Epidemiologia de la influença porcina: Estudis seroepidemiològics i dinàmica de la infecció en explotacions porcines

Tesi doctoral presentada per Meritxell Simon Grifé 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. Jordi Casal i Fàbrega.

Bellaterra, 2012
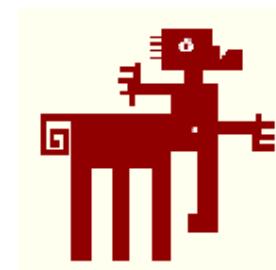
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Certifica:

Que la memòria titulada, ***"Epidemiologia de la influença porcina: Estudis seroepidemiològics i dinàmica de la infecció en explotacions porcines"***, presentada per Meritxell Simon Grifé per l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la seva direcció al Centre de Recerca en Sanitat Animal (CReSA).

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra, a 17 de Setembre de 2012.

Dr. Jordi Casal i Fàbrega   Meritxell Simon Grifé

Director   Doctoranda

Dedicada a tots aquells que m'estimen perquè gràcies a l'energia, força i ànims que m'han donat he arribat fins aquí.

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Resum

Des de l'any 1931, s'han aïllat diferents tipus de virus influença en l'espècie porcina i en l'actualitat es considera que certs subtipus circulen de forma endèmica entre la població porcina mundial.

Degut a que els porcs poden infectar-se tant per virus influença d'origen aviar com d'origen humà, l'espècie porcina es considera la font de generació de nous virus influença recombinants, que podrien incloure gens de virus de diferents orígens. Per aquest motiu, més enllà de les conseqüències productives que comporta la malaltia pel sector porcí, la grip porcina prèn importància degut a les implicacions que pot tenir per la salut pública.

En el primer estudi d'aquesta tesi es va examinar la seroprevalença enfront els virus influença en el porcí d'Espanya i els factors de risc associats. Es van recollir informació i mostres de sèrum (2.151 animals) de 98 explotacions distribuïdes arreu del país. Els resultats obtinguts mitjançant la tècnica d' inhibició de la hemaglutinació (IH), utilitzant els subtipus H1N1, H1N2 i H3N2 com antígens, van mostrar que el 75.4% dels animals presentaven anticossos enfront algun dels subtipus. A totes les granges estudiades es va detectar com a mínim un animal seropositiu enfront algun dels subtipus, però només el 9% van reportar simptomatologia compatible amb grip durant l'últim any. Per últim, tres factors de risc van resultar associats a la infecció: el percentatge de reposició, les separacions discontínues entre corrals i un accés no controlat a l'explotació. Els resultats obtinguts en aquest estudi mostren una àmplia disseminació dels virus influença en la població porcina d'Espanya. Així mateix, ressalten la importància de les mesures de bioseguretat així com del disseny de les instal·lacions a l'hora de minimitzar la prevalença dels virus influença en les explotacions porcines.

En el segon estudi es van seguir serològicament i virològicament dos lots de producció de dues granges comercials, per tal d'explorar la dinàmica de la

infecció dels virus influença en el porc. En una de les explotacions es van detectar quatre onades víriques, la primera es va observar a les 3 i 4 setmanes de vida dels garrins en presència d'anticossos maternals. L'anàlisi filogenètic va mostrar que havien cocirculat endèmicament dues variants del virus H1N1. A més, en dos animals, es va aïllar la mateixa soca viral en dos moments diferents separats entre sí per com a mínim 4 setmanes. En l'altre explotació en canvi, només es va aïllar una soca del subtipus H1N2 que es va detectar en una única onada vírica en la que el 93.7% dels animals van resultar RRT-PCR positius. Els resultats obtinguts més rellevants d'aquest estudi són: a) la infecció per virus influença en les granges porcines comercials pot donar-se tant de forma epidèmica com de forma endèmica. b) els virus influença poden infectar els garrins amb anticossos maternals. c) la protecció homòloga generada després d'una primera infecció podria no prevenir enfront una segona infecció amb la mateixa soca o una molt similar. d) diferents variants de virus influença poden circular conjuntament entre els animals d'una explotació durant llargs períodes de temps.

En el tercer estudi s'explorava si el virus pandèmic H1N1 (pH1N1) continuava circulant, entre 22-26 mesos després de la infecció original, en 3 explotacions porcines d'Austràlia on s'havia detectat el virus l'any 2009. També es valorava el potencial de disseminació del virus a altres explotacions a través del moviment de persones i animals. Per portar-lo a terme, es van analitzar els sèrums de 55 animals de cada granja mitjançant l'IH per detectar anticossos específics enfront el virus pH1N1. Els resultats obtinguts mostren que almenys en una de les explotacions el virus pH1N1 havia circulat recentment.

Resumen

Desde el año 1931, se han aislado diferentes tipos de virus influenza en la especie porcina y en la actualidad se considera que determinados subtipos circulan de forma endémica entre la población porcina mundial.

Debido a que los cerdos pueden infectarse tanto por virus de origen aviar como de origen humano, la especie porcina se considera la fuente de generación de nuevos virus influenza recombinantes, que podrían combinar genes de virus de distintos orígenes. Por este motivo, más allá de las consecuencias productivas que conlleva la enfermedad para el sector porcino, la gripe porcina toma relevancia debido a las implicaciones que podría tener para la salud pública.

En el primer estudio de esta tesis se examinó la seroprevalencia frente los virus influenza en el porcino de España y los factores de riesgo asociados. Se recogió información y muestras de suero (2.151 animales) de 98 explotaciones distribuidas por todo el territorio español. Los resultados obtenidos mediante la técnica de inhibición de la hemaglutinación (IH), utilizando los subtipos H1N1, H1N2 y H3N2 como antígenos, mostraron que el 75.4% de los animales presentaban anticuerpos frente alguno de los subtipos. Todas las granjas estudiadas presentaron como mínimo un animal seropositivo frente alguno de los subtipos. Sin embargo, sólo el 9% reportaron sintomatología compatible con gripe durante el último año. Por último, tres factores de riesgo resultaron asociados a la infección: el porcentaje de reposición, las separaciones discontinuas entre corrales y un acceso no controlado a la explotación. Los resultados obtenidos en este estudio muestran una amplia diseminación de los virus influenza en la población porcina de España. Del mismo modo, señalan la importancia de las medidas de bioseguridad así como del diseño de las instalaciones para minimizar la prevalencia de los virus influenza en las explotaciones porcinas.

En el segundo estudio se siguió serológicamente y virológicamente dos lotes de producción de dos granjas comerciales con el fin de explorar la dinámica de la infección de los virus influenza en el cerdo. En una de las

explotaciones se detectaron cuatro oleadas víricas, la primera se observó a las 3 y 4 semanas de vida de los lechones en presencia de anticuerpos maternales. El análisis filogenético demostró que habían cocirculado dos variantes del virus H1N1. Además, en dos animales, se aisló la misma cepa viral en dos momentos distintos separados entre ellos por como mínimo 4 semanas. En la otra explotación en cambio, sólo se aisló una cepa del subtipo H1N2 que se detectó en una única oleada vírica en la que el 93.7% de los animales resultaron RRT-PCR positivos. Los resultados obtenidos más relevantes de este estudio son: a) la infección por virus influenza en las granjas porcinas comerciales puede darse tanto de forma epidémica como de forma endémica. b) los virus influenza pueden infectar los lechones con anticuerpos maternales. c) la protección homóloga generada después de una primera infección podría no proteger frente a una segunda infección con la misma cepa o una muy similar. d) diferentes variantes de virus influenza pueden circular conjuntamente entre los animales de una explotación durante largos períodos de tiempo.

En el tercer estudio se exploraba si el virus pandémico H1N1 (pH1N1) continuaba circulando, entre 22-26 meses después de la infección original, en 3 explotaciones porcinas de Australia donde se había detectado el virus en el año 2009. También se valoraba el potencial de diseminación del virus a otras explotaciones a través del movimiento de personas y animales. Para llevarlo a cabo, se analizaron los sueros de 55 animales de cada granja mediante la IH para detectar anticuerpos específicos frente el virus pH1N1. Los resultados obtenidos muestran que al menos en una de las explotaciones el virus pH1N1 había circulado recientemente.

Summary

Since 1931, different types of swine influenza virus (SIV) have been isolated in swine and nowadays it is assumed that certain subtypes are endemically circulating among pigs population worldwide.

Pigs can be infected by both avian and human influenza virus, and as a consequence, swine is considered the source of generation for new reassortant influenza virus, which virus could combine genes from different origins. For this reason, beyond the consequences that involved the disease for pig sector, swine influenza is taking special importance because of the implications it could have on public health.

In the first study of this thesis seroprevalence and risk factors of SIV in swine population from Spain were examined. Data and serum samples (2,151 animals) were collected from 98 herds located throughout the Spanish territory. The results obtained by hemagglutination inhibition (HI), using H1N1, H1N2 and H3N2 SIV subtypes as antigens, showed that 75.4% of the animals had antibodies against at least one of the subtypes examined. All farms studied had at least one seropositive animal against one of the HI tests. However, only 9% reported clinical signs compatible with swine influenza during the previous year. Finally, three risk factors were associated with the infection: increasement of replacement rates, existence of open partitions between pens and uncontrolled entrance to the farm. The results obtained in this study indicate a widespread exposure to SIV in swine population from Spain. Furthermore, these results highlight the importance of biosecurity measures and facilities design in order to minimize the SIV prevalence is pig farms.

In the second study serological and virological follow-ups were conducted in two whole batches of pigs from two commercial pig farms in order to assess the dynamics of SIV infection in pigs. In one farm, four viral waves were observed; the first one took place in the presence of colostral-derived antibodies when the piglets had 3-4 weeks of age. Phylogenetic analysis showed that two H1N1 variants circulated in that farm. Moreover, in two pigs, the same strain was isolated in two non-consecutive sampling points separated

at least 4 weeks. In contrast in the other farm, only one strain of H1N2 subtype was detected in one viral wave in which 93.7% of the animals were RRT-PCR positive. The most relevant results of this study are: a) the SIV infection in pigs from commercial herds can occur both in epidemics and endemics form. b) SIV infection can occur in piglets in presence of colostral-derived antibodies. c) homologous protection after infection with one strain could not prevent a infection with the same strain or closely related one. d) several SIV may co circulate for extended periods of time.

In the third study, freedom for pandemic H1N1 (pH1N1) virus, 22-26 months following the original infection, in three piggeries from Australia where the pH1N1 were detected in 2009 was assessed. In addition, the potential spread of the virus to other piggeries through people and animal movement was investigated. To accomplish this aim, serum samples from 55 pigs from each piggery were analyzed by HI to detect specific antibodies against pH1N1 virus. The results obtained show that at least in one piggery pH1N1 had recently circulated among pigs.

Els resultants de la present tesi han estat publicats o enviats per publicar en les següents revistes científiques internacionals:

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.

Simon-Grifé M, Hernàndez-Jover M, Holyoake P, Ward MP. Pandemic H1N1 influenza A virus in Australian piggeries: Serological follow-up study and investigation of the potential spread of the virus by people and animal movements

Taula de continguts

Abreviatures i símbols

ADN: àcid desoxiribonucleic

AIAO: all-in/ all-out

ANCOVA: analysis of covariance (estudi 1)

ARN: àcid ribonucleic

BIP: broncho-intersticial pneumonia (estudi 2)

CEEAH: Animal and Human Ethics experimentation Committee (estudi 2)

CI: confidence interval

CRC: Cooperative Research Centre (estudi 3)

ECE: embryonated chicken eggs

ELISA: enzime-linked immunosorbent assay

EUA: Estats Units d'Amèrica

F1: Farm 1 (estudi 2)

F2: Farm 2 (estudi 2)

GEE: generalized estimating equations (estudi 1)

GEP: Good Experimental Practices (estudi 2)

HA: hemaglutinina

HU: hemagglutinin units

IAAP: influença aviar alta patogenicitat

IABP: influença aviar de baixa patogenicitat

IFN-α: interferó alfa

IgA: immunoglobulina A

IgG: immunoglobulina G

IgM: immunoglobulina M

IH: inhibició de la hemaglutinació

IHQ: immunohistoquímica

IL-1: interleuquina 1

IL-6: interleuquina 6

IN: inhibició de la neuraminidassa

M1: proteïna de matriu 1

M2: proteïna de matriu 2

MDA: maternally-derived antibodies

MDCK: Madin-Darby Canine Kidney

NA: neuraminidasa

NK: natural killers

NP: nucleoproteïna

NS1: proteïnes no estructural 1

NS2: proteïnes no estructural 2

OMS: organització mundial de la salut

OR: odds ratio

P1: piggery 1 (estudi 3)

P2: piggery 2(estudi 3)

P3: piggery 3 (estudi 3)

PA: polimerasa A

PB1: polimerasa B1

PB2: polimerasa B2

pH1N1: pandemic H1N1 influenza A

PI: post-infecció

Q1: lower quartile

Q3: upper quartile

QIC: quasi-likelihood under independence model criterion

RBC: red blood cells

RDE: receptor-destroying enzime

RRT-PCR: reacció de transcripció en cadena de la polimerasa en temps real

RT-PCR: reacció de transcripció en cadena de la polimerasa

S/P: sample-to-positive control ratio

SI: swine influenza

SIV: swine influenza virus

SNA: social network analysis (estudi 3)

SPF: specific pathogen free

TNF-α: factor de necrosis tumor alfa

VSRRP: virus de la síndrome respiratòria i reproductiva porcina

Capítol 1. Introducció

1.1 Virus Influença A

1.1.1 Introducció als virus influença A

Els virus Influença pertanyen a la família *Orthomyxoviridae.* Aquesta família està formada per virus amb embolcall que presenten el seu genoma segmentat en diferents fragments d'àcid ribonucleic (ARN) de sentit negatiu. La família *Orthomyxoviridae* inclou cinc gèneres de virus, influenzavirus A, influenzavirus B, influenzavirus C, thogotovius i isavirus (Wright *et al.*, 2007). Els virus del gènere influenzavirus C pràcticament només infecten als humans. Els virus influença del tipus B infecten els humans però l'espècie porcina també és susceptible a infectar-se per aquests virus i esporàdicament s'han pogut aïllar en brots clínics que han afectat a porcs (Takátsy *et al.*, 1967; Takátsy *et al.*, 1969). Els virus influença del tipus A poden infectar a una gran varietat d'hostes com són els humans, èquids, suids, entre altres mamífers, així com una gran varietat d'aus domèstiques i salvatges i, per tant, són els virus influença de més importància veterinària.

Els virus influença tipus A són pleomòrfics i de mida mitjana (entre 80 i 120 nm de diàmetre) (Wright *et al.*, 2007). El genoma dels virus influença està format per 8 segments, cadascun dels quals codifica per una proteïna excepte els segments 7 i 8 que codifiquen per dues (Taula 1). Així doncs, 10 proteïnes conformen els virus influença A; tres polimerases: PB1, PB2 i PA, dues glicoproteïnes transmembranals: hemaglutinina (HA) i neuraminidasa (NA), nucleoproteïna (NP), proteïna de matriu (M1) de la qual deriva una tercera proteïna transmembranal (M2), i dues proteïnes no estructurals: (NS1 i NS2). (Figura 1).

L'HA i la NA són funcionalment molt importants ja que són les responsables de l'adhesió i penetració a la cèl·lula hoste (HA) i de l'alliberament de les partícules víriques (NA) un cop el virus ha replicat. Actualment, es coneixen 16 tipus diferents de HA (H1-H16) i 9 tipus diferents de NA (N1-N9) (Olsen *et al.*, 2006). Els virus d'influença de tipus A es classifiquen en subtipus en funció de l'HA i la NA que posseeixen, de forma que la combinació dels diferents tipus de HA i NA dóna lloc als diferents subtipus de virus influença tipus A, ex: H1N1, H1N2 o bé H3N2. Les diferents soques de virus influença tipus A s'anomenen

seguint la nomenclatura establerta per la Organització Mundial de la Salut (OMS, 1980) de la següent forma: A/ espècie origen/ localització geogràfica de l'aïllat/ número d'aïllat/ any de l'aïllament i seguidament el subtipus entre parèntesis (excepte en el cas del aïllats humans, en els quals l'espècie no es menciona), (ex: A/swine/Spain/1/2010 (H1N2)).

La particular segmentació del genoma dels virus d'influença A, possibilita que la infecció simultània de la mateixa cèl·lula per més d'un virus influença pugui resultar en l'intercanvi de fragments del genoma i com a conseqüència, en la generació d'un nou virus recombinant que combini fragments genòmics dels virus originals (Olsen, 2002).

Taula 1. Segments del genoma dels virus d'influença tipus A, proteïnes per les que codifiquen i funció que se'ls atribueix.

| Segment	Nº Nucleòtids	Proteïna	Funció
1	2341	Polimerasa PB2	Replicació
2	2341	Polimerasa PB1	Replicació
3	2233	Polimerasa PA	Replicació
4	1778	Hemaglutinina HA	Adhesió, penetració
5	1565	Nucleoproteïna NP	Replicació, transport ARN
6	1413	Neuraminidasa NA	Alliberament
7	1027	Proteïna matriu M1	Ensamblatge
		Proteïna matriu M2	Penetració
8	890	Proteïna no estructural NS1	**Desconegut**
		Proteïna no estructural NS2	**Desconegut**
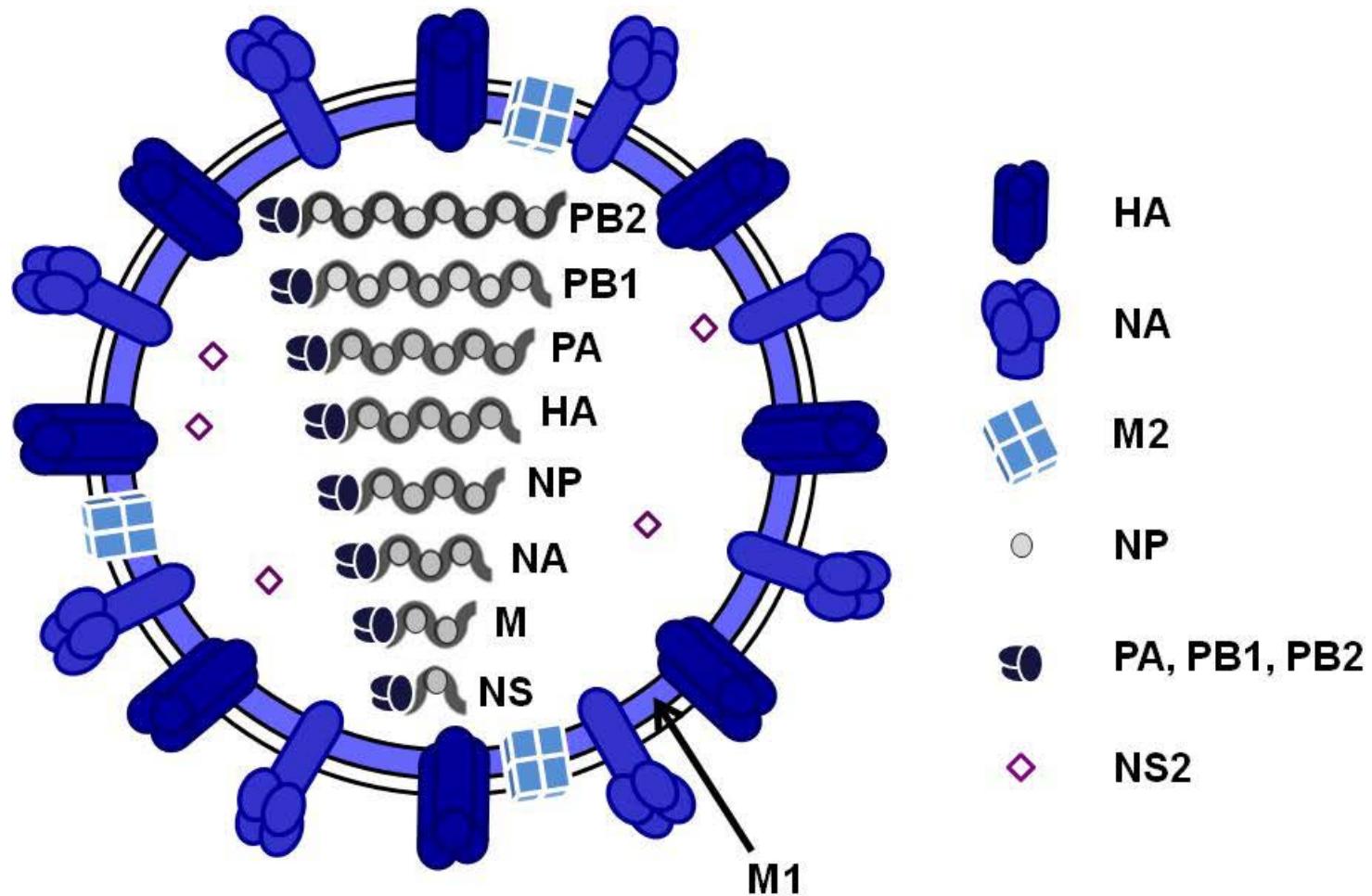
Figura 1. Representació esquemàtica dels virus influença tipus A (adaptació de Taubenberger i Kash, 2010).

1.1.2 Hostes dels virus Influença A i transmissió entre espècies

Les aus aquàtiques són el principal hoste i reservori dels virus influença A En aquest sentit, és important destacar que tots els tipus de HA i NA que avui dia es coneixen, s'han pogut aïllar en aus (Alexander, 2007). De fet, es considera que els virus que s'han aïllat en aus i mamífers es van originar en les aus aquàtiques (Webster *et al.*, 1992). En aus aquàtiques, els virus influença normalment produeixen una infecció entèrica que tot i que acostuma a ser asimptomàtica resulta en la excreció d'una gran quantitat de virus en femtes (Webster *et al.*, 1978; Kida *et al.*, 1980).

