Universitat Autònoma de Barcelona

Epidemiological surveillance of swine influenza viruses in pig farms

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Tesis doctoral presentada por Silvana Nelly Sosa Portugal para acceder al grado de Doctora en el marco del programa de Doctorado en Medicina y Sanidad Animal de la Facultad de Veterinaria de la Universidad Autónoma de Barcelona, bajo la co-dirección del Dr. Enric Mateu de Antonio y el Dr. Gerard Martín-Valls.

Bellaterra, 2020

El Dr. Enrique M. Mateu de Antonio, profesor titular del Departamento de Sanidad y Anatomía Animal de la Facultad de Veterinaria, y el Dr. Gerard Eduard Martín Valls, investigador titular del Departamento de Sanidad y Anatomía Animal de la Facultad de Veterinaria,

Declaran,

Que la memoria titulada, "Epidemiological surveillance of swine influenza viruses in pig farms" presentada por Silvana Nelly Sosa Portugal para la obtención del grado de Doctora en Veterinaria, se ha realizado bajo su dirección dentro del programa de doctorado del Departamento de Sanidad y Anatomía Animal, opción Sanidad Animal.

Y per tal que conste a efectos oportunos, firman el presente certificado en Bellaterra, 16 de Julio de 2020.

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Table of contents

Acknowledgements

Remembering my good friend Anna's thesis, she compared the experience of the PhD with a football match, even though it seemed very accurate for her, I didn't think that would work for me. So, it got me thinking, and one of the first things that popped in my mind was hiking. Over the last year, Sean and I, had developed the taste for a good long hike, it could either be in the beautiful mountains of Garraf, in the forever cold North of England, or even in the middle of the rain in Germany. What they all had in common were three things: a naïve start, an exhausting middle, and an end. I started the PhD as the same way we plan a hike, thinking 'Alright, this doesn't look too bad, of course we can hike all across a mountain, cross a field, get to the other side of the lake on a ferry that only runs every hour, get back before sunset and even have spare time to take some pics', I couldn't have been more wrong. This was one of those things that you don't know what you are getting into, until you actually do. Within the first couple of months I found myself getting bombarded by boxes with nasal swabs, RNA extractions that would take so long that whenever I would finish one in a Friday afternoon, when leaving the lab looked like I had woken up in a zombie apocalypse, and of course, how could I ever forget 'The meetings' in Enric and Gerard's office, looking like as if I had seen a ghost and trying to write a thousand words per minute so I could remember everything I had to do. But time went by, and the lab didn't look like a daunting place anymore, because I realised that I wasn't alone. Thanks to everyone in the team (you know who you are), for all the laughs, the gossip, the help, the advice, the hugs, the knowledge and the tricks, all to make the hours in the lab go as smoothly as possible. I really thank you all for that.

Now, this is where we get to the second part of the hike, the exhausting middle, of course any long project will get to a point where you run out of energy, your under eye bags look worse than ever, and where no matter how hard you are trying, it feels like you have not made any progress at all. So, this is when you have been hiking for hours, and you should have crossed the mountain, or at least caught a sight of the field, and on top of that, you are not even sure you are actually on the right path, or even worse, it starts raining. Those days when you feel that you are going to start crying if someone says there's a big white box in the lab 287 with your name on it, but you have to go anyway, with a cool face and your notebook ready to start processing all those samples, even if that meant 400 nasal swabs and 200 blood samples. And even if at the time I didn't show all the stress I was

feeling, having a good friend say to me *'Ànims reina, que ets una campeona'*, really made a difference.

Anyone who has been through this must be thinking 'Been there, done that', and they are right. Once you decide to get back on your feet and continue walking, then just then, you can actually see the field, and even some of the lake shining in the distance. So now, I have crossed the mountain, the field, seen the lake, got on the ferry, and I'm currently waiting to reach the shore. Hopefully, I am not jinxing this by writing it when there are still a few loose ends to take care of. But even if I do, I want to thank Enric and Gerard, for giving me the opportunity to take part of this project, for having loads of patience with me, for not getting angry at my mistakes, for always having the time to hear my doubts, for those online Teams meetings, and for helping me finish this even amidst a global pandemic. To everyone in the infectious diseases and epidemiology groups, a big heartfelt thank you. I would also like to thank the people from Ceva for sponsoring this project and giving me the opportunity to share my results in conferences. As well as all the collaborating veterinarians and especially to Selecció Batallé, for helping make the studies possible.

And how could I forget to thank the person who did all those crazy hikes with me, who helped me cross streams, rocky paths, and held my hand when going down slippery trails. Thank you Sean for all you do for me, and all you have done over the last years. For waiting for me every day to come back home with a warm meal on the stove and a smile on your face. And finally, I want to thank my parents, for always telling me I can do whatever I set my mind to, even if that means leaving my home, my friends and my family, flying over the Atlantic with my cat (#bestcatever) and move to Spain to fulfil my dream of becoming a Vet. Nothing, but really, nothing, would have been possible without your support, your constant whatsapp messages, and your motivational quotes that I would suspiciously always receive when I was commuting back home. Thank you.

Silvana

List of abbreviations

ADCC: antibody-dependent cellular cytotoxicity

ADCP: antibody-dependent cell-mediated phagocytosis

AH: absolute humidity

av: avian

BAL: bronchoalveolar lavage

cRNA: complementary RNA

csH1N1: classical H1N1

ECE: embryonated chicken eggs

ELISA: enzyme-linked immunosorbent assay

ELLA: Enzyme-Linked Lectin Assay

ER: endoplasmic reticulum

FA: fluorescent antibody eest

H3N2v: H3N2 variant

HA: haemagglutinin

HEF: haemagglutinin–esterase–fusion

HI: haemagglutinin inhibition

hu: human

IAV: influenza A virus

Ig: immunoglobulin

IHC: immunohistochemistry

IL: interleukin

INF: interferon

IVPM: influenza virus proteins

LAIV: live-attenuated influenza virus

M: matrix

MDA: maternally-derived antibody

MDCK: Madin-Darby canine kidney

mRNA: messenger RNA

NA: neuraminidase

NAI: neuraminidase-inhibition assay

NEP: nuclear export protein

NGS: next generation sequencing

NI: neuraminidase inhibition

NK: natural killer

NP: nucleoprotein

NS: nasal swabs

NS1: non-structural protein 1

NS2: non-structural protein 2

OF: oral fluids

OIE: World Organisation for Animal Health

PA: polymerase acidic protein

PB1: polymerase basic protein 1

PB2: polymerase basic protein 2

PCR: polymerase chain reaction

PRDC: porcine respiratory disease complex

PRRSV: porcine reproductive and respiratory syndrome virus

R: reproduction ratio

RBC: red blood cells

RBS: receptor binding site

RH: relative humidity

RT PCR: reverse transcriptase polymerase chain reaction

SA: sialic acid

SN: serum neutralisation assay

SRH: single radial haemolysis

TEM: transmission electron micrograph

TMD: terminal transmembrane domain

TNF: tumour necrosis factor

tr: triple reassortant

TRIG: triple reassortant internal gene cassette

USDA: U.S. Department of Agriculture

VAERD: vaccine associated enhanced respiratory disease

VE: vaccine efficacy

VN: virus neutralisation

vRNA: viral RNA

vRNP: viral ribonucleoprotein complex

WHO: World Health Organization

WIV: Whole inactivated virus

Summary

In the **first study** of the present thesis, we investigated outbreaks of respiratory disease (n=211) compatible with influenza A virus (IAV) as well as farms without overt respiratory disease (n=19) for the presence of IAV. In the outbreak investigations, nasal swabs were taken from animals with respiratory signs and fever (≥40°C) while in the farms with no evident respiratory disease, nasal swabs were randomly taken from suckling piglets, weaners and fatteners (20 animals per phase). Presence of IAV and lineage determination were assessed by RT-qPCR and isolation was attempted in selected samples using MDCK cells. Isolates were sequenced (full genome) by using Illumina Miseq technology. IAV participation was confirmed in 145 (68.7%) of the outbreaks, and in 15 (78.9%) of the farms without overt disease. The most commonly detected lineages were H1avN2hu (33.6%), H1avN1av (24.3%) and H1huN2hu (18.7%). Sixty IAV isolates were obtained and the genomes were fully sequenced. Genotypes D and A, H1avN2hu and H1avN1av, respectively, were predominant but up to 14 genotypes were identified, of which seven had not been previously reported. Four isolates containing a new H3hu lineage derived from a human seasonal virus were detected, and isolates containing genes from the pandemic virus represented a 31.7 % of the total. In the **second study** of the present thesis, the transmission dynamics of IAV in the nurseries from an endemic farm were assessed before and after the application of different vaccination schemes for sows. Three follow-up periods were examined: before vaccination, after vaccination with a commercial inactivated polyvalent H1N1-H1N2-H3N2 and after vaccination with a monovalent pandemic H1N1. Nasal swabs of piglets were taken weekly from 3-9 weeks of age and blood samples were taken at three, six and nine weeks of age. In the first follow-up before vaccination, the basal IAV circulation was assessed by sampling 50 piglets in 4 batches. In the second longitudinal study, sows were blanket vaccinated with the polyvalent vaccine (control group) and half of them received an extra dose 3 weeks pre-farrowing (treatment group). A random cohort of 10 sows in each group was selected and 5 piglets per sow were weekly followed. The trial was replicated in 4 consecutive batches. In the third follow-up period, the procedure was the same as in the second, but using a pandemic H1N1 inactivated vaccine. Nasal swabs were examined by RT-qPCR and serum samples were analysed using a commercial ELISA (Civtest-Suis Influenza). Incidences and beta values per week and pen were calculated after the RT-qPCR results. Before applying any vaccination scheme, the farm was found to be

endemically infected with an H1avN1av virus. Patterns of incidence were diverse in the examined pens but often viral circulation was detected as early as 4 weeks of age. Weekly beta-values for this first period were mostly below 5 and rarely exceeded 7.5. At three weeks of age, most of the analysed animals were positive with high S/P ratios. In the second follow-up period, after the application of the first vaccination scheme including an H1avN1av vaccine, the onset of infection was delayed by two weeks compared to the previous observations. Also, in both the treatment and the control groups, beta-values increased significantly, reaching frequently values >7.5 at six weeks of age, suggesting that weaners were only infected after a period in the nursery premises and probably, after the fading out of maternally-derived passive protection. Since viral circulation did not fully stop, the reactivity of the virus circulating in the farm in front of the anti-vaccine antiserum was tested by the hemagglutination inhibition test. Surprisingly, the anti-vaccine H1avN1av antiserum (1:320 homologous titre) only reacted at 1:40 with the farm strain while an anti H1N1pdm09 sera of similar homologous titre reacted at 1:320. Accordingly, a second vaccination trial with the same scheme but using an H1N1pdm09 virus was used. In that third follow-up period, the onset of infection shifted to 3 weeks of age or before as revealed by RT-qPCR without significant differences between treatments and controls. In all of the three studies, animals that shed virus in two and even three consecutive sampling times were detected. However, this was significantly higher when the H1avN1av vaccine was used (41%) compared to the other two periods (31% before vaccinating, 11% in the second vaccination trial, $p<0.05$). Also, a proportion between 1-5% of the animals were reinfected after the cease of a previous shedding. Neither the prolonged shedding nor the cases of reinfection could be related to the levels of maternally-derived antibodies at weaning. Interestingly, an H1avN1av virus was initially detected in the farm, but during the third study, a H3huN2hu was found circulating in the batches, carrying a new H3 human-like derived from human seasonal virus.

Resumen

En el **primer estudio** de la presente tesis, se estudiaron brotes de enfermedad respiratoria compatible con virus de la influenza de tipo A (IAV) así como granjas que no mostraban sintomatología clínica. Para el estudio de los brotes, se recogieron muestras de hisopos nasales de animales con signos respiratorios y fiebre (≥40°C), mientras que en las granjas sin sintomatología clínica, se recogieron hisopos nasales de lechones de maternidad, transición y cerdos de engorde (20 por grupo). Se estudió un total de 211 brotes y 19 granjas aparentemente subclínicas. La presencia y linaje se determinaron por RT-qPCR, y se hizo el aislamiento de muestras seleccionadas usando células MDCK. Los aislados fueron secuenciados (genoma completo) mediante la tecnología Illumina Miseq. Se confirmó la presencia de IAV en 145 casos de brotes (68.7%), y en 15 granjas aparentemente subclínicas (78.9%). Los linajes mayormente detectados fueron H1avN2hu (33.6%), H1avN1av (24.3%) y H1huN2hu (18.7%). Se obtuvo un total de 60 aislados, y sus genomas fueron completamente secuenciados. Los genotipos mayoritariamente detectados fueron el tipo D y el A, que se corresponden a los linajes H1avN2hu y H1avN1av, respectivamente. Se detectaron un total de 14 genotipos diferentes, de los cuales, 7 de ellos no habían sido previamente reportados. Se detectaron cuatro aislados que contenían un nuevo linaje de H3hu derivado de un virus de la gripe estacional humana, y además, se detectaron aislados que contenían genes del virus pandémico en un 31.7% del total las secuencias. En el **segundo estudio** de la presente tesis, se estudió la dinámica de transmisión de IAV en las transiciones de una granja endémica antes y después de la aplicación de diferentes esquemas de vacunación en las cerdas. Se realizaron un total de tres estudios longitudinales: antes de la vacunación, después de la vacunación con una vacuna comercial polivalente inactivada H1N1-H1N2-H3N2 y después de la vacunación con una vacuna comercial monovalente pandémica H1N1. Se recogieron muestras semanales de hisopos nasales de los lechones desde las 3-9 semanas de vida, y muestras de sangre a las 3, 6 y 9 semanas de vida. En el primer longitudinal antes de la vacunación, se evaluó la circulación vírica basal en 50 lechones de 4 lotes consecutivos. En el segundo longitudinal, se realizó vacunación en sábana de cerdas usando la vacuna comercial polivalente (grupo control) y la mitad de estas fueron revacunadas 3 semanas antes del parto (grupo tratamiento). Se seleccionó un grupo aleatorio de 10 cerdas de cada grupo y se hizo el seguimiento semanal de 5 lechones por cerda. El estudio fue repetido en 4 lotes consecutivos. En el tercer estudio longitudinal,

el procedimiento fue el mismo que en el anterior, pero usando la vacuna inactivada pandémica H1N1. Hisopos nasales fueron examinados por RT-qPCR y los sueros fueron analizados usando un ELISA comercial (Civtest-Suis Influenza). Después de los resultados de RT-qPCR, se calcularon las incidencias y valores beta por semana y corral. Antes de la aplicación de cualquier esquema de vacunación, se confirmó que la granja estaba infectada endémicamente con un virus H1avN1av. Los patrones de incidencia fueron diversos en los corrales evaluados y a menudo, la detección de circulación vírica se detectó tan temprano como a las 4 semanas de vida. Los valores beta semanales para el primer periodo fueron mayormente por debajo de 5 y raramente excedieron 7.5. A las 3 semanas de vida, la mayoría de los animales analizados fueron positivos con elevados ratios S/P. En el segundo longitudinal después de la aplicación de la primera vacuna que incluía un H1avN1av, se observó que el inicio de la infección se retrasó en dos semanas en comparación con las observaciones previas. Asimismo, en ambos grupos de tratamiento y control, los valores beta aumentaron significativamente, alcanzando frecuentemente valores >7.5 a las seis semanas de vida, sugiriendo que los lechones destetados solo se infectaban después de un periodo en las instalaciones de transición y probablemente, después de la desaparición de la protección pasiva de los anticuerpos maternales derivados. Ya que la circulación vírica no se detuvo completamente, se evaluó por inhibición de la hemaglutinación la reactividad del virus circulante en la granja en frente del antisuero anti-vacunal. Sorprendentemente, el antisuero de la anti-vacuna H1avN1av (1:320 título homólogo) solo reaccionó a 1:40 con la cepa de la granja mientras que el suero anti H1N1pdm09 de título homólogo semejante, reaccionó a 1:320. De igual manera, se realizó un segundo ensayo de vacunación con el mismo esquema pero usando un virus H1N1pdm09. En ese tercer estudio longitudinal, el inicio de la infección se desplazó a las 3 semanas de edad o antes, observado por RT-qPCR sin diferencias significativas entre los grupos tratamiento y control. En los tres estudios, se detectaron animales que excretaron virus en dos o hasta en tres muestreos consecutivos. Sin embargo, esto fue significativamente mayor cuando se usó la vacuna H1avN1av (41%), en comparación con los otros dos periodos (31% antes de vacunar, 11% en el segundo ensayo de vacunación, $p<0.05$). Igualmente, una proporción de entre 1 – 5% de los animales se re-infectaron después de una parada en la excreción previa. Ni los animales con excreción prolongada ni los casos de re-infección pudieron ser relacionados con los niveles de anticuerpos maternales derivados en el destete. El linaje presente en la granja durante los dos primeros estudios longitudinales correspondió a un H1avN1av. Sin

embargo, durante el tercer estudio, se detectó circulando en todos los lotes de animales un H3huN2hu, que llevaba un nuevo linaje de H3 humano derivado de un virus de la gripe estacional humana.

Part I:
Thesis background

Chapter 1. Introduction

1. Introduction

Influenza A virus (IAV) is a major cause of acute respiratory disease outbreaks in pigs. The agent is easily transmissible and when introduced for the first time in a farm it can rapidly infect the entire population and oftentimes establish as an endemic pathogen. The productive and economic impact of the disease is high but IAV is also a public health concern. One of the key features of this virus is its huge genetic and antigenic diversity that allows its establishment and maintenance in the population. The knowledge of the patterns that drive such diversity is key for the understanding of the epidemiology of IAV. The present introduction aims to review the most important features related to this virus.

1.1. A brief history of influenza

1.1.1. Influenza pandemics

Historically, influenza has been one of the infectious diseases with the highest impact on human lives. Its name comes from the Italian term "influenza" because in Medieval and Renaissance times, colds and similar diseases were thought to be the result of the "influence" of some evil alignment of the stars.

It is impossible to determine with precision when did influenza viruses emerge, but there is evidence that can trace influenza pandemics back to, at least, the 16th century although some data suggests a previous pandemic in the winter of 1173-74 by Hirsch in 1860 (*reviewed in* Potter, 2008). There is a general agreement on the fact that the respiratory disease pandemic of 1580 that started in Asia and spread to North Africa, Europe and America was influenza (Daly et al., 2007) and further influenza pandemics probably occurred again in 1650, 1781 and 1830 (Potter, 2001).

However, when discussing influenza pandemics, the 1918 "Spanish flu" is the paradigm. The "Spanish flu" spread throughout the world and became the deadliest influenza pandemic in history. It affected an estimate of 500 million people and caused the death of approximately 50 million, mainly young adults between the ages of 20 and 40. Pigs were affected in the same manner as humans, and H1N1 viruses deriving from the 1918 pandemic were found circulating among pigs. The 1918 pandemic virus was fully avian

and spread in three waves, the last two being the most severe (Taubenberger & Morens, 2006).

The next influenza pandemic occurred in 1957, the so-called "Asian-flu". On this occasion, people under 70 years old who had not experienced the 1918 pandemic, were the ones who suffered the most, as they had not been exposed and did not have previous immunity to protect them (Kilbourne, 2006). The 1957 pandemic was due to a H2N2 virus, which was a reassortant of a previously circulating H1N1 with avian H2/N2 viruses. A few years later, in 1968, another influenza pandemic arose, this time reported in Hong Kong, and it was caused by a H3N2 virus that maintained the neuraminidase (NA) gene from the Asian influenza pandemic, thus cross-protection was demonstrated (Eickhoff & Meiklejohn, 1969).

In 1977, a virus H1N1 re-emerged, spreading mainly in the former Soviet Union as well as in China and received the name of "Russian flu". Although not fully proven, it is suspected that the 1977 H1N1 was a leak from a laboratory (Zimmer & Burke, 2009) of a virus related to the ones circulating in humans in the 1950s (Kilbourne, 2006). In this case, young people, under 25 were mostly affected, although in a mild manner.

The most recent flu pandemic happened in 2009. An increase in cases of respiratory disease resembling influenza were first reported in April of that year. Soon thereafter, two human cases of flu were reported in the United States that were attributed to a novel H1N1 virus of swine origin. The infection was then detected in Canada, occurring in people with a history of travel to Mexico (WHO, 2009), and shortly after it turned into a worldwide pandemic affecting people from all ages and a pandemic alert was raised by the World Health Organisation (WHO) (Dawood et al., 2009). In a matter of a few weeks, the virus spread all over the world.

