaldma K, Ustav M, Sikut R (2009) Elicitation of broad CTL response against HIV-1 by the DNA vaccine encoding artificial multi-component fusion protein MultiHIV–study in domestic pigs. Vaccine 28: 293–298.
17. Christensen JP, Doherty PC, Branum KC, Riberdy JM (2000) Profound protection against respiratory challenge with a lethal H7N7 influenza A virus by increasing the magnitude of CD8(+) T-cell memory. J Virol 74: 11690–11696.
18. Riberdy JM, Flynn KJ, Stech J, Webster RG, Altman JD, et al. (1999) Protection against a lethal avian influenza A virus in a mammalian system. J Virol 73: 1453–1459.
19. Hillaire ML, van Trierum SE, Kreijtz JH, Bodewes R, Geelhoed-Mieras MM, et al. (2011) Cross-protective immunity to influenza pH1N1 2009 viruses induced by seasonal A(H3N2) virus is mediated by virus-specific T cells. J Gen Virol.
20. Stanekova Z, Vareckova E (2010) Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. Virol J 7: 351.
21. Watanabe Y, Ibrahim MS, Ellakany HF, Kawashita N, Mizuike R, et al. (2011) Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. PLoS Pathog 7: e1002068.
22. Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoint. A J Hygiene 27: 493–497.
23. World Organisation for Animal Health, OIE (2008) Manual for Diagnostic Tests and Vaccines for Terrestrial Animals. OIE 1130–1131.
24. Sirskyj D, Weltzin R, Golshani A, Anderson D, Bozic J, et al. (2010) Detection of influenza A and B neutralizing antibodies in vaccinated ferrets and macaques using specific biotin-streptavidin conjugated antibodies. J Virol Methods 163: 459–464.
25. Argilaguet JM, Perez-Martin E, Gallardo C, Salguero FJ, Borrego B, et al. (2011) Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. Vaccine 29: 5379–5385.
26. Ballester M, Rodriguez-Carino C, Perez M, Gallardo C, Rodriguez JM, et al. (2011) Disruption of Nuclear Organization during the Initial Phase of African Swine Fever Virus Infection. J Virol 85: 8263–8269.