Els virus influença de les aus domèstiques es poden classificar en dos "patotips" diferents segons la virulència que provoquen en infectar pollastres, de forma que podem distingir els virus d'influença aviar d'alta patogenicitat (IAAP) i els virus influença aviar de baixa patogenicitat (IABP) (Swayne i Suarez, 2000). Els virus IABP causen infeccions localitzades a l'aparell respiratori i intestinal, en canvi els virus IAAP produeixen una infecció sistèmica incloent el sistema nerviós central causant mortalitats que poden ser del 100%. Els virus IAAP només s'han associat als subtipus H5 i H7, encara que no tots els virus d'aquests subtipus corresponen a IAAP. L' aparició de virus IAAP podria ser deguda a mutacions dels virus IABP que es produeixen en infectar aus domèstiques (Alexander, 2007). Els virus d'influença A s'han transmès des de les aus aquàtiques a moltes espècies entre elles els suids, els èquids, aus domèstiques i mamífers aquàtics. Alhora, també s'han transmès des de l'home al porc i a la inversa i des de les aus domèstiques a l'home (Ducatez *et al.*, 2008)

Els virus influença que s'han establert en la població humana es limiten als subtipus H1N1, H1N2, H2N2 i H3N2 (Taubenberguer i Kash, 2010). Com a conseqüència la població és vulnerable a patir infeccions greus per altres subtipus enfront els quals no seria immunològicament eficient. En aquest sentit, durant els últims anys s'han documentat un elevat nombre de casos d'influença en humans provocats per altres subtipus com H5N1 (Peiris *et al.*, 2007), H7N3 (Tweed *et al.*, 2004), H7N7 (Fouchier *et al.*, 2004) i H9N2 (Lin *et al.*, 2000),

encara que no hi ha evidències que aquets subtipus s'hagin establert circulant de forma permanent en la població humana.

Els virus d'influença de tipus A són juntament amb els virus de tipus B els responsables de les epidèmies anuals recurrents o grips estacionals (Hay *et al.*, 2001). Els virus influença són la causa més freqüent d'infeccions respiratòries en humans degut a l'elevada morbilitat que presenta la malaltia. A més a més, la infecció dels virus d'influença pot provocar significatives mortalitats en determinats grups de la població com són les persones d'edat avançada, els infants i les persones amb malalties cròniques (Taubenberguer i Kash, 2010). Per altra banda, a més dels brots d'influença anuals associats a les èpoques fredes, ocasionalment poden emergir virus d'influença pandèmics. En els últims 100 anys els virus influença han estat responsables de cinc pandèmies els anys 1918, 1957, 1968, 1977 i més recentment l'any 2009. La pandèmia de l'any 1918 va ser la més devastadora de tota la història provocant més de 50 milions de morts a nivell mundial (Johnson i Mueller, 2002).

1.2 La influença porcina

1.2.1 Els virus influença en porcs

Els primers casos clínics d'influença porcina es van detectar l'any 1918 (Koen, 1919) coincidint amb la pandèmia d'influença que va afectar a la població humana el mateix any. El virus causant d'aquest primer brot clínic molt probablement va ser transmès des de la població humana cap a l'espècie porcina, ja que els casos detectats en suids van donar-se cronològicament just després de la malaltia en humans (Brown, 2000). L'any 1931 es va poder aïllar la primera soca del virus en porcs que va correspondre al subtipus H1N1 i es va denominar com A/swine/Iowa/30 o virus H1N1 clàssic (Shope, 1931).

Des de l'any 1931 fins a dia d'avui s'han aïllat diferents subtipus d'influença en el porc, alguns només han causat brots limitats, però en canvi altres s'han establert de forma endèmica. En aquest sentit, actualment els subtipus H1N1, H1N2 i H3N2 s'han pogut evidenciar en porcí pràcticament a nivell mundial (Brown, 2000; Kuntz-Simon i Madec, 2009). Cal dir però, que existeixen

diferències en els virus d'influença que actualment circulen en l'espècie porcina, depenent de la zona geogràfica a la que ens referim. En aquest sentit a Europa, el subtipus H1N1 és predominantment d'origen aviar (avian-like H1N1). Aquest subtipus es va detectar per primera vegada en porcs l'any 1979 (Pensaert *et al.*, 1981) i es va disseminar ràpidament entre la població porcina europea reemplaçant, a partir dels anys 90, el virus H1N1 clàssic que fins aleshores havia estat predominant (Brown, 2000). El subtipus H3N2 es va aïllar per primera vegada en porcs al continent Asiàtic a principis dels anys 1970 poc després de la pandèmia d'influença que va afectar la població humana l'any 1968. Aquest virus d'origen humà (human-like H3N2) va estendre's ràpidament entre l'espècie porcina de forma que a Europa existeixen evidències serològiques de la circulació d'aquest virus en porcs des de principis dels anys 70 (Harkness *et al.*, 1972; Popovici *et al.*, 1972). Aquest virus va continuar circulant entre els porcs europeus fins l'any 1984 quan va emergir un nou H3N2 recombinant més virulent que l'anterior (Castrucci *et al.*, 1993) que va disseminar-se desplaçant el virus human-like H3N2 original de la població porcina europea fins a fer-lo desaparèixer. El nou virus H3N2 recombinant contenia l'HA i la NA del virus H3N2 original però tots els gens interns eren d'origen aviar (Campitelli *et al.*, 1997). El primer aïllat en la espècie porcina del virus H1N2 a Europa es va obtenir a França l'any 1987 (Gourreau *et al.*, 1994). Aquest virus, que contenia la HA d'origen aviar i la NA d'origen humà va aparèixer com a conseqüència de la recombinació entre el virus avian-like H1N1 i el virus human-like H3N2. Cal dir però que aquest nou subtipus no va disseminar-se eficientment entre la població porcina europea i va ser reemplaçat per un altre virus H1N2 reportat per primera vegada a Gran Bretanya l'any 1994 (Brown *et al.*, 1995). Aquest nou virus H1N2 també es va originar com a conseqüència fenòmens de recombinació, de forma que l'HA d'aquest virus derivava del virus H1N1 human-like que circulava en porcs des de la pandèmia de l'any 1977 i la NA i els gens interns provenien del virus H3N2 recombinant (Brown *et al.*, 1998). Anys després es va detectar una tercera generació de virus H1N2 recombinants a França que havien intercanviat l'HA human-like per l'HA provinent d'origen aviar del virus avian-like H1N1 (Marozin *et al.*, 2002).

A Nord Amèrica els virus influença que circulen en l'espècie porcina presenten diferències genètiques i antigèniques rellevants en comparació amb els virus que circulen a la població porcina d'Europa. Així doncs, a Nord Amèrica els virus del subtipus H1N1 predominants corresponen al virus H1N1 clàssic (Vincent *et al.*, 2006). Pel que fa als virus H3N2, des de finals de la dècada dels noranta, el virus H3N2 triple recombinant ha establert un llinatge estable en l'espècie porcina de Estats Units i Canadà (Webby *et al.*, 2000). El virus H3N2 triple recombinant es va generar a partir de la combinació de fragments de genoma de virus influença d'origen humà (HA, NA, PB1) porcí (NS, NP, M) i aviar (PB2 i PA) (Zhow *et al.*, 1999). A partir de la introducció del subtipus H3N2 a Nord Amèrica es van produir fenòmens de recombinació entre els virus H3N2 triple recombinant i el virus H1N1 clàssic que van donar lloc a l'aparició del subtipus H1N2 que va establir-se també de forma endèmica en el continent (Karasin, 2002).

Altres subtipus de virus influença s'han pogut aïllar de forma esporàdica en els suids sense que arribin a establir-se en l'espècie porcina com per exemple els subtipus H1N7, H4N6, H9N2, H3N3, H5N1, H3N1, H2N3 (Brown *et al.*, 1997; Karasin *et al.*, 2000; Xu *et al.*, 2004; Karasin *et al.*, 2004; Choi *et al.*, 2005; Lekcharoensuk *et al.*, 2006; Ma *et al.*, 2007).

1.2.2 Epidemiologia

Històricament, es considerava que la influença porcina seguia un patró estacional, de forma que els brots d'influença porcina s'associaven a èpoques fredes i humides. En l'actualitat en canvi, s'ha demostrat que els virus influença circulen durant tot l'any (Olsen *et al.*, 2000), probablement degut als sistemes de producció actuals, que confinen els animals en instal·lacions on les oscil·lacions tèrmiques estan controlades.

Els virus influença en el porc s'excreten a través de les secrecions nasals de forma que, la transmissió dels virus influença entre l'espècie porcina es dóna per contacte directe o bé per via aerògena (Olsen *et al.*, 2006). Un cop els virus influença s'han introduït en una granja, aquests circularien entre els

animals susceptibles de forma que, la morbilitat de la malaltia generalment és molt elevada arribant a afectar al 100% dels animals d'una explotació. Per contra, la mortalitat associada a la malaltia acostuma a ser molt baixa (< 1%).

1.2.3 Patogènia

La infecció per virus influença és generalment limitada al tracte respiratori i la presència del virus s'ha pogut demostrar a les cèl·lules epitelials de la cavitat nasal, tonsil·les, tràquea, pulmons i limfonodes traqueobronquials (Olsen *et al.*, 2006). La virèmia només s'ha demostrat en escasses ocasions i en qualsevol cas sempre han estat virèmies de baix títol i curta durada (Brown, 1993).

La influença porcina és una infecció aguda, de forma que en la majoria d'estudis experimentals que s'han dut a terme l'excreció vírica s'ha detectat des del dia 1 fins aproximadament el dia 7 post-infecció (PI). Els pulmons són l'òrgan diana del virus que té un específic tropisme per l'epiteli bronquiolar. Fins al 100% de l'epiteli de revestiment dels bronquis i bronquíols així com un alt percentatge de les cèl·lules epitelials alveolars poden resultar infectades (Van Reeth *et al.*, 1998). Normalment, a partir dels 2-3 dies PI el nombre de cèl·lules infectades i et títol víric a nivell pulmonar comença a declinar.

Les citoquines proinflamatòries que produeix l'hoste durant la infecció s'han suggerit com les responsables de la patogènesis i manifestació clínica de la infecció. En concret les citoquines, interferó alfa (IFN-α), el factor de necrosis tumor alfa (TNF-α), la interleuquina 1 (IL-1) i la interleuquina 6 (IL-6) que indueixen disfunció pulmonar, febre i pèrdua de la gana entre altres efectes, s'han observat en elevats nivells al mateix temps que els animals es trobaven en el pic del títol víric i dels signes clínics (Van Reeth, 2000, Van Reeth *et al.*, 2002).

1.2.4 Signes clínics

Els signes clínics associats a la infecció dels virus influença apareixen a partir del dia 1-3 PI. La simptomatologia clínica provocada pels virus influença es caracteritza per febre, letargia, anorèxia, pèrdua de pes, tos, respiració dificultosa, conjuntivitis i secrecions nasals (Olsen *et al.*, 2006; OIE, 2010). En les truges gestants poden observar-se alteracions reproductives, com per exemple avortaments, degut a la piròxia que provoca la infecció (OIE, 2010). Generalment, al cap de 5-7 dies després de l'inici dels signes clínics els animals comencen a recuperar-se.

Els signes clínics associats a la infecció, s'observen en els animals que no presenten cap tipus d'immunitat a la malaltia (Olsen *et al.*, 2006). Per tant, la manifestació clínica de la malaltia s'observa amb més freqüència en els porcs d'engreix després que aquests perdin la immunitat maternal, fet que succeeix entre les 8 i les 12 setmanes de vida (Loeffen *et al.*, 2003a). Per altra banda, la influença porcina també pot manifestar-se de forma asimptomàtica. La presentació subclínica de la malaltia, sembla més freqüent que la forma clínica, tenint en compte l'elevada disseminació dels virus influença que han mostrat els estudis serològics que s'han dut a terme i que semblen indicar que la infecció és més freqüent que la manifestació clínica de la malaltia (Van Reeth *et al.*, 2008). La presentació clínica de la malaltia així com la gravetat de la simptomatologia depèn de molts factors, com són l'edat, la pressió d'infecció, altres infeccions concomitants o factors climàtics (Olsen *et al.*, 2006).

1.2.5 Lesions

Les lesions macroscòpiques que provoca la infecció dels virus influença en porcs, sense altres agents associats, es corresponen amb una pneumònia viral (Olsen *et al.*, 2006). Tot i que en alguns estudis experimentals les lesions provocades pels virus influença han afectat més del 50% del teixit pulmonar (Richt *et al.*, 2003), freqüentment es limiten als lòbuls apicals i cardíacs dels pulmons. Les àrees afectades presenten una consistència més sòlida i una coloració violeta i normalment existeix una línia molt marcada entre les zones afectades i les zones normals del pulmó. Les vies respiratòries poden presentar

contingut sanguinolent i exsudats fibrinosos en el seu interior i els limfonodes bronquials i mediastínics poden estar augmentats de tamany.

Microscòpicament, la infecció per virus influença provoca una necrosis de l'epiteli pulmonar i una descamació de l'epiteli bronquiolar (Haesebrouck i Pensaert, 1986). Les vies aèries apareixen obstruïdes degut a la presència de cèl·lules epitelials necròtiques i cèl·lules inflamatòries de tipus neutrofílic majoritàriament (Van Reeth *et al.*, 1998). En estadis més avançats s'observen infiltrats limfocitaris a les zones perivasculars i peribronquials (Richt *et al.*, 2003). Alhora, els virus influença provoquen destrucció dels cilis bronquiolars i vacuolització de les cèl·lules epitelials, fets que afavoririen les infeccions bacterianes secundàries (Rubin, 2002). Per altra banda, la infecció dels virus influença s'ha associat amb pneumònies necrotico-proliferativa tot i que altres estudis han determinat que l'agent etiològic clau que desencadena aquestes lesions és el virus de la síndrome respiratòria i reproductiva porcina (VSRRP) (Drolet *et al.*, 2003). Les lesions associades a la infecció dels virus influença poden ser lleus o fins i tot inexistents, tal i com hem indicat en l'aparició dels signes clínics.

1.2.6 Immunitat

Els anticossos específics que es generen després de la infecció per virus influença estan principalment dirigits enfront l'HA, la NA i la NP i les proteïnes de la matriu (M1 i M2) (Kitikoon *et al.*, 2008). Només els anticossos dirigits enfront l'HA són capaços d'evitar la unió del virus amb la cèl·lula hoste i per tant, evitar la infecció (Andrew i Coupar, 1988; Webster *et al.*, 1991). Després de la infecció viral es generen en primera instància immunoglobulines (Ig) M i seguidament es produeixen els isotips IgA i IgG. Els isotips IgG i IgM predominen a nivell sanguini, mentre que el isotip IgA és predominant a nivell mucosal (Heinen *et al.*, 2000; Larsen *et al.*, 2000).

Generalment la resposta immunitària és molt eficient i és capaç d'eliminar el virus durant els 7 dies posteriors a la infecció (Olsen *et al.*, 2006). Estudis experimentals han detectat, mitjançant la tècnica d'inhibició de l'hemaglutinació

(IH), la presència d'anticossos en sèrum a partir dels 7-10 dies PI; els títols més alts es mostren a les 2-3 setmanes PI i poden mantenir-se elevats fins a les 8-10 setmanes quan comencen a declinar (Heinen *et al.*, 2000; Larsen *et al.*, 2000). La durada dels anticossos després de la infecció és molt variable de forma que s'han pogut detectar fins a 28 mesos després de la infecció (Desrosiers, 2004). Estudis experimentals han mostrat que després d'una primera infecció, hi ha una protecció eficaç enfront a la infecció pel mateix virus o per soques víriques similars tant a nivell pulmonar com a nivell nasal (Heinen *et al.*, 2000; Larsen *et al.*, 2000). Amb tot i això la durada d'aquesta protecció homòloga és desconeguda. Pel que fa a la protecció heteròloga, a nivell experimental s'han observat certs nivells de protecció creuada que s'han traduït en una disminució de l'excreció vírica i en una presentació clínica més lleu (Heinen *et al.*, 2001a; Van Reeth *et al.*, 2003).

La presència d'anticossos maternals protegeix enfront a la infecció pulmonar i per tant, enfront a la presentació clínica però sembla no ser totalment eficient en la prevenció de la infecció a nivell nasal i per tant, no evita l'excreció vírica (Loeffen *et al.*, 2003b). Per altra banda, és important destacar que la presència d'immunitat maternal interfereix en el desenvolupament d'una resposta adequada després de la vacunació i per tant, la durada dels anticossos maternals s'ha de contemplar a l'hora d'aplicar estratègies vacunals (Kitikoon *et al.*, 2006).

La infecció per virus influença també desenvolupa una resposta immunitària de tipus cel·lular. Inicialment, es generen les citoquines IFN-α, TNF-α, i IL-1 i seguidament es generen IL-6 i citoquines quimiotàctiques i els nivells més alts en totes elles s'observa als 2-3 dies PI (Van Reeth, 2000, Van Reeth *et al.*, 2002). La resposta immunitària cel·lular té un paper clau en la regulació de la replicació vírica, ja que les citoquines indueixen l'activació dels limfòcits T citotòxics i la diferenciació dels limfòcits B alhora que estimulen les cèl·lules "natural killers" (NK), responsables de l'eliminació de les cèl·lules infectades (Rincón *et al.*, 1997; Brassard *et al.*, 2002).

1.2.7 Diagnòstic laboratorial

La influença porcina no és diagnosticable de forma concloent a partir de la clínica i lesions observades, degut a que la malaltia no desenvolupa signes patognomònics. Per aquest motiu, el diagnòstic s'ha de realitzar al laboratori mitjançant l'aïllament del virus o la detecció de proteïnes o material genètic d'aquest, o bé la detecció d'anticossos específics generats enfront al virus.

L'aïllament del virus pot realitzar-se a partir de la inoculació de les mostres obtingudes (mitjançant hisops nasals/orofaríngics o a partir d'homogeneïtzats de teixit respiratori) en ous de pollastre embrionats o en cultius cel·lulars. En el primer cas la inoculació es realitza al líquid al·lantoideu d'ous embrionats d'uns 10-11 dies d'edat i la presència vírica es confirma pel test d'hemaglutinació (OIE, 2010). Cal dir però, que el creixement d'alguns virus influença del porc és pobre sinó nul en ous. En aquests casos és recomanable utilitzar cultius cel·lulars. La línia cel·lular Madin-Darby Canine Kidney (MDCK) és la línia cel·lular d'elecció per al creixement dels virus influença, tot i que també es poden utilitzar altres línies primàries, derivades de ronyó, testicle, pulmó o tràquea porcins, (OIE, 2010). Les cèl·lules MDCK expressen els receptors de l'àcid siàlic amb unió a galactosa α2,6 i α2,3 fet que permet que s'hi uneixin virus influença d'origen humà, aviar i porcí (Seo *et al.*, 2001). La presència d'efecte citopàtic és indicativa de creixement víric tot i que és necessària la confirmació mitjançant el test d'hemaglutinació o la reacció de transcripció en cadena de la polimerasa (RT-PCR) (OIE, 2010).

La determinació del subtipus dels virus influença es pot realitzar mitjançant l'IH i a través de la inhibició de la neuraminidassa (IN). La detecció dels virus influença i la seva subtipificació també es pot realitzar per tècniques de biologia molecular. En aquest sentit, hi ha metodologies de RT-PCR en temps real (RRT-PCR) que són capaces de detectar de forma genèrica els Influenzavirus A, ja que estan dirigides al gen de la matriu, una zona altament conservada dels virus influença (Spackman i Suarez, 2008; Slomka *et al.*, 2010). Complementàriament, hi ha altres RT-PCR convencionals o en temps real que detecten l'HA i la NA i que per tant, permeten subtipificar els virus influença

(Choi *et al.*, 2002; Chiapponi *et al.*, 2003; Mallinga *et al.*, 2010). La presència vírica també pot ser detectada mitjançant d'altres tècniques com la immunoflorescència directa sobre teixit respiratori o a per tècniques d' immunohistoquímica (IHQ) en teixits fixats.

La infecció per virus influença també es pot demostrar a partir de tècniques serològiques que detectin anticossos específics enfront a la malaltia. Tot i que hi ha diferents kits comercials d'ELISA (enzime-linked immunosorbent assay), la tècnica de referència per a la detecció d'anticossos enfront al virus influença és l'IH (OIE, 2010). L'IH és una tècnica que discrimina entre els anticossos produïts enfront diferents subtipus i per tant, que permet distingir les infeccions produïdes pels diferents subtipus de virus influença (Van Reeth *et al.*, 2006). Degut a que el sèrum porcí pot contenir inhibidors de l'hemaglutinació no específics, els sèrums s'han de pretractar per tal d'eliminar-los amb receptor-destroying enzime (RDE). Alhora, és necessari absorbir els sèrums en eritròcits de pollastre i d'aquesta manera destruir l'activitat de factors d'aglutinació no específics (OIE, 2010). Per tal d'optimitzar la sensibilitat de l'IH cal utilitzar soques víriques representatives de la zona geogràfica i alhora contemporànies en relació a les mostres que s'analitzen.

1.2.8 Prevenció i control

1.2.8.1 Mesures de bioseguretat

L'aplicació d'un programa de bioseguretat adequat ajuda a prevenir la introducció i disseminació dels virus influença a les explotacions porcines. En aquest sentit, l'aplicació d'una quarantena adequada als animals de reposició, que s'ha suggerit com una via freqüent d'entrada de virus influença en les explotacions (Alexander, 2007), i restringir l'accés de les aus silvestres a les instal·lacions o als aliments, són mesures de bioseguretat necessàries per reduir el risc d'introducció del virus a les explotacions porcines. Per altra banda, l'espècie humana pot actuar com una font d'introducció del virus a les granges porcines i per tant, les mesures de bioseguretat dirigides a evitar la introducció de malalties a través de les persones també podrien reduir el risc d'entrada dels virus influença.