1.1.2. The discovery of influenza a virus (where the pig played a major role)

In the late 19th century, Pfeiffer claimed that influenza was caused by a gramnegative rod –named by him *Bacillus influenzae*– that was commonly isolated from nasal swabs from flu patients (Pfeiffer, 1892). Notwithstanding, the viral nature of influenza became evident after the "Spanish flu" pandemic of 1918. Olitsky and Gates reproduced the

disease in rabbits by inoculation with filtered nasal secretions from flu patients, and showed that the infection was caused by a filterable agent (Olitsky & Gates, 1921). However, they failed to recognise the true nature of the agent and believed to have found *Bacterium pneumosintes,* an unusual filterable bacterium.

Pigs were also affected by the Spanish flu and soon it became evident that both humans and pigs suffered the same disease. The initial studies on pigs focused on the isolation of bacteria and similarly to what Pfeiffer did, Lewis and Shope recovered from the respiratory tract of flu-affected pigs a bacterium that they named *Hemophilus influenzae var suis*. Nonetheless, inoculation of that bacterium in healthy pigs did not result in any significant disease (Lewis & Shope, 1931).

Finally, in 1930 Shope isolated influenza virus from pig secretions and lung tissue and showed that a filterable agent was the cause of influenza (Shope, 1931). Later on, it was demonstrated that the serum obtained from people who survived the 1918 pandemic neutralised the infectivity of the pig's virus, proving that the swine virus was a descendant of the 1918 Spanish flu (Shope, 1936; Smith et al., 1935).

The work of those pioneers was followed by many others; nevertheless, there are still many aspects of the epidemiology of this disease that we do not fully understand. We have a long road ahead before the time comes when influenza pandemics can be seen as just a thing of the past.

1.2. The influenza viruses

1.2.1. Taxonomy and general features of the virus structure

1.2.1.1. Taxonomy

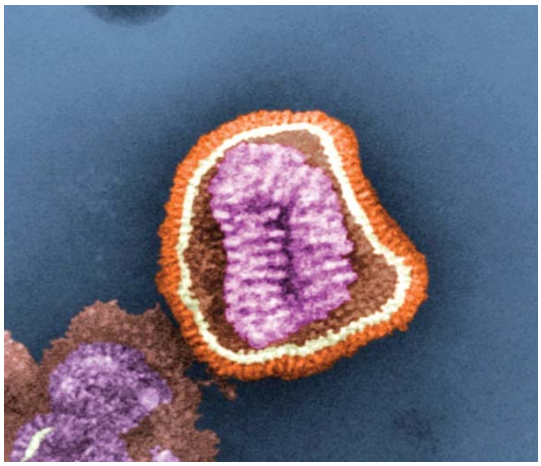
Influenza viruses belong to the *Orthomyxoviridae* family. They are enveloped, single stranded negative-sense RNA viruses. Influenza viruses are classified into four types/species: A, B, C and D based on the antigenic type of the nucleoprotein. In pigs, influenza is caused by the *Influenza A virus* (IAV) the only species in the *Alphainfluenzavirus* genus. Human influenza can be caused by IAV but also by Influenza B or C. Occasional infections of pigs with type B virus have been reported (Tsai & Tsai,

2019). Influenza D has been reported in cows and evidence suggest that pigs and poultry could be the potential hosts of the virus (Bailey et al., 2020; Gorin et al., 2019; Lee et al., 2019; Yilmaz et al., 2020).

1.2.1.2. General features of the virus structure

As explained above, IAV are enveloped viruses (Figure 1). They have two main surface glycoproteins, the haemagglutinin (HA), a protein essential for viral entrance into the host cell, and the neuraminidase (NA), essential for the release of newly formed virions. Influenza C and D viruses have a single glycoprotein, haemagglutinin–esterase–fusion (HEF) (Russel, 2008). The Matrix protein conforms the scaffold of the virus structure. The genome is within the nucleocapsid made of the nucleoprotein (NP).

Figure 1. Viral structure of IAV. Pseudocoloured, negative-stained (false-coloured) transmission electron micrograph (TEM) depicting the ultrastructural details of an influenza virus particle.



The external HA and NA proteins are coloured in orange. The M is coloured in yellow, and the NP and viral genome complex are in violet.

Photo Credit: Cynthia Goldsmith Content Providers(s): CDC/ Dr. Erskine. L. Palmer; Dr. M. L. Martin - This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #10073.

The genome is divided into 8 independent segments that codify for at least 13 viral proteins and 3 non-structural proteins (Table 1): a) the polymerase subunit 2 (PB2), involved in the mRNA cap recognition; b) the polymerase subunit 1 (PB1) involved in the RNA elongation process; c) an accessory protein PB1-F2 with pro-apoptotic activity; d) the acid polymerase (PA), which has a protease activity; e) the haemagglutinin (HA), which is a glycoprotein involved in receptor binding and fusion activity; f) the nucleoprotein (NP) which is a basic protein, important for the RNA binding, replication process and nuclear import and defines the species; g) the neuraminidase (NA) which is a glycoprotein crucial for virus release; h) the matrix protein 1 (M1) that regulates nuclear

export and is involved in viral budding; i) the matrix protein 2 (M2) that has ion channel activity; j) the non-structural protein 1 (NS1) that regulates host gene expression and, k) the nuclear export protein (NEP; also known as non-structural protein 2 (NS2) that mediates the nuclear export of RNA (Shaw & Palese, 2008). Additionally, Wise et al. (2009) found a protein derived from the PB1, called PB1 N40, which is not essential for viral viability but which absence can affect replication. More recently, a new protein called PA-X has been reported. It seems to have some effects regarding the cellular genetic expression and the immunological response (Jagger et al., 2012).

Table 1. Influenza A virus genome. Summary of the genome segments, the encoded viral proteins and their respective function(s).

Segment	Proteins encoded	Function(s)
1	PB2	Involved in mRNA cap recognition
2	PB1	RNA elongation process
	PB1-F2	Pro-apoptotic activity
3	PA	Protease activity
4	HA	Involved in receptor binding and fusion activity
5	NP	Important for the RNA binding
6	NA	Crucial for virus release
7	M1	Regulates nuclear export and is involved in viral budding
	M2	Ion channel activity
8	NS1	Regulates host gene expression
	NEP (NS2)	Mediates the nuclear export of RNA

1.2.2. Replication of influenza viruses

The cycle of replication of influenza A viruses may be divided in several phases: entry into the host cell; entry of viral ribonucleoprotein complex (vRNP) into the nucleus; transcription and replication of the viral genome; export of the vRNPs from the nucleus; and assembly and budding at the host cell plasma membrane (figure 2) (*reviewed in* Samji, 2009).

1.2.2.1. Binding, entry and transport to the nucleus

The infection starts with the binding of the HA receptor-binding domain from the virus to sialic acid (SA) residues in glycosylated proteins. The precise receptor is unknown.

SA belongs to a diverse family of sugars terminally linked to carbohydrates, mainly galactose, either by α-2,3 (SAα-2,3Gal) or α-2,6 (SAα-2,6Gal) glycosidic bond (Weis et al., 1988). Different HA subtypes will have preferences in binding to those linkages. For example, avian influenza HA mostly binds to SAα-2,3Gal, whereas human adapted HA subtypes will prefer SAα-2,6Gal (Matrosovich et al., 1997; Rogers et al., 1983). It is thought that these preferences are related to the 3D structure of the sialic acid residues. While α-2,3 are linear, α-2,6 are more bent-shaped and this seems to affect the specificity/avidity of the binding (Gambaryan et al., 1997; Matrosovich et al., 2000). This seems to affect effective transmission between avian and mammals (Matrosovich et al., 2000).

The binding to the receptor triggers the endocytosis of the virion. This endocytosis may occur in two ways. The first, similarly to other viruses, by a clathrin-dependent endocytosis; the second is by micropinocytosis (Chen & Zhuang, 2008; de Vries et al., 2011). In any case, the virus is then transported within the endosome along microtubules, where the low pH will activate the M2 ion channel. This will produce a conformational change in the HA that will expose a segment of the HA known as HA2 or fusion peptide. This fusion peptide is essential for delivering the viral genome. Once exposed, the cell proteases may cleave the subunits HA1 and HA2 (Maeda et al., 1981). When exposed, this fusion peptide inserts the N-terminal domain into the endosomal membrane while the C-terminal transmembrane domain (TMD) anchors it to the viral membrane. This structure creates a hairpin and promotes the formation of the fusion pore which releases the vRNPs into the cytosol. These vRNP are: NP, PA, PB1 and PB2. Since replication takes place in the nucleus, the vRNP will have to gain entry to the nucleoplasm (*reviewed in* Dou et al., 2018; Wiley & Skehel, 1987). Transport of the vRNP to the nucleus is possible since vRNPs have nuclear localisation signals that can activate the machinery of the nucleus (*reviewed in* Boulo et al., 2007). Several cell proteins are known to be involved in that transport but an essential one has not yet been recognised.

1.2.2.2. RNA replication and transcription

Once in the nucleus, the viral RNA (vRNA) must be replicated. Since influenza viruses are negative sense RNA viruses, a positive sense copy has to be firstly made. The replication may initiate without the need of priming for the RNA-polymerase. This is possible because 5´and 3´ ends of the viral genes are partially complementary creating

pairs of bases that are enough to start this replication. Again, the precise mechanism is not yet fully known.

Transcription takes place by "cap-snatching" where the PB2 subunit binds to the 5′ cap of a host mRNA (Guilligay et al., 2008; Reich et al., 2014). After cleavage, a conformational shift repositions the mRNA capped primer to the PB1 subunit where the 3′ end base-pairs with a complimentary sequence at the vRNA 3′ end. Following the priming event, the viral polymerase extends the mRNA transcript (Plotch et al., 1981; Robertson et al., 1981). Viral mRNAs encoding the membrane proteins NA, HA, and M2 are exported for translation by cytosolic ribosomes (Shapiro et al., 1987).

Newly synthesized viral polymerase subunits (PA, PB1, and PB2) and nucleoprotein (NP) are imported back into the nucleus. The polymerase transcribes vRNA copies from the positive strand in the cRNPs and these assemble into new vRNPs by association with a new viral polymerase (PA, PB1, and PB2) and NP (*reviewed in* Dou et al., 2018). Once assembled, the new vRNPs can transcribe additional viral mRNAs, transcribe new cRNA copies, or associate with the newly synthesized viral proteins M1 and NS2 (Copeland et al., 1986; Huet et al., 2010; Shaw & Palese, 2008).

The presence of positive-sense and negative-sense copies of the viral genome in the nucleus requires a mechanism for selecting only negative-sense copies to be assembled in new virions. The most commonly accepted mechanism is that vRNP bind to viral M1 that, in turn, binds to nuclear export protein that makes the vRNP exit the nucleus. Viral mRNAs encoding the membrane proteins NA, HA, and M2 are exported for translation by cytosolic ribosomes (*reviewed in* Boulo et al., 2007).

1.2.2.3. Virion assembly and budding

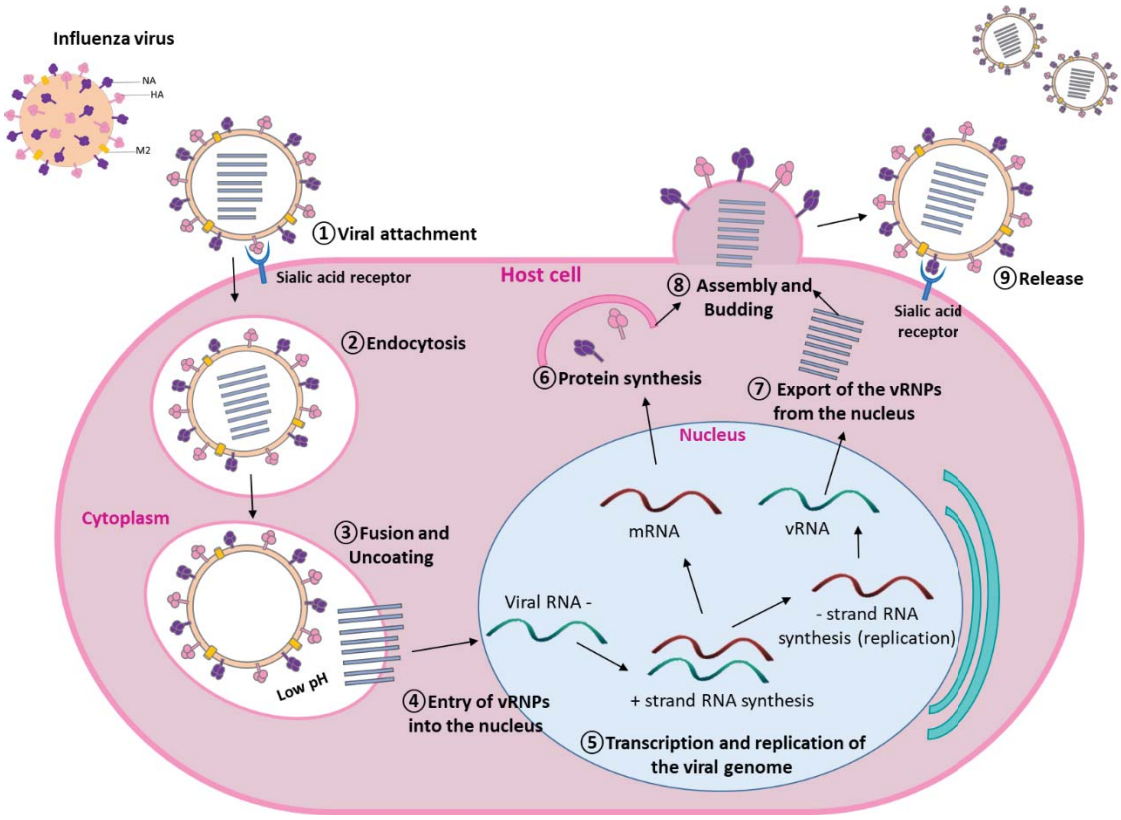
Following synthesis at the endoplasmic reticulum (ER), the proteins are trafficked through the Golgi apparatus to the budding regions in the plasma membrane (Copeland et al., 1988). With viral mRNAs being translated into viral proteins and vRNP in the cytoplasm, then it is necessary to assemble the virion and to release it to the extracellular compartment. It is unclear how the virions assemble.

For assembly and budding, firstly HA, NA and M2 are transported to the cell membrane. Then, the virions assemble. Two hypotheses have been proposed: specific packaging and unspecific packaging (Bancroft & Parslow, 2002; Smith & Hay, 1982). The first proposes that packaging of viral genome segments is random while the second suggests that the viral segments have specific signals for packaging. There is evidence for both theories. Once assembled, M2 and M1 will produce the closing of the virion and the budding with the cell membranes.

Following budding, progeny virus can remain attached to the infected cell's surface through HA binding to SA (Palese et al., 1974). NA promotes release of the virus by hydrolyzing the glycosidic bond and removing them, this facilitates the release of the virus during budding. As the viral components bud from the cell, NA cleaves sialic acids from receptors near the budding site to prevent virions binding back to the dying cell and also prevents newly budded virus from clumping together (Basak et al., 1985; Palese et al., 1974). Both these functions enable efficient release of the nascent virions from the cell. Only after this, the newly created viral particles can infect neighbouring cells.

Figure 2. Summary of the replication cycle of IAV.

①Viral attachment: the HA from the virus binds to the SA receptors on the host cell surface; ②Endocytosis: the virion enters into the cytoplasm; ③Fusion and Uncoating: Low pH activates the M2 ion channel producing a conformational change in the HA exposing the fusion peptide, then cleavage of subunits HA1 and HA2 creates a fusion pore from which the viral vRNPs will be released into the cytosol for transport into the nucleus; ④Entry of vRNPs into the nucleus: is necessary for viral RNA replication; ⑤Transcription and replication of the viral genome: first a positive copy is made, then transcription takes place by "cap-snatching", translation of viral mRNAs, the polymerase transcribes vRNA copies, assembly into new vRNPs, the new vRNPs can transcribe additional viral mRNAs; ⑥Protein synthesis: new viral proteins are synthesized for the virion; ⑦Export of the vRNPs from the nucleus: negative-sense vRNPs are selected and bind to the nuclear export protein to exit the nucleus; ⑧Assembly and Budding: the new virion is assembled and then the budding with the cell membranes; ⑨Release: NA cleaves sialic acids from receptors near the budding site to prevent virions binding back to the dying cell and also prevents the newly budded virus from clumping together.



1.2.3. Role of HA and NA and contribution to pathogenesis and immunity

HA and NA are viral surface glycoproteins embedded in the lipid envelope and are found along with the M2 ion channel. The HA is a type I glycoprotein that must undergo a maturation process involving the cleavage of its two domains, the C-terminus for HA1 and the N-terminus for HA2 (the role of HA2 is explained above). This cleavage is absolutely essential for HA function and cell infection and determines both tropism and the clinical outcome of an influenza infection (Klenk & Garten, 1994; Rott et al., 1995). The three domains in the globular head of HA1 form the receptor binding site (RBS). Mutations within the RBS determine the specificity of binding to avian-like α-2,3-SA or mammalian-like α-2,6-SA receptors. The stability of the HA protein at various pH is associated with transmission in different hosts, with mammalian viruses having a lower optimum pH of conformational stability than avian viruses (Joseph et al., 2017; Skehel & Wiley, 2000).

Influenza virus ecology is strongly influenced by the adaptation of the virus to its host. The number of glycosylation sites in the mature HA protein vary among different virus species. This feature holds an impact on the antigenic properties as HA mediates binding

to the sialic acid receptors on the cell surface (Schulze, 1997). As explained above, the SA receptors are found in α-2,3 and α-2,6 conformations and this determines the host range and tropism of the virus (Ito et al., 1998). While α-2,6 is common in ciliated human cells of the respiratory tract, α-2,3 is present mostly in alveolar and conjunctival cells (*reviewed in* Kumlin et al., 2008). Avian influenza viruses cannot be easily transmitted to humans and although the infection is possible, viral replication might be limited due to a non-optimal cellular tropism (Matrosovich et al., 2004). Interestingly, an important characteristic of pigs, is their susceptibility to infection by human and avian influenza viruses. This is because they possess α-2,3 and α-2,6-SA receptors on the upper respiratory tract epithelia. Experimentally, avian H1N1 virus has proven to adapt from birds to pigs and become infective to other pigs. In the same study, it was also shown that avian viruses could successfully replicate in ferrets and cats (Hinshaw et al., 1981).

The NA is a tetramer type II glycoprotein, whose presence is critical for the release of the newly formed viral progeny after viral replication and the posterior infection of other cells through the spread of progeny virions (Palese et al., 1974). Thus, preventing reinfection into the same cell and viral spread within the host. NA is synthesized as a single polypeptide chain but unlike HA no post-translational cleavage occurs. It has four monomers, each fold into four distinct structural domains: the highly conserved short cytoplasmic tail, the hydrophobic transmembrane region that provides the anchor for the stalk, and the globular catalytic head. This globular head domain forms a 6-bladed propeller structure, where each blade is comprised of four antiparallel β-sheets that are stabilized by disulfide bonds (Joseph et al., 2017; Shaw & Palese, 2008).

While HA binds to SA receptors to initiate virus infection, NA cleaves SA from cellular receptors to facilitate progeny virus release, otherwise viral aggregation would occur. An optimal balance between the HA and NA protein function is required for effective infection and transmission of IAV. An excess of NA proteins can hamper the binding of HA to the host cell receptors, whereas insufficient NA causes a diminished virus spread (Wagner et al., 2002). Avian NA proteins preferentially cleave α-2,3-SA residues, whereas mammalian NA proteins can cleave both α-2,3-SA and α-2,6-SA residues, indicating host-specific adaptations of the protein. This balance can be disturbed by reassortment, virus transmission to a new host, or by therapeutic inhibition of NA by

drugs such as oseltamivir and zanamivr, two clinically licensed anti-influenza drugs (Matrosovich et al., 2004; Russel, 2008).