L'aplicació de sistemes de filtració d'aire a les explotacions porcines han demostrat ser efectives en la reducció de la transmissió de malalties a través d'aerosols (Dee *et al.*, 2005) i alguns estudis han mostrat que també podrien reduir significativament la incidència de la infecció de virus influença (Loeffen *et al.*, 2008).

1.2.8.2 Estratègies vacunals

Les vacunes enfront a la influença porcina s'apliquen per via intramuscular en dues dosis separades entre 2 i 4 setmanes i es recomana la revacunació cada dos anys en el cas de truges. L'eficàcia de la vacunació depèn de molts factors com l'estat de salut de l'animal i el moment d'administració de la vacuna ja que la presència d'anticossos maternals interfereix en el desenvolupament d'immunitat activa (Kitikoon *et al.*, 2006).

Degut a les diferències genètiques i antigèniques que existeixen entre els virus influença que circulen a Europa i Nord Amèrica, les soques víriques que componen les vacunes comercials també difereixen entre continents.

A Europa, la primera vacuna enfront la grip porcina va obtenir la llicència als anys 80 i contenia els subtipus H1N1 i H3N2. Tot i que aquesta vacuna bivalent conté soques aïllades als anys 70, estudis experimentals han mostrat que segueix oferint protecció clínica i virològica enfront soques més recents (Heinen *et al.*, 2001b; Van Reeth *et al.*, 2001). Amb tot i això, les vacunes bivalents no són protectives enfront al subtipus H1N2 (Van Reeth *et al.*, 2003). Recentment, a Europa s'ha aprovat la comercialització d'una nova vacuna trivalent que incorpora soques actuals dels subtipus H1N1, H1N2 i H3N2. La taula 2 mostra les vacunes que actualment es comercialitzen a Europa.

Als Estats Units d'Amèrica (EUA) l'any 1993 es va introduir la primera vacuna monovalent enfront el subtipus H1N1. Posteriorment, quan va emergir el subtipus H3N2 entre la població porcina dels EUA va estar disponible la vacuna bivalent que també incorporava aquest subtipus. Les vacunes que es comercialitzen actualment a EUA contenen el subtipus H3N2 recombinant, el subtipus H1N1 clàssic i, en el cas de les vacunes trivalents més recents, també el subtipus H1N1 recombinant. Recentment, els EUA han aprovat la

comercialització d'una vacuna que incorpora el subtipus H1N1 responsable de la pandèmia humana de l'any 2009 (USDA, 2009).

Les vacunes anomenades "de nova generació" s'han testat experimentalment i els resultats obtinguts són contradictoris, de forma que encara que les vacunes d'ADN (àcid desoxiribonucleic) i les vacunes recombinats generen resposta immunitària no semblen protegir enfront a la infecció o a la malaltia (Olsen, 2000; Larsen *et al.*, 2001; Heinen *et al.*, 2002).

Empresa productora	Nom del producte	Soques de virus influença
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)

Taula 2. Vacunes d'influença porcina comercialitzades a Europa.

1.2.9 Implicacions de la influença porcina en la salut pública.

En humans les infeccions causades per virus influença porcina han estat reportades als EUA, Canadà, Europa i Àsia (Myers *et al.*, 2007). La majoria de casos han estat reportats a EUA i causats pel subtipus H1N1 clàssic tot i que també s'ha aïllat el subtipus H3N2 en algun dels pacients. La major part de les persones afectades mantenien contacte estret amb l'espècie porcina, a més a més estudis serològics han mostrat que les persones amb una ocupació que implica exposició a l'espècie porcina tenen un risc més elevat d'infectar-se per virus influença (Olsen *et al.*, 2002; Myers *et al.*, 2006; Ramirez *et al.*, 2006).

Generalment, els virus influença porcins no es transmeten amb facilitat entre l'espècie humana encara que hi ha hagut excepcions com l'episodi que va transcórrer a Fort Dix l'any 1976, en el que fins a 230 soldats van infectar-se pel virus (Top i Russell, 1977).

Els porcs poden infectar-se per virus influença humans i aviaris (Hinshaw *et al.*, 1981; Landolt *et al.*, 2003) ja que l'espècie porcina presenta en el seu aparell respiratori receptors de l'àcid siàlic amb unió a galactosa α2,6 i receptors de l'àcid siàlic amb unió a galactosa α2,3, que els virus influença humans i aviaris respectivament, utilitzen de forma preferencial per unir-se a les cèl·lules hostes (Rogers i Paulson, 1983; Rogers *et al.*, 1983). Per aquest motiu, tradicionalment s'ha proposat l'espècie porcina com a possible font de generació de nous virus recombinants que combinin gens provinents de virus humans i aviaris. Més recentment, estudis realitzats també han demostrat l'abundant presència de receptors de l'àcid siàlic amb unió a galactosa del tipus α2,3 en el tracte respiratori inferior dels humans, fet que facilita la replicació de virus influença d'origen aviar (Shinya *et al.*, 2006). Per tant, l'espècie humana també podria actuar com a font de generació de nous virus recombinants.

1.3 Pandèmies humanes causades per virus influença A

L'any 1918 va emergir una pandèmia, també anomenada grip espanyola, que va afectar a més d'un terç de la població mundial (Frost, 1920). La malaltia va ser excepcionalment greu amb letalitats superiors al 2.5%. El virus influença

responsable d'aquesta pandèmia procedia dels virus H1N1 aviaris (Taubenberger *et al.*, 2005).

Després del brot pandèmic dels anys 1918 i 1919 la influença va tornar a manifestar-se amb el seu patró comú, amb brots epidèmics regionals de menys gravetat fins que l'any 1957 es va detectar un brot a Hong Kong que va afectar a més de 250.000 persones. El virus responsable d'aquesta pandèmia corresponia al subtipus H2N2. Aquest nou virus, era un descendent lineal del virus H1N1 de l'any 1918 que havia adquirit 3 segments genètics (HA, NA, PB1) d'origen aviar (Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989). L'espècie de l'hoste en la qual es va produir aquesta recombinació genètica continua sent desconeguda. Aquest virus va esdevenir estacional i endèmic en la població humana fins que al cap d'uns 11 anys va desaparèixer i no ha retornat, de fet es considera l'únic virus pandèmic no establert de forma endèmica en l'espècie humana (Taubenberger *et al.*, 2010).

L'any 1968 un nou virus pandèmic va aparèixer al Sud-est asiàtic. Aquest virus corresponia al subtipus H3N2 i va originar-se com a resultat de la recombinació entre el virus H2N2, aleshores circulant en humans, i virus d'origen aviar dels que va incorporar els fragments genètics de l'HA i de la PB1 (Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989). Aquest virus ràpidament va esdevenir endèmic i estacional i encara circula actualment entre la població humana.

L'any 1977 va reemergir una variant del virus H1N1 responsable de la pandèmia de l'any 1918 després d'haver estat absent durant més de 20 anys. Estudis filogenètics han demostrat que el virus era molt similar a les soques del virus H1N1 aïllades durant els anys 1950 (Nakajima *et al.*, 1978). Aquest virus ha continuat circulant juntament amb el virus H3N2, i la coinfecció d'aquests dos virus ha generat un nou virus recombinant que correspon al subtipus H1N2.

Més recentment, a l'Abril de l'any 2009 es van detectar 2 casos en humans a Califòrnia del Sud causats per un nou virus influença A corresponent al subtipus H1N1 (CDC, 2009). L'anàlisi genòmica va mostrar que el nou virus del subtipus H1N1 era fruit de la recombinació de diferents virus circulants en

l'espècie porcina. Concretament, el virus H1N1 recombinant contenia els segments genètics corresponents a la NA i a la proteïna M provinents dels virus avian-like H1N1 del llinatge europeu i els 6 fragments genètics restants procedents del virus H1N2 triple recombinant del llinatge de Nord Amèrica (Smith *et al*., 2009). El virus es va disseminar eficientment entre la població humana obligant a la Organització Mundial de la Salut (OMS) a declarar l'alerta de nivell 6. Posteriorment, es va detectar el virus H1N1 pandèmic en porcs domèstics de diferents països. La majoria d'estudis epidemiològics duts a terme van suggerir la introducció del virus pandèmic en les explotacions porcines a partir dels humans (Hofshagen *et al*., 2009; Howden *et al*., 2009; Pereda *et al*., 2010; Welsh *et al*., 2010; Wong i Deng, 2011). Recentment, s'han detectat en el porc noves soques de virus influença derivades de la recombinació del virus H1N1 pandèmic amb altres virus influença endèmics en l'espècie porcina Vijaykrishna *et al*, 2010; Howard *et al*, 2011; Kitikoon *et al*, 2011; Moreno *et al*, 2011)

La figura 3 mostra les relacions genètiques entre els virus influença que han causat pandèmies (adaptació de Morens *et al*, 2009).
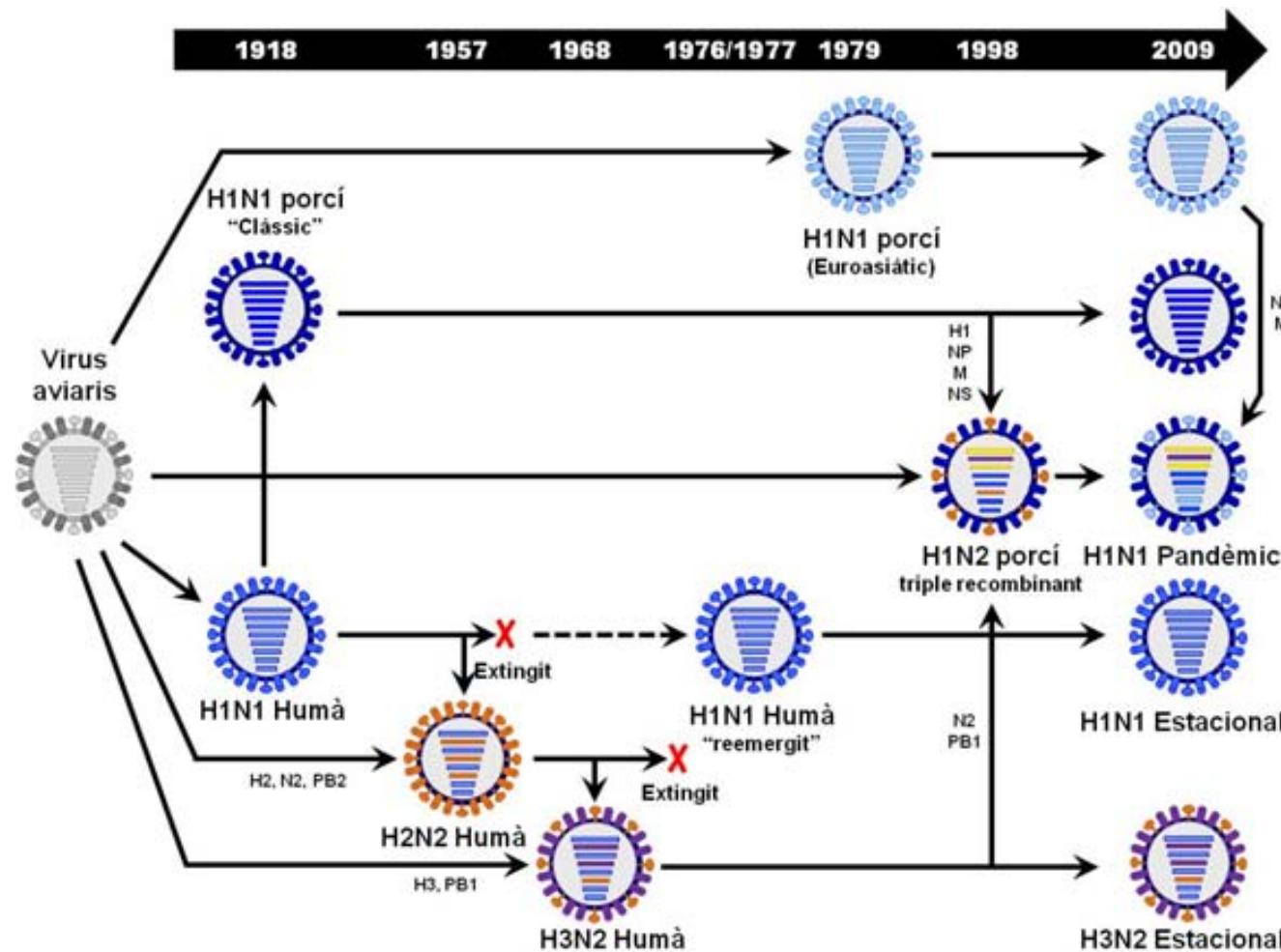
Capítol 2. Objectius de la tesi

Objectius de la tesi

La complexitat de l'epidemiologia dels virus influença, el potencial zoonòtic que han demostrat al llarg de la història juntament amb la importància que té en el sector porcí posen de manifest la necessitat d'un millor coneixement de la situació de la influença en l'espècie porcina.

La importància de la indústria porcina a l'Estat Espanyol, que actualment és el segon productor de la Unió Europea, denota la necessitat de conèixer els virus que actualment circulen en la població porcina espanyola. A més a més, un millor coneixement de l'epidemiologia així com els factors de risc de la influença porcina contribuirien a prevenir i limitar l'entrada i disseminació dels virus influença en les explotacions porcines.

L'objectiu genèric de la tesi és aportar informació sobre la disseminació dels virus influença a Espanya i investigar l'epidemiologia de la malaltia en les explotacions porcines.

A continuació es defineixen els objectius específics de la tesi;

1) Identificar els virus influença que actualment circulen en les àrees de major producció porcina de l'Estat Espanyol així com els factors que predisposen a la infecció en la espècie porcina.

2) Investigar la dinàmica d'infecció dels virus influença en les explotacions porcines. Més específicament, es pretén avaluar quin és el patró de circulació dels virus influença i la simptomatologia clínica associada a la infecció en garrins i porcs d'engreix de granges porcines comercials.

3) Determinar si el virus pandèmic H1N1 es manté circulant a les explotacions porcines que es van infectar durant el brot ocorregut a Austràlia i examinar la potencial disseminació del virus a altres explotacions a través del moviment de persones i animals.

Capítol 3. Estudis

3.1 Estudi 1

Seroprevalença i factors de risc de la influença porcina a Espanya

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 associate 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, 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 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.*, 2010) 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 ±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. 3.1.1.
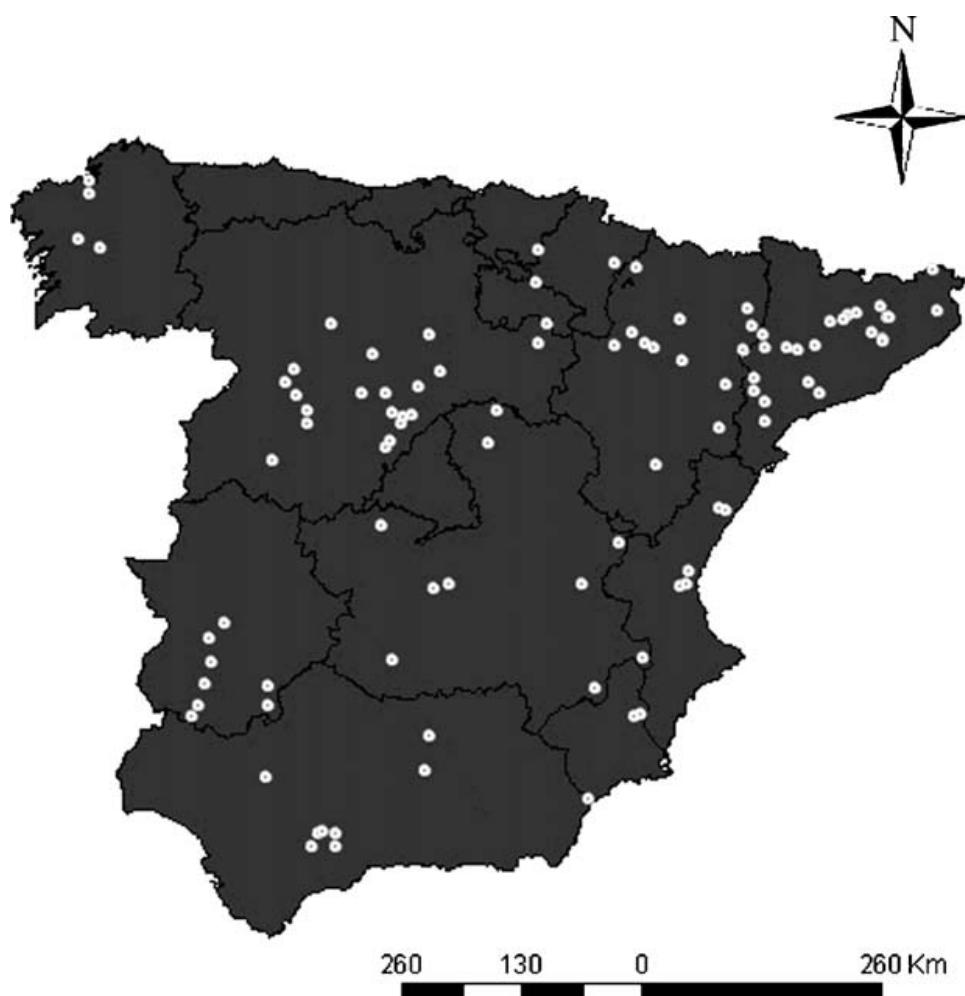

Fig. 3.1.1. Distribution of analysed farms (white dots) in Spain.

2.2. Data collection: the questionnaire

Epidemiological data were gathered through an on farm interview with the farmer. The questionnaire (provided in Annex 1) 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 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 ºC) within 24–48 h of sampling. Blood samples were centrifuged at 400 x g for 15 min at 4 ºC and sera were stored at -80 ºC 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 ºC for 60 min. Subsequently, sera were adsorbed using a 50% chicken red blood cells (RBC) suspension at 4 ºC. 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 X^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 3.1.1).

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.*, 2003a). 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 3.1.1. 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*	Number of nearby pig farms**
	Type of control system for ventilation in maternity*
	Type of control system for ventilation in pregnancy*
	Type of ventilation in maternity*
	Sow census**
Replacement rate value**	Controlled access to farm*
	Presence of pregnancy yards*
	Type of ventilation in maternity*
	Sow census**
Type of ventilation in pregnancy*	Type of control system for ventilation in maternity*
	Type of control system for ventilation in pregnancy*
	Type of ventilation in maternity*
	Sow census**
Fattening pigs model	
All in/all out performance in fatteners*	All in/all out performance in nursery*
Number of nearby pig farms**	
Controlled farm access*	
Partitions between pens in fattening units*	Partitions between pens in nursery*
Presence of bird-proof nets*	
Presence of other livestock animals than pigs in the farm*	
Type of control system for ventilation in fattening*	Type of ventilation in fattening*
	Type of control system in nursery*
	Type of control hitting in nursery*
Type of farm*	

* Corresponding to categorical variables.

** 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 3.1.2 and 3.1.3.

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.

Table 3.1.2. 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 *					
	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

* Cut-off = 1:20

Table 3.1.3. Number of animals (n = 2,151) and age groups (sows: n = 1,302; fattening pigs: n =849) with HI antibodies to one or more SIV subtypes.

Virus subtype(s)	Number and percentage of seropositive animals*					
	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

* Cut-off = 1:20

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 log2; $CI_{95\%}$: 6.5–6.7) than titres against either H1N1 or H1N2 (6.2 log2; $CI_{95\%}$: 6.2–6.3 and 6.2 log2; $CI_{95\%}$: 6.1–6.4, respectively). Likewise, H1N1 and H3N2 titres were significantly higher in sows (6.4 log2; $CI_{95\%}$: 6.3–6.5 and 6.8 log2; $CI_{95\%}$: 6.7–6.9, respectively) than in fattening pigs (5.7 log2; $CI_{95\%}$: 5.5–5.9; and 6.0 log2; $CI_{95\%}$: 5.8–6.2, respectively). There were no significant differences between those two age groups with regards to H1N2 subtype. Fig. 3.1.2 (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.

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; CI95%: 0.41–0.49; p < 0.05). Forty-four farms had at least one animal negative to HI and positive to ELISA.
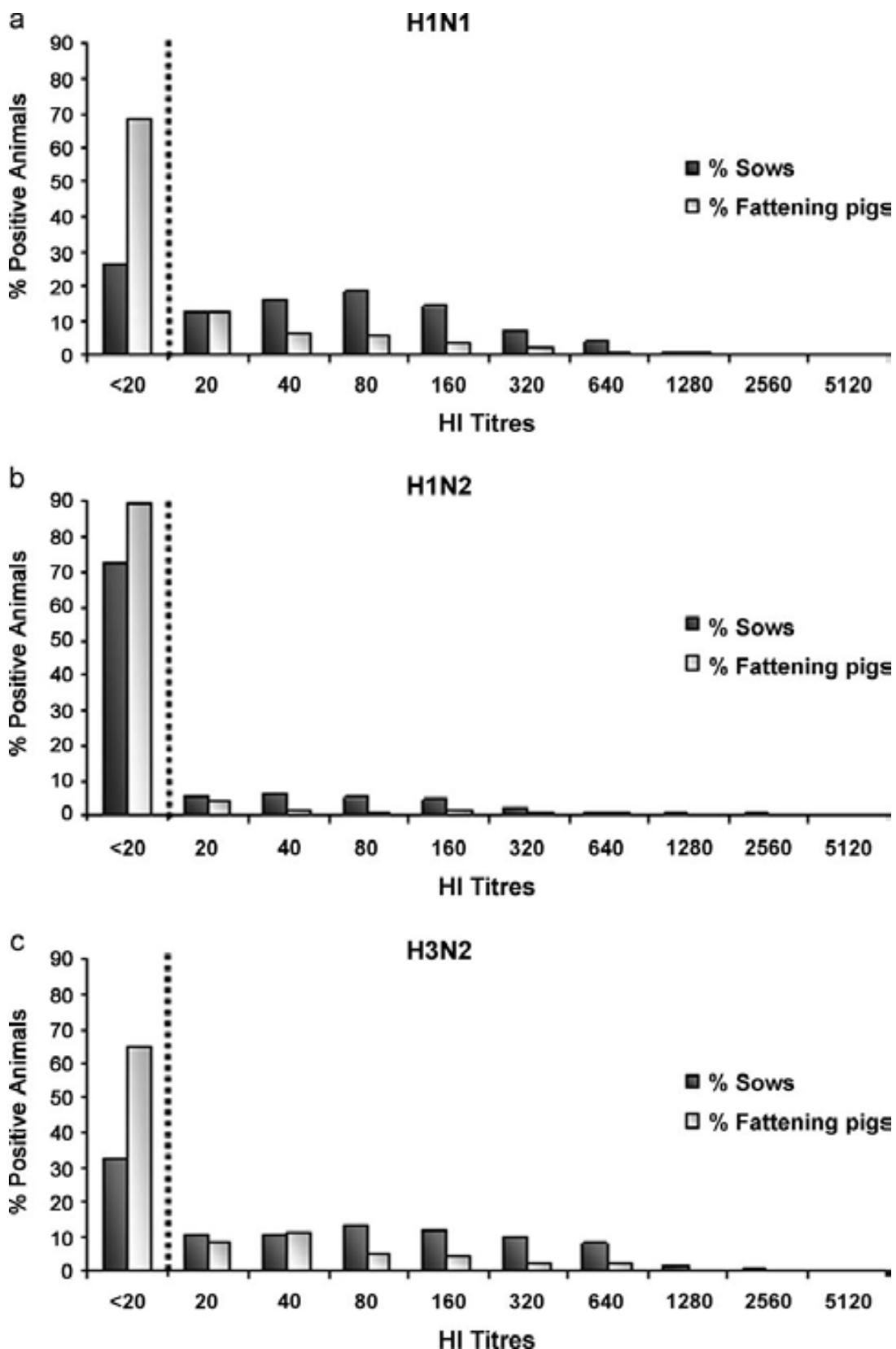
Fig. 3.1.2. Distribution of antibody titres against H1N1 (section a), H1N2 (section b) and H3N2 (section c). Dotted line represents the cut-off.

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 3.1.1). 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 3.1.4 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).

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.

Table 3.1.4. 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% C.I OR	pvalue	OR	95% C.I OR	pvalue	OR	95% C.I OR	pvalue
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

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 *et al.* (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 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-Grifé *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 noncontemporary 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 influenzavirus 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.

3.2 Estudi 2

Dinàmica d'infecció dels virus influença en dues granges porcines; Resultats d'un estudi longitudinal

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.

1. 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 (Van Reeth *et al.*, 2007; OIE, 2008). Sneezing, conjunctivitis, nasal discharge and abortions may also be observed (OIE, 2008). SI-associated gross lung lesions observed in pigs are mainly those of a viral pneumonia, and are characterized by a broncho-intersticial pneumonia (BIP) (Olsen *et al.*, 2006).

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 (Castrucci *et al.*, 1993; Ito *et al.*, 1998). 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 (Smith *et al.*, 2009).

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 (Olsen *et al.*, 2006). However, Swine Influenza Virus (SIV) seems to be more widespread in pigs than previously thought (Brown, 2000). 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 (Maldonado *et al.*, 2006; Van Reeth *et al.*, 2008; Simon-Grifé *et al.*, 2011). On the other hand, although the persistence of SIV activity after an acute outbreak has been described (Madec *et al.*, 1985), and the existence of endemically infected herds has been postulated (Olsen *et al.*, 2006; Brown, 2000), 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-tofinish pig farms.

2. Materials and methods

2.1 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.

2.2 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 (Simon-Grifé *et al.*, 2011), 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.

2.3 Farm facilities and biosecurity practices

2.3.1 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.

2.3.2 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.

2.4 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.

2.5 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 ½ dilution, and known positive and negative samples were used as test controls (Busquets *et al.*, 2010).

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 (OIE, 2008) with 4 hemagglutinin units (HU) per well. Cut-off of HI was set to ≥1:20 as reported before (Van Reeth *et al.*, 2008; Kyriakis *et al.*, 2009). Three SIV strains that belonged to Eurasian clusters circulating in Europe were used for HI: A/swine/Neth/Best/96 (avianlike 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.

2.6 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 (Busquets *et al.*, 2010) 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 (OIE, 2008) 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.

2.7 Subtyping and phylogenetic analysis

Viral isolates were subtyped by multiplex RT-PCR described by Chiapponi *et al.* (Chiapponi *et al.*, 2003) 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 Annexe 3:

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 Annexe 4.

2.8 Statistical analysis

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

3. Results

3.1 Farm 1

3.1.1 Antibodies against influenza A viruses

Figure 3.2.1 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.

Table 3.2.1 shows the proportion of pigs positive by the hemagglutination inhibition test ($\geq$ 1:20) to H1N1, H1 N2 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.
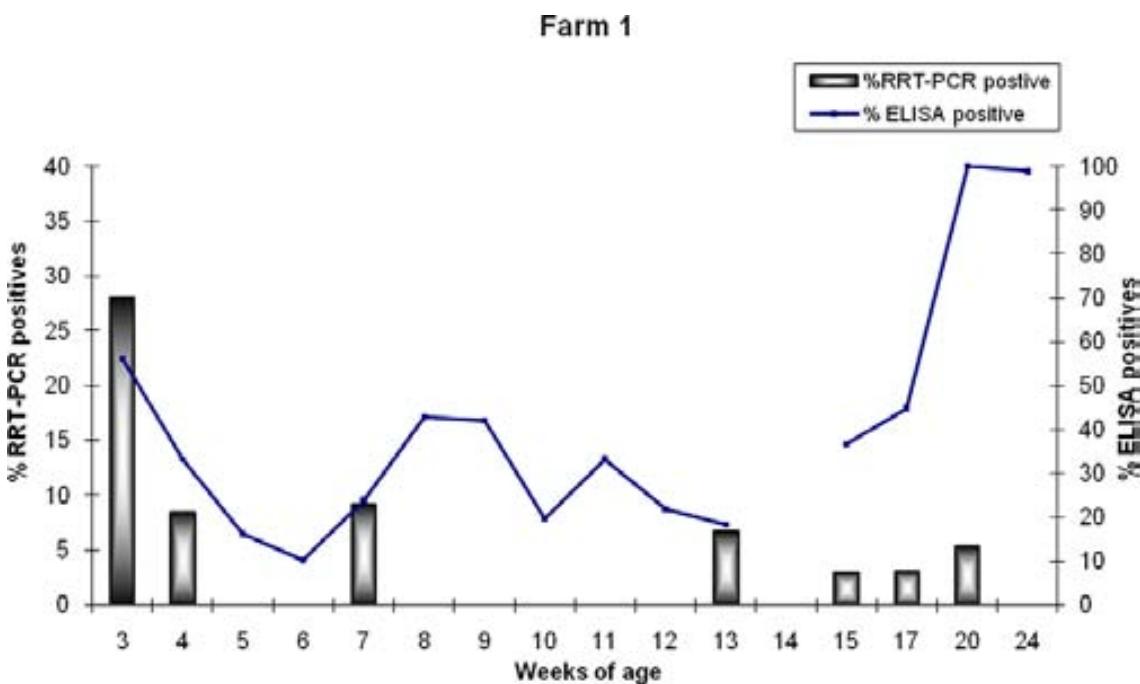

Figure 3.2.1. Seroprevalence and incidence of SIV in Farm 1. Antibodies against SIV were analyzed by ELISA (line) and nasal shedders were determined by RRT-PCR (bars) at each sampling time.

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.

Table 3.2.1. Seroprevalence of antibodies against H1N1, H1N2 and H3N2 in Farm 1 obtained by HI test.

Age (weeks)	Percentage of seropositive animals*					
	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

3.1.2 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.

SIV 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 (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 3.2.2). 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).
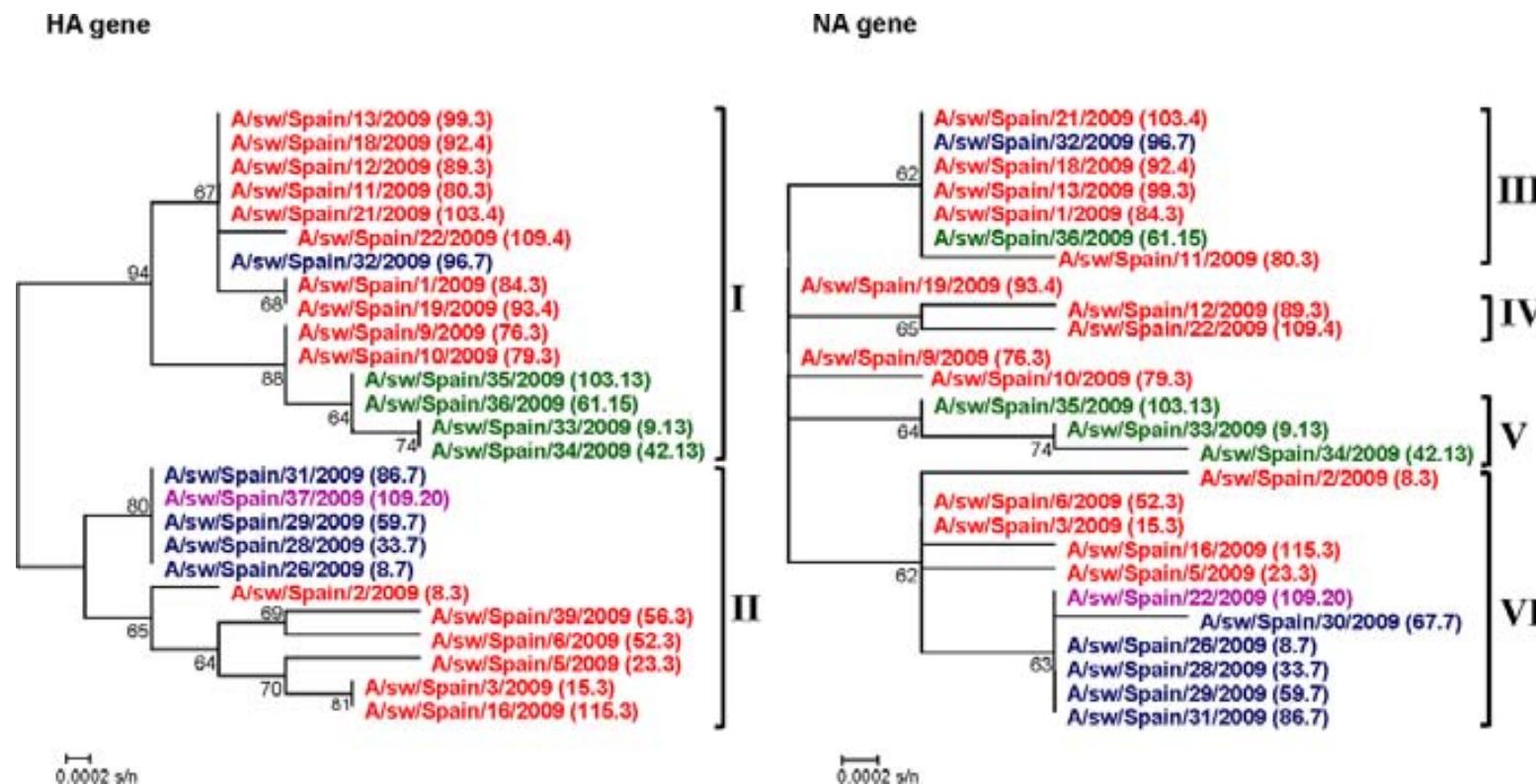
Figure 3.2.2. Phylogenetic tree of the HA1 and NA1 genes of SIV isolates from Farm 1. The accession numbers of sequence data of influenz virus were deposited in GenBank under the accession numbers [GenBank: JF960169, JF960172 - JF960174, JF960176, JF960177, JF960180 JF960184, JF960187, JF960189, JF960190, 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 isolate 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; an cluster VI, VI.

All the isolates from this farm were subtyped as H1N1 and grouped with other European H1N1 SIV of an avian-like clade (Figure 3.2.3).

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 3.2.4.

3.1.3 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 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.
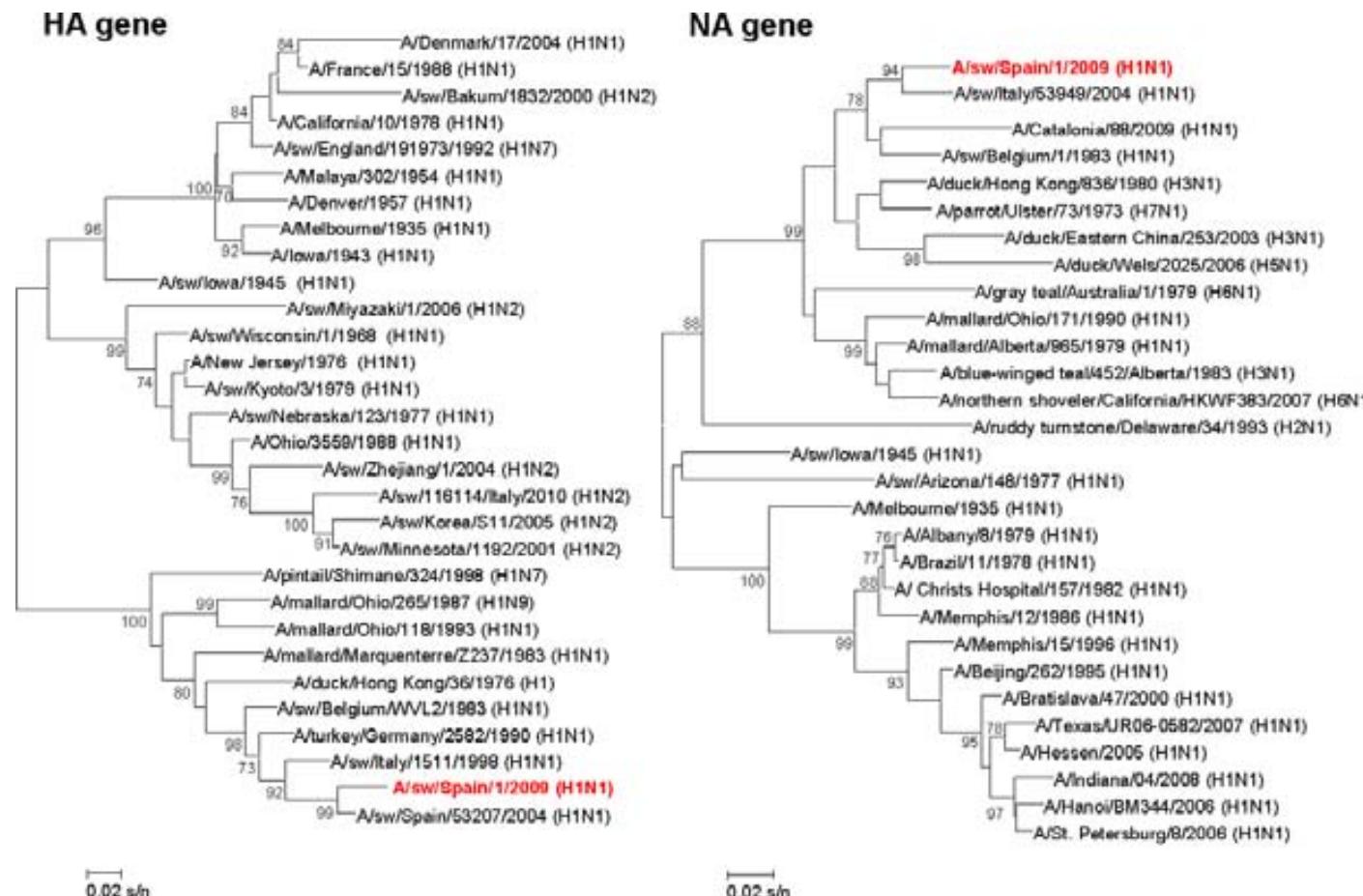
Figure 3.2.3 Phylogenetic analysis for HA and NA of SIV isolates retrieved in Farm 1. The strains isolated in our study are highlighted in red. Unrooted bootstrapped neighbour-joining trees of nucleotide sequences of hemagglutinin and neuraminidase. Bootstrap values, calculated on 1000 replicated trees, are shown if ≥ 70 percent. Scale bars indicate substitutions per site. The accession numbers of sequence data are provided in Annexe 4.
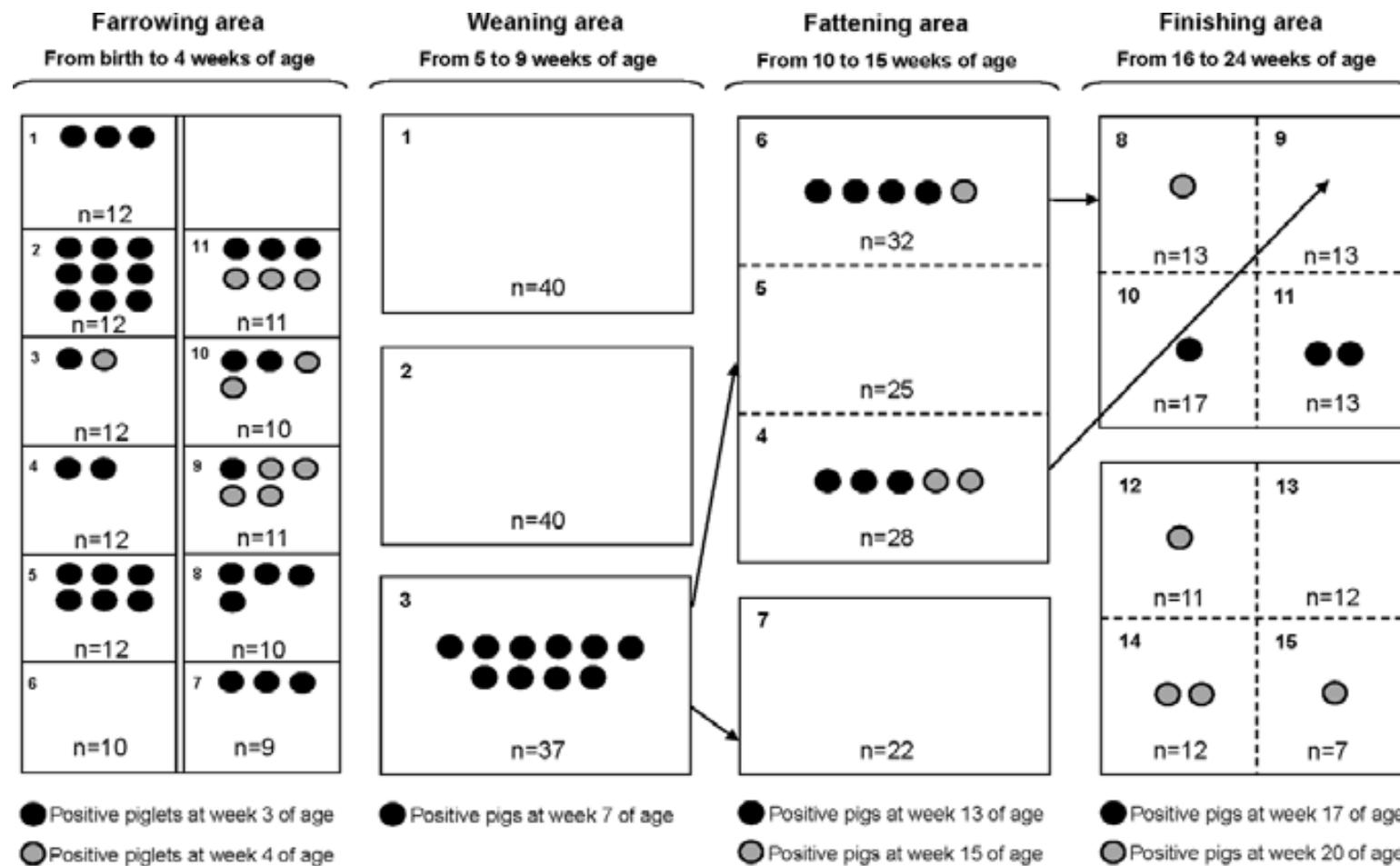
Figure 3.2.4 Distribution of viral shedders according to RRT-PCR results in Farm 1. Open separations between pens are represented as dashed lines; closed separations between pens are shown with solid lines. Arrows show movements of RRT-PCR positive pigs.