In regards to immunity, inhibition of receptor binding is an important component of neutralisation. The RBS of the HA is a pocket at the distal end of the molecule, composed of amino acid residues which are largely conserved in various strains of the virus (Wilson et al., 1981). For the H3 subtype, four antigenic sites (A, B, C and D) were described; and for the H1 subtype, five antigenic regions were detected on the surface of the protein on the three-dimensional structure of the HA (Caton et al., 1982; Wiley et al., 1981). These antibody-binding sites in HA vary depending on whether it is found in its acid or neutral conformation. Antibodies may recognise the acid, the neutral or both forms (Copeland et al., 1986).

The antigenic sites are on the distal surface of HA1 domain, surrounding the receptor-binding sites (Caton et al., 1982). Portions of the HA surface are covered by carbohydrate side chains that help mask them. During antigenic drift (Laver et al., 1981), amino acid substitutions create new oligosaccharide attachment sites resistant to antibody binding (Bizebard et al., 1995). Changes at one or more amino acid positions in the HA1 subunit have a direct effect on the receptor-binding activity (Naeve et al., 1984; Rogers et al., 1983; Underwood et al., 1987).

1.3. Evolutionary mechanisms of IAV

Evolution of influenza viruses is driven by mutation and reassortment events, and shaped by selective pressure, such as immunity in a given population. As seen before in this chapter, the viral RNA-polymerases of RNA viruses lack proofreading capacities, which enables the accumulation of mutations in each copy of the viral genome. The error rate of the influenza A polymerase is about $10^{-3}/10^{-4}$ (Ahlquist, 2002; Chen & Holmes, 2006) which in practical terms, results in all viral copies harbouring more than one mutation. If these mutations affect the viral antigenic recognition (especially important for the HA and NA), an antigenic variant may emerge. This phenomenon of small but continued accumulation of mutations in relevant antigenic sites is known as antigenic drift. It has been proposed that sows, as living longer and being more exposed to different seasonal IAV, could exert some degree of immune pressure and a potential antigenic drift event (de Jong et al., 2007).

Reassortment events happen when a cell is simultaneously infected by two different influenza viruses (of the same or different subtype). Newly produced virions may assemble genome segments from the two parental viruses infecting the cell. As a consequence, an entirely new combination of genes (and respective proteins) may appear. This phenomenon represents a major antigenic change and is known under the name of antigenic shift. These reassortments happen most commonly among human, avian or swine-origin viruses, and despite a frequent generation of new reassortants only a few can persist (Vijaykrishna et al., 2011). In the case of human seasonal IAV, reassortments are rare, as virus evolution in the human population is mainly a result of genetic drift and antibody-mediated selection, such as history of exposure to the virus and vaccination (Zell et al., 2020).

Instead, as it will be exposed in the next section, these events are highly important in pigs. Under experimental conditions, it has been demonstrated that antigenic drift can happen shortly after infection and that this is not greatly affected by the immune status of the animal (Diaz et al., 2013). It has also been shown that detection of reassortants can continue to occur even several years after the initial detection of a parental reassortant virus was reported (Chastagner et al., 2019), and it has been proposed that reassortment is more important for the evolution of IAV in swine than in humans, as well as more unpredictable with a slower lineage turnover (Vijaykrishna et al., 2011; Zell et al., 2020). There is an annual introduction into pigs populations of the human seasonal IAV derived from the pandemic lineage, this has potentiated the virus diversity in swine (Anderson et al., 2020). All this has implications towards the vaccination of pigs, as it has been seen that fewer amino acid substitutions are needed in pigs compared to humans, to change the antigenic properties of the HA, as seen with the H3N2 viruses (de Jong et al., 2001), and surveillance should be performed to periodically verify the antigenic match of current vaccines (de Jong et al., 2007).

1.3.1. Classification of IAV isolates under the rank of species and nomenclature

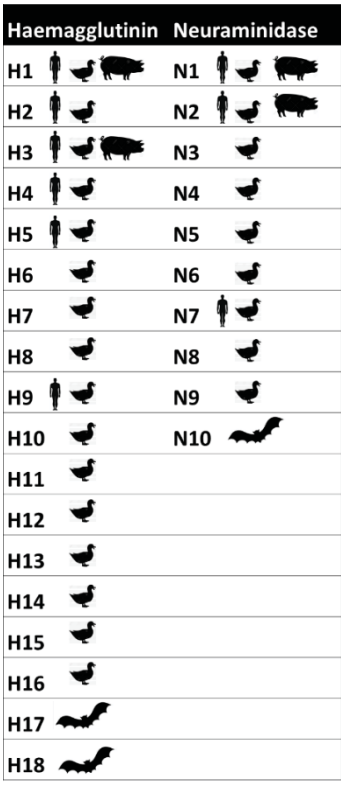
Type A influenza viruses are further classified into subtypes based on the antigenic properties of their surface antigens, haemagglutinin (HA) and neuraminidase (NA). Currently, 18 types of HA (H1-H18) and 11 types of NA (N1-N11) have been described (Russel, 2008; Tong et al., 2012). The HA and NA are the most variable proteins in the

virus, due to the fact that they are constantly exposed at the surface and so they suffer from the antigenic pressure (Röhm et al., 1996). Almost all combinations of HA and NA have been found in avian species while for mammals, the range of subtypes circulating in each species seems to be limited. In humans, domestic birds, horses and pigs, IAV can become adapted and establish subtypes and variants. Figure 3 shows the most common species distribution (pig, humans and birds) for the different H and N.

Influenza virus strains are named following a specific order, first, the subtype should be stated (A, B, or C), then the host species from which the virus was isolated (this information is omitted if the source is from humans), the location from where the virus was isolated, the identification number of the isolate and the year of isolation; for the case of the influenza viruses type A, the HA and NA subtype should be mentioned in parenthesis at the end (Russel, 2008; Shaw & Palese, 2008). For example, A/swine/Spain/001/2017(H3N2) references a virus type A that was isolated in swine in Spain, with an isolate identification number 001, which was isolated in the year 2017, and belongs to the H3N2 subtype. Other examples that could be found in databases: A/turkey/Netherlands/543301/1999(H1N1), A/Madrid/INS573/2011(H1N1), etc.

For humans, H1, H2 and H3 have been reported as well as N1 and N2 although other combinations have been reported were introduced from avian species (Khuntirat et al., 2011; Morens et al., 2010). In the pig, the major subtypes are H1N1, H1N2 and H3N2 and have been introduced from birds and humans. Nevertheless, depending on the geographic area, variations can be found within the same subtype. In pigs, the genetic and antigenic diversity of IAV makes it necessary to further subdivide the isolates within a subtype. This characterisation refers to the origin of one or more viral proteins of a group of strains within a given subtype.

Figure 3. Most common distribution of haemagglutinins and neuraminidases in humans, pigs, birds and bats. H4, H5 and H9 and N7 viruses of humans are the result of zoonotic transmission events.



1.3.2. Classification of swine influenza A viruses

Despite only three major subtypes circulating in pigs (H1N1, H3N2 and H1N2), the HA and NA genes show great diversity. This is the product of two-way transmissions between humans and swine (Lewis et al., 2016; Ma et al., 2009; Nelson et al., 2012; Rajão et al., 2018), followed by antigenic drift and shift within swine host populations, and viral spread through animal transport and trade.

1.3.2.1. Main events implicated in the generation of endemic IAV in swine

There are, at least, six human IAV strains that have been introduced in different times and geographical areas to swine, and, at least, two from birds. These introductions have been adapted to the swine population, have established as endemic variants, and have generated the current diversity of IAV in pigs (Figures 4 and 5).

The first introduction occurred in the beginning of the 20th century. The first lineage of swine influenza viruses was the one grouping the H1N1 strains, which originated from the 1918 Spanish flu (the so-called *classical* H1N1; csH1N1). This introduction occurred first in North America, in 1919 "the Hog Flu"; (Koen, 1919) and later on in Europe (late 1930s). In both regions, the virus spread and established as endemic. Interestingly, the csH1N1 from North America was antigenically distinct from the one detected in European swine. This virus was introduced soon after the pandemic in North America and remained antigenically stable until the late 1960s (Sheerar et al., 1989). Later, by the time the csH1N1 was introduced in Europe, the virus had been evolving by antigenic drift as a seasonal flu in humans for the past 15-18 years. Therefore, at that point, there were two lineages of csH1N1 circulating in two different geographical areas. From that moment onwards, the diversity of swine IAV has evolved differently in these regions.

Focusing in Europe, the North American csH1N1 was confirmed in European pigs in the year 1976. This introduction occurred after an influenza outbreak in pigs from Italy which had been imported from the United States. These strains showed close antigenic relationship to the classical H1N1 Iowa and New Jersey strains (Nardelli et al., 1978). The second introduction of a human IAV into the pig population occurred after the Russian flu of 1977, a human H1N1 virus was circulating in pigs in Europe and was named "human-like swine H1N1" (huH1N1) (Webster et al., 1992). This virus circulated in pigs during the 80s in Europe. Soon after that, in 1979, inter-species transmission from birds to pigs occurred when an H1N1 of avian origin was wholly introduced directly from wild ducks and successfully adapted to pigs (Pensaert et al., 1981). This virus was called the Eurasian "avian-like" H1N1 (H1avN1av) and could be considered the first event of an introduction from birds to pigs, replacing the csH1N1 in Europe. Outbreaks were reported in Belgium and Germany, and subsequently, it became widespread throughout the continent. To this date, that H1avN1av represented the dominant strain among H1N1 circulating in the European swine. A year after, in 1980, Ottis & Bachmann (1980), reported the isolation from adult mallard ducks in Southern Germany of a strain antigenically related to the classical swine influenza H1N1. This suggested reverse pig-to-bird inter-species transmission of IAV.

The third human-to-pig introduction occurred in the years following the Hong Kong pandemic of 1968, when a H3N2 virus adapted to pigs and was detected in several

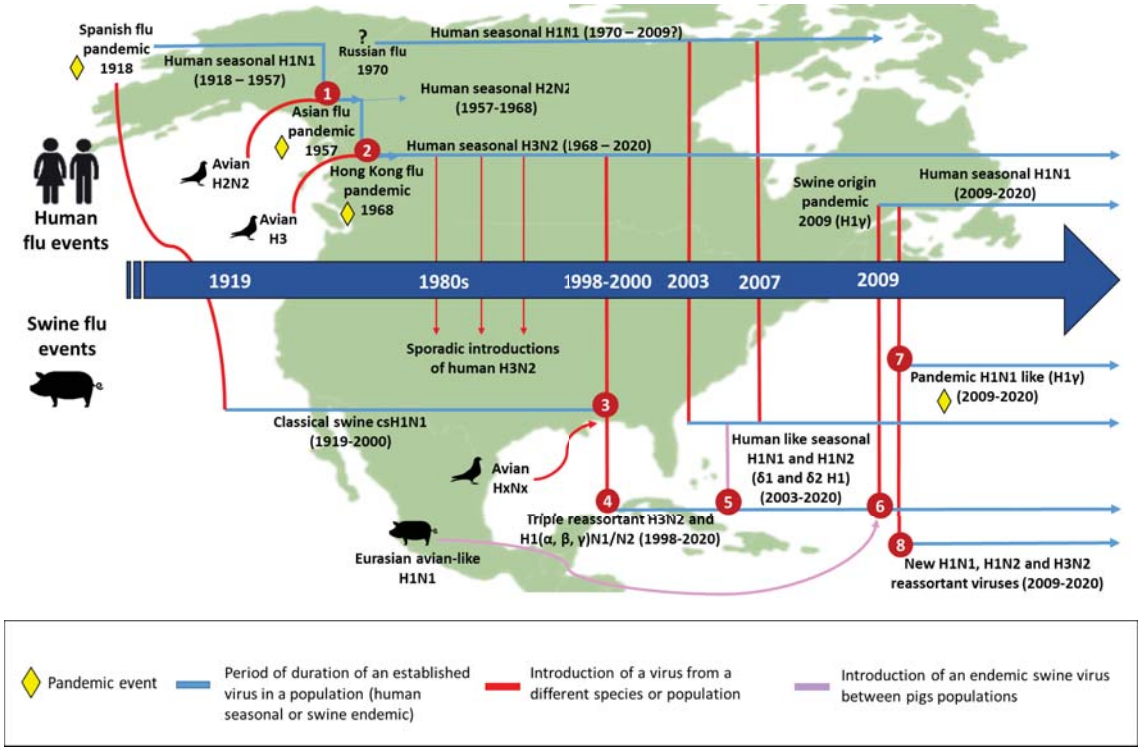
European countries (human-like H3N2; H3huN2hu). In 1984, a reassortment of the human-like swine H3N2 virus and the H1avN1av took place, with cases reported in France and Italy (Madec et al., 1984; Castrucci et al., 1993). This H3N2 virus had the HA and NA from the human H3huN2hu and the internal gene cassette (PB2, PB1, PA, NP, M and NS) of avian origin present in the H1avN1av (Campitelli et al., 1997), and replaced the original human H3N2 virus in pigs. Nevertheless, its spread was limited and was soon replaced by a novel human-like H1huN2hu initially detected in pigs in the United Kingdom in 1994 (Brown et al., 1995). This virus was found to have a HA closely related to the huH1N1 that circulated during the 1980s while the NA was related to the original H3huN2hu. The internal genes were of avian origin which had been circulating in pigs in northern Europe (Brown et al., 1998). An avian-like H1N2 swine influenza virus was generated by the reassortment of the HA gene of the Eurasian avian-like H1N1 and the NA gene of the swine-adapted H3N2, this virus was initially detected in Denmark in 2003 and has since then established in the Danish pig population (Trebbien et al., 2013; Ryt-Hansen et al., 2019b). These viruses have shown to be antigenically and genetically different from the prevalent human-like H1N2 (Trebbien et al., 2013).

Moving back to North America, during the 80s there were several introductions of the human seasonal H3N2 derived from the 1968 pandemic. These were sporadic introductions and represented a very low prevalence when compared with the csH1N1. Between 1998 and 2000, several reassortment events occurred between this H3huN2hu, the csH1N1 and an avian virus. These reassortment events were associated with an increase of outbreaks in pig farms of USA, and led to the generation of the first stable H3huN2hu lineage of swine in North America; the triple reassortant H3huN2hu (trH3N2). This virus contained the HA, NA and the internal gene PB1 from the human seasonal H3N2, the NP, M, and NS genes from the csH1N1, and the PB2 and PA genes from an avian IAV (Zhou et al., 1999). These were the fourth and the second introduction from human-to-pig and avian-to-pig, respectively. In the following years, new reassortments occurred between the csH1N1 and the trH3N2, generating new trH1N1 and trH1N2 viruses containing the internal cassette of trH3N2 (Anderson et al., 2020; Vincent et al., 2008). After these reassortments, there was a genetic and antigenic differentiation on the HA of these H1 viruses, that resulted in a new classification; α, β and γ H1's. Afterwards, from 2003 and 2007, two new introductions occurred in North America from human seasonal H1N1 to pigs, representing a fifth human-to-pig introduction. These

H1N1 incorporated two new H1 variants in the swine IAV constellation, the sub-clusters δ1 and δ2. The HA from the δ-cluster viruses had most likely emerged from two separate human introductions from the seasonal H1N2 and H1N1 viruses, respectively, and they could be phylogenetically differentiated (Lorusso et al., 2013; Walia et al., 2019).

Around 2009, a new reassortment occurred in pigs, between the trH1N1 including the γ-H1 and the N1 and M segments from the swine Eurasian H1avN1av. This virus jumped to humans and caused the 2009 human pandemic (H1pdmN1pdm). This strain was re-introduced into the swine population worldwide. The first to be documented was an outbreak in Canada in April (Howden et al., 2009), and in Europe in Northern Ireland in September 2009, followed shortly by two other outbreaks (Welsh et al., 2010). This novel H1N1 had a particular gene combination that had not been reported before (Smith et al., 2009). This virus revealed a combination of avian, human and swine viruses from at least two different geographical regions; Europe and America. This mixture of genes occurred most probably in North American swine over the previous 30 years at least. The phylogenetic analysis showed that each segment of the genome could be nested within well-established swine influenza lineages before the outbreak. Interestingly, the virus has become endemic in many pig producing countries, and has resulted in many different reassortants, known as ‘‘H1N1pdm-like reassortant viruses; such as H1pdmN1av, H3huN1pdm, among others, as well as the exchange with genes from the pandemic internal cassette like the H1huN2hu with a full pandemic internal cassette (Simon et al., 2014; Watson et al., 2015).

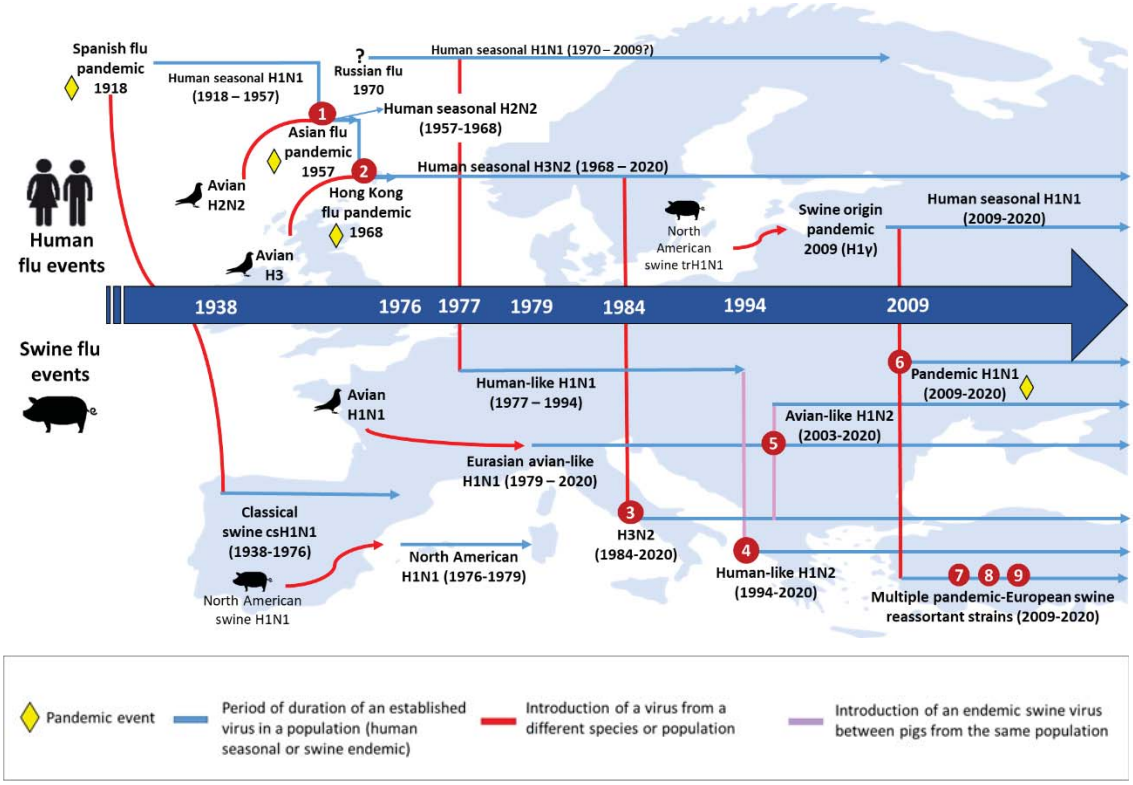
Figure 4. Events involved in the evolution of the North American IAV



①Asian flu: H2N2 virus, which was a reassortant of a human seasonal H1N1 with avian H2/N2 viruses, and internal genes from the H1N1. ②Hong Kong flu: H3N2 reassortment between the NA from the Asian flu and the human seasonal H3N2, and avian internal genes. ③Triple reassortant trH3N2: contains the HA, NA and PB1 from the human seasonal H3N2, the NP, M, and NS from the csH1N1, and the PB2 and PA from an avian HxNx. This combination created a stable trH3N2 lineage and the emergence of the TRIG cassette. The HA of the trH3N2 viruses can be further classified into 4 distinct genetic clusters (I, II, III and IV). ④trH1N1 and trH1N2: product of the reassortments between the csH1N1 and the trH3N2. The trH1N1 contains the HA and NA from the csH1N1, but with the TRIG cassette; and the trH1N2 contains the HA from the csH1N1, the NA from the trH3N2 and the TRIG cassette. The H1 viruses evolved and differentiated genetically and antigenically into α, β and γ. ⑤Human-like H1N1 and H1N2: two independent introductions of human seasonal H1 creating a separate cluster (δ) that was later divided into two sub-clusters (δ1 and δ2). ⑥ and ⑦ The 2009 pandemic reassortant: A new reassortant in pigs between the trH1N1 including the γ-H1 and the Eurasian avian-like. This strain contains the HA, NP and NS derived from the csH1N1, the NA and M from the Eurasian avian-like H1N1 and the PB2, PB1 and PA of the TRIG cassette. This virus later jumped to humans and caused the 2009 human pandemic (H1pdmN1pdm) and then the strain was re-introduced into the swine population. ⑧Other new reassortants: Other

variants of the dominant subtypes can be found circulating among pigs, such as new H1N1, H1N2 and H3N2.