3.2 Farm 2

3.2.1 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 3.2.5 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.
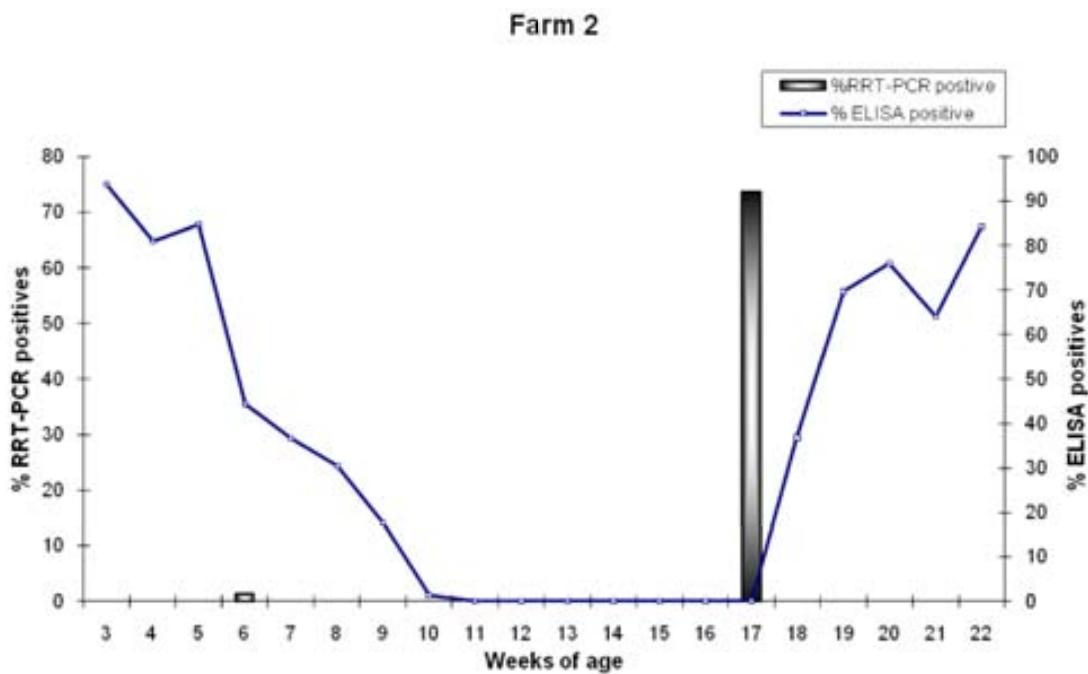
Figure 3.2.5. Seroprevalence and incidence of SIV in Farm 2. The techniques and symbols used are the same as those used in Figure 3.2.1.

3.2.2 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.

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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 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 3.2.6). Positive animals detected in week 17th were distributed among all the pens that housed fatteners.

3.2.3 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%.
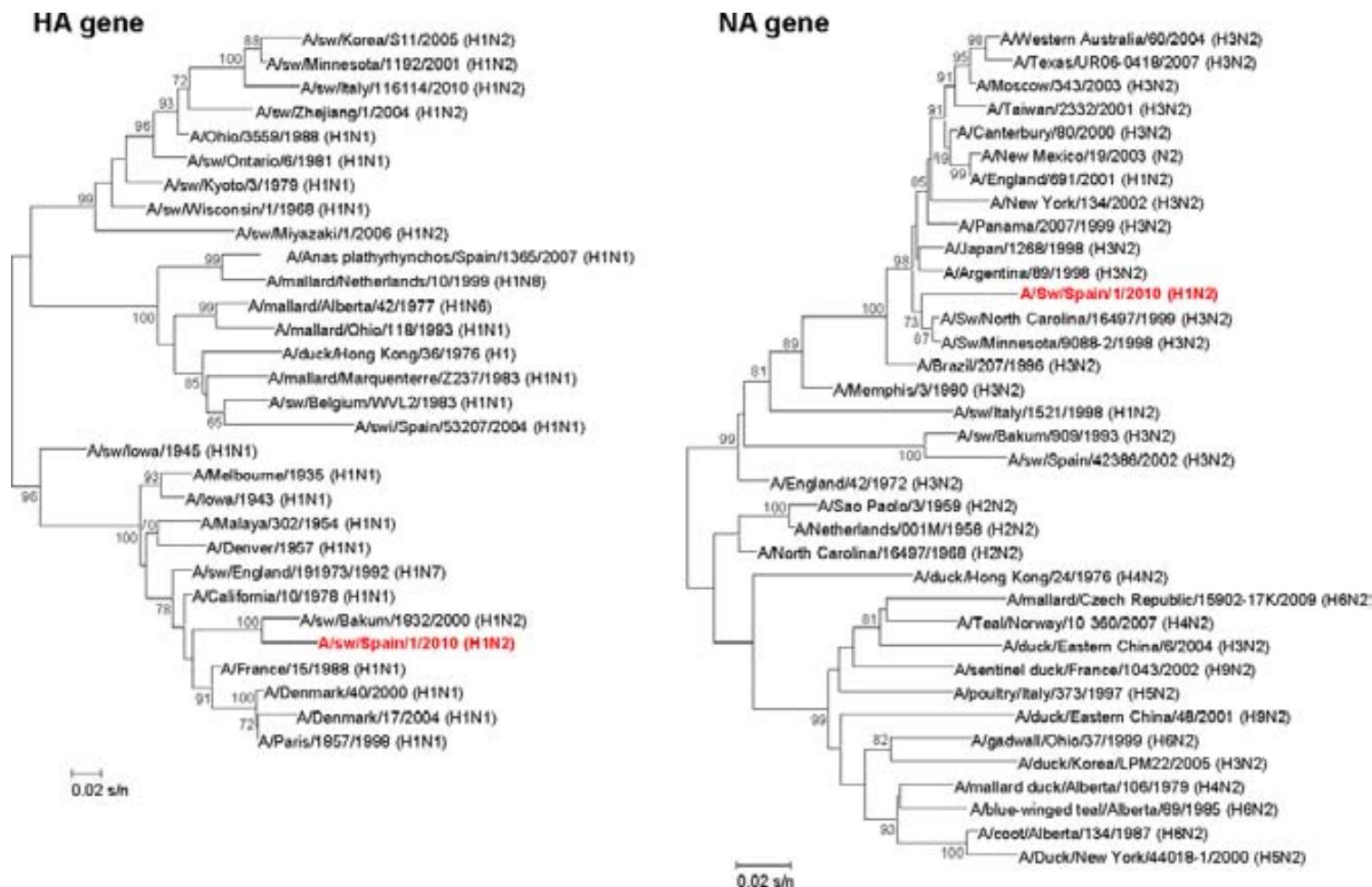
Figure 3.2.6. Phylogenetic analysis for HA and NA of SIV isolates retrieved in Farm 2. Color scheme, rooting, and scale are the same as thos used in Figure 3.2.3. The accession numbers of sequence data are provided in Annexe 4.

4. 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 maternally-derived antibodies (MDA) which occurs after 10 weeks of age (Loeffen *et al.*, 2003a; Loeffen *et al.*, 2009). On the other hand, mucosal IgA is considered to be a correlate of protection against virus replicating in the upper airways (Tomoda *et al.*, 1995; Renegar *et al.*, 2004). In the present case, piglets with MDA 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 (Easterday, 1972; Macklin *et al.*, 1998; Loeffen *et al.*, 2003b; Choi *et al.*, 2004; Kitikoon *et al.*, 2006). 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 (Brookes *et al.*, 2010).

Humoral protection against influenza viruses is mainly mediated by antibody responses to HA (Murphy and Clements, 1989). 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 intrasubtype 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 (Nelson *et al.*, 2008). 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 (Palese, 2004) 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 (Olsen *et al.*, 2006). 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 (Maes *et al.*, 2000) 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.* (Liu *et al.*, 2009), 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 (Dupont *et al.*, 2009; Simon-Grifé *et al.*, 2011). 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 (Lu *et al.*, 2005) or in MDCK cells cultures (Price *et al.*, 1997; Schultz *et al.*, 1998) 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 (Martín-Valls *et al.*, 2010) 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) (Maldonado, 2007), 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.

Acknowledgements

3.3 Estudi 3

Virus influença H1N1 pandèmic a les granges porcines australianes: Seguiment serològic i investigació del potencial de disseminació del virus a través del moviment de persones i animals

Abstract

In April 2009, a novel H1N1 influenza A (pH1N1) virus was identified in Mexico. The virus has since spread throughout the world and has caused an influenza pandemic. In July 2009 the first incursion of pH1N1 virus into a pig herd in Australia was reported and the virus was subsequently identified in a small number of other pig herds in this country. The aims of the present study were to assess freedom from pandemic infection on three of these affected piggeries (P1, P2 and P3) - 22-26 months following the original infection - and the potential spread of the virus to other piggeries through people and animal movement. We qualitatively evaluated the impact of the outbreak on piggery workers, pig productivity and on-piggery biosecurity practices using a questionnaire completed during each piggery visit. During June 2011, serum samples from fifty-five pigs from each piggery were analysed by a hemagglutination inhibition (HI) test to detect specific antibodies against pH1N1 virus. Our results show that at least in one piggery (P2) pH1N1 virus had circulated recently among pigs. After the outbreak, biosecurity practices targeting human-borne disease introduction were enhanced in all piggeries. However, only P1 adopted compulsory vaccination of their employees against seasonal flu (including pH1N1). Moreover, pH1N1 virus transmission between piggeries via animal and people movements were likely to have occurred. This study highlights the need for further research on influenza virus epidemiology in piggeries in Australia to inform future disease control and response plans.

1. Introduction

In April 2009, a novel H1N1 influenza A (pH1N1) virus was isolated in Mexico (CDC, 2009). This virus spread quickly in the human population, causing the World Health Organization to raise their alert to the highest pandemic phase level on 11 June 2009.

Pig herds in Australia are considered free of *classical* swine influenza (SI) subtypes A viruses (DAFF, 2008a). However, during the pandemic influenza in 2009 Australia was the third country to report pH1N1 virus in domestic pigs, following Canada and Argentina (OIE, 2009a; OIE, 2009b; OIE, 2009c). The detection of the pH1N1 virus in piggeries in Australia represented the first clinical swine influenza virus (SIV) infection in pigs reported in that country. The first affected herd was located in New South Wales (NSW) and was reported in July 2009 (OIE, 2009c). Additional outbreaks were reported in two piggeries in Victoria and two piggeries in Queensland. Australian authorities implemented several measures to control the outbreak, such as restricted movement of animals onto and off the infected piggeries for between 2 and 4 weeks and limited people access (AHA, 2009). Moreover, pigs from the infected piggeries were monitored until the outbreaks were resolved.

Several experimental studies have shown that pigs are susceptible to infection with the pH1N1 virus and that the virus can be transmitted among them (Lange *et al.*, 2009; Brookes *et al.*, 2010). However, investigations conducted in commercial piggeries affected by the pH1N1 virus in countries such as Canada, Norway and Cameroon has suggested that transmission from humans was the source of infection (Howden *et al.*, 2009; Hofshagen *et al.*, 2009; Wong and Deng, 2011; Njabo *et al.*, 2012).

Traditionally, SI outbreaks are characterized by respiratory signs. Most pigs in a herd are affected, with morbidity up to 100% (Olsen *et al.*, 2006). Nonetheless, the widespread exposure to SIV and the low number of reported clinical outbreaks suggest that most of the SIV infections are subclinical (Simon-Grifé *et al.*, 2011). Furthermore, it is likely that herds will become persistently-infected when naïve replacement breeding stock and/or grower stock are routinely introduced (Simon-Grifé *et al.*, 2011; Simon-Grifé *et al.*, 2012). In this

sense, pH1N1 virus could still be circulating in Australian piggeries affected during the 2009 pandemic outbreak.

The research objectives of the present study were to: 1. assess freedom from pandemic infection in three piggeries that were infected during the pH1N1virus outbreak in Australia; 2. describe the impact of the outbreak on piggery workers, piggery productivity and on biosecurity practices; and 3. investigate the potential spread of the virus to other piggeries through animal and people movements.

2. Materials and methods

2.1 Study design

The Departments of Primary Industries in the three Australian states where the five pH1N1 infected herds were located, were contacted (by email) to introduce the study and to seek their approval and participation. Approval was obtained from two states and therefore the three piggeries located in these states (P1, P2 and P3) were included in the study.

The sample size required to detect infection using an imperfect diagnostic test (sensitivity and specificity < 1) was calculated using Freecalc version 2 software. Different input parameter values were used for the calculation of the sample size according to different assumptions: (1) Sensitivity and specificity of the diagnostic test of 0.98; (2) Population size of 1,000 and 2,000 animals; (3) Expected minimum prevalence of disease of 0.10, 0.15 and 0.20; (4) Probability of detection of 0.95; and, (5) Precision of 0.05. The sample size obtained according to the expected minimum prevalence of disease (0.1, 0.15 and 0.20) was 64, 36 and 28 animals, respectively. The sample size required did not vary according to assumed population size.

2.1 Sample collection

The three piggeries were visited in June 2011 to collect blood samples from the animals and to gather information regarding the pandemic influenza outbreak in 2009/2010. In each piggery, 55 pigs were randomly selected. Pigs over 12 weeks of age were targeted, when the presence of maternally-derived

antibodies (MDA) is less likely to occur (Loeffen *et al.*, 2003a). However, pigs of this age were not available in one of herds (P3) and the oldest growing pigs (8-weeks old) were sampled.

Blood samples were collected by jugular venipuncture by two experienced veterinarians (MS-G, TH) using a sterile collection system (Vacutainer®, Becton-Dickinson, USA). Blood samples were transported to the reference laboratory (Elizabeth MacArthur Agriculture Institute, EMAI) and subsequently centrifuged at 400 × g for 15 min at 4 ºC. Sera were stored at -80 ºC until further analysis.

2.3 Serologic testing

Serum samples were analysed by hemagglutination inhibition (HI) test at EMAI. The HI was performed according to standard procedures (OIE, 2008) and four hemagglutinin units (HU) per well were used. Sera were treated overnight (18 h) with receptor-destroying enzyme (RDE) from Vibrio cholerae to remove nonspecific inhibitors and were inactivated at 56 ºC for 60 min. Subsequently, sera were treated with chicken red blood cells (RBC) suspension at room temperature to remove natural serum agglutinins. The starting dilution for testing sera was 1:10. Cut-off of HI was set to ≥1:10. The SIV strain used as antigen in the HI was A/Auckland/1/2009 (H1N1). Viral stocks were cultured in Madin-Darby Canine Kidney (MDCK) cells.

2.4 Questionnaire design

A four-page questionnaire was developed to gather information on piggery husbandry and biosecurity practices, and the impact of the pH1N1 outbreak in the selected piggeries. The questionnaire was similar in format and content to a questionnaire previously used in a study on pig producers' perceptions of the pH1N1virus outbreak (Hernàndez-Jover *et al.*, manuscript in preparation).

The questionnaire, written in English, consisted of 48 mostly closed questions organized into 4 sections. Questions were expressed in a simple and clear format to minimize confusion and maximize accuracy (Thrusfield, 1995;

Dohoo *et al.*, 2003b). Section 1 focused on piggery husbandry and biosecurity practices. Sections 2 and 3 sought to determine the changes in the biosecurity practices since the outbreak and the impact of the outbreak on animals and personnel. Section 4 investigated the potential dissemination of the virus to other piggeries through animal and people movement. In this last section, information on the number of movements of pigs off the piggery, destination of these pigs and piggery visitors during the 6- month period prior to the outbreak were recorded.

The questionnaire (Annex 2) was designed to be administered during face-to-face interviews with the pig owners and took up to 45 min to complete. During the interview, certain records such as book for visitors and receipts for livestock purchases were checked in order to support answers to the questionnaire.

2.5 Network components

Social Network Analysis (SNA) describes the relationship between individuals within a group. Recently, SNA tools have been increasingly applied in veterinary medicine to identify individuals or populations (such as herds) involved in disease spread (Martínez-López *et al.*, 2009).

An SNA approach was used to investigate the potential spread of pH1N1 virus from the infected piggeries during 2009/2010 to other piggeries or abattoirs. Network data, such as information on contact between the index piggeries and other piggeries and abattoirs (nodes of the network) through animal and people movements, were collected from the three index piggeries included in this study. Each contact between nodes could potentially result in the transmission of the pH1N1 virus. Contact-tracing data on all pig movements - including the location by postcode of the destination premises and number of pigs per movement - during the month prior to the study were collected. In addition, information on visitors to each piggery during the 6 months prior to the study was also collected. Piggery visitors were subsequently contacted to gather information on the number and location of other piggeries they visited one month after contact with the index piggery.

2.6 Data analysis

Data from the questionnaire and the serological results obtained by HI diagnostic test were entered into an Excel (Windows XP 2007) spreadsheet. The seroprevalence and 95% of confidence interval (CI) of SIV were calculated (SPSS 17.0. SPSS Inc., Chicago, IL, USA).

Network data for all contacts for each herd were imported into UCINET version 6.350 software (Analytic Technologies; http://www.analytictech.com) for analysis. Network graphs were created and visualized using NetDraw version 2.113 (Analytic Technologies). Following this, results obtained from these applications were exported to ArcGISTM 154 9.3 (ESRI Inc., Redland, CA, USA) to allow geographical mapping. Note that for reasons of commercial confidentiality, the actual geographic locations of the piggeries are not shown.

3 Results

3.1 Piggery descriptions and biosecurity practices

Piggery 1 (P1) operated a 250 sow farrow-to-finish piggery and employed 4 workers; whereas P2 and P3 were farrow-to-weaning piggeries with 1,000 and 1,200 sows, and had 7 and 10 employees, respectively. P1 and P2 were relatively isolated with no other piggeries within a 5 km radius. In contrast, another farrow- to-finish piggery was located approximately 200 metres from P3. The biosecurity practices that were in place at the time of the piggery visits are described in Table 3.3.1. The three piggeries were accredited within the Australian pig industry quality assurance program (Australian Pork Industry Quality, APIQ√™). P2 and P3 had quarantine facilities for replacement s tock. Although P1 bred their own replacement stock, a new boar was introduced once a year and it was quarantined prior to its introduction to the main herd.

Despite all piggeries having a dressing room with showers, none of them required a compulsory shower for visitors before entering the piggery. Only the workers from one piggery (P1) were vaccinated against seasonal flu (including pH1N1).

Table 3.3.1. Husbandry and biosecurity practices currently applied in three piggeries in Australia which reported pH1N1 virus during the 2009/2010 outbreak.

On-piggery practice	Piggery 1	Piggery 2	Piggery 3
No other piggeries in ≤ 5km	✓	✓	
Perimeter fence around the piggery	✓	✓	✓
Application of quarantine measures for replacement stock	✓	✓	✓
No contact of pigs with other livestock animals	✓	✓	✓
Pigs housed in indoor facilities	✓	✓	✓
Record of visitors	✓	✓	✓
Visitors are provided with on-piggery overalls and boots	✓	✓	✓
Visitors are required to shower before entering the piggery			
Piggery staff is not allowed to contact other piggeries	✓	✓	✓
Mandatory staff vaccination against seasonal flu (including pandemic H1N1)	✓	✓[a]	
Control program for rodents	✓	✓	✓
Use of a veterinary consultant	✓	✓	✓
APIQ-accredited	✓	✓	✓

The symbol "✓" indicates that the biosecurity practice is applied in the piggery
[a] Only half of piggery staff workers were vaccinated against human influenza

3.2 Biosecurity changes since the H1N1/09 outbreak

Management on all three piggeries enhanced biosecurity procedures to reduce the likelihood of introduction of human-borne disease to their herds in response to the outbreak. Family members were allowed to access the piggery and be in contact with the animals at P1 before the outbreak; however, after the outbreak only piggery personnel could contact the pigs. Additionally, commercial representatives were not allowed into P2 after the outbreak and the owner from P3 started to keep records of visitors. Moreover, all piggery owners stated that staff were not allowed to go to work if they had flu-like symptoms. Vaccination against seasonal flu before the outbreak was not a requirement for staff working in any of the three piggeries, and only P1 adopted compulsory vaccination of their employees against seasonal flu (including pH1N1).

3.3 Impact and management of the pH1N1 virus outbreak

Although pig movement restrictions were implemented at the three piggeries to avoid pH1N1 virus spread, none of the piggeries reported major problems due to overcrowding. Two piggeries (P2 and P3) reported having production losses due to increased medication costs. Moreover, P3 owners stated that there was an increased incidence of other diseases such as proliferative enteropathy or erysipelas and a slightly decreased fertility rate.

The owners of P1 and P3 reported being emotionally stressed due to the excessive media attention. For example, one of these piggeries had media helicopters flying over the piggery property on several days during the pH1N1 outbreak. Moreover, the piggery employees were concerned about their own health and the health of their families due to the lack of understanding of the actual risk of transmission of the virus from pigs and the severity of the disease.

Piggery owners believed that sample collection by the animal health authorities was conducted appropriately. However, one of the piggeries reported some lack of communication between the pig industry representative body and the government, because in their opinion they received some inconsistent messages. Overall, the three piggeries agreed that the pH1N1 outbreak was appropriately managed by the authorities.

3.4 Antibodies against pH1N1 virus

Pigs from two (P2 and P3) of the three study herds were found to be seropositive (titre ≥1:10) to pH1N1 virus from samples collected in June 2011. The proportion of pigs with pH1N1-specific antibodies was 0% (n = 0; $CI_{95\%}$: 0-8.1), 56.4% (n = 31; $CI_{95\%}$: 42.4-69.4) and 74.5% (n = 41; $CI_{95\%}$: 60.7-84.9) for P1, P2 and P3, respectively. Figure 3.3.1 shows the antibody titre distribution of the sampled pigs in each herd. When the number of positive reactors is used to evaluate freedom of disease in the population, results indicate that P1 is free from disease at 99.9% confidence interval and, in absence of maternal antibodies, that pig populations in P2 and P3 had recently been infected with pH1N1. (Freecalc v.2).
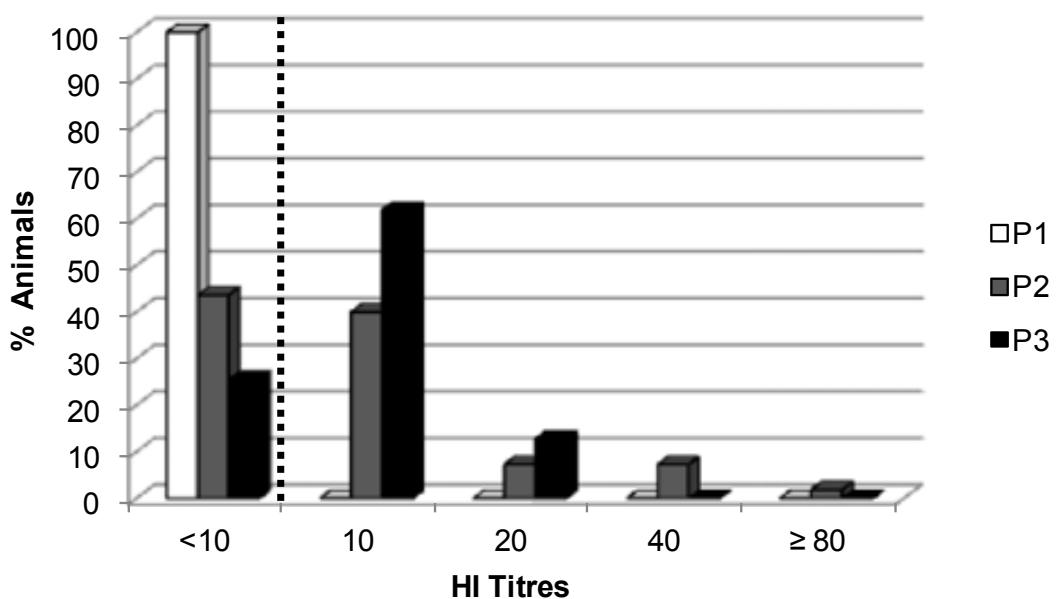
Figure 3.3.1. Distribution of antibody titres against pH1N1 virus in the three piggeries studied (P1, P2 and P3) from Australia in June 2011, and which were infected in 2009 (P1 and P3) and 2010 (P2). Dotted line represents the cut-off value used for the hemagglutination inhibition test.

3.5 Potential of pH1N1 spread through people and animal movements.

Animal movements off the index piggeries are represented in Figure 3.3.2. Slaughter pigs from P1 were sent to two different abattoirs. Approximately

360 pigs per month were sent to an abattoir located 192 km South of the piggery and 30 pigs per month were sent to an abattoir located 96 km North-East of the piggery. Thirty cull breeders per month from P2 and 23 from P3 were sent to the same abattoir, which was located 305 and 50 km from P2 and P3, respectively. In addition, P2 sent 1,400 weaners per month to a finishing piggery located at a distance of 156 km and P3 sent 1,720 weaners per month to another finishing piggery located at a distance of 80 km.
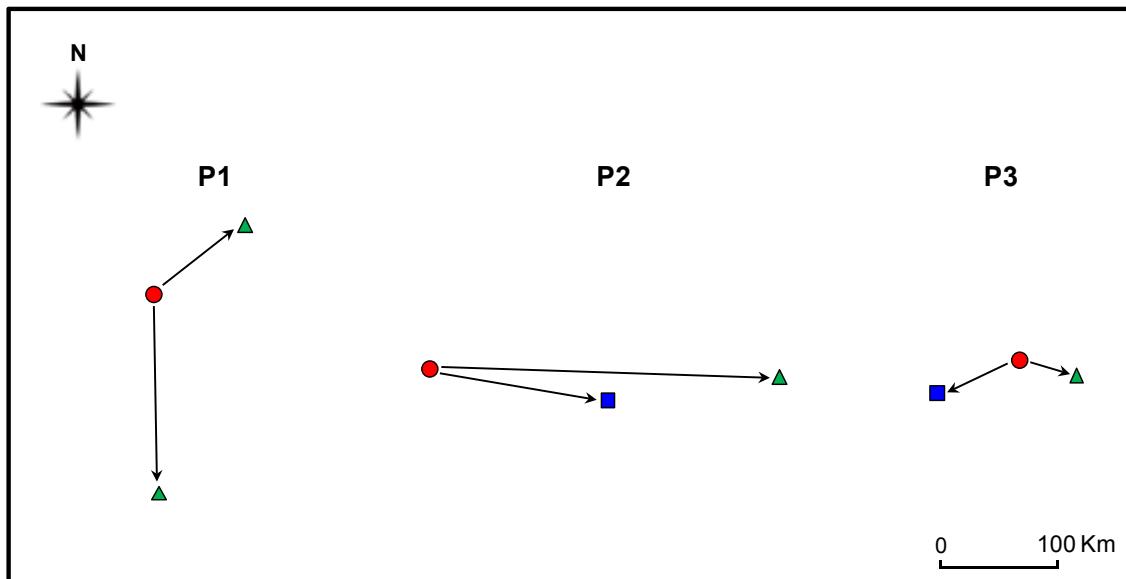
Figure 3.3.2. Representation of the distances between, and the geographic position of, 3 piggeries that were infected by pH1N1 virus in Australia in 2009 (P1 and P3) and 2010 (P2) (circles) and other piggeries (squares) and abattoirs (triangles) connected to these 3 piggeries via animal movement.

Figure 3.3.3 represents people movement off the index piggeries. P1 was visited by one commercial representative and the veterinary consultant responsible for the pig health. Three other piggeries were visited by the same veterinarian during the following month after visiting P1; these were located at 120, 151 and 245 km from P1. The commercial representative visited five piggeries during the month after visiting P1; these were located between 579 and 1,013 km from P1. In addition, the owner of P1 visited another piggery (536 km north) to source a replacement boar. P1 had no contact with private transport companies or feed supplier companies because animal transport to the abattoir was undertaken by the piggery owner using the piggery's truck and feed was prepared on the piggery. P2 was visited by the veterinary consultant, one commercial representative, staff from the company providing replacement

gilts and the drivers of the animal transport company and the feed supplier company. Moreover, this piggery was visited by a veterinarian from the government to sample the pigs during the outbreak. The same government veterinarian visited another piggery located 143 km from P2 two weeks after visiting this piggery.
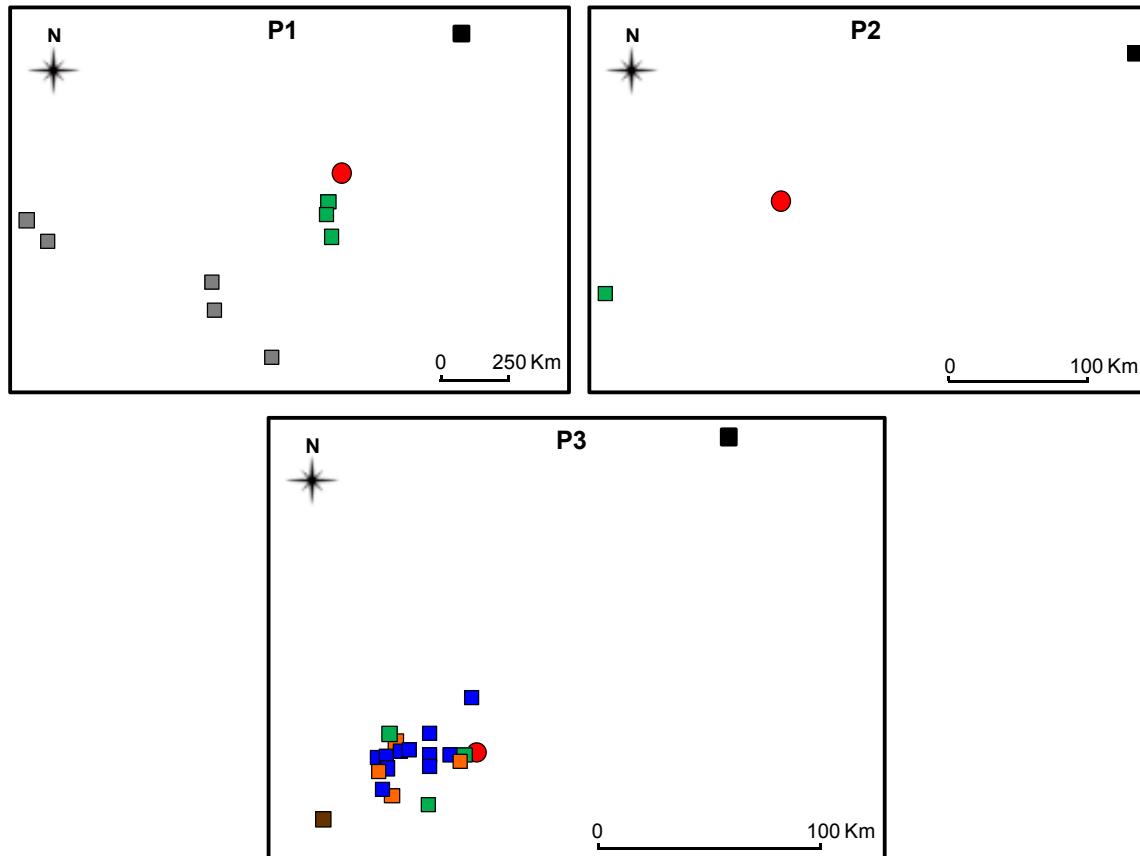
Figure 3.3.3. Representation of the distances between, and the geographic position of, 3 piggeries (P1, P2 and P3) that were infected by pH1N1 virus in Australia in 2009 (P1 and P3) and 2010 (P2) (circles) and other piggeries (squares) connected via people movement. The colour of the squares shows the occupation (black - replacement gilts transport; green - veterinarian; grey - commercial representative; blue - feed supplier company; brown - animal transport company; and orange - feed supplier and animal transport company) of the people who visit the piggery.

Due to confidentiality issues and lack of willingness to participate in the study, information about contacts with other piggeries from the veterinary consultant, the commercial representative and the animal transport and feed supplier companies was unavailable. P3 was in some way linked with 24 other piggeries via movement of visitors, including the veterinary consultant, staff from the company providing the replacement stock, the animal transport

company and the feed supplier company (Figure 3.3.3). Most of piggeries linked with P3 were visited by the same truck of the feed supplier company, which provided feed for 15 piggeries, located between 20 and 50 km from P3. The veterinary consultant visited three more - located between 6 and 38 km from this piggery - the month after visiting P3.

4. Discussion

The pig population in Australia is considered free of classical swine influenza. Freedom from disease is based on passive surveillance (reporting) of clinical signs. Serological monitoring of pig herds in Australia has not been assessed since 1948 (Pullar, 1948). It should be noted that, in this 1948 study, only a small number of blood samples from pigs with typical pneumonia were examined for influenza antibodies.

In the current study, we investigated three piggeries affected during the pH1N1 outbreak in Australia in 2009 and the potential spread of pH1N1 virus to other piggeries by people and animal movements. The results obtained demonstrate that pH1N1 virus was circulating in at least one of the piggeries studied more than 18 months after the outbreak. Furthermore, there is the potential for the pH1N1 virus to be transmitted from the studied piggeries to a considerable number of other piggeries via established pathways - people and animal movements.

Antibodies against pH1N1 were detected in two (P2 and P3) of the three piggeries studied. In one of these piggeries (P3), pigs were sampled at 8 weeks of age, so that the possibility of MDA presence cannot be discarded. Passively-acquired antibodies in the serum of piglets, derived from the sow, may be present until 12 weeks of age (Loeffen *et al.*, 2003a). Considering that antibody persistence in sows after natural infection with SIV can last up to 28 months (Desrosiers, 2004) and that samples analysed in this study from P3 were collected only 2 years after the outbreak, antibodies detected in these 8-wk old pigs could have been transferred from sows. However, the MDA presence in these pigs without pH1N1 virus circulation after the outbreak seems unlikely due to the high percentage of seropositive animals (>70%) detected in this piggery. In contrast, pigs sampled from P2 were >12 weeks of age and

therefore antibody presence indicates a recent circulation of pH1N1 virus among the pig population in this piggery.

Two hypotheses could explain the current circulation of pH1N1 virus among pigs at P2. Firstly, this virus may have been reintroduced into the piggery from infected people, most probably piggery personnel. Secondly, it is possible that pH1N1 virus has been circulating among the pig population since the outbreak in 2010. The latter has been suggested in a previous study, in which circulation of SIV during an extended period of time within the same herd was confirmed (Simon-Grifé *et al.*, 2012). Interestingly, clinical signs compatible with pH1N1 had not been observed at P2 since the 2010 outbreak. However, SIV infection could be subclinical, which is very common in pigs (Hinshaw *et al.*, 1978; Van Reeth *et al.*, 2007). Studies carried out in Europe showed that a high percentage of pig population may become infected with one or more subtypes without ever showing clinical signs (Simon-Grifé *et al.*, 2011).

A limitation of the current study was that confirmation of virus presence and subsequent sequencing of the isolates was not conducted. Although collection of nasal swabs at the seropositives piggeries for further PCR testing to detect influenza RNA was initially planned, consent from the piggery owners to collect further samples was not granted. This decision by owners might have been based, in part, on the negative impacts experienced during the outbreak in 2009/2010.

Whether other influenza viruses distinct from pH1N1 exist in domestic pigs in Australia is unknown. Recently, the generation of novel reassortant viruses derived from the combination of genes from pH1N1 and other endemic swine influenza viruses have been reported (Vijaykrishna *et al.*, 2010; Howard *et al.*, 2011; Kitikoon *et al.*, 2011). Therefore, the potential generation of new reassortant virus cannot be discarded in Australia. Movement of people between piggeries has been a frequent transmission pathway identified in infectious disease outbreaks in pigs. Some examples include the classical swine fever outbreaks that occurred in the Netherlands and Spain in 1997-1998 and in 2001, respectively (Elbers *et al.*, 1999; Allepuz *et al.*, 2007). Regarding influenza viruses, although the virus can be introduced into a piggery via airborne spread from nearby infected piggeries, epidemiological studies suggest

the transmission from the humans to pigs is the main source of pH1N1 virus introduction into pig herds (Hofshagen *et al.*, 2009; Howden *et al.*, 2009; Wong and Deng, 2011; Njabo *et al.*, 2012). For this reason, we investigated the movements of people who had visited infected piggeries and who therefore could have potentially spread pH1N1 virus to other piggeries. Four main groups of people visited the three infected piggeries: veterinarians, commercial representatives, and truck drivers of animal transport and feed supplier companies. Veterinarians are the group of visitors who might pose the highest risk of transmission because they are more likely to have direct contact with animals than the other 3 groups. Although veterinarians are more likely than other visitors to follow biosecurity practices to prevent spread of diseases between piggeries, transmission of the pH1N1 virus from infected humans can occur during the incubation (subclinical) period of infection. Commercial representatives do not usually have access to the animal holdings, thus posing a lower risk of disease transmission. If an adequate biosecurity program is implemented, trucks of feed supplier companies must make deliveries outside the perimeter fence and pig transporters should be not allowed have contact with the piggery facilities (Pritchard *et al*, 2005). However, in some instances truck drivers transporting animals are involved with the loading of pigs into the truck. We estimate that data on people movements from our study represents approximately 70% of these movements, so results could be underestimating the potential disease transmission to other piggeries. Regarding disease transmission by movements of animals, the pH1N1 virus could potentially have been transmitted to other finishing piggeries which received pigs from P2 and P3. This is important for disease control, especially for P2, because serological results suggest that pigs from this piggery have recently been exposed to the pH1N1 virus. Clinical signs associated with pH1N1 infection were not observed at P2, therefore pH1N1 could be transmitted to other piggeries by the movement of subclinically-infected pigs.

The swine-origin of pH1N1 has highlighted that pigs are a mixing vessel for the generation of novel reassorted viruses, from the combination of mammalian and avian influenza, with pandemic potential (Smith *et al.*, 2009). Therefore, reducing the likelihood of introduction of influenza viruses from birds and humans into pig populations is crucial to prevent emerging reassortant

viruses. In this sense, although all the studied piggeries did not allow personnel to go to work with flu-like symptoms after the outbreak, this measure may not be sufficient to prevent human-to-pig virus transmission given that, often influenza virus infection in human is mild or even subclinical (Gray *et al.*, 2007). Interestingly, P1 in this study - in which all pigs sampled were seronegative against pH1N1 virus - was the only piggery where staff vaccination against seasonal flu was mandatory after the outbreak.

5. Conclusion

Surveillance for pH1N1 in susceptible animal species, in particular pigs, has been recommended by FAO for the early detection of risks to human health (FAO, 2010). Results of this limited study suggest that the pH1N1 virus is currently circulating among the pig population in Australia and that transmission of the virus between piggeries via animal and people movement may occur. This study also highlights the need for further research on influenza virus ecology in the pig population in Australia, which should be a component of a global strategy for surveillance of influenza virus in pigs.

Acknowledgments

Capítol 4. Discussió general

4. Discussió general

En aquest capítol es discutiran els resultats obtinguts en relació a la situació dels virus influença a les explotacions porcines d'Espanya i els factors de risc associats a la infecció. També, es debatran els resultats obtinguts en tres explotacions porcines d'Austràlia on l'any 2009 es va detectar el virus influença A H1N1 pandèmic. Finalment es discutirà la dinàmica d'infecció dels virus influença observada en dues explotacions porcines de cicle tancat.

Virus influença en les explotacions porcines d'Espanya

Les seroprevalences obtingudes enfront als subtipus de virus influença H1N1, H1N2 i H3N2 mostren una àmplia disseminació d'aquests virus influença en les explotacions porcines d'Espanya (estudi 1). Les seroprevalences obtingudes enfront H1N1 (57.1%) i H3N2 (54.4%) van ser elevades i similars a les observades en altres països de la Unió Europea com Bèlgica, Alemanya o Itàlia (Van Reeth *et al.*, 2008). En canvi, el percentatge d'animals seropositius enfront el subtipus H1N2 va ser molt menor (20.6%) que per als subtipus H1N1 i H3N2 indicant una menor disseminació d'aquest subtipus de virus influença entre les explotacions porcines d'Espanya.

Un elevat percentatge de granges (89.9%) i d'animals (44.6%) van resultar seropostius enfront dos o més subtipus de virus influença indicant cocirculació de diferents virus ja no només en una mateixa explotació sinó també en el mateix animal. Les probes serològiques no permet discriminar si aquesta elevada cocirculació de diferents virus es produeixen consecutivament en el temps o és deguda a infeccions mixtes produïdes en un mateix moment. Aquest últim escenari proporcionaria, en cas que els virus infectéssin una mateixa cèl·lula, la oportunitat de generació de nous virus influença recombinants que combinarien fragments genètics de virus ja existents, com va succeir amb el virus que va causar la pandèmia l'any 2009 (Smith *et al.*, 2009).

Des d'un punt de vista metodològic, les soques de virus influença utilitzades com antigen en la tècnica de l'IH no eren autòctones ni contemporànies al període de realització de l'estudi. Tot i que l'evolució antigènica dels virus influença porcins és menor a la que es dóna en els virus influença humans (Sugita *et al.*, 1991; de Jong *et al.*, 1999; de Jong *et al.*, 2001; de Jong *et al.*, 2007), aquest fet podria disminuir la sensibilitat de la tècnica. Amb tot i això, les soques utilitzades com antigen pels subtipus H1N1 i H1N2, A/swine/Neth/Best/96 (H1N1) i A/swine/Gent/7625/99 (H1N2), serien representatives dels virus influença que circulen de forma predominant a Europa (Van Reeth *et al.*, 2006; Gerard-Martín *et al.*, 2010) i per tant, almenys pels subtipus H1N1 i H1N2, els resultats obtinguts serien representatius de la situació real dels virus influença a les explotacions porcines d'Espanya. En canvi, les seroprevalences reals podrien estar subestimades en el cas del subtipus H3N2.

En l'espècie porcina esporàdicament s'han pogut aïllar altres subtipus de virus influença com els subtipus H3N3, H7N7 o H5N1 entre altres, tot i que cap d'ells ha estat capaç d'establir-se en la població porcina degut a la baixa capacitat que tenien per transmetre's entre l'espècie porcina (Karasin *et al*, 2004; Loeffen *et al.*, 2004; Choi *et al.*, 2005). En l'estudi 1 es va observar discordança entre els resultats obtinguts per algunes mostres que eren ELISA positives però negatives per la tècnica de l'IH. Si bé aquests resultats poden ser deguts a una falta de sensibilitat de la tècnica de l'IH tal i com s'ha discutit en el punt anterior, algun d'ells podria estar evidenciant la circulació de subtipus de virus influença diferents a H1N1, H1N2 i H3N2. Desafortunadament, no vam testar aquests sèrums utilitzant altres subtipus de virus influença com antígens i per tant, no podem aportar informació pel que fa al subtipus enfront els quals s'haurien generat.

Factors de risc de la infecció dels virus influença

En l'estudi 1 es van identificar tres factors de risc associats a una elevada seroprevalença enfront els virus de la influença porcina: El percentatge de reposició, les separacions discontínues entre corrals i un accés no controlat

a l'explotació. Els dos primers posarien de manifest les vies principals d'entrada i transmissió dels virus influença que tradicionalment s'han postulat (Olsen *et al.*, 2006). Així doncs, els animals de reposició s'han suggerit com factors de risc de la influença (Poljak *et al.*, 2008), ja que un percentatge elevat de reposició comportaria una major entrada de nous animals a l'explotació que podrien actuar per una banda, com a fonts d'introducció de nous virus influença a la granja i per l'altre com animals susceptibles a infectar-se pels virus ja circulants a l'explotació. Per altra banda, les separacions discontínues permetrien el contacte físic directe entre animals de diferents corrals facilitant la disseminació del virus a través de les secrecions nasals. En l'estudi 2 els virus influença no van transmetre's entre els animals de diferents corrals amb separacions contínues indicant que aquest disseny d' instal·lacions és aconsellable per reduir la disseminació de la infecció de virus influença.

La densitat porcina ha estat identificada com un factor de risc de la influença porcina (Maes *et al.*, 2000; Poljak *et al.*, 2008; Suriya *et al.*, 2008) degut a que un nombre elevat de granges o de porcs en una zona facilitaria la transmissió dels virus influença. En aquest sentit, en el estudi 2 s'ha suggerit que l'explotació que va presentar una major incidència de la infecció podria haver-se infectat a partir de la transmissió del virus per via aèria des d'una granja pròxima, ja que aquesta explotació es trobava ubicada en una zona d'elevada densitat porcina.