Figure 5. Events involved in the evolution of the European IAV.



①Asian flu: H2N2 virus, which was a reassortant of a human seasonal H1N1 with avian H2/N2 viruses, and internal genes from the H1N1.②Hong Kong flu: H3N2 reassortment between the NA from the Asian flu and the human seasonal H3N2, and avian internal genes. ③Human-like H3N2: with the HA and NA from the initial human H3N2 and all internal genes of avian origin. ④Human-like H1N2: contains a HA closely related to the huH1N1, with a NA of the H3N2 and internal genes of avian origin. ⑤Avian-like H1N2: contains the HA of the Eurasian avian-like H1N1, the NA of the swine-adapted H3N2 and internal genes of avian origin. ⑥The 2009 pandemic reassortant: A new reassortant in pigs between the trH1N1 including the γ-H1 and the Eurasian avian-like, was first detected in Europe in Northern Ireland in September 2009. ⑦, ⑧ and ⑨ Novel reassortant viruses: H1N1pdm-like viruses or reassortant viruses that have at least one gene from the pandemic strain; such as H1pdmN1av, H3huN1pdm and H1huN2hu with a fully pandemic internal cassette, etc.

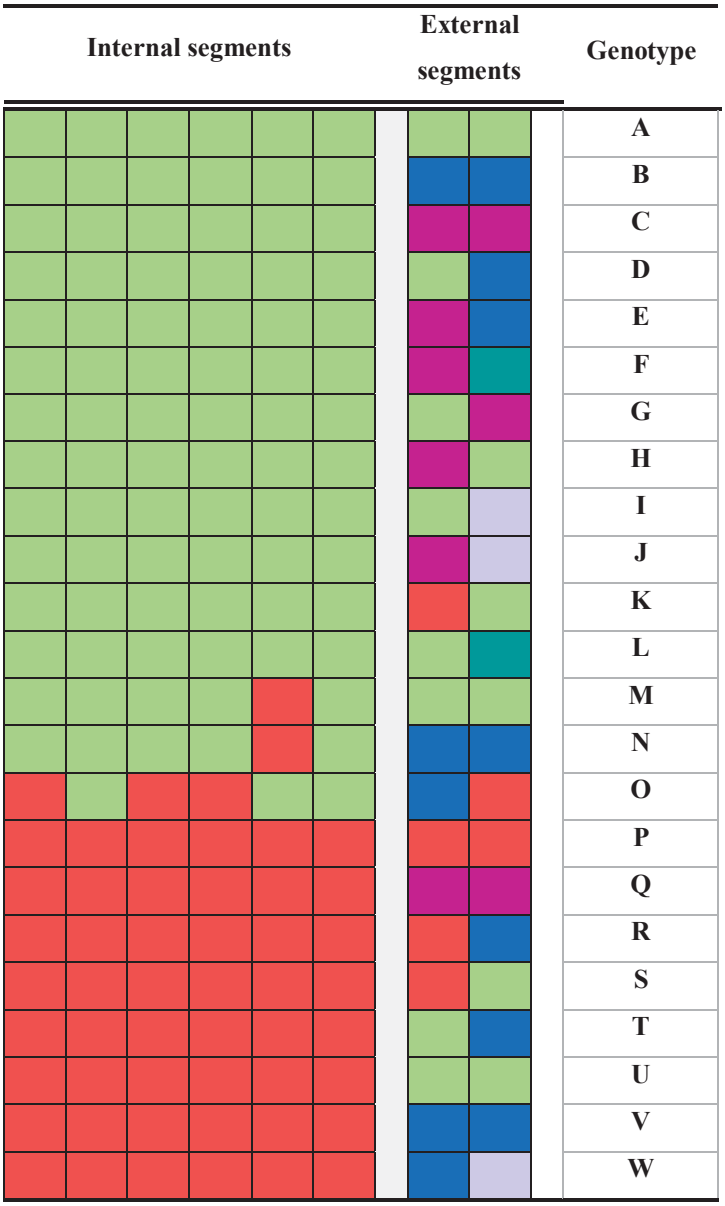
1.3.2.2. Classification of lineages and genotypes

Considering that the HA is the major surface glycoprotein with importance in the immune recognition, and that H1 is extremely diverse in swine, it has been proposed a phylogeny-based global nomenclature system for the haemagglutinin H1 (Anderson et al., 2016). In summary, 1A includes the HAs related with the csH1 (α, β, γ and the pandemic H1 variants), 1B includes the human-seasonal related H1s (European H1hu and the δ1 and δ2 H1s), and finally, the 1C that includes all the Eurasian avian-like H1s (H1avN1 and N2). Similarly, the N1s can be classified as csN1, avN1 and pdmN1 (Lorusso et al., 2011). For the H3 lineage, there are 6 clades within the 1990s (IV, IV-A, IV-B, IV-C, IV-D, IV-E, and IV-F) and 1 clade within the 2010s (human-like H3) (Walia et al., 2019). The 1A HAs can be found along a N1 from the csH1, pandemic or with a N2 genes derived from the 1998 or 2002 human seasonal lineages (Nelson et al., 2011; Walia et al., 2019). This classification makes sense as these differences are not only at genetic level but also at antigenic level. It is known that the viruses from 1A show limited or null cross-reactivity in front 1B and 1C clusters, and vice versa (Lorusso et al., 2011). Also, sub-classifications within each group are necessary due to differences in cross-reactivity, this is heterologous within avian-like viruses (Lewis et al., 2016; Lorusso et al., 2011), or within clade IB, with the δ1 and δ2 viruses. The H1huN2hu does not present any cross-reactivity with the HA from the H1avN1av, thus they can both be serologically differentiated (Brown et al., 1998). The European H1avN1av and H3huN2hu show some degree of cross-reactivity with IAV from the 1980s (Van Reeth, 2007). It has been shown as well that European IAV viruses have serologic cross-reactivity in front of the H1pdmN1pdm strains (Kyriakis et al., 2010), this suggests that the internal genes could be also playing a role in terms of immunity and cross-reactivity.

Regarding the internal genes, until 2009 there was no need for classification in swine IAV. The viruses circulating in Europe remained stable, including only the avian-like cassette. In a similar way, since the reassortment events occurred in North American strains, the triple reassortant cassette was predominant in North America. However, due to the introduction in 2009 of the H1pdmN1pdm, new reassortants including both avian-like or triple reassortant internal genes and pandemic internal genes appeared in multiple combinations. In 2015, Watson et al. published the results of an epidemiologic surveillance study that took place between the years 2009 and 2013 in 14 European countries where they performed a genomic characterization of 290 swine influenza

viruses. They proposed a genotyping system to classify viruses of the different lineages that were being detected across Europe (Figure 6). The scheme considered the origin of their two external and six internal gene segments (avian, human or pandemic). At that moment, they detected 23 genotypes (designated with a letter from A to W).

Figure 6. Genotype classification system proposed by Watson et al. (2015) copied and adapted from: "Molecular epidemiology and evolution of influenza viruses circulating within European swine between 2009 and 2013". Genes are colour-coded to help indicate their origin or strain reference.



As different genotypes can be determined based on the combinations of the internal and external genes. Examples: Nº1; represents genotype A where all segments have an Eurasian avian-like origin, Nº 2; represents genotype D where the HA has an H1av origin, a NA that has a H3N2 1984-like origin and an internal cassette of avian origin, Nº 3;

represents genotype P where all segments descend from the 2009 Pandemic virus, and finally, Nº 4; represents genotype O where the HA has a H3N2 1984-like origin, a NA that descends from the 2009 Pandemic virus and an internal cassette with 3 segments from avian origin and the other 3 from the 2009 pandemic virus.

Internal segments						External segments		Genotype	Example Nº
PB	PB	PA	NP	MP	NS	HA	NA		
								A	1
								D	2
								P	3
								O	4

1.3.2.3. Other strains of importance

There are other reported strains that have been detected in pigs. As is the case of an H1avN2hu that appeared in Brittany in 1987-88. That virus had a HA of avian origin and a NA of human origin, and it was the product of the reassortment of the H1avN1av and the H3huN2hu viruses (*reviewed in* Moreno et al., 2013).

Also, other strains such as H9N2 in Southeast Asia apparently introduced from poultry (Brown, 2000), an H1N7 was isolated in 1992 from pigs in England with an H1 closely related to a human strain and an N7 related to an equine H7N7 (Brown et al., 1997). An H2N3 genetically similar avian/swine virus reassortant that was isolated from diseased swine from two farms in the United States (Ma et al., 2007).

In Denmark, an H1avN2hu subtype has established in the Danish pig population since 2003, this subtype is the result of a reassortant between a swine "avian-like" H1N1 and H3N2. This subtype differs from most other reported H1N2 viruses in Europe (Trebbien et al., 2013) and is still to this date the most prevalent subtype in Denmark (Ryt-Hansen et al., 2019b). Recently, a new "human-like" H3 that resembles a human seasonal influenza from 2004-2005 (Krog et al., 2017) has been detected in Danish farms.

1.4. Epidemiology

1.4.1. Prevalence of swine IAV

The prevalence of different subtypes, lineages and genotypes varies depending on the continent as well as the region therein. There are several studies that evaluate the presence and prevalence in these different areas by surveillance and seroprevalence of the different circulating IAV. In the following sections, the distribution of these viruses will be summarised.

1.4.1.1. IAV in Europe

IAV is considered endemic in pig populations in many parts of Western Europe and a distinction is made regarding the different circulating subtypes, H1N1, H1N2 and H3N2. Van Reeth et al. (2008) in collaboration with the European Surveillance Network for Influenza in Pigs 1, conducted a seroprevalence study for these three subtypes in Belgium, the Czech Republic, Germany, Italy, Ireland, Poland and Spain. They evaluated a total of 4190 sow sera from 651 farms during 2002 and 2003. In Belgium, Germany, Italy and Spain they found high (30%) to very high (50%) seroprevalence rates to each of the three subtypes, except for a lower H1N2 seroprevalence rate in Italy (13.8%). These countries have in common large pig populations and intense farming, the opposite of what is seen in Ireland, the Czech Republic and Poland, where swine farming is less intensive thus a lower pig density and also possible differences in the structure of the pig industry. Furthermore, in the case of Ireland, despite being in Occidental Europe, it has a geographical isolated location, and in Poland, despite being the largest producer in Central Europe, it is still far behind the other Occidental countries. Due to these possible explanations, in Ireland, the Czech Republic and Poland, they found that H1N1 was the dominant subtype (8.0–11.7%) and H1N2 and H3N2 antibodies were rare (0–4.2%).

In a virological surveillance conducted by Kyriakis et al. (2011) in Belgium, UK, Italy, France and Spain from 2006 to 2008. They found H1N1 to be the predominant subtype (48.3%), followed by H1N2 (29.6%) and H3N2 (21.3%). The UK and the Brittany region of France, where the only places where H3N2 was not detected, possibly absent or circulating at very low levels, meanwhile in the other countries all three subtypes were found.

Particularly, in Spain, there have been prevalence studies showing the endemic presence of IAV in pig farms and of seropositive animals for one or more subtypes. Spain - together with Germany - is the leading pork producing country in Europe and among the top 5 in the world (Eurostat, 2017). López-Soria et al. (2010), found 85% of sows and 80% of fatteners seropositive for one or more strains of IAV. A year later, Simon-Grifé et al. (2011), found H1N1 to be the predominant circulating subtype (92.9%), followed by H3N2 (92.9%) and H1N2 (64.3%). Moreover, they found a high percentage (87.8%) of farms and 44.6% of pigs positive to more than one subtype, indicating co-circulation of different subtypes. Interestingly, for the case of the H3N2 subtype, in a recent study the prevalence was found to be 7.5%, it is important to remark that this study used RT-PCR from nasal swabs instead of HI from serum samples. Nevertheless, the prevalence for that particular subtype seems to have greatly decreased in a matter of ten years. Unfortunately, there is no available data regarding the prevalence of any of these subtypes in Portugal, this could be due to the low density of pigs and therefore a lesser important pork production.

Up until 2009, seroprevalence studies to detect the subtype were performed using HI, but after the introduction of the pandemic lineage, this technique has become partially limited due to the fact that there is some level of cross-reactivity between this pandemic and avian H1N1 (Kyriakis et al., 2010) and this makes interpretation difficult for each subtype. This is the main reason why it is important to characterise by lineages and not only by the subtype.

Moving to the detected lineages, during a surveillance study in Belgium, UK, Italy, France and Spain from 2006 to 2008, by Kyriakis et al. (2011), they found predominantly avian-like H1N1 (47.9%), human-like H1N2 (27.8%) and human-like H3N2 (21.3%). They also detected five novel reassortants; two H1N1 with a human-like HA and three H1N2 with an avian-like HA. In a more recent study by Simon et al. (2014) mainly conducted through passive surveillance programs, they examined more than 9000 herds in 17 European countries. They observed a variation on the distribution of circulating lineages, most likely related to the introduction of the pandemic H1N1. The predominantly detected lineage was the avian-like H1N1 (53.6%), followed by the human-like reassortant swine H1N2 (13%), pandemic H1N1 (10.3%) and human-like

reassortant swine H3N2 (9.1%). They also detected a percentage of viruses that were the product of reassortments between these four lineages (13.9%).

The proportion of H3huN2hu viruses identified from 2010 to 2013 was lower than the one previously reported (Van Reeth et al., 2008). They were considered to be absent or circulating at undetectable levels in the UK, France and Denmark (Kyriakis et al., 2011; Simon et al., 2014). This could have been the result of a spread in the circulation of H1huN2hu or the reassortant H1avN2hu which could have led to a decrease in the circulation of H3huN2hu. Interestingly, Simon et al. (2014), did not detect any H1pdmN1pdm in Spain, Belgium and The Netherlands. Nevertheless, this pandemic lineage has increased in prevalence in some countries from indicating an establishment in the European pig population (Simon et al., 2014). In a recent study in France (Hervé et al., 2019), they detected a predominance of H1avN1av, followed by H1huN2hu virus, and interestingly, they found a higher proportion of H1pdmN1pdm in the winter and in the areas with the smallest pig populations. In a surveillance study conducted in Spain from 2017-2019, the pandemic lineage had a prevalence around 6% based on the HA and NA.

Martín-Valls et al. (2014) detected outbreaks in Spain caused by H1avN1av, H3huN2hu, H1huN2hu and H1pdmN1pdm, and in a big proportion of the sequenced cases found indications of reassortment events, such as H1huN1av or a new H1huN2hu including a new seasonal human NA. This new reassortant including the N2 from a human seasonal origin was also detected in another study from Italy (Moreno et al., 2011). In regards to the presence of reassortants with the pandemic lineage, these have been reported in Germany and Denmark (Breum et al., 2013; Harder et al., 2013; Lange et al., 2013) which mostly consisted of pandemic H1N1 with a N2hu. Also in Germany and Hungary, a pandemic virus acquired an avian-like N1 (Banyai et al., 2012; Starick et al., 2011). Other various reassortants have been reported between the pandemic and European lineages (Chiapponi et al., 2013; Howard et al., 2011). The results obtained in these studies, suggest that reassortment events do not only affect the HA and NA, but also other parts of the genome. For example, despite detecting a prevalence of 6% for H1pdmN1pdm in Spain, in the same study a prevalence of 31.7 % of isolates that contained internal genes from the pandemic virus was detected. This fact highlights the importance of taking into consideration the eight segments instead of only the external ones.

As previously mentioned, the introduction of the pandemic strain completely changed the classification of the European swine IAV. This is the reason why Watson et al. (2015), proposed a tool to help researchers classify all these different genotypes. In the study they detected that genotypes A to L, accounted for 67% of the isolates and all of them contained an internal gene cassette derived from the Eurasian avian-like lineage. Strains containing a reassortant internal gene cassette (with both Eurasian and 2009 pandemic genes) represented 6% of the isolates (genotypes M, N and O) and the ones containing an internal cassette derived from the H1N1pdm09 lineage represented 27% of the isolates (genotypes P to W). Only four genotypes, A, B, C and P, were found to be panEuropean, whereas A, B and C represented the classical lineages H1avN1av, H3huN2hu and the H1huN2, P was an H1pdmN1pdm that included a fully pandemic internal gene cassette.

Some geographical distribution trends could be seen during the study. Interestingly, while in mainland Europe, Eurasian-based genotypes (A to L) were predominant, in the UK, A(H1N1)pdm09-based genotypes (P to W) were more prevalent. Furthermore, in mainland Europe they observed the following frequencies. The Eurasian avian-like H1N1 (genotype A) was detected at an average of 37%, with Belgium having the highest rate (58%). Gent/84-like H3N2 (genotype B) had an average frequency rate of 15%, with the highest detection in Spain (36%) and Hungary (33%). The Scot/94-like H1huN2 (genotype C), had an average of 7% with the highest frequency in France (30%) followed by Belgium and Germany. In the case of Denmark, a reassortant H1avN2hu (genotype D) was found to be the predominant isolate with a 47%. In the UK, genotype B was not detected, genotype A only represented 15% of the isolates and genotype C had a 7%.

Following the introduction of the pandemic strain, in 2010 a new genotype was identified in the UK, which was the product of a reassortment between the pandemic virus and the Scot/94-derived H1huN2, acquiring the external glycoproteins (genotype Q), this new genotype replaced the circulation of genotype C during three years, and became the most frequent isolate (54%) in that area. Meanwhile in mainland Europe, genotype Q was not detected, but a reassortant between A(H1N1)pdm09 and the H3huN2hu (genotype B), acquiring the N2 segment (genotype R) was predominantly isolated in Germany, where it represented 26% of the isolates, followed by Italy and The Netherlands.

Since 2015, some new genotypes have been reported in Europe. As is the case of Italy, where two different genotypes that were similar to the genotype F but possessed a M gene from the A(H1N1)pdm09 lineage representing a novel reassortant genotype (Beato et al., 2016). Also in France, a triple reassortant of an H1avN2hu with a M segment from A(H1N1)pdm09 (Chastagner et al., 2019). In a study in the Iberian Peninsula from the period 2017 – 2019, from a total of 60 isolates, 7 new genotypes were reported, representing 11.7%, which is a high percentage considering the time comprised.

1.4.1.2. IAV in North America

Anderson et al. (2013), reported a decrease in the pandemic virus along with an increase in the frequency of H1δ1 and an increased diversification of the H3 cluster IV, representing 25% of isolations. Using deep genome sequencing, Diaz et al. (2017) differentiated 13 distinct viral genomes in the US, demonstrating the molecular complexity of the virus during natural infection. Similarly, Rajão et al. (2017), reported the circulation of different genomic constellations in pigs, product of reassortments with the pandemic virus, they also identified 44 different genotypes, the most common containing a clade IV-A HA gene, a 2002-lineage NA gene, a M-pdm09 gene, and gene segments of the triple reassortant internal gene (TRIG) origin, this represented the 32.3% of isolations. Exhibition swine in agricultural fairs are important events in North America, these create the opportunity for virus introduction and widespread within regions, Nelson et al. (2016), studied the evolution and diversity in fairs in Ohio and Indiana from 2009-2013, and they identified the introductions of human variant H3N2 (H3N2v) virus, they also reported the introduction of 10 viruses in one single year.

In Western Canada, Grgić et al. (2017), when evaluating outbreaks of respiratory disease in pigs, H3N2 viruses belonged to clusters IV and IV-C and after characterisation of the these viruses they observed reassortment of gene segments between the North American swine trH3N2 from Cluster IV and the A(H1N1)pdm09, as well as unique groups of H3N2 reassortants, based on the H3N2/H1N1pdm09 gene combinations. In Mexico, Nelson & Vincent (2015), after phylogenetic analysis, they found four novel lineages originated from human seasonal virus, two different lineages related to North America, as a result of migration from US or Canadian herds, as well as multiple lineages that had been circulating undetected, and repeated introductions of the pandemic virus.

1.4.1.3. IAV in Asia

The current circulating diversity of IAV in this continent is the result of a mix of European and North American strains. Virological surveillance revealed that csH1N1, human-origin H3N2, Eurasian viruses, triple recombinant and their reassortant variants co-circulate in pigs in this region (Choi et al., 2013; Vijaykrishna et al., 2011; Zhu et al., 2013). The H3N2 reassortant was isolated in Hong Kong from pigs imported from southern China, it was antigenically and genetically distinct from the human-like H3N2 that was circulating in pigs (Zhu et al., 2013). Most human-like H3N2 seem to have difficulty in establishing in pigs as they do not form an independent group or sublineage in the evolutionary trees (Vijaykrishna et al., 2011). In 1998, there was a reassortment event and H3N2 and H1N2 triple reassortant viruses were generated in North America and then arrived to Asia through pig movement (Karasin et al., 2006; Zhou et al., 1999).

The first detection of Eurasian viruses occurred in 2001 in Hong Kong (Smith et al., 2009; Vijaykrishna et al., 2011), it was isolated from pigs imported from southern China. Since then, the virus has co-circulated with the other strains until it became predominant (Choi et al., 2013; Zhu et al., 2013). Since the introduction of the pandemic virus, this has been reported in many Asian countries (Vijaykrishna et al., 2010) presumably from direct introductions from humans to pigs (Zhu et al., 2013). It has been reported that gene segments of the pandemic lineage are greatly distributed, causing reassortments and currently circulating in Japanese pig populations (Okuya et al., 2018) as well as in farms in Thailand (Nonthabenjawan et al., 2015).

Avian influenza viruses have frequently been isolated or detected in pigs in Asia, being the most frequent H9N2 and H5N1, which are enzootic in poultry (Vijaykrishna et al., 2011; Zhu et al., 2013). Avian-like H9N2 was detected in China and Korea, and avian-like H5N1 in China, Vietnam and Indonesia (Nguyen et al., 2005; Nidom et al., 2010; Yu et al., 2011). This is most likely due to physical closeness of poultry and pigs and the subsequent direct contact in farms.

1.4.1.4. IAV in other regions

There are other regions where cases are reported, but there is not much scientific information regarding the situation. For example, in Africa, the introduction of the pandemic virus and its reassortments has been detected in Togo, which is characterised

by small farms and free range pigs, possibly as a result from human-to-swine transmission (Ducatez et al., 2015). Equally, in Nigeria and Cameroon, they have found serological evidence of this virus circulating in pigs (Njabo et al., 2012; Snoeck et al., 2015). Also, reverse zoonotic transmission from humans to pigs in Nigeria in intensive farms has been reported (Meseko et al., 2019).

In South America, in backyard pigs in Southern Brazil, serological surveillance showed low prevalences of IAV and the predominant lineage was H1pdmN1pdm (Souza et al., 2019). In Argentina, IAV is considered widespread among pig farms, subtypes reported are H1pdmN1pdm, H3N2, δ1H1N1, and δ2H1N2, as well as coinfections with two or more subtypes in 80.5% of positive pigs (Dibárbora et al., 2013). In Peru, farming of swine and domestic birds, such as chickens or ducks, is a common practice in some areas, and can be also accompanied by suboptimal biosecurity and hygiene practices by farmers (McCune et al., 2012). In Chile, 3 novel clades of H3N2 and H1N1 viruses of human origin have been reported, possibly due to independent introductions from humans (Nelson & Vincent, 2015). Overall, more surveillance studies should be conducted in these continents to know which strains are circulating in pigs, as backyard farming is not uncommon, and this could pose a risk to public health in the future.

1.4.2. Transmission of IAV

1.4.2.1. Routes of infection

Transmission can occur via aerosols, large droplets, and direct contact with secretions or contaminated fomites (Tellier, 2006). The primary route is direct contact with oronasal secretions (Van Reeth & Vincent, 2019). Influenza virus is not transmitted through semen and blood-borne transmission via insects, as the virus does not cause viremia (*reviewed in* Torremorell et al., 2012).

Aerosols are the consequence of particles that are expelled when coughing and/or sneezing, which turn into virus suspensions by evaporation. It has been demonstrated under experimental conditions that virus infectivity has a direct relation to the relative humidity (RH), showing an increased survival at low RH (15-40%) and an exponential decay when RH is >40% (*reviewed in* Tellier, 2006). In relation to the transmission in pig production facilities, a study in North America by Neira et al. (2016), quantified and characterised the level of IAV in samples of aerosols and surfaces during acute outbreaks

of influenza infections. They found presence of IAV in the air with levels that could be sustained for periods of 20 days, and they also detected a correlation between these levels, the number of positive samples and the quantity of virus in the swine oral fluids as well as in the air. Demonstrating air can be an important route of IAV transmission in swine production facilities. Also, respiratory secretions can remain infectious even for one week (Larsen et al., 2000), and it can remain viable on hard nonporous dry surfaces for 24 - 48 hours (Bean et al., 1982) what makes fomite-mediated transmission an important mean in influenza virus transmission. Contaminated water with bird faeces can also play a role in the indirect transmission (Karasin et al., 2000; 2004).

Fomites that have been in contact with infected pigs are a confirmed way of transmission between and within pig populations (Allerson et al., 2013). Emphasizing the importance of a biosecurity protocol in a farm and education of the farm workers. Transport of infected and subclinically infected animals plays an important role in the transmission and movement of virus strains, as it has been seen in the case of Asia, particularly in China (Vijaykrishna et al., 2011).

1.4.2.2. Dynamics of infection in a pig farm

Transmission of influenza virus may happen either by contact with clinically or subclinically infected animals, highlighting the importance of controlled animal movement practices (Torremorell et al., 2012). Once an influenza outbreak occurs in a farm, it can progress in the entire herd within 2-3 weeks (Easterday, 1980; Hinshaw et al., 1981; Reynolds et al., 2014). The virus will be then maintained by susceptible piglets and through the ones that recovered from the infection but later on became susceptible once again, and this process will result in the farm becoming endemic (Reynolds et al., 2014).

In the herd, influenza infections can follow either an epidemic or and endemic presentation.

1.4.2.2.1. Epidemic presentation

The epidemic form is a consequence of the introduction of an IAV strain for which the population of animals only had low or non-existent levels of immunity. In those cases, there is usually an overt disease. Given the short incubation period (1-3 days) and the

high transmissibility of the infection, the disease is seen as an abrupt outbreak affecting most of the animals present in the farm. Affected animals will show signs of high fever (40.5 – 41.5 °C), anorexia, inactivity and coughing, reaching morbidities up to 100% (Van Reeth & Vincent, 2019). The IAV incidence in epidemic circulation have been shown to be over 70% in a given population within one week (Simon-Grifé et al., 2012). It will take around two weeks for the animals to fully recover their previous body condition. The fatality rate is low (usually less than 1%) with initial recovery within 3-7 days, except in those cases where bacterial co-infections are present (Olsen et al., 2006). These situations are sporadic but may cause an important impact if pregnant sows are affected as abortions or stillbirths could happen subsequently from the infection (Janke, 2014; Van Reeth & Vincent, 2019). No evidence for transplacental transmission in pigs has been found (Kwit et al., 2014).

1.4.2.2.2. Endemic presentation

The initial outbreak usually gives rise to an endemic situation where influenza can be recurrently detected. This form of IAV is characterised by a lower incidence sustained across time. Susceptible subpopulations will play a major role in the persistence of the virus in each farm. There are three populations that need to be considered; 1) the gilts and young sows, 2) the suckling piglets and, 3) the weaners. If unvaccinated or uninfected by the IAV strain of the herd, gilts and young sows (first parturition) are more susceptible to the infection when compared to older sows, who most presumably have been in contact with the herd isolate or vaccinated. In a study carried out in Denmark (Larsen et al., 2010), it was shown that young sows are more predisposed to have IAV positive litters during lactation, probably due to a lower level of maternally derived antibodies. Similarly, these sows are likely to seroconvert during their stay in the maternities, indicating a poor immunisation process.

In a study conducted in the Midwestern US (Diaz et al., 2015), they found that replacement animals resident on-farm for less than 4 weeks (new gilts) and piglets (less than 21 days of age) had higher odds of testing positive compared to replacement animals with a stay of more than 4 weeks (gilts). Suggesting that new gilts and piglets may be the most epidemiologically significant reservoirs for IAV in swine breeding herds.

Suckling piglets have been proposed to play an important role in the endemic circulation of IAV in a herd. There are several studies showing the presence of IAV at these early

ages (Larsen et al., 2010; Simon-Grifé et al. 2012; Allerson et al., 2013; Diaz et al., 2015). Moreover, they can become infected in the presence of maternally-derived antibodies (Diaz et al., 2013). MDA positive animals may be prolonged IAV shedders. This phenomenon has been reported in both experimental and longitudinal field studies, where animals have shown to excrete virus for more than 2 weeks (Ryt-Hansen et al., 2019b). On the other hand, MDA can confer partial protection in front clinical signs but weaners with maternally-derived immunity without overt disease can nevertheless generate infectious aerosols (Corzo et al., 2014) therefore they are capable of spreading the infection. Despite the fact that clinical signs do not seem to show in the pre-weaning period, the presence of IAV in farrowing units has been associated with an increase of post-weaning mortality (Alvarez et al., 2015). Finally, animals infected in the presence of MDAs may remain susceptible afterwards (Loeffen et al., 2003). Indeed, consecutive infections by the same or similar IAV strain in the same pig have been demonstrated in experimental studies (Cador et al., 2016a; Loeffen et al., 2003) and have been described in longitudinal field studies (Ryt-Hansen et al., 2019b; Simon-Grifé et al., 2012).

Finally, weaners play an important role in the persistence of IAV in the herd. They generally become infected between 6-8 weeks of age (Gillespie, 1999; Rose et al., 2013; Simon-Grifé et al., 2012), coinciding with the decay of the MDA. Also, at this stage, some animals can be recurrently infected (Ferreira et al., 2017). Moreover, most swine farms have a constant turnover of piglets, generating animals on a weekly basis in many cases. Furthermore, IAV can "jump" from one batch to the other, this has two main consequences; 1) the virus can persist in the farm even if the batch stays positive for a short period of time, and 2) clinical disease generally appears at weaning, affecting piglets of similar age groups (6-8 weeks of age) batch after batch. Therefore, animals showing clinical disease, pigs subclinically infected, and the continued introduction of susceptible animals play an important role in the maintenance, transmission and dissemination of influenza virus at population level (Brown, 2000).

Commonly, the same strain will persist in the farm until replaced by a new one most fitted to that situation (no previous immunity, better adaptation to pigs, etc.) (Pitzer et al., 2016). However, several studies describe more than one IAV strain circulating at a same time in a same farm and in the same herd in endemic circulations (Anderson et al., 2015; López-Soria et al., 2010; Martín-Valls et al., 2014; Rose et al., 2013), suggesting that one virus

will not always replace the previous one, but that they can co-exist. This could be the ideal scenario for the occurrence of reassortment events. Endemic circulation has an impact on the increase of mortality at weaning (around 2%), on the average daily gain, on the increase of secondary bacterial diseases and also on the increase of medication costs (Cador et al., 2016a; Gillespie, 1999; Torremorell et al., 2009).

1.4.2.2.3. Transmission parameters of IAV

The spread of infection in a determined population can be estimated according to the reproduction ratio (R). The basic reproduction ratio is defined as the average number of secondary cases in a completely susceptible population caused by one infectious individual during its entire infectious period (Diekmann et al., 1990). As it has a threshold value that equals 1, when R is >1, implies that an infection may spread in a population, for it is ideal to keep with the correct control measures and maintain R below 1 (Velthuis et al., 2007).

Is IAV a virus that spreads fast in a given population? The transmission parameters vary depending on the characteristics of each farm and the type of circulation for the particular virus and the immune status of the affected population. An easy way to understand the spread of a given pathogen is to compare it with another common pathogen. In a study conducted in Spain by Pileri et al. (2017), they assessed the basic reproductive rate (R) and the dynamics of transmission of IAV and porcine reproductive and respiratory syndrome virus (PRRSV) in two commercial pig farms endemically infected. They observed that in farm 1, an H1N1 circulated endemically. The transmission of IAV and PRRSV was similar under these circumstances, with R values ranging from 1 to 3. These values were observed mostly in the farrowing units and in nurseries with some level of previous passive immunity. In the case of IAV, seroconversion was observed in a limited number of animals and an important proportion of the animals repeated the infection in different sampling times. These results suggested that a first infection in presence of MDAs blocked the adaptive immune response and animals remained susceptible. On the other hand, a higher R ranging from 3 to 6 was observed when a new IAV virus was introduced in the fattening units without presence of previous immunity. This R was observed in both farm 1 and 2. Also in farm 2, PRRSV transmission was faster, showing R values around 5 and 7. However it is worth noting that for PRRSV the infectious period is 3 weeks and for IAV only 6-7 days. Considering this, for a shorter period of time, IAV

transmission is more efficient than the one observed for PRRSV. A good example of this is what was observed in farm 2; PRRSV and IAV infected almost 100% of the animals, showing similar R values, but when PRRSV needed more than 4 weeks to reach the highest incidence, IAV spread to more than 70% of the present animals in only one week.

1.4.3. Factors associated with the presence and transmission of IAV in pig farms

1.4.3.1. Risk factors

In a logistic regressions study (Fablet et al., 2013), they identified factors associated to a seropositivity to H1N1 or H1N2. It was found that, for both subtypes, the odds for being positive increased when more than two pig herds were in the vicinity. For the case of H1N1, the odds were higher when large numbers of pigs were allocated per pen in the post-weaning room, regarding room temperature they found that when the heating device in the farrowing room was below 25ºC, and the ventilation controller below 24ºC, also the transport of pigs to the fattening facility passing by a room that has older pigs. For H1N2, they found that a short period in the farrowing room, a small floor area per animal in the post-weaning pen, and for the fattening unit, they identified that a large room, not performing an all-in all-out management, and a range of less than 5ºC controlling the ventilation temperature were associated with seropositivity. It has also been suggested that IAV levels in herd can be affected by the time of the year in a cyclical pattern, increasing during autumn and reaching a peak in early winter and late spring, to finally decrease in the summer, this could be partially explained by the absolute humidity (AH) of the air and temperature changes (Chamba Pardo et al., 2017).

Breed-to-wean farms have been suggested to favour IAV spread because suckling piglets not only maintain but also allow diversification and transmission during weaning (Chamba Pardo et al., 2018). According to a study in Spain (Simon-Grifé et al., 2011), there are three risk factors associated with seroprevalences of IAV and its spread; these are 1) an increased replacement rate in pregnancy units which was represented by an increased seropositivity against H1N2, H3N2, and to farms with co-circulation of subtypes, 2) the lack of barriers between pens and 3) an uncontrolled access to the farm facilities. They also found a correlation between farms that had and did not have a bird-proof net, as the former showed a protective effect against IAV and a lower

seroprevalence. Their results propose the gilt replacement rate to potentially behave as a source of introduction of the virus through an influx of susceptible and subclinically infected animals as well as a source of spread in the farm. The introduction of infected gilts has also been associated with higher IAV seroprevalence in other studies (Corzo et al., 2014; Serafini Poeta Silva et al., 2019; White et al., 2017). In acclimatisation units, animals can adapt to the microorganisms and pathogens circulating in the farm and prepare for breeding. Also, gilts that have been infected by IAV before or during the acclimatization period will have time to recover from the infection before being transferred to the barn. Therefore, it has been associated with a lower seroprevalence (Serafini Poeta Silva et al., 2019). Effective quarantine, based on the early detection of infected units alone, could have the largest impact in limiting influenza outbreaks in swine populations with barely any spread to humans (Dorjee et al., 2016).

1.4.3.2. How does previous immunity affect IAV transmission?

A study to assess the effect of heterologous and homologous vaccines on susceptibility to infection by the H1N1 triple reassortant (Romagosa et al., 2011), showed that vaccination can reduce transmission, delay replication and decrease the susceptibility to infection. However, the efficacy of the vaccination varied whether the vaccine was heterologous or homologous, the former could not completely prevent transmission (R=1 compared with R=10 in unvaccinated animals), whereas with the latter, it could not be detected (R=0).

Another experiment (Cador et al., 2016b), assessed the impact of MDAs on the transmission and duration of infection and its spread in young piglets in the absence and presence of MDAs. They found that the presence of MDAs in weaned piglets significantly reduced transmission, with an R three times lower than in animals without the MDAs. However, the resulting transmission was still over 1, offering limited protection against the spread of the virus. As a downside, the dissemination was slower which could play a detrimental role in allowing infected animals to pass unnoticed for a longer period. Deblanc et al. (2018), analysed the impact of different levels of residual MDAs in relation with virus excretion and the immune response in piglets born from sows with and without MDAs (MDA+, MDA-). In both cases virus excretion was not prevented but they had a

lower infectious potential in piglets MDA+. Also in this group, humoral response was affected but they showed protection against a second infection with the same virus.

The sow has an epitheliochorial placenta, which does not allow any significant transfer of antibodies to the foetus and, as a result, all maternally-derived systemic protection depends on the ingestion of colostrum. This might lead to the silent spread of the virus in the first weeks of age, which could partly explain the recurrence of epidemics after passive immunity waning. In addition, MDA may interfere with the development of active immunity upon vaccination or infection (Loeffen et al., 2003).

Reynolds et al. (2014), developed a mathematical model to assess the impact of vaccination strategies, they calculated that a homologous vaccine administered to the entire population after the loss of MDA eliminated influenza, but a heterologous vaccine with a partial protection had little effect on the infection levels; although these results are only a product of a simulation study and when applied to a real scenario the outcome might be different. In regards to the mechanisms involved in spread and persistence in farrow-to-finish farms, Cador et al. (2016b), developed a metapopulation model to represent the population dynamics in two subpopulations (breeding sows and growing pigs) in a batch-rearing system coupled with an epidemiological model, for partial passive immunity protection in neonatal piglets and an immunity boost in re-infected animals. They found that piglets with maternally derived partial immunity could extend the duration of epidemics and favour the transmission, having as a consequence an endemic persistence of IAV in a herd.

1.4.4. Transmission from pigs to humans

The transmission flow from this virus has been, in its great majority, from humans to pigs, and after human pandemics, those viruses have become predominant in pig populations. Nevertheless, when comparing the frequency of human-to-swine and swine-to-human transmission, there are differences regarding the surveillance, which varies between countries, continents, and species, as it will not be performed with the same exhaustiveness in pigs as in humans (*reviewed in* Nelson & Vincent, 2015). Reports of transmission have a low global rate –and most likely many go unreported-, but it does not mean that they lack epidemiological importance. It is possible that if there are enough transmission events, then some of these viruses could significantly affect humans.

One of the most known cases of transmission happened in 1976, at a military base in Fort Dix, New Jersey. Where there was a large-scale transmission of more than 200 cases of human-to-human of IAV, and soldiers were seropositive to H1N1 (*reviewed in* Krueger & Gray, 2013). The isolate A/New Jersey/76, was similar to the virus believed at the time to be the cause of the 1918 pandemic (*reviewed in* Sencer & Millar, 2006). Risk factors for transmission from pigs include the modernisation of farms with a more intensive industrialised production, to work in a laboratory where there is exposure to the virus, swine shows at agricultural fairs (Krueger & Gray, 2013), and naturally, to be a farm worker in close contact with pigs, as happened in 2008 in Teruel (Spain) where a woman farmer tested positive to H1N1 virus (Adiego Sancho et al., 2008).

In the US, agricultural fairs are popular events in which exhibition swine and people gather to spend time in close contact, they represent a risk for transmission. There are multiple reports of human cases due to participation in these events, such as more than 300 cases due to H3N2 variant virus (H3N2v) in Ohio the period from 2009 - 2011 (Bowman et al., 2012). This variant that was isolated from exhibition swine was antigenically similar to the H3N2 circulating in swine in farms, and it has been suggested that events like this can predispose the emergence of variants, and even though the risk is suspected to be small for people attending the fair without close contact, it still poses a risk because of the large population that participate in them (Feng et al., 2013; Wong et al., 2013). Some of the risk factors related to the fairs are shows with larger pigs, as they are more likely to be infected, as well as the presence of open-classes and breeding shows, which is why blanket vaccination of animals before attending the event is recommended (Bowman et al., 2014).