Dinàmica d'infecció dels virus influença en explotacions porcines

Tradicionalment, s'ha considerat que després de l'entrada d'un nou virus influença en una explotació, el virus es disseminaria de forma epidèmica transmetent-se ràpidament entre els animals de la granja i continuaria circulant mentre existíssin animals susceptibles (Olsen *et al.*, 2006). S'ha postulat l'existència de granges infectades de forma endèmica (Brown, 2000; Olsen *et al.*, 2006).

Els resultats obtinguts en l'estudi 2 han demostrat que els virus influença poden comportar-se seguint ambdós patrons en les explotacions porcines, tant

una forma epidèmica com una forma endèmica. En una de les explotacions la dinàmica d'infecció dels virus influença en el lot estudiat va seguir el patró tradicionalment acceptat, amb una disseminació epidèmica, infectant més del 70% dels animals a les 17 setmanes de vida i restant indetectable durant la resta de mostrejos que es varen dur a terme. En canvi, en l'altra explotació estudiada en l'estudi 2, dues variants del subtipus de virus influença H1N1 van circular conjuntament entre el lot d'animals durant més de 15 setmanes. L'elevada similitud genètica que presentaven les dues variants víriques i l'agrupació de les diferents variants en funció de l'edat dels animals en els quals havien estat aïllades suggereixen que durant l'estudi van ocórrer fenòmens de "drift" que haurien donat lloc a les diferents variants víriques. Encara que la detecció simultània de les diferents variants víriques en el mateix animal no es va evidenciar, la circulació de diferents virus influença en un mateix lot d'animals posa de manifest altra vegada la possible importància de l'espècie porcina en la generació de nous virus recombinants.

El major percentatge d'animals infectats (36.6%) es va detectar a les tres i quatre setmanes de vida, quan alguns dels garrins RRT-PCR positius encara presentaven anticossos maternals i sense que cap dels animals d'aquesta edat presentés simptomatologia compatible amb influença. Aquests resultats semblen suggerir que la presència d'anticossos maternals enfront els virus influença podria no protegir els animals enfront la infecció però si enfront la replicació i invasió dels virus a nivell pulmonar. Els resultats obtinguts reforçarien la idea dels garrins com a font de manteniment dels virus influença en les explotacions (Loeffen *et al.*, 2003a; Loeffen *et al.*, 2009) de forma que serien un grup d'edat que podrien infectar-se i excretar virus sense manifestar simptomatologia clínica, actuant com a font de virus influença per a la resta d'animals de l'explotació. A més, els garrins amb anticossos maternals que s'infectéssin amb virus influença podrien excretar els virus de forma més perllongada que els garrins sense anticossos maternals (Loeffen *et al.*, 2003b). Des d'aquest punt de vista, la presència de truges i garrins com a fonts de virus influença, explicaria el fet que en les explotacions de cicle tancat les infeccions per virus influença en els animals d'engreix es donin de forma més precoç que en les granges on només s'hi allotgen animals d'engreix (Loeffen *et al.*, 2009).

En l'estudi 2 nou animals van resultar RRT-PCR positius en diferents punts del mostreig, separats com a mínim quatre setmanes l'un de l'altre. Aquest fet podria tenir dues explicacions, la primera seria una infecció continuada dels animals que nosaltres només hauríem pogut detectar en determinats moments, però aquesta possibilitat sembla poc probable ja que mai s'ha demostrat una excreció de virus influença tan perllongada. Per altra banda, aquests resultats podrien ser l'evidència d'una reinfecció viral. Aquest segon escenari també generaria certa controvèrsia, donat que tradicionalment s'accepta que després d'una primera infecció amb un virus influença l'individu quedaria protegit enfront reinfeccions causades pel mateix virus o per virus similars (Olsen *et al.*, 2006). En dos d'aquests animals, els virus que es van aïllar corresponien filogenèticament al mateix clúster i per tant, a la mateixa variant vírica tot i que en el moment de la reinfecció els animals tenien títols d'anticossos protectius (>1:20) enfront aquesta variant. Aquests resultats també posarien en qüestió el títol d'anticossos que actualment és assumit com a protectiu enfront la infecció per una soca homòloga.

Virus influença com a causant de malaltia respiratòria

En l'estudi 1 només el 9% de les explotacions van reportar simptomatologia clínica compatible amb influença durant l'últim any, tot i que la totalitat de les explotacions estudiades va presentar com a mínim un animal seropositiu enfront algun dels subtipus analitzats. En les dues explotacions estudiades en l'estudi 2 només un baix percentatge d'animals van mostrar clínica compatible amb influença durant l'estudi. Per últim, en una de les explotacions estudiades en l'estudi 3, on els resultats obtinguts suggerien que recentment el virus H1N1 pandèmic (pH1N1) havia circulat, tampoc es va observar simptomatologia compatible amb la infecció. L'absència de signes clínics associats a la infecció per virus influença estaria suggerint que sovint cursa de forma subclínica passant totalment desapercebuda per la majoria dels ramaders. Els garrins amb anticossos maternals serien el grup d'edat on la forma subclínica de la malaltia podria ser més freqüent ja que el virus podria infectar les vies altes dels animals però la invasió a nivell pulmonar estaria més

restringida i per tant, la simptomatologia seria poc evident (Loeffen *et al.*, 2003b). Sense dubte però, caldria conèixer el possible paper d'aquestes infeccions en relació a la productivitat o les coinfeccions amb altres agents infecciosos.

Influença a les explotacions porcines d'Austràlia

Les explotacions porcines d'Austràlia es consideraven lliures de virus influença fins l'any 2009 quan el virus pH1N1 es va detectar en algunes explotacions sent els humans la font d'introducció suggerida. Amb tot i això, l'absència de virus influença en les granges australianes es basava en la manca d'estudis al respecte i en l'absència de casos clínics compatibles amb la infecció més que per evidències científiques. A més a més, la introducció del virus pH1N1 en les granges porcines australianes fa qüestionar-se perquè prèviament altres virus influença presents en la població humana d'Austràlia no haurien pogut estar capaços d'introduir-se en l'espècie porcina. Si bé la baixa densitat porcina d'Austràlia, amb només 3 milions de porcs aproximadament repartits entre els seus 7 estats (DAFF, 2008b), podria dificultar la transmissió de virus influença entre granges, hi ha altres vies d'introducció de virus influença en les explotacions porcines com els humans o les aus i per tant, l'absència d'infeccions per virus influença en els porcs de les granges australianes seria difícil d'assumir.

La durada dels anticossos maternals dels garrins depèn principalment de l'estatus immunitari de la mare, el qual és molt variable en funció de la granja i fins i tot de la truja en particular. El títol d'anticossos de les truges d'una explotació, vindria condicionat per diferents factors tals com, el pla vacunal i l'edat de la truja entre altres. Per aquest motiu el títol d'anticossos i l'edat en que el nivell d'anticossos començaria a declinar pot ser molt variable en funció de la garrinada. Amb tot i això, es considera que els anticossos maternals enfront la influença porcina podrien estar presents fins als 3 o 4 mesos d'edat com a màxim (Loeffen *et al.*, 2003a). En dues de les tres explotacions estudiades en l'estudi 3 vam detectar animals seropositius enfront el virus pH1N1. En una d'aquestes explotacions els animals que es van mostrejar

tenien 8 setmanes d'edat, i per tant, els anticossos detectats podrien ser d'origen maternal. En l'altra granja es van mostrejar animals de més de 12 setmanes de vida (aproximadament 15 setmanes) i el 56,4% dels animals van resultar seropositius enfront el virus pH1N1. Prenent aquests resultats conjuntament estarien indicant una circulació recent del virus pH1N1 en aquesta explotació. Així doncs, el virus pH1N1 hauria circulat un any i mig després de la seva primera introducció en aquesta granja. El virus hauria pogut continuar circulant des de la seva primera introducció entre els animals susceptibles de la granja, de forma endèmica, tal i com succeïa en una de les granges estudiades en l'estudi 2. Per altra banda, el virus pH1N1 hauria pogut introduir-se de nou a l'explotació com va ocórrer l'any 2010. També, ambdós fets, tant la persistència del virus pH1N1 a la granja com la reintroducció d'aquest, haurien pogut contribuir conjuntament a que el virus encara estigués circulant en l'explotació. Per últim, si bé es cert que els resultats obtinguts en l'estudi 3. suggereixen que el virus pH1N1 hauria pogut disseminar-se a altres granges a través del moviment de persones i animals, el caràcter exploratori de l'estudi i les limitacions d'aquest condicionen que aquest fet no pugui afirmar-se.

Capítol 5. Conclusions

Conclusions

1. En una mostra representativa de les granges d'Espanya, totes les explotacions varen ser seropositives enfront algun dels subtipus de virus influença examinats, demostrant que els virus influença estan àmpliament distribuïts en les granges porcines.

2. S'han identificat tres factors de risc associats a la seroprevalença dels virus influença porcins: el percentatge de reposició, les particions discontínues entre corrals i els accessos no controlats a l'explotació.

3. La infecció per virus influença en les explotacions porcines pot donar-se tant de forma epidèmica, afectant un elevat percentatge d'animals durant un període curt de temps, com de forma endèmica persistint en les explotacions durant llargs períodes de temps.

4. En un mateix lot d'animals hi poden circular, de manera simultània o en diferents moments al llarg de l'engreix, diverses variants de virus influença, en la majoria dels casos sense presentació clínica.

5. Una proporció important de garrins amb anticossos maternals s'ha infectat amb virus influença de forma subclínica.

6. La detecció del mateix virus en dos moments diferents, separats com a mínim quatre setmanes entre ells, en tres animals demostra que els

animals es poden infectar dues vegades amb la mateixa soca viral o una soca molt similar.

7. El virus influença H1N1 pandèmic continuava circulant 1 any i mig després de que el virus es detectés per primera vegada en una explotació porcina d'Austràlia.

Capítol 6. Bibliografia

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Capítol 7. Annexos

7.1 Annex 1

CReSA
Centre de Recerca en Sanitat Animal

Data realització enquesta: ..../......../.........   Enquesta núm. ☐☐☐

DADES GENERALS DE L'EXPLOTACIÓ

• Explotació:.............................. • Marca oficial.................... • Telèfon del titular: ....
• Població:... • Comarca : ....
• Província:.. • Comunitat Autònoma : ....
• Veterinari:.. • Telèfon del veterinari: ....
• Nombre de persones que treballen a la granja: ....
• Quines persones han tingut contacte amb els animals de la granja durant l'últim mes?
....
• Es permet el consum de productes d'origen porcí dins de la granja? ☐ si   ☐ no
• Tenen algun altre tipus de bestiar dins l'explotació?
    ☐ No
    ☐ Si. Quin?
     ☐ Aus, nº....... ☐ Boví, nº....... ☐ Oví, nº...... ☐ Gossos, nº...... ☐ Gats, nº..... ☐ Altres............................
• Pertany a una integradora?   ☐ Si   ☐ No

CARACTERÍSTIQUES DE LA GRANJA

• Classificació de l'explotació:
    cicle tancat ☐   producció de garrins ☐   múltiples fases ☐ (*Especificar tipus*)
• Tipus de maneig:
    tot dins tot fora estricte a :   maternitat   ☐
                               transició   ☐
                                 engreix   ☐
                              quarantena   ☐
• Cens actual:
    Nº de truges productives *(s'inclouen llavores cobertes):* ....
    Nº de verros: ....
    Nº de porcs de transició: ....
    Nº de porcs d'engreix: ....

• Distància a la granja més propera: ....
    Cens de truges..
    Cens de porcs d'engreix....................................
• Nº de granges a 1Km a la rodona: ....

PRODUCCIÓ

• Quina és la mitjana de desmamats per truja/any? ....
• Quin és el percentatge habitual de mortalitat en:
    Maternitat: ....   Transició:.....................   Engreix: ....

• Quin és el % de reposició anual de la seva granja? ....

• Totes aquestes dades estan informatitzades?   ☐ Si   ☐ No

INSTAL·LACIONS

	Gestació	*Maternitat*	*Transició*	*Engreix*
Terra de la nau	☐Slat/Ciment ......%/.......% ☐Ciment ☐Plàstic ☐Altres, Quin? -------------------	☐Slat/Ciment ......%/.......% ☐Ciment ☐Plàstic ☐Altres, Quin? -------------------	☐Slat/Ciment ......%/.......% ☐Ciment ☐Plàstic ☐Altres, Quin? -------------------	☐Slat/Ciment ......%/.......% ☐Ciment ☐Plàstic ☐Altres, Quin? -------------------
Control de la ventilació	☐Sense control ☐Automàtic ☐Manual	☐Sense control ☐Automàtic ☐Manual	☐Sense control ☐Automàtic ☐Manual	☐Sense control ☐Automàtic ☐Manual
Tipus de ventilació	☐Forçada ☐Natural ☐Mixta	☐Forçada ☐Natural ☐Mixta	☐Forçada ☐Natural ☐Mixta	☐Forçada ☐Natural ☐Mixta
Sistema de calefacció	☐Radiador ☐Placa ☐Altres..........	☐Radiador ☐Placa ☐Altres..........	☐Radiador ☐Placa ☐Altres..........	☐Radiador ☐Placa ☐Altres..........
Particions entre corrals			☐Murs ☐Barrots ☐Altres. Quina?	☐Murs ☐Barrots ☐Altres. Quina? ....
Disposen de pati?	☐Si ☐No		☐Si ☐No	☐Si ☐No

	Transició	*Engreix*
Particions entre corrals	☐Continua ☐Discontinua ☐Altres, Quina?	☐Continua ☐Discontinua ☐Altres, Quina? ....
L'abeurador és tipus?	☐Xumet ☐Cassoleta ☐Altre. Quin?......................	☐Xumet ☐Cassoleta ☐Altre. Quin?......................
Abeurador dins la menjadora	☐Si ☐No	☐Si ☐No
El pinso es dispensa de forma:	☐Automàtica ☐Manual. Com?...................	☐Automàtica ☐Manual. Com?...................
La menjadora dels animals és de:	☐Metall ☐Plàstic ☐Formigó	☐Metall ☐Plàstic ☐Formigó

MESURES DE BIOSEGURETAT

- Nº de molls de càrrega i descàrrega a la granja?
- Distància del moll de càrrega i descàrrega a la nau més pròxima: ....m
- Cada quan es produeixen les entrades de reposició: cada .... setmanes
- Origen de les llavores:
  - ☐ Propi
  - ☐ Extern   Quants? ....
  - Es realitza quarentena? ☐Si   ☐No Existeix sala específica de quarentena? ☐Si   ☐No
    - A quina distància es troba dels edificis principals: ....
    - Quant de temps dura? ....
- Es netegen les sales de: Es desinfecten les sales de:
  - maternitat   ☐   maternitat   ☐
  - transició   ☐   transició   ☐
  - engreix   ☐   engreix   ☐
  - quarentena   ☐   quarentena   ☐
- Quin sistema utilitzen per netejar?
  - ☐ Aigua a pressió calenta
  - ☐ Aigua a pressió freda
  - ☐ Raspall
  - ☐ Altres (especificar) ....
- Quin tipus de desinfectant fan servir (*pot indicar-ne el nom comercial*)?
....
- Fossa de purins: ☐ Fossa única   ☐ Fosses múltiples
- El drenatge dels purins és a cel obert?   ☐ Si   ☐ No
- Tenen xarxes per evitar l'entrada d'aus a les naus?   ☐ Sí   ☐ No
- Les portes de les naus estan sempre tancades?   ☐ Sí   ☐ No
- Realitzen plans de desratització? ☐ No
  - ☐ Si   Qui el realitza? ☐ Propietari
    - ☐ Empresa comercial
- Realitzen plans de desinsectació: ☐ No
  - ☐ Si Qui el realitza? ☐ Propietari
    - ☐ Empresa comercial

- D'on prové l'aigua de beguda? ☐ Pou
  - ☐ Xarxa
    - ☐ Altres:...
- Fan cloració de l'aigua? ☐ No
  - ☐ Si. Quin sistema de cloració utilitzen? ☐ Automàtic
    - ☐ Manual
- Cada quan la fan ? ....
- Cada quan verifiquen la potabilitat de l'aigua? ....
- Com fan la verificació? ....
- Hi ha gual sanitari?   ☐ si   ☐ no
- Hi ha arc de desinfecció?   ☐ si   ☐ no
- El moll està dissenyat de forma que impedeix l'entrada de líquids i sòlids i la re-entrada d'animals que ja han sortit? ☐ si   ☐ no
- Hi ha alguna tanca que separi físicament el perímetre de la granja de l'exterior ☐ si   ☐ no
- Els accessos o entrades a la granja estan sempre tancats?   ☐ si   ☐ no
- Hi ha un timbre a l'entrada?   ☐ si   ☐ no
- Hi ha una zona d'aparcament pels cotxes a l'exterior de la granja?   ☐ si   ☐ no
- Es porta un registre de les visites?   ☐ si   ☐ no

• Existeix una oficina on es reben les visites?   ☐ si   ☐ no
• Té una sala de vestuari i dutxes d'ús diari?   ☐ si   ☐ no
• Dins de la sala de vestuari existeix una separació clara entre la zona "bruta" (on el visitant deixa roba i sabates) i la zona "neta" (on es posen les botes i el mono de explotació)?
☐ si   ☐ no
• Es facilita roba de treball a les visites? ☐ si   ☐ no
• Es faciliten botes a les visites? ☐ si   ☐ no
• Es porta un registre d'entrada de vehicles que transporten(especificar):
      ☐ aliment
      ☐ animals
      ☐ purins
      ☐ altres materials (palla, serradures, materials de construcció...)
• El vehicle que transporta l'aliment per els animals entra dins de la granja? ☐ si   ☐ no
• El vehicle que transporta purins entra a l'explotació? ☐ si   ☐ no
• El vehicle de recollida de cadàvers entra dins de la granja? ☐ si   ☐ no
• Si els treballadors mengen a la granja, existeix alguna zona destinada a cantina? ☐ si   ☐ no
• El dipòsit de purins està situat fora del perímetre de la granja? ☐ si   ☐ no

ESTAT SANITARI

	Seropositiu	Seronegatiu
Aujeszky	☐	☐
PRRS	☐	☐

Altres: ....

Programa de vacunació de les truges: indicar el moment de la vacunació

PRRS	☐	....
E.coli	☐	....
Clostridium	☐	....
Altres:		....

Programa de vacunació dels garrins i porcs d'engreix: indicar el moment de vacunació

PRRS	☐	....
Mycoplasma	☐	....
Altres	☐	....

Programa de vacunació per Influença:
    ☐No
    S'ha vacunat en algun període anterior ☐ No   ☐ Si
    ☐Si (indicar moment de vacunació)
    Maternitat   ....
    Garrins i engreix   ....

• Han patit algun problema diarreic durant l'últim any?
    ☐ No
    ☐ Si   A quina edat?..
        • Es va realitzar algun tipus de diagnòstic? ☐Clínic   ☐Necròpsia   ☐ Laboratorial
          • Quin va ser el diagnòstic...
          • Quin tractament va usar per controlar-ho?...
          • I per quina via es va aplicar? ☐ Pinso   ☐ Aigua   ☐ Injectable

• Han patit algun problema respiratori durant l'últim any? (*sobretot si pot estar relacionat amb Influença):*

☐ No

☐ Si   A quina edat?..

- Es va realitzar algun tipus de diagnòstic? ☐Clínic   ☐Necròpsia   ☐ Laboratorial
- Quin va ser el diagnòstic..
- Quin tractament va usar per controlar-ho?..
- I per quina via es va aplicar? ☐ Pinso   ☐ Aigua   ☐ Injectable

• Presencia d'infermera: ☐No   ☐ Engreix ☐ Transició

• Localització d'infermera: ☐ Dins de les naus ☐ Fora de les naus

7.2 Annex 2

Faculty of Veterinary Science

Study of H1N1 Pandemic in pigs

Private & Confidential: Your experience during the H1N1 pandemic is valuable and will provide information to improve exotic disease management. However, your response is voluntary.

Farm Location (locality/nearest town/postcode):

Interview Date:

Husbandry practices

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1. As of today, how many sows do you have?

2. Approximately how many pigs to you wean each week?

3. What age do you sell pigs (weeks)?

4. Where do you market your pigs (abattoir/saleyard/other)

	Abattoir	Saleyard	Others
Where is it?			
How often do you send them there?			
How many pigs each consignment?			

What biosecurity procedures do you have to prevent diseases tracking back from these places?

5. Do you bring in live pigs to your farm? ☐ Yes ☐ No

If yes;

	Breeder	Another producer	Saleyard
Where is it?			
How often do you buy them there?			
How many pigs do you buy each time?			

6. Do you have quarantine area?   ☐ Yes ☐ No

If yes, where is it?   _____

7. How many piggeries are within 5 km of your property?

Large-scale (>20 sows)   _____

Small-scale (20 or less sows/pigs)   _____

8. In Kilometers, how far is your piggery to the nearest:

   a. Abbatoir   _____

   (specify the name)   _____

   b. Saleyards/ Livestock market   _____

   (specify the name)   _____

   c. Feed Store   _____

   (specify the name)   _____

9. Do your pigs have contact with any of the following on your property?

   a. Poultry   ☐ Yes ☐ No

   b.Ruminants   ☐ Yes ☐ No

10. Do you run any pigs outdoors?   ☐ Yes ☐ No

11. Do you have a perimeter fence around your farm?   ☐ Yes ☐ No

12. Have you ever seen feral pigs on or near your property?   ☐ Yes ☐ No

13. How many people work with the pigs on your farm?   _____

Of these, how many are employed staff?   _____

14. Do farm workers contact pigs from other piggeries?   ☐ Yes ☐ No

If yes, where are these piggeries located?   _____

15. Is the farm staff vaccinated against seasonal flu (including) pandemic H1N1? ☐ Yes ☐ No

16. Do you supply visitors with overalls and boots? ☐ Yes ☐ No

17. Are you APIQ-accredited (or seeking to become?) ☐ Yes ☐ No

18. How is the carcass diposal done on your piggery?

    Burn/Bury in your own farm ☐ Yes ☐ No

    A company collect carcasses ☐ Yes ☐ No

If yes;
a.Which company is providing this service? _____

    b. How many times did this company visit   your piggery in the last 6 months? _____

19. How is the effluent removed from your piggery?

    The effluent is treated on the farm ☐ Yes ☐ No

    Another producer collects the effluent ☐ Yes ☐ No

If yes;
a. Who is this producer? _____

    b. How many times did this producer visit your piggery in the last 6 months?