The most important event was the 2009 pandemic H1N1 virus, this was the product of a triple reassortant between avian, swine and human influenza viruses. This pandemic affected more than 214 countries and caused the death of over 18,449 people (WHO, 2010), but more recent estimations attribute a global death toll related to respiratory and cardiovascular compromise of between 151,700 and 575,500 deaths during the first twelve months of the pandemic (Dawood et al., 2012). Due to influenza virus behaviour, it is likely that another pandemic will arise in a few years, hopefully of a lesser magnitude.

1.5. Clinical course and immunity

1.5.1. Clinical signs and lesions

In the epidemic forms, the infection most often has an overt clinical course because animals in the farm do not have previous immunity against the strain. Infected animals show coughing, fever (40.5ºC), sneezing, lethargy, dyspnoea, nasal discharge and conjunctivitis. Pregnant sows may have abortions due to the high fever. During the third and fourth day of disease, pigs will develop a harsh deep cough as a result from an extensive bronchitis and bronchiolitis (Janke, 2014; Loeffen et al., 1999). Bacterial complications and interaction with other respiratory agents in the farm are common (Brockmeier et al., 2002; Van Reeth & Pensaert, 1994). In endemic situations the disease is more insidious. Usually the main sign is a recurrent cough that appears in the weaned piglet's batch after batch (Brockmeier et al., 2002), in the framework of the porcine respiratory disease complex.

The most consistent macroscopic lesion is a cranioventral bronchial-interstitial-pneumonia affecting the lobules in various degrees. The hallmark microscopic lesion is a necrotizing bronchitis and bronchiolitis. Within the first 24 to 48 hours of infection, neutrophils accumulate in the vasculature adjacent to bronchioles, there is a necrosis and sloughing of airway epithelial cells and neutrophil transmigration into airway lumens with vascular congestion and oedema. After this period, epithelial cells become flattened, macrophages become predominant and there is a lymphocyte accumulation around airways. Cell death by necrosis stimulates a strong inflammatory response through cytokine induction. Injury and destruction of the cells lining the respiratory tract are a result of both direct virus infection and cytokines of the innate immune response (Janke, 2014).

1.5.2. Immune response of pigs against IAV

During an influenza infection, the innate and adaptive immune responses participate to fight the virus. The first one is crucial in early stages and controls different mechanisms, such as the viral replication with the aid of natural killer cells (NKC), alveolar macrophages, and dendritic cells, and the regulation of the virus-specific adaptive immune responses (McGill et al., 2009). After initial infection there is an important inflammatory response due to the cellular necrosis in the lung tissue and direct stimulation

of the virus to release cytokines (Van Reeth et al., 1999). Main cytokines involved correspond to interferon-α and -γ (IFN-α; -γ) and tumour necrosis factor-α (TNF-α); interleukin-6 (IL-6) is related to the acute clinical signs and pathology, and it seems that interleukin-1 (IL-1) has minimal participation (Van Reeth et al., 2002). Within the first 18–24 h, pro-inflammatory cytokines reach a peak, and this increase is in direct correlation with the virus dose and replication and the intensity of clinical signs (Charley et al., 2006; Van Reeth et al., 1999). On the other hand, macrophage levels and infiltration of neutrophils in the lung appear to be low (Charley et al., 2006).

The adaptive immune response is regulated by a virus-specific humoral response and cell-mediated immunity. This humoral response is targeted to the surface proteins of the virus, mainly HA and NA. Also, mucosal IgA is important for local protection and viral neutralisation during early infection (Charley et al., 2006; Loving et al., 2012). Cell-mediated immunity intervenes in the resolution and clearance of the infection. This can be achieved by T CD4+ cells, which help activate B cells and antibody production, or either by T CD8+, who are directed towards more conserved epitopes in the virus and differentiate into cytotoxic T lymphocytes and mediate lysis of the infected cells (Rajão & Vincent, 2015; Van Reeth & Vincent, 2019).

Protection in case of infection is provided by the antigenic similarity and homologous features of the strains (de Jong et al., 2001), as it is the case of the cross-protection and subsequent immunity after the prior infection with H1avN1av in front of the pandemic virus, which can substantially reduce shedding and viral load in respiratory tissues (Busquets et al., 2010; De Vleeschauwer et al., 2011). Immunity against H1N1 or H3N2 has protective effects against H1N2 virus replication and shedding, and if the immunity is against both, then animals do not show clinical disease and viral replication is severely reduced (Van Reeth et al., 2003). The HA from the H1huN2hu has low antigenic and genetic homology with H1av and H3hu, but it has been seen that previous infections can boost a strong immunity response against these strains, even after one vaccination event (Van Reeth et al., 2006).

1.5.2.1. Correlates of protection

The HA is the major target of neutralising antibodies, and antibodies that inhibit virus haemagglutination by HI are considered correlates of protection (Vincent et al., 2017).

The HI titre of a serum corresponds closely with its neutralising activity against the infectivity of the homologous virus. In humans, specifically healthy young adults, there is an established cut-off value with HI of 1:40 to achieve a 50% of protection, but this value is not necessarily accurate for children nor the elderly (*reviewed in* Ward et al., 2018). Antibodies play a key role in protective immunity to IAV and are considered to be the best-defined correlate of protection (Holzer et al., 2019). The HI test can discriminate between infection with European H1N1, H3N2, and H1N2 (Van Reeth et al., 2006). Since then, the HI titre has been used as a correlate for serum HI antibodies against IAV infection (Krammer et al., 2020).

Correlates of protection (Table 2) such as IFN-γ secreting cells, cross reactive CD4+ and CD8+ T cells, serum neuraminidase inhibition (NI) antibody titres, antibodies measured by single radial haemolysis (SRH), and antibodies that target the HA stalk domain are immunological markers and they are often based on conserved viral epitopes. T cell immunity is directed against conserved epitopes of both surface and internal viral proteins. Other markers that are currently being investigated to establish their correlation with protection are the following: antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP), mucosal IgA levels, antibodies to the ectodomain of the matrix 2 ion channel (M2), matrix protein 1 (M1) or nucleoprotein (NP), influenza virus proteins (IVPM), among others (Holzer et al., 2019; *reviewed in* Krammer et al., 2020).

Table 2. Summary of the different assays for correlation of protection. (Copied and adapted from Krammer et al. Meeting report and review: Immunological assays and correlates of protection for next-generation influenza vaccines. 2020. *Influenza Other Respir Viruses*, 14**:** 237-243.)

Correlate of protection	**Assay**
Serum haemagglutination inhibition antibodies	HI assay
Nasal IgA Serum anti-neuraminidase binding antibody HA stalk-specific antibodies	Enzyme-linked immunosorbent assay (ELISA)
Serum virus neutralisation antibody titre	Virus neutralisation (VN) assay
IFN-γ secreting cells	IFN-γ Elispot
Antibodies to the ectodomain of the matrix 2 ion channel (M2), matrix protein 1 (M1) or nucleoprotein (NP)	Cell-based ELISA or flow Cytometry
Serum neuraminidase inhibition (NI) antibody titres	Enzyme-Linked Lectin assay (ELLA)
Cross reactive CD4+ and CD8+ T cells	Virus-specific cytotoxicity, Interferon gamma ELISPOT
Serum single radial haemolysis antibodies (SRH)	Single radial haemolysis assay
Antibody-dependent cellular cytotoxicity (ADCC) Antibody-dependent cell-mediated phagocytosis (ADCP)	Antibody-dependent cellular cytotoxicity (ADCC); antibody-dependent cell-mediated phagocytosis (ADCP)
Influenza virus proteins	Influenza virus protein arrays (IVPM)

Broadly neutralising antibodies against the conserved stalk of the HA protein, offer protection even between influenza subtypes. It has been suggested that broadly cross-protective immunity can be antibody and T cell-mediated (Holzer et al., 2019).

Despite HI often showing a good correlation with other assays, there are a few limitations of this technique and with VN. For example: reference materials like serum and virus strains should be defined, sample collection and reagents should be standardised, as well as the methodologies and protocols between laboratories to avoid variation in the titres and allow an accurate reproducibility (*reviewed in* Ward et al., 2018; *reviewed in* Krammer et al., 2020).

1.6. Diagnostics

1.6.1. Submission of samples

Various samples can be collected for influenza testing, such as, serum, lung tissue, nasal swabs, tissue swabs, and oral fluids. Detection of IAV, viral antigen, nucleic acid, or antibodies is going to depend on the correct time of infection, collection of the appropriate sample type, the quality, and the proper storage and handling of the sample (Culhane & Detmer, 2014). Samples should be collected from an acutely affected pig that is febrile, has a distinctive cough, and has not been medically treated (Swenson et al., 2001). Nasal swabs have been over the years, the reference sample for IAV detection and isolation (Goodell et al., 2013; Van Reeth & Vincent, 2019) and in recent years, oral fluids have become the sample of choice for large groups of weaned pigs because it is easy to collect (Garrido-Mantilla et al., 2019; Goodell et al., 2013). Group and environmental sampling strategies using oral fluids, sow udder skin wipes, surface wipes and airborne particle deposition, have been found in a study to be better than individual samples like nasal swabs, nasal wipes and oropharyngeal swabs for active surveillance, but these do not replace individual samples when it comes to obtaining a viral isolate or for sequencing (Garrido-Mantilla et al., 2019). Sampling of air or surfaces has also been tested in studies to assess the risk of influenza exposure in pig workers (Choi et al., 2015) or for other pigs (Neira et al., 2016).

1.6.1.1. Ante mortem samples

1.6.1.1.1. Nasal Swabs

Nasal swabs (NS) collected for detection by isolation or RT-PCR should be taken from clinically ill animals during the febrile period of illness (Van Reeth & Vincent, 2019). For this, the pig should be restrained with the head upwards, and a polyester swab inserted into the nostril and then slowly withdrawn with a rotating motion, this process should be repeated for the other nostril using the same swab. Finally, the tip of the swab should be put into a vial containing 2-3 ml of transport medium and kept at 4 °C for up to 48 h. If processing is not to be immediate, storage at −70 °C is recommended because the virus is not stable at −20 °C (Van Reeth & Vincent, 2019; WHO, 2011). However, if done too aggressively or if the swab is inserted too deeply, blood may be produced and this could interfere with the tests (Swenson et al., 2001), so caution is recommended. Collection of

NS is laborious and not cost-effective for surveillance purposes, but still is the best sample for viral isolation (Goodell et al., 2013).

1.6.1.1.2. Oral Fluids

Oral fluids (OF) can be collected from single animals or from groups of pigs by hanging ropes over the pens, these should be at shoulder height for the pig and away from feed and water. After 20–30 min, the OF are extracted from the rope either by placing a plastic bag over it and squeezing the fluid into the bag or by cutting off the rope, placing it into a bag and squeezing out the fluid. This technique is easy, sensitive and effective for the detection of IAV at pen-level, including pre-wean piglets, using rapid testing methods such as RT-qPCR (Detmer et al., 2011; Goodell et al., 2013; Panyasing et al., 2014; Ramirez et al., 2012; Romagosa et al., 2012). However, obtaining a viral isolate or a sequence from oral fluids can be challenging due to the quality of the sample (Garrido-Mantilla et al., 2019).

1.6.1.1.3. Serum

Serology can be used to demonstrate the presence of specific antibodies against IAV, to determine the immune status of a herd, the levels of MDA in piglets and their kinetics, and to assess post-vaccination antibody titres (Van Reeth & Vincent, 2019; WHO, 2011). For this, a volume between 3–5 ml of blood via jugular venepuncture is recommended for collection. Following clotting, it should be centrifuged to separate the serum and then stored at 4ºC for immediate processing or at −20°C for further storage (WHO, 2011). The diagnosis of acute infection requires the use of paired, both acute and convalescent, serum samples. It has been recommended for samples to be collected at the time of infection and then at 3–4 weeks later for comparison (Culhane & Detmer, 2014; Van Reeth & Vincent, 2019; WHO, 2011). One week after infection, pigs may have titres of at least 1:80. These titres can increase to 1:320 to 1:640 when sampled 14–21 days after infection (Janke, 2000).

1.6.1.1.4. Snout Wipes

This method is performed by using a disposable cleaning pad soaked in saline, which is rubbed over a pig's nose and then placed in a plastic bag. Then, a corner of the bag is cut, and the pad is squeezed so the liquid falls into a tube for laboratory submission (WHO, 2011). One case study compared diagnostic results from testing nasal swabs versus snout

wiping and they found virus isolation and sequencing to be successful (Dobesh et al., 2013). This method is not accepted in the USDA Surveillance Program (Sandbulte et al., 2015), although it has been recommended for monitoring the virus in agricultural fairs (Edwards et al., 2014).

1.6.1.2. Post mortem samples

1.6.1.2.1. Bronchoalveolar fluids

Bronchoalveolar fluids (BAL) can be obtained by extracting the lung lobes and trachea intact from the thoracic cavity. Then a hot spatula is pressed on the outside of the trachea, an incision is made between the tracheal rings with a sterile scalpel, and then aseptically infused with approximately 100 ml of virus transport media. The lung lobes are then gently massaged and as much fluid as possible is removed and collected in sterile tubes (WHO, 2011).

1.6.1.2.2. Lung and trachea tissue

Tissue samples can be taken following BAL collection. Samples should be preferably addressed to the left middle lobe or lobes with signs of lesions, such as purple areas, or with firm consolidation. Portions of the lung lobes and trachea should be shipped at 4°C for nucleic acid detection or virus isolation. Remaining tissue portions can be fixed in formalin and shipped for histopathology and immunohistochemistry (WHO, 2011). The localisation of IAV may differ depending the area of the lungs which is being sampled and this could affect the interpretation of the assays performed (Swenson et al., 2001). Lungs can be processed for RT-qPCR, virus isolation, immunohistochemistry and type A antigen-capture enzyme-linked immunosorbent assays (ELISAs) (OIE, 2018).

1.6.2. Diagnostic methods

Diagnosis is only possible by isolation of virus, detection of viral proteins or nucleic acid, or by demonstration of virus-specific antibodies (Van Reeth & Vincent, 2019). An ideal diagnostic method, should be accurate, cost effective and should give a result in a rapid manner, also, sample selection is a critical factor in making an accurate diagnosis (WHO, 2011). Diagnostic tests can be used in a direct (antigen) or indirect (antibodies) manner. Specific IgG and IgM against the HA can be detected in the respiratory tract starting 5 days post-infection (Lee et al., 1995), and in the serum, IgG can be detected from the first

week up to the second week post- infection with an increasing tendency (Larsen et al., 2000).

1.6.2.1. Viral isolation

Despite the development of molecular techniques, viral isolation still is one of the basic techniques in the study of influenza viruses. Isolation permits to test the antigenic and biological properties of a strain and often is required to produce sequences of quality.

One of the classical approaches is to combine the inoculation of a sample in a cell culture or in embryonated eggs with the use of the haemagglutination assay (HA) to screen for the successful isolation of the virus. The HA test is relatively quick and can detect both live and inactivated influenza viruses (WHO, 2011). The HA assay is not an identification assay, as other agents also have hemagglutinating properties.

1.6.2.2. Madin–Darby canine kidney (MDCK) cells

IAV can be successfully isolated in cell culture from lung tissue and nasal swabs (OIE, 2018). MDCK cells are permissive for various subtypes and strains, but primary swine kidney, swine testicle, swine lung, or swine tracheal cells and other mammalian cell lines can also be used (OIE, 2018), and it was found that adding trypsin to the MDCK culture favoured the growth and efficiency of IAV (Tobita, 1975). Currently, MDCK cells are the most commonly used cell line for isolation, propagation, and titration of IAV (WHO, 2011; Zhang & Gauger, 2014). Nasal swabs are the preferred over lung for virus isolation (Clavijo et al., 2002).

The advantages of isolation in MDCK is that the virus can be further used for antigenic and genetic characterisation, vaccine development and drug-susceptibility tests (WHO, 2011). It is highly efficient for conducting plaque assays (Gaush & Smith, 1968), and it has optimal sensitivity for influenza viruses, even greater than Vero and MRC-5 cell lines (Frank et al., 1979; Reina et al., 1997). Also, compared to other cell cultures, haemadsorption may not be necessary for the recognition of influenza viruses, due to its distinctive CPE, and finally, is more suitable for the study of viral shedding than primary cell lines, which vary in sensitivity (Meguro et al., 1979). Katz & Webster (1992), reported that primary isolation of IAV H3N2 in MDCK cells resulted in a HA identical to that of the original replicating virus from the infected individual, whereas isolation in

ECE resulted in the selection of variants with amino acid substitutions in the globular head region of the HA molecule.

Isolation has its limitations, as it can be time-consuming, and also, because cells might lose their susceptibility to respiratory viruses after a number of passages. But most importantly, this method requires a sufficient amount of viable virus in the sample that capable of causing CPE (Zhang & Gauger, 2014).

1.6.2.3. Embryonated chicken eggs (ECE)

This method has been considered one of the best methods for influenza isolation due to its sensitivity (WHO, 2011). Virus isolation in ECE or in different cell lines offer alternative options when isolation in MDCK cells is unsuccessful (Zhang & Gauger, 2014). Nevertheless, there are a few drawbacks to this method, like the fact that it can take a few days to have a result, it can be expensive to maintain the supply of high-quality eggs, also, cultivation of IAV in eggs can lead to the selection of variants with amino acid substitutions near the receptor-binding site of the HA molecule with antigenic and structural changes, whereas it remains identical to that of the viruses grown in mammalian cell cultures (Gambaryan et al., 1999; Zhang & Gauger, 2014). Most importantly, unlike avian viruses, some porcine viruses grow poorly in eggs, especially if inoculated by the allantoic route alone, so it is not always accurate. In such instances, MDCK cells are a useful additional approach to isolating virus (Swenson et al., 2001; WHO, 2011). It has been proposed that due to the high variability of influenza viruses, both ECE and cell culture should be used together primary isolation of IAV (Clavijo et al., 2002).

1.6.2.4. Molecular diagnostics

1.6.2.4.1. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR assays (traditional and real-time technologies) provide highly analytically sensitive and specific detection of viral nucleic acid extracted from clinical sample preparations. Some of them are limited to only detecting the presence of IAV, these assays are suitable for initial screening of clinical samples (Spackman et al., 2002). Others, can provide information regarding the subtype and even the lineage, these multiplex RT-qPCR assays primer mixtures specifically designed to detect and identify different HA

and NA subtypes (Chang et al., 2008; Goecke et al., 2018; Henritzi et al., 2016, 2019). These subtyping assays are usually of slightly lower analytical sensitivity and less useful for primary screening (Van Reeth & Vincent, 2019).

This technique may be performed on lung tissue, nasal swabs and oral fluids for a qualitative result or for determining the subtype. A real-time RT-qPCR assay on nasal swab samples was determined to be highly specific at 100% with sensitivity ranging from 88% to 100% (Landolt et al., 2005). Molecular techniques include RT-qPCR and conventional PCR; targeted to the highly conserved matrix protein (Busquets et al., 2010; Fouchier et al., 2000) or the nucleoprotein for detecting infection with IAV (OIE, 2018).

One major obstacle for PCR techniques aimed at influenza diagnosis is that the virus is evolving continuously and, as a consequence, primers for detection and subtyping need to be continually validated and updated. Current testing strategies rely on targeting relatively well conserved nucleotide sequences for the primers. However, some level of mismatching is almost impossible to avoid and therefore, samples with relatively low vial loads may not be detectable by PCR (particularly by subtyping PCRs) and it may be necessary to attempt virus isolation prior to identifying the subtype (OIE, 2018).

1.6.2.5. Other techniques for IAV detection

1.6.2.5.1. Fluorescent Antibody Test (FA)

FA detects IAV antigens on lung tissue, and H1N1 and H3N2 subtypes can be detected (OIE, 2018). However, the test does have its limitations, as the antigens are only present in lung tissue for a short time following infection, also there may be variations among different samples from the same specimen due to the multifocal distribution of the virus, and the need to have fresh tissues with minimal autolytic changes (Vincent et al., 1997). This technique is highly dependent on the use of reference reagents representative of circulating viruses in the region and on skilled readers (OIE, 2018).

1.6.2.5.2. Immunohistochemistry (IHC)

IHC is an inexpensive, rapid and easy to perform test for detecting H1N1 and H3N2 IAV antigens on slides from formalin-fixed tissue or from nasal swabs. It has been shown to have sensitivity equivalent to virus isolation and greater than FA, as well as a direct

correlation between the presence of viral antigen and microscopic lesions in tissues, and is useful for performing retrospective studies when the original fresh tissue is no longer available (Vincent et al., 1997). A disadvantage is that antigens are only present in the lung tissue for a short time following infection, which limits the ability to detect the infection (WHO, 2011; OIE, 2018).

1.6.2.5.3. Antigen-capture enzyme-linked immunosorbent assays

Antigen-capture enzyme-linked immunosorbent assays are commercially available to detect influenza in nasal swabs and lung tissue. However, excess blood and mucus on nasal swabs or freezing lung tissue may reduce its sensitivity (WHO, 2011; OIE, 2018).

1.6.3. Serology

Serological diagnosis for influenza is mostly useful with a retrospective aim or to ascertain the negative status of a pig source. However, they are of limited usefulness for the diagnosis of acutely infected animals. As a matter of fact, infected animals resolve the infection when they develop a specific immune response. On the other hand, as explained above, in young animals, MDA may be present but cannot fully protect against the infection.

1.6.3.1. Haemagglutination inhibition Test (HI)

The specific attachment of antibody to the antigenic sites on the HA molecule interferes with the binding between the virus and the receptors on the red blood cells (RBCs). This effect inhibits haemagglutination and is the basis for the HI test (WHO, 2011). This test is conducted by adding serial dilutions of the submitted serum samples to a known concentration of virus. The titre is determined by the degree to which antibodies in the serum bind to the virus, preventing agglutination of erythrocytes (Pedersen, 2014). Paired serum samples collected 10–21 days apart are ideal. A fourfold titre increase between the acute and convalescent serum is considered diagnostically positive for that influenza type/subtype. A standard HI procedure was proposed by Hirst (1941), and after some modifications, HI has become the test of choice for WHO global influenza surveillance (WHO, 2011; OIE, 2018).

The HI test is extremely reliable, provided reference antisera are available to all subtypes. Serum HI antibodies are also considered the gold-standard correlate of protection from

inactivated IAV vaccines. A study by Skibbe et al. (2004), showed that the ELISA had 82.8–86.7% agreement with the HI for detecting swine influenza antibodies. Disadvantages of the HI test include the need to remove nonspecific inhibitors from the serum that could lead to false results, to standardise reference serum and virus antigens each time a test is performed, and the experienced interpretation of the test (Kitikoon et al., 2014; Leuwerke et al., 2008). Serum from some birds such as turkeys, chickens, and geese may cause nonspecific agglutination of chicken RBCs (Pedersen, 2014).

Amino acid changes located in and around the receptor binding pocket of the HA molecule sometimes result in a loss of sensitivity to certain RBCs. Chicken RBCs are frequently chosen for HI testing because inhibition patterns are clearer, and the cells are readily available. But, some strains of influenza during initial and early passage may not haemagglutinate chicken RBCs. Certain virus strains haemagglutinate turkey rather than chicken RBCs to greater or lesser degrees (OIE, 2018). Therefore, it may be necessary to choose the species based on the strains circulating in a given area (WHO, 2011; OIE, 2018).

1.6.3.2. Serum neutralisation assay (SN)

This assay is based on the capability of certain antibodies to block the infectivity of the virus. This assay has the advantage of detecting functional antibodies to a specific influenza virus showing protective immunity after vaccination or post-infection. Also, it is less cross-reactive between antigenically related viruses than HI and when used together, neutralisation tests provide additional information on the identity of the infecting virus. Finally, tests that use acute and convalescent serum samples may be used to indicate recent exposure (Gauger & Vincent, 2014). However, SN tests do not distinguish between natural exposure and vaccination status of the animals under evaluation (Gauger & Vincent, 2014). Besides this, the neutralisation assays with influenza viruses present a series of difficulties. The most important one is the fact that trypsin is needed for assuring viral infectivity. The protein present in serum may block trypsin and, as a result, the classical SN assays are not reliable (they are based on the evaluation of cytopathic effect or not when a mixture of a fixed amount of virus and variable dilutions of serum are inoculated onto susceptible cells). Alternative methods have been developed. These methods are mainly based in the use of relatively high amounts of trypsin-treated virus that are mixed with the dilutions of the serum, inoculated in the cell cultures and the reaction is revealed by different methods after approximately

one cycle of replication (that is well before the development of any cytopathic effect). The OIE proposes a method based on revealing the reaction by the addition of a peroxidase-labelled antibody and an ELISA substrate in a neutralisation-ELISA format (OIE, 2018).

1.6.3.3. Enzyme-linked immunosorbent assays (ELISA)

Two main types of ELISAs may be found in the market. The first group of assays detect antibody in front of a highly conserved antigen, such as the NP. These tests generally have good diagnostic sensitivity (Ciacci-Zanella et al., 2010) and are useful as a screening assay to determine herd status, but they do not differentiate between virus subtypes (Van Reeth & Vincent, 2019). The second group of ELISAs detect subtype-specific antibodies. These assays might offer lower diagnostic sensitivity than the HI test if the circulating strains are antigenically divergent from the viral antigen used in the commercial ELISA test (Barbé et al., 2009; Gauger et al., 2014; Leuwerke et al., 2008) but may have application in studies where status to a specific virus subtype/strain is required. Commercial assays offer the advantage of test standardisation and large sample analysis (WHO, 2011). In general, ELISAs are less specific, but more sensitive, than HI or SN, depending on the antibody isotype and immune compartment being sampled (Gauger et al., 2014).

1.6.3.4. Neuramindase assay (NA) and Neuraminidase-inhibition assay (NAI)

Inhibition of the action of NA with antibodies or specific inhibitors restricts the virus to a single round of replication showing that the NA has no role in attachment, fusion, replication, assembly or budding. Reference antisera are designed to distinguish between different NA subtypes but are broadly cross-reactive to detect as many different variants as possible within a certain NA subtype. There are some advantages to serological tests using NAI assay in that few sera contain nonspecific inhibitors to NA whereas many sera contain inhibitors to HA. A good policy is to use both HI and NAI assays (WHO, 2011).

1.6.4. Sequencing

In essence, sequencing is not a diagnostic method but at present, it is an essential tool for characterising influenza viruses. It provides very complete phylogenetic information regarding possible reassortants and emerging mutations across all genes to help

surveillance programmes study their evolutionary patterns, as well as information on intra-host diversity of the virus (McGinnis et al., 2016). Sanger sequencing from a conventional RT-PCR product has been the standard method for decades, but it had the disadvantage of using primers directed to the ends of the gene segments, and this process of finding a correct set required a trial and error approach (Quiñones-Mateu et al., 2014; Spackman, 2014). Moreover, errors derived from the RT-PCR could bias the final results, particularly when sequencing from cloned DNA (Marston et al., 2013).

Nowadays, sequencing technology has improved, and these problems can be avoided with Next Generation Sequencing (NGS). Deep sequencing is highly efficient, it can generate an enormous amount of information, it allows detection and characterisation of any pathogen in the sample, and factors such as cost and time have been reduced (Marston et al., 2013; Quiñones-Mateu et al., 2014). Disadvantages for NGS are that depending on the sequencing method, the instrument used, and the assembly software, sequencing errors may lead to misidentification, also, when analysing the data, sequence impurities caused by the presence of nucleic acid from other species should be corrected (McGinnis et al., 2016; Schirmer et al., 2015). Finally, the difficulty of obtaining high percentages of viral RNA when there are high levels of host RNA (Marston et al., 2013). For influenza, a previous step of amplification of the viral genetic material in the sample, prior to performing NGS, is necessary to overcome preferential sequencing (McGinnis et al., 2016). One of the crucial features for sequencing success is how the nucleic acid must be purified, whether it be RNA or DNA. As viral preparations are usually heavily contaminated by host nucleic acid, and as much of this should be removed to ensure that a good amount of sequence reads are of viral rather than host origin (Radford et al., 2012).

There are many options of NGS technologies, such as Roche 454, SOLiD, Illumina, Helicos, PacBio and Ion Torrent. Deciding which technology is best depends on the specific experiment being planned, factors to take into consideration should include the size of the genome, its complexity, and the depth of coverage and accuracy required (Radford et al., 2012). In the case of MiSeq platform Illumina, amplification occurs on by a process termed ‘bridge amplification’, then, successive rounds of PCR result in the generation of clusters of amplified molecules which serve as clones for subsequent sequencing using fluorescently labelled reversible terminators for the reactions (Radford et al., 2012).

1.7. Control and prevention

1.7.1. Husbandry and biosecurity practices

Biosecurity and vaccination practices are the cornerstone for control and prevention of IAV transmission. Measures such as placing new replacement animals in isolation before mixing them with the rest of the herd "gilt acclimatisation units", conduction of tests on them to confirm their health status, use of air filtration systems when possible, use of bird-proof nets to prevent contact between wild birds and pigs and good instruction of the farm personnel, among others are known practices in most farms (Serafini Poeta Silva et al., 2019; Torremorell et al., 2012).

Pitzer et al. (2016), used an epidemiological model to analyse critical herd size and the relation with IAV persistence and showed that the virus could persist in relatively small populations, highlighting the importance of a high population turnover and constant influx of new susceptible pigs, and its posterior implications for management of swine and for overall patterns of genetic diversity of IAV. White et al. (2017) proposed a stochastic model representing the infection dynamics of IAV in a farrow-to-wean production unit and found that the combination of frequent homologous mass vaccination, early weaning, gilt separation and their vaccination, as well as longer periods between gilt introductions, reduced the overall endemic prevalence. They also suggested the pivotal role piglets play in IAV persistence. Cador et al. (2017) conducted a stochastic metapopulation model to assess their relative efficacy regarding viral persistence. They implemented three vaccination schemes; batch to batch vaccination of breeding sows (pre-farrowing vaccination) to induce a high antibody level in colostrum and further transfer to piglets, batch to batch vaccination in growing pigs to reduce the infection pressure in growing pig facilities, and mass vaccination of the breeding sows in service to reduce infection pressure in breeding sow facilities. The export of consecutive piglet batches was identified as the most efficient measure for infection fade-out. This correlates with the role piglets take in IAV persistence. Batch-to-batch vaccination had a beneficial effect in breeding sows by reducing the persistence of IAV within this subpopulation, none of the vaccination strategies achieved IAV fade-out within the entire farrow-to-finish pig herd.

Control and possible eradication of infection requires the combination of adequate vaccination programs and husbandry practices aimed at revealing which factors, practices and procedures are important in piglets at weaning to prevent IAV spread. Biosecurity measures designed to prevent IAV from reaching piglets combined with strain-targeted (homologous) vaccines are the best option for producers to control IAV in their herds (White et al., 2017).

1.7.2. Influenza vaccines

1.7.2.1. Types of influenza vaccines for pigs

Current vaccines for swine influenza are mostly made of whole inactivated virus (WIV) produced in embryonated hen eggs or in cell culture. Inactivation is usually performed by chemical means using agents such as formaldehyde or binary ethylenimine. Influenza vaccines needs an adequate adjuvant to be immunogenic enough. All inactivated influenza vaccines for pigs are aimed to be used by intramuscular injection (OIE, 2018; Van Reeth & Ma, 2013).

Antibody responses in pigs after the administration of WIV vaccines are mainly directed towards HA, and to a lesser extent, against NA, M, and NP (Holzer et al., 2019; Van Reeth & Ma, 2013). WIV vaccines induce high titres of neutralising antibodies that target the immunodominant HA head domain, thereby inhibiting virus entry into host cells (Heinen et al., 2001). In a study conducted by Van Reeth et al. (2006), they observed that when animals had been previously exposed, this previous encounter would prime their future antibody response after one single dose of inactivated vaccine; if then these were infected by the same strain they had been exposed to, then titres ≥160 could be found, and if infection was by a different strain, some serologic cross-reaction would be detected as well, although much lower (10–20). This is of importance in the field, as knowledge of the vaccination status of the herd will help to interpret HI results, especially in the case of H1N2, where they observed that despite not ever being previously infected by it, animals could have antibody titres ranging from 10 to 360, if they had been vaccinated against H1N1.

Inducing high titres in sows is highly important for the colostrum and the level of protection their offspring can acquire and therefore, the viral circulation levels in the farm.

Unfortunately, there is not enough information in regards to the role of CD4+ and CD8+ T cells against influenza infection in pigs (Holzer et al., 2019).

Live-attenuated influenza virus (LAIV) vaccines are administered by the intranasal route, and the virus replicates in the upper respiratory tract where induces a balanced mucosal and systemic immune response. A single dose of LAIV administered to MDA-positive pigs has shown to provide partial protection, making it safer for young pigs under field conditions, where sows are routinely vaccinated and diverse IAV strains are in circulation (*reviewed in* Sandbulte et al., 2015; Vincent et al., 2012). They have also proven to reduce shedding of viable virus for vaccinated newborn piglets with or without MDA (Genzow et al., 2018). One concern about live-virus vaccines would be possible reassortment between field strains and the vaccine virus, producing new reassortant viruses (Thacker & Janke, 2008). Indeed, a reassortment between live attenuated strain and a field strain has been recently described in USA (Sharma et al., 2020).

Vectored vaccines using baculovirus, alphavirus, or adenovirus also are have shown promising results after challenged with viruses with matched and mismatched HA (Abente et al., 2018; Hernandez et al., 2016; Wesley et al., 2004), although they are under investigation and not currently in the market. DNA vaccines also have been studied, the theoretical advantage of these vaccines is the production of viral protein with normal conformation, without the risks associated with the use of live virus. In one study they have shown to reduce viral shedding and to elicit a strong immune response (Sisteré-Oró et al., 2019), although they have been associated with significantly severe clinical signs and even death when challenged (Heinen et al., 2002). Universal influenza virus vaccines that target conserved regions, such as the HA stalk domain, the ectodomain of the M2 ion channel or the internal matrix and NP are in development (Nachbagauer & Krammer, 2017).

1.7.2.2. Vaccine efficacy

Vaccine efficacy (VE) can be described as the reduction in individual susceptibility as a consequence of protection derived from a vaccine, directed towards the infectiousness of the infected individuals (Farrington, 2003). Since the theoretical efficacy of a vaccine can be considerably decreased due to several factors, it is critical to identify them prior the establishment of any vaccination protocol in farms (Rose & Andraud, 2017). The

population impact of a vaccination programme depends on three main factors: First, on the epidemiology of the infection, more specifically on its transmission potential "R0" (Diekmann et al., 1990; Dietz, 1993). Second, on the impact of the vaccine on the ability of individuals to contribute to the transmission of the infection "vaccine efficacy". Third, on the vaccine coverage in the population (Farrington, 2003).

VE can be critically affected by mismatches between circulating and vaccine strains, the variation from season to season and the mutations in HA antigenic sites (Belongia et al., 2009; Chambers et al., 2016). As well as, mutations related to egg adaptation (Skowronski et al., 2014). Chen et al. (2019) concluded that the adaptative distance and VE could be strongly affected by passage adaptation in embryonated eggs and a contributor to low VE. As mentioned before, anti-HA antibodies neutralise virus infectivity, and amino acid substitutions in the HA change their structure and create an antigenic variation. For this reason, circulating strains have to be monitored to ensure that commercial vaccines are as closely as possible related to the circulating viruses.

In Europe, since the introduction of the pandemic virus, old and newly established IAV lineages, along with the numerous reassortants and genotypes that have risen from this, can be found circulating in different countries (Simon et al., 2014; Watson et al., 2015). In North America, there is a diversification specially observed in the H1's (Lorusso et al., 2011; 2013; Van Reeth & Ma, 2013), including the reassortment events occurring between them, the appearance of these different clades (α, β, γ or δ) make the efficacy prediction difficult. Mismatches between circulating strains and commercial vaccines represent an important factor when assessing VE in a herd (Van Reeth & Ma, 2013). For this purpose, influenza surveillance in pigs is essential for knowing the majoritarian strains that are present and evaluating the current available vaccines in the market.

1.7.2.3. Influenza vaccines in Europe and North America

In Europe, commercial influenza vaccines for pigs are based on WIV and they work reducing clinical disease against homologous and antigenically related viruses, by inducing an antibody response against the surface glycoprotein proteins (Van Reeth & Ma, 2013). They can either have H1N1 and H3N2 or the three pig subtypes (H1N1, H3N2 and H1N2) (Table 3).

Table 3. Summary of the licensed IAV vaccines available in Europe in 2020.

Product name	Manufacturer	IAV strains included	Adjuvant
Respiporc FLU	IDT Biologika GmbH	Sw/Belgium/230/92 (H1N1) Sw/Belgium/220/92 (H3N2)	Aluminum Hydroxide-Oil
Respiporc FLU3	IDT Biologika GmbH	Sw/Haselunne/ 2617/03 (H1N1) Sw/Bakum/1769/03 (H3N2) Sw/Bakum/1832/00 (H1N2)	Carbomer
Respiporc FLUpan H1N1	IDT Biologika GmbH	A/Jena/VI5258/2009(H1N1)pdm09	Carbomer
Hipra	Gripork	A(H1N1)OLL A(H3N2)G	*Liquid Paraffin*-Aluminum Hydroxide

In a study performed by Kyriakis et al. (2010), the efficacy of four commercial vaccines containing different H1N1 strains was compared against the H1N1 field isolate Sw/Ghent/112/07. They found that the vaccine that contained the most recent H1N1 strain with the closest genetic homology to the challenge virus failed to protect against the challenge strain, while two vaccines including older, antigenically more distant strains produced significant levels of protection. The reason for those differences could not be established but it was proposed that they could be related to the different amounts of viral antigens in the different vaccines.

In the USA, both WIV and LAIV are marketed. Most vaccines licensed in the U.S. contain WIV of the H1 and H3 subtypes (Vincent et al., 2008). In Europe this type of autogenous vaccines for influenza are not authorised. These can be given to newborn piglets, as early as 1 day of age, and have proven protection and reduction of shedding of viable virus for at least 12 weeks, critical time for the piglet (Kaiser et al., 2019). Very recently, a reassortment incident has been reported after the use of LAIV (Sharma et al., 2020). When comparing the protection conferred by WIV and LAIV against antigenically distinct H3N2, LAIV offered a complete protection and no signs of virus replication, unlike as it was with the WIV (Abente et al., 2018). Nevertheless, in situations where commercial vaccines are ineffective or unavailable, farmers are authorised to use autogenous

vaccines, which are custom-made according to the herd (*reviewed in* Sandbulte et al., 2015)

Table 4. Summary of the licensed IAV vaccines available in the U.S. as of March 2020.

Product name	Manufacturer	IAV strains/clusters included	Adjuvant
FluSure XP® Whole/inactivated	Zoetis	Gamma H1N1 Delta-1 H1N2 Cluster IV-A H3N2 Cluster IV-B H3N2	Amphigen®
FluSure® Pandemic Whole/inactivated	Zoetis	A/California/04/2009 (H1N1)	Amphigen®
Swine Influenza Vaccine, RNA Whole/inactivated	Harrisvaccines	Cluster IV H3N2	None
Ingelvac Provenza™ Live attenuated influenza vaccine	Boehringer Ingelheim	H1N1 H3N2	None

1.7.2.4. Vaccination strategies and limitations of vaccination

Before implementing any vaccination plan it should be determined the overall goals for the farm: is it targeted for piglet protection to reduce virus propagation at that age? Is it to limit pathogen circulation in the sows that are acting as permanent reservoirs and sources of reinfection? Or is it aim towards a global eradication at the herd level? (Rose & Andraud, 2017).

Vaccination is commonly performed in sows with two main objectives: to limit abortions in gestating sows and to promote MDA (Cador et al., 2017). Influenza-associated abortion in sows is usually related to high fever (Littauer et al., 2017; Yoon & Janke, 2002), although, the virus has not been consistently proven to reach the uterus or to infect foetuses. Thus, with regards to this aspect, vaccination would be efficacious as far as to prevent fever. In a recent study, it was shown that vaccination against the H1pdmN1pdm virus in an endemic area resulted in the reduction of the abortion rate in farms were vaccination was implemented in sows (Gumbert et al., 2020). In a simulation study conducted by White et al. (2017), they concluded that homologous mass vaccination

paired with biosecurity measures, such as early weaning, were the most efficacious interventions.