20. Do you have a pest control program in the piggery? ☐ Yes ☐ No

If yes, how is the pest control program applied?

    You perform it yourself ☐ Yes ☐ No

    A company performs the pest control program ☐ Yes ☐ No

If yes;
a. Which company is providing this service? _____

    b. How many times did this company visit your piggery in the last 6 months?

21. Do you consult a veterinarian for the health of your pigs? ☐ Yes ☐ No

If yes;
a. How many times did the vet visit your piggery in the last 6 months?
b. When was the last time the vet visited your pigs? _____

c. Who is your veterinarian? _____

22. Do you have a feed store supplying pig feed to your piggery? ☐ Yes ☐ No

If yes; a. How is the feed delivered?

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b. Which feed store supplies your piggery? _____

c. How many times did they visit your piggery in the last 6 months?

23. Do commercial representatives/consultants visit your farm? ☐ Yes ☐ No

If yes;
a. Which companies do they represent? _____

b. How many times did they visit your piggery in the last 6 months?

Introduced changes since H1N1 Pandemic outobreak

Which of the following statements relating to biosecurity have been true on your farm BEFORE and AFTER the human swine flu A/H1N1 outbreak?

	BEFORE		AFTER	
1. I have a controlled entry of visitors	☐ Yes	☐ No	☐ Yes	☐ No
2. I display quarantine signs for visitors at my farm entry	☐ Yes	☐ No	☐ Yes	☐ No
3. I allow my staff to contact pigs other than my own	☐ Yes	☐ No	☐ Yes	☐ No
4. I allow staff to come to work if they have flu-like symptoms	☐ Yes	☐ No	☐ Yes	☐ No
5. I require the use of clothes worn only on the farm	☐ Yes	☐ No	☐ Yes	☐ No
6. I require the use of boots or shoes worn only on the farm	☐ Yes	☐ No	☐ Yes	☐ No
7. I keep records of visitors to my pig farm	☐ Yes	☐ No	☐ Yes	☐ No

8. I ask visitors if they have recently been overseas   ☐ Yes   ☐ No   ☐ Yes   ☐ No

9. I make visitors wash their hands and scrub fingernails   ☐ Yes   ☐ No   ☐ Yes   ☐ No

10. I record movements of pigs on and off my farm   ☐ Yes   ☐ No   ☐ Yes   ☐ No

11. I train workers in emergency disease recognition   ☐ Yes   ☐ No   ☐ Yes   ☐ No

12. I inspect my herd regularly for unusual signs of disease   ☐ Yes   ☐ No   ☐ Yes   ☐ No

13. I regularly consult my vet or pig health specialist   ☐ Yes   ☐ No   ☐ Yes   ☐ No

14. I require a shower before vistors entry onto the farm   ☐ Yes   ☐ No   ☐ Yes   ☐ No

15. I make my staff vaccinate against seasonals influenza strains   ☐ Yes   ☐ No   ☐ Yes   ☐ No

Any comments :

Impact of H1N1 Pandemic outbreak

Which of the following affected you during the H1N1 Pandemic?

1. Animal movement restrictions   ☐ Yes   ☐ No

If yes, how many days were pigs movement restricted?   _____

Any comments:

2. People movements were restricted   ☐ Yes   ☐ No

Any comments:

3. Restriction of vehicles (including feed trucks) access onto the farm ☐ Yes ☐ No

Any comments:

4. Which of the following statements relating to production losses have been true on your farm due to the H1N1 pandemic outbreak?

Selling pigs heavier resulted in increased P2 backfat ☐ Yes ☐ No

Movement standstill meant overcrowding issues ☐ Yes ☐ No

Growth performance sufferred (reduced growth/FCE/increased deaths) ☐ Yes ☐ No

Increased incidence of other diseases ☐ Yes ☐ No

Increased medication costs ☐ Yes ☐ No

Increased vet/diagnostic costs ☐ Yes ☐ No

Reduced reproductive performance ☐ Yes ☐ No

5. What is your perception about how the regulators handled the situation during the H1N1 outbreak?

Communication with you ☐ GOOD ☐ BAD

Collection of samples for diagnostics ☐ GOOD ☐ BAD

How do you think handling of the situation during H1N1 Pandemic outbreak could have improved?

6. Was staff morale affected? ☐ Yes ☐ No

If yes, how?

7. Was staff affected by clinically infected animals?   ☐ Yes   ☐ No

Thank you for your participation in this study!

7.3 Annex 3

Primer set used to amplify each segment of the SIV. Primer set used to amplify each segment of the SIV and information aboutthe 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.

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	
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
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
NS	1-AGCAAAAGCAGGGTG-15	AGTAGAAACAAGGGTGTTTTTTA

7.4 Annex 4

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.

	GenBank Accession	Country of Isolation	Year of Isolation	Isolate (subtype)
1	CY009326	Australia	1935	A/Melbourne/1935 (H1N1)
2	CY009324	Australia	1935	A/Melbourne/1935 (H1N1)
3	CY020461	USA	1943	A/Iowa/1943 (H1N1)
4	EU139834	USA	1945	A/swine/Iowa/1945 (H1N1)
5	EU139824	USA	1945	A/swine/Iowa/1945 (H1N1)
6	CY021053	Malaysia	1954	A/Malaya/302/1954 (H1N1)
7	CY008988	USA	1957	A/Denver/1957 (H1N1)
8	CY077742	Netherlands	1958	A/Netherlands/001M/1958 (H2N2)
9	AY209901	Brazil	1959	A/Sao Paolo/3/1959 (H2N2)
10	EU139825	USA	1968	A/swine/Wisconsin/1/1968 (H1N1)
11	CY031589	USA	1968	A/North Carolina/1/1968 (H2N2)
12	AY210132	UK	1972	A/England/42/1972 (H3N2)
13	K02252	Ireland	1973	A/parrot/Ulster/1973 (H7N1)
14	CY021957	USA	1976	A/New Jersey/1976 (H1N1)
15	D00838	Japan	1976	A/duck/Hong Kong/36/1976 (H1)
16	D21184	Japan	1976	A/duck/Hong Kong/24/1976 (H4N2)
17	CY022373	USA	1977	A/swine/Nebraska/123/1977 (H1N1)
18	CY025004	USA	1977	A/swine/Arizona/148/1977 (H1N1)
19	CY004458	USA	1977	A/mallard/Alberta/42/1977 (H1N6)
20	CY021717	USA	1978	A/California/10/1978 (H1N1)
21	CY020295	Brazil	1978	A/Brazil/11/1978 (H1N1)
22	CY014624	Australia	1979	A/gray teal/Australia/1/1979 (H6N1)
23	CY004492	USA	1979	A/mallard/Alberta/965/1979 (H1N1)
24	CY026413	Albany	1979	A/Albany/8/1979 (H1N1)
25	AB434384	Japan	1979	A/swine/Kyoto/3/1979 (H1N1)
26	CY004792	USA	1979	A/mallard duck/Alberta/106/1979 (H4N2)
27	AB292403	Japan	1980	A/duck/Hong Kong/836/1980 (H3N1)
28	CY008470	USA	1980	A/Memphis/3/1980 (H3N2)
29	CY026451	Canada	1981	A/swine/Ontario/6/1981 (H1N1)
30	CY021039	UK	1982	A/Christs Hospital/157/1982 (H1N1)
31	CY004636	USA	1983	A/blue-winged teal/Alberta/452/1983
32	AJ412690	Belgium	1983	A/swine/Belgium/1/1983 (H1N1)
33	GU066779	France	1983	A/mallard/Marquenterre/Z237/2007
34	CY037967	Belgium	1983	A/swine/Belgium/WVL2/1983 (H1N1)
35	CY004196	USA	1985	A/blue-winged teal/Alberta/69/1985
36	CY019103	USA	1986	A/Memphis/12/1986 (H1N1)
37	CY017275	USA	1987	A/mallard/Ohio/265/1987 (H1N9)
38	CY004517	Alberta	1987	A/coot/Alberta/134/1987 (H6N2)
39	CY024925	USA	1988	A/Ohio/3559/1988 (H1N1)
40	L19019	France	1988	A/France/15/1988 (H1N1)
41	U96766	Germany	1990	A/turkey/Germany/2482/1190 (H1N1)

42	CY012802	USA	1990	A/mallard/Ohio/171/1990 (H1N1)
43	U85986	UK	1992	A/swine/England/191973/1992 (H1N7)
44	CY015137	USA	1993	A/ruddy turnstone/Delaware/81 1993
45	CY018885	USA	1993	A/mallard/Ohio/118/1993 (H1N1)
46	EF409255	Germany	1993	A/swine/Bakum/909/1993 (H3N2)
47	CY033616	China	1995	A/Beijing/262/1995 (H1N1)
48	CY021007	USA	1996	A/Memphis/15/1996 (H1N1)
49	EF584357	Brazil	1996	A/Brazil/207/1996 (H3N2)
50	CY020231	Italy	1997	A/poultry/Italy/373/1997 (H5N2)
51	AB274304	Japan	1998	A/pintail/Shimane/324/1998 (H1N7)
52	AJ344016	Italy	1998	A/swine/Italy/1511/1998 (H1N1)
53	AJ457892	France	1998	A/Paris/1857/1998 (H1N1)
54	AF153238	USA	1998	A/Swine/Minnesota/9088-2/1998 (H3N2)
55	EF584359	Japan	1998	A/Japan/1268/1998 (H3N2)
56	EF584374	Argentina	1998	A/Argentina/89/1998 (H3N2)
57	AJ412701	Italy	1998	A/swine/Italy/1521/1998 (H1N2)
58	CY060206	Netherlands	1999	A/mallard/Netherlands/10/1999 (H1N8)
59	AF268139	USA	1999	A/Swine/North Carolina/16497/1999
60	CY016158	USA	1999	A/gadwall/Ohio/37/1999 (H6N2)
61	DQ487337	Panama	1999	A/Panama/2007/1999 (H3N2)
62	EU053148	Germany	2000	A/swine/Bakum/1832/2000 (H1N2)
63	AJ518093	Slovakia	2000	A/Bratislava/47/2000 (H1N1)
64	EU097942	Denmark	2000	A/Denmark/40/2000 (H1N1)
65	AY300933	USA	2000	A/Duck/New York/44018-1/2000 (H5N2)
66	CY008782	UK	2000	A/Canterbury/80/2000 (H3N2)
67	EU139828	USA	2001	A/swine/Minnesota/1192/2001 (H1N2)
68	EU429707	China	2001	A/duck/Eastern China/48/2001 (H9N2)
69	DQ249253	Taiwan	2001	A/Taiwan/2332/2001 (H3N2)
70	AJ489847	UK	2001	A/England/691/2001 (H1N2)
71	AM157359	France	2002	A/sentinel duck/France/1043/2002
72	CY000443	USA	2002	A/New York/134/2002 (H3N2)
73	CY020503	Spain	2002	A/swine/Spain/42386/2002 (H3N2)
74	EU429754	China	2003	A/duck/Eastern China/253/2003 (H3N1)
75	EU100577	Mexico	2003	A/New Mexico/19/2003 (N2)
76	DQ091199	Russia	2003	A/Moscow/343/2003 (H3N2)
77	EU045388	Italy	2004	A/swine/Italy/53949/2004 (H1N1)
78	DQ139320	China	2004	A/swine/Zhejiang/1/2004 (H1N2)
79	EU097943	Denmark	2004	A/Denmark/17/2004 (H1N1)
80	CY010580	Spain	2004	A/swine/Spain/53207/2004 (H1N1)
81	EU429745	China	2004	A/duck/Eastern China/6/2004 (H3N2)
82	CY015950	Australia	2004	A/Western Australia/60/2004 (H3N2)
83	FJ231817	Germany	2005	A/Hessen/4/2005 (H1N1)
84	DQ666933	Korea	2005	A/swine/Korea/S11/2005 (H1N2)
85	EU301289	Korea	2005	A/duck/Korea/LPM22/2005 (H3N2)
86	AB286007	Vietnam	2006	A/Hanoi/BM344/2006 (H1N1)
87	CY035128	Russia	2006	A/St. Petersburg/8/2006 (H1N1)
88	GQ355843	Austria	2006	A/duck/Wels/2025/2006 (H5N1)
89	AB441170	Japan	2006	A/swine/Miyazaki/1/2006 (H1N2)
90	CY032714	USA	2007	A/northern shoveler/California/2007

91	CY026717	USA	2007	A/Texas/UR06-0582/2007 (H1N1)
92	FN386463	Spain	2007	A/Anas plathyrhynchos/Spain/
93	CY025853	USA	2007	A/Texas/UR06-0418/2007 (H3N2)
94	FN773069	Norway	2007	A/Teal/ A/Teal/Norway/10 1360/2007
95	EU779649	USA	2008	A/Indiana/04/2008 (H1N1)
96	GQ166189	Spain	2009	A/Catalonia/88/2009 (H1N1)
97	HQ244432	Czech Republic	2009	A/mallard/Czech Republic/15902-
98	JF960169	Spain	2009	A/swine/Spain/1/2009 (H1N1)
99	JF960172	Spain	2009	A/swine/Spain/1/2009 (H1N1)
100	JF960173	Spain	2009	A/swine/Spain/2/2009 (H1N1)
101	JQ301920	Spain	2009	A/swine/Spain/2/2009 (H1N1)
102	JF960174	Spain	2009	A/swine/Spain/3/2009 (H1N1)
103	JQ301921	Spain	2009	A/swine/Spain/3/2009 (H1N1)
104	JF960175	Spain	2009	A/swine/Spain/4/2009 (H1N1)
105	JQ301945	Spain	2009	A/swine/Spain/4/2009 (H1N1)
106	JF960176	Spain	2009	A/swine/Spain/5/2009 (H1N1)
107	JQ301922	Spain	2009	A/swine/Spain/5/2009 (H1N1)
108	JF960177	Spain	2009	A/swine/Spain/6/2009 (H1N1)
109	JQ301923	Spain	2009	A/swine/Spain/6/2009 (H1N1)
110	JF960178	Spain	2009	A/swine/Spain/7/2009 (H1N1)
111	JQ301946	Spain	2009	A/swine/Spain/7/2009 (H1N1)
112	JF960179	Spain	2009	A/swine/Spain/8/2009 (H1N1)
113	JQ301947	Spain	2009	A/swine/Spain/8/2009 (H1N1)
114	JF960180	Spain	2009	A/swine/Spain/9/2009 (H1N1)
115	JQ301924	Spain	2009	A/swine/Spain/9/2009 (H1N1)
116	JF960181	Spain	2009	A/swine/Spain/10/2009 (H1N1)
117	JQ301925	Spain	2009	A/swine/Spain/10/2009 (H1N1)
118	JF960182	Spain	2009	A/swine/Spain/11/2009 (H1N1)
119	JQ301926	Spain	2009	A/swine/Spain/11/2009 (H1N1)
120	JF960183	Spain	2009	A/swine/Spain/12/2009 (H1N1)
121	JQ301927	Spain	2009	A/swine/Spain/12/2009 (H1N1)
123	JF960184	Spain	2009	A/swine/Spain/13/2009 (H1N1)
124	JQ301928	Spain	2009	A/swine/Spain/13/2009 (H1N1)
125	JQ301959	Spain	2009	A/swine/Spain/13/2009 (H1N1)
128	JQ301963	Spain	2009	A/swine/Spain/13/2009 (H1N1)
129	JQ301966	Spain	2009	A/swine/Spain/13/2009 (H1N1)
130	JQ301966	Spain	2009	A/swine/Spain/13/2009 (H1N1)
131	JQ301972	Spain	2009	A/swine/Spain/13/2009 (H1N1)
132	JQ301975	Spain	2009	A/swine/Spain/13/2009 (H1N1)
133	JQ301948	Spain	2009	A/swine/Spain/14/2009 (H1)
134	JQ301949	Spain	2009	A/swine/Spain/15/2009 (H1)
135	JF960187	Spain	2009	A/swine/Spain/16/2009 (H1N1)
136	JQ301929	Spain	2009	A/swine/Spain/16/2009 (H1N1)
137	JF960188	Spain	2009	A/swine/Spain/17/2009 (H1N1)
138	JQ301950	Spain	2009	A/swine/Spain/17/2009 (H1N1)
139	JF960189	Spain	2009	A/swine/Spain/18/2009 (H1N1)
140	JQ301930	Spain	2009	A/swine/Spain/18/2009 (H1N1)
141	JF960190	Spain	2009	A/swine/Spain/19/2009 (H1N1)
143	JQ301931	Spain	2009	A/swine/Spain/19/2009 (H1N1)

144	JF960191	Spain	2009	A/swine/Spain/20/2009 (H1N1)
145	JQ301951	Spain	2009	A/swine/Spain/20/2009 (H1N1)
147	JF960192	Spain	2009	A/swine/Spain/21/2009 (H1N1)
148	JQ301932	Spain	2009	A/swine/Spain/21/2009 (H1N1)
149	JF960193	Spain	2009	A/swine/Spain/22/2009 (H1N1)
150	JQ301933	Spain	2009	A/swine/Spain/22/2009 (H1N1)
151	JQ301952	Spain	2009	A/swine/Spain/23/2009 (H1)
152	JF960195	Spain	2009	A/swine/Spain/24/2009 (H1N1)
153	JQ301953	Spain	2009	A/swine/Spain/24/2009 (H1N1)
154	JQ301954	Spain	2009	A/swine/Spain/25/2009 (H1)
155	JF960197	Spain	2009	A/swine/Spain/26/2009 (H1N1)
156	JQ301934	Spain	2009	A/swine/Spain/26/2009 (H1N1)
157	JF960198	Spain	2009	A/swine/Spain/27/2009 (H1N1)
158	JQ301955	Spain	2009	A/swine/Spain/27/2009 (H1N1)
159	JF960199	Spain	2009	A/swine/Spain/28/2009 (H1N1)
160	JQ301935	Spain	2009	A/swine/Spain/28/2009 (H1N1)
161	JF960200	Spain	2009	A/swine/Spain/29/2009 (H1N1)
162	JQ301936	Spain	2009	A/swine/Spain/29/2009 (H1N1)
163	JF960201	Spain	2009	A/swine/Spain/30/2009 (H1N1)
164	JQ301956	Spain	2009	A/swine/Spain/30/2009 (H1N1)
165	JF960202	Spain	2009	A/swine/Spain/31/2009 (H1N1)
166	JQ301937	Spain	2009	A/swine/Spain/31/2009 (H1N1)
167	JQ301960	Spain	2009	A/swine/Spain/31/2009 (H1N1)
168	JQ301964	Spain	2009	A/swine/Spain/31/2009 (H1N1)
169	JQ301967	Spain	2009	A/swine/Spain/31/2009 (H1N1)
170	JQ301970	Spain	2009	A/swine/Spain/31/2009 (H1N1)
171	JQ301973	Spain	2009	A/swine/Spain/31/2009 (H1N1)
172	JQ301976	Spain	2009	A/swine/Spain/31/2009 (H1N1)
173	JF960203	Spain	2009	A/swine/Spain/32/2009 (H1N1)
174	JQ301938	Spain	2009	A/swine/Spain/32/2009 (H1N1)
175	JF960204	Spain	2009	A/swine/Spain/33/2009 (H1N1)
176	JQ301939	Spain	2009	A/swine/Spain/33/2009 (H1N1)
177	JF960205	Spain	2009	A/swine/Spain/34/2009 (H1N1)
178	JQ301940	Spain	2009	A/swine/Spain/34/2009 (H1N1)
179	JQ301961	Spain	2009	A/swine/Spain/34/2009 (H1N1)
180	JQ301965	Spain	2009	A/swine/Spain/34/2009 (H1N1)
181	JQ301968	Spain	2009	A/swine/Spain/34/2009 (H1N1)
182	JQ301971	Spain	2009	A/swine/Spain/34/2009 (H1N1)
183	JQ301974	Spain	2009	A/swine/Spain/34/2009 (H1N1)
184	JQ301977	Spain	2009	A/swine/Spain/34/2009 (H1N1)
185	JF960206	Spain	2009	A/swine/Spain/35/2009 (H1N1)
186	JQ301941	Spain	2009	A/swine/Spain/35/2009 (H1N1)
187	JF960207	Spain	2009	A/swine/Spain/36/2009 (H1N1)
188	JQ301942	Spain	2009	A/swine/Spain/36/2009 (H1N1)
189	JF960208	Spain	2009	A/swine/Spain/37/2009 (H1N1)
190	JQ301943	Spain	2009	A/swine/Spain/37/2009 (H1N1)
191	JQ301958	Spain	2009	A/swine/Spain/38/2009 (H1N1)
192	JQ301962	Spain	2009	A/swine/Spain/38/2009 (H1N1)
193	JQ301944	Spain	2009	A/swine/Spain/39/2009 (H1)

194	JQ301957	Spain	2009	A/swine/Spain/40/2009 (H1)
195	CY067662	Italy	2010	A/swine/Italy/116114/2010 (H1N2)
196	JF960170	Spain	2010	A/swine/Spain/1/2010 (H1N2)
197	JF960171	Spain	2010	A/swine/Spain/1/2010 (H1N2)