Regarding the vaccination of sows with the aim to increase the MDA transfer to piglets, several trials have been published. Corzo et al. (2012) reported the effects of mass vaccination in a breeding farm using a killed commercial vaccine. Viral shedding was monitored in sows and piglets. The follow-up of the farm showed a gradual decrease in the proportion positive animals until the virus was no longer detected. This correlated with an increase of HI titres that were determined against five different strains. The reduction in incidence was clear three weeks after the second dose when the virus was only found in the 21 day-old piglets. However, at six weeks of age, pigs became infected and this was attributed to the decline in MDA and horizontal transmission due to the farm's dynamics. It has been seen that another important point is to reduce the number of infected piglets at weaning, and this can be achieved through sow vaccination. Presence of MDA with HI titres of ≥40 at weaning has been associated with a prevalence decrease in nursery, and a delay and or shortening of the infectious period. Suggesting that high levels of strain-specific MDA can help reduce IAV circulation in piglets (Chamba Pardo et al., 2019).

Sow vaccination can provide some protection to the offspring but shows some limitations. The first is the need of a close antigenic match between the vaccine strain and the circulating strain to be effective to stop transmission (Sandbulte et al., 2015). Moreover, the levels of MDA in the piglet population depend importantly on the management of the colostrum intake in the farm. Sow vaccination strategies (mass and pre-farrow vaccination) are widely applied for ensuring uptake of MDAs in the piglets (Rajão et al., 2014). However, the protection obtained through MDAs can be sub-optimal and therefore different approaches as vaccination of piglets can be needed. For example, Gillespie (1999), studied a farm that presented atypical signs of acute IAV infection starting at 5-7 days post-weaning with a later worsening of the animals and a 3.5% mortality rate due to a secondary complication with *S. suis* causing meningitis. Using a commercial vaccine, he performed vaccination of sows at 5 and 2 weeks prior to the farrowing date, and for the case of piglets that were born to unvaccinated sows, they received a first dose at 5-7 days old, and a second at weaning (approximately at 18 days old). He obtained good results for both vaccination schemes, shown by the average daily gain and the feed:gain

ratio, especially for the case of the unvaccinated piglets born to vaccinated sows when they entered the nurseries and showed limited clinical signs. In a recent study conducted in Denmark (Ryt-Hansen et al., 2019a), piglets were vaccinated at the time of castration using a quarter of a dose from an inactivated commercial vaccine and contrasted the results in front of a control group that received sterile saline. They did not find an overall difference between both groups, and IAV prevalence was high in both scenarios. Possible explanations for this were the early age of infection of these piglets, which did not allow for a vaccine response to be generated, plus the low doses that they received. Other reasons were an inhibitory effect of MDA, as well as a mismatch between the field and the vaccine strain, also, the circulating strain had five pandemic internal genes and the vaccine used does not include any pandemic strain.

Colostrum antibodies can interfere with active humoral and cellular immune responses to vaccine and may lead to vaccine-associated enhanced respiratory disease (VAERD) when pigs are vaccinated with WIV in the presence of MDA and then exposed to heterologous virus. In these cases, the vaccine has proved to potentiate clinical signs, inflammation and pneumonia following challenge with antigenically divergent heterologous IAV of the same HA subtype (Gauger et al., 2011; 2012; Kitikoon et al., 2006). Nevertheless, it was later shown that it does not negatively interfere with the induction of an immune response and elevated local adaptive cytokine levels despite the severe clinical disease and underlying lung pathology (Gauger et al., 2013).

Chapter 2. Hypothesis and objectives

Information regarding the diversity of circulating strains in Spanish pig farms has not been updated in the last seven years. For this reason, it is important to have up-to-date knowledge of how this diversity is changing, not only in terms of lineages, but also in genotypes, and to assess where this evolution is leading us.

Furthermore, farms considered to be endemically infected can pose a real challenge when it comes to prevention of IAV infection. As vaccination is the only strategy that could prevent infection, it would be of interest to study the impact for this particular age group on viral transmission by the implementation of a vaccination programme in the sows, using commercial vaccines.

Hypothesis:

1) The diversity of lineages and genotypes currently circulating in Spanish farms is expected to be different than what it has been previously reported in the last ten years.
2) The implementation of a pre-farrowing vaccination scheme in the sows will modify the virus transmission dynamics in the offspring, making it more efficient when compared to the offspring from the ones that only received blanket vaccination and from unvaccinated sows.

Objectives:

1) To determine the diversity of circulating strains of IAV in pigs with overt respiratory disease and in pigs from sub-clinically infected farms in Spain and Portugal, in all three levels of depth: subtype, lineage and genotype.

2) To determine the transmission dynamics in the offspring of sows that were only blanket vaccinated and sows that received an extra dose pre-farrowing.

Part II: Studies

Chapter 3.
Study 1

Title: Diversity of influenza A viruses retrieved from respiratory disease outbreaks and subclinically infected herds in Spain (2017-2019)

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Summary

The present study was aimed to assess the diversity of influenza A viruses (IAV) circulating in pig farms in the Iberian Peninsula. The study included two different situations: farms suffering respiratory disease outbreaks compatible with IAV (n= 211) and randomly selected farms without overt respiratory disease (n=19). Initially, presence of IAV and lineage determination were assessed by RT-qPCR using nasal swabs. IAV was confirmed in 145 outbreaks (68.7%), mostly in nurseries (53/145; 36.5%). Subtyping by RT-qPCR was possible in 94 of those cases being H1avN2hu (33.6%), H1avN1av (24.3%) and H1huN2hu (18.7%) the most common lineages. H3huN2hu and H1pdmN1pdm represented 7.5 and 6.5% of the cases, respectively. As for the randomly selected farms, 15/19 (78.9%) were positive for IAV. Again, the virus was mostly found in nurseries and H1avN2hu was the predominant lineage. Virus isolation in MDCK cells was attempted from positive cases. Sixty of the isolates were fully sequenced with Illumina MiSeq®. Within those 60 isolates, the most frequent genotypes had internal genes of avian origin, and these were D (19/60; 31.7%) and A (11/60; 18.3%), H1avN2hu and H1avN1av, respectively. In addition, seven previously unreported genotypes were identified. In two samples more than one H or N were found and it was not possible to precisely establish their genotypes. A great diversity was observed in the phylogenetic analysis. Notably four H3 sequences clustered with human isolates from 2004-05 (Malaysia and Denmark) that were considered uncommon in pigs. Overall, this study indicates that IAV is a very common agent in respiratory disease outbreaks in Spanish pig farms. The genetic diversity of this virus is continuously expanding with clear changes in the predominant subtypes and lineages in relatively short periods of time. The current genotyping scheme has to be enlarged to include the new genotypes that could be found in the future.

Keywords: Epidemiology, influenza A, lineages, pig, sequencing.

1. Introduction

Influenza A virus (IAV) is one of the most important respiratory pathogens in pigs being a key agent in the porcine respiratory disease complex (PRDC) of weaners and growers. For many years, IAV infection in pigs, was mainly associated with epidemic outbreaks of acute respiratory disease affecting most animals in the farm. However, in the last years, increasing evidence indicate that most farms in Europe are endemically infected by one or more IAV viruses (Kyriakis et al., 2011, 2013; Simon-Grifé et al., 2012; Van Reeth et al., 2008) and that reassortment events are not uncommon (Martín-Valls et al., 2014). In those farms, the main circulation of the virus takes place in the farrowing units and nurseries (Ferreira et al., 2017; Ryt-Hansen et al., 2019), with occasional generalised outbreaks when a new IAV strain enters the farm for the first time.

The IAV genome consists of eight segments, which encode for up to 14 proteins, among them five structural ones: haemagglutinin (HA), neuraminidase (NA), two matrix proteins (M1 and M2) and the nucleoprotein (NP). The combination of haemagglutinin (HA) and neuraminidase (NA) determines the subtype. At present, 18 types of HA and 11 types of NA are known in mammals and birds, but only three subtypes predominate in pigs: H1N1, H1N2 and H3N2. However, the genetic and antigenic diversity within those subtypes is considerable. In Europe this diversity is particularly high for swine H1N1 and H1N2 viruses. Furthermore, these viruses have evolved into distinct genetic lineages in different geographic locations. Since the 2009 pandemic, four lineages are predominantly found in pig farms: Eurasian avian-like H1N1 (H1avN1av), human-like H1N2 (H1huN2hu), human-like H3huN2hu, and the pandemic lineage H1N1 (H1pdmN1pdm).

Whilst the haemagglutination inhibition test (HI) may distinguish isolates of different phylogenetic origin within a given subtype (Van Reeth et al., 2006), cross reactivity amongst strains belonging to subtypes and lineages different to H1 have been reported for North American H1 and classical swine H1(Kyriakis et al., 2010). Also, some degree of cross-protection may exist between H1pdmN1pdm and H1avN1av (Busquets et al., 2010; Kyriakis et al., 2010). Altogether, this indicates the need for additional classification schemes.

Watson et al. (2015) proposed a genotyping system based on the origin of their two external (HA and NA) and six internal gene segments (NP, M, the three polymerase genes and the non-structural protein, also known as internal gene cassette) to classify the swine IAV lineages detected in Europe. At the time of that publication, they had classified 23 genotypes, but only four of them presented a pan-European distribution. In that study, most isolates contained an internal gene cassette derived from the Eurasian avian-like lineage (67%) or the H1pdmN1pdm lineage (27%).

IAV diversity in pigs has been related to the intensification of the production system (Van Reeth et al., 2008). In this sense, it has been suggested that factors such as the continuous introduction of animals from different sources can contribute to the introduction of new viruses into a farm and the subsequent reassortment amongst strains. These reassortment events seem to be very common in Europe (Beato et al., 2016; Howard et al., 2011; Lange et al., 2013; Moreno et al., 2009, 2011; Simon-Grifé et al., 2011).

At present, Spain is an ideal scenario for the examination of such IAV diversity in the European context. As it is one of the few countries in Europe where pig population increased in the last years. In 2019, with 2.2 million sows, a total census of 29 million pigs and imports of about 1 million animals (Ministerio de Agricultura, Pesca y Alimentación, 2019), Spain - together with Germany - are the leading pork producing countries in Europe and among the top 5 in the world (Eurostat, 2019).

Epidemiological information paired with sequencing and detailed phylogenetic analysis are tools that can help understand the circulation routes and mutation patterns of these viruses. The aim of the present study was to gain a better insight into the diversity of IAV in Spanish pig farms with outbreaks of respiratory disease or farms with sub-clinical infections.

2. Materials and Methods

2.1.Data collection

The study took place between February 2017 and March 2019 and included two different situations: farms suffering respiratory disease and samples from subclinical farms that did not report respiratory disease cases.

For the farms suffering respiratory disease, a case was defined as a situation where respiratory disease was noticeable in at least one age group of pigs with predominant signs of cough and fever. Shipping of suspected influenza cases (nasal swabs) was requested from field veterinarians in Spain and Portugal. In order to accept a submission, a data sheet indicating clinical signs of the animals had to be fulfilled. Sampling had to include at least 10 nasal swabs from feverish animals. Samples were collected in a virus transport medium (Virocult®, Sigma-Aldrich), kept at 4ºC during transport and immediately processed upon arrival. Occasionally, if dead animals were found, lungs were also sent for analysis.

To assess the circulation of IAV in subclinical farms (no obvious respiratory disease), sampling was performed in 19 farms in Catalonia. The selection of these farms was done randomly from a group of different pig producing enterprises representing 20% of the Catalan pig industry. The included farms fulfilled two criteria; 1) farms that were not suffering from respiratory outbreaks in any production phase and 2) farms that were farrow-to-finish. In those farms, nasal swabs were taken randomly from suckling pigs, weaners and fatteners (n=20 each). Samples were collected using the aforementioned virus transport medium and under the mentioned storage conditions. That sampling strategy would allow us to detect viral circulation in at least one positive farm assuming a 15% herd prevalence of subclinical herds and considering a 95% confidence interval. Similarly, the sampling method allowed us to detect at least one positive assuming a prevalence of 15% of IAV in each sampled age (95% confidence interval).

2.2.Processing of samples

Upon arrival to the laboratory, the Virocoult® transport media containing the nasal swabs were vortexed and the medium was transferred to a sterile 1.5 ml tube. Then, tubes were centrifuged at 4,000 g for 5 min and the supernatant was recovered for further processing.

Viral RNA was extracted from 150 µl of the supernatant using a commercial kit (NucleoSpin® RNA virus, Macherey-Nagel) according to the manufacturer's instructions. Initial detection of the virus was done by means of a RT-qPCR targeted to the IAV matrix gene (Busquets et al., 2010) using the AgPath-ID™ One-Step RT-PCR reagents (ThermoFisher). Positive samples underwent a second RT-qPCR for identifying subtypes and European lineages of H and N, including: H1av, H1hu, H1pdm, H3hu, N1av, N1pdm and N2hu (Henritzi et al., 2016, 2019).

2.3. Virus isolation

Madin-Darby canine kidney (MDCK) cells (ATCC CRL-2936™) were used for the isolation of IAV from RT-qPCR-positive (M gene) nasal swabs. Viral isolation was attempted from up to three RT-qPCR positive samples per case (from both respiratory outbreaks and subclinical sampled farms), to obtain a high-quality isolate for further sequencing. For the isolation process, 100 µl of the sample supernatant was mixed with 900 µl of Minimum Essential Medium Eagle (MEM) supplemented with L-glutamine (200 mM), penicillin-streptomycin (10,000 units and 10 mg/mL, respectively) and trypsin TPCK-treated for a final concentration of 10 µg/ml of trypsin. Trypsin-treated samples were inoculated in 25cm^2 cell culture flasks. After 1.5 h of incubation at 37ºC, the flasks were filled with 9 ml of medium without trypsin (final concentration of trypsin in the culture 1µg/ml) and cultures were incubated for up to 5 days.

Cells were observed every 24 hours to check for the development of cytopathic effect (CPE). Once CPE was observed flasks were frozen at -80ºC, thawed and, after centrifugation, the cell culture supernatant was examined by RT-qPCR (M gene) to confirm the replication of the virus (lower Cq values compared to the initial inoculum). Samples were discarded when CPE was not observed after a third blind passage. Up to three samples per case were attempted for viral isolation.

2.4. Next Generation Sequencing

In order to have a more precise picture of the IAV circulating in the studied population, the genome of 60 representative isolates were fully sequenced using next generation sequencing technology (enough to detect at least one genotype with an occurrence of ≥5% in the cases, 95% confidence). These cases were selected from the MDCK isolated viruses, this included one isolate per farm as maximum. The total RNA extraction from

the 60 selected viruses isolated in MDCK cells was sequenced using an Illumina® MiSeq platform at *Parc de Genòmica i Recerca-UAB*. The output reads in fastaq format (doubled paired) were checked for quality using Trimmomatic (matching of forward and reverse sequences and quality index>20). Next, high quality reads were filtered using IAV sequence references belonging to all known lineages for each of the eight genome segments (Supplementary material l). Genome consensuses were generated using the Simple Consensus maker tool (available at: https://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html) when a viral quasi-species was obtained. The segments were then manually aligned and trimmed using BioEdit sequence alignment editor for windows (Hall, 1999). When filtering did not yield a viral quasi-species, consensus sequence for that gene was forced using QuASAR software (available at: https://github.com/piquelab/QuASAR). The resulting consensus sequence was blasted against available sequences in GenBank and the closest sequence was used to re-filter the original fastaq file.

2.5. Phylogenetic analysis and genotyping

Phylogenetic analysis was performed using the MEGA X software (available for download at: https://www.megasoftware.net/). An initial database was built using all sequences available in GenBank of Euroasian swine IAV for each genome segment. At first, an initial removal of identical sequences (100% of identity in identity matrix) was done. A preliminary phylogenetic analysis (Neighbour-Joining) allowed the removal of redundant sequences within a given cluster. For each cluster, this corresponded to highly similar isolates (>97% identity). Finally, in order to improve the resolution of each tree, a BLAST was done using each of the sequences obtained in the present paper, and the output was used for completing the database for each gene with sequences not initially included. Final databases for each gene contained about 150-250 sequences representing the significant clusters in the preliminary analysis. Relevant non-European strain sequences were included when needed. Supplementary file 2 shows the GenBank accession numbers of the included sequences. Sequences obtained in the present study were added to the corresponding databases for each gene. Final phylogenetic trees were built using the maximum likelihood method (1,000 iterations) using the general time reversible model (GTR) and subtree pruning and regrafting moves (SPR) option. Once the subtype, lineage and phylogenetic clustering for each isolate and gene was identified, the genotyping was done according to the classification of Watson et al. (2015). Briefly,

for internal genes (PB2, PB1, PA, NP, M and NS), genes were classified as “avian-like” or “pandemic”. For haemagglutinins, there was a differentiation between the three H1s; avian-like, human-like and pandemic. In literature there is only one type of H3. For neuraminidases, N1 differentiation was also done depending if they clustered with avian-like isolates and pandemic isolates, and for N2 only the human like was considered. The combination of the 8 segments defined the genotype, previously reported genotypes have been named according to Watson et al., (2015) proposal, following the alphabetical order. Previously unreported genotypes were assigned as UG - standing for “unreported genotype” - followed by a number.

3. Results

3.1. Cases of respiratory disease

Table 1 summarises the results for the submitted cases. During the study period, 211 submissions fulfilled the requirements and were considered respiratory disease cases. Cases were distributed in all regions of Spain with significant pig production, plus four cases submitted from Portugal. Most of the submissions corresponded to nursery pigs (39.8%), suckling piglets and fatteners had a considerable number of submissions (17.5% and 13.3%, respectively). While only 7.1% were sow samples, accounting this latter group for the lowest percentage of positive submissions (p=0.02). Unfortunately, in 22.3% of submissions, the age information could not be provided to us by different reasons. Of the 211 submissions, in 145 (68.7%, $CI_{95\%}$:60.5-73.8%), IAV positive animals (M gene RT-qPCR) were present. Supplementary file 3 shows the geographic distribution of the positive cases for which the precise location of the farm was recorded. The Cq values in IAV positive cases versus the proportion of infected animals was also examined (Figure 1). The results showed that the higher the proportion of infected animals, the lower the average Cq in positive animals (r=-0.33, $CI_{95\%}$ = -0.49 to -0.15; $p<0.0001$).

a) Assessment of apparently subclinical farms

Apparently healthy animals of 17 farrow-to-finish farms and 2 farms without fattening unit were examined. Of these, in 15 farms (78.9%) animals tested positive for IAV by RT-PCR for the M gene (Table 2). The distribution of positive animals did not show

differences between suckling piglets and weaners, as 12 farms were detected as positive in each of these two phases. In 7 farms, both phases were positive at the same time of sampling. There was one farm that tested positive for the 3 production phases, being the only one having positive results not only for suckling piglets and weaners, but also for the fattening unit.

b) Lineages detected

Among the 145 cases with positive samples for the M gene, 94 (85.4%; $CI_{95\%}$:78.1-90.6%) could be subtyped using the subtyping RT-qPCR (Table 3). All typable samples yielded Cq values <30 for the M gene. Isolation of the virus was successful in 11/51 cases, in which RT-qPCR typing was not possible (21.5%; $CI_{95\%}$:11.8-35.7%). Those 11 samples were sequenced by NGS and belonged to different subtypes and lineages (H1avN1av, H1avN2hu, H3huN1av and H3huN2hu). For the 40 remaining cases, the complete lineage could not be obtained by RT-qPCR and attempts of isolation in cell culture were unsuccessful. No geographical pattern in the distribution of the lineages was found, except for the H3huN1av isolates that were detected in two farms of the same company in the same province.

Regarding the assessment of apparently subclinical farms, the lineage of the IAV virus circulating in the farm could be determined by RT-qPCR in 12 cases while in three farms only the H or N lineage could be determined. The lineages detected were as follows: H1avN2hu, H1huN2hu, H1avN1av, H1huN1av and for the three partially subtyped, these were HxN1av, HxN2hu and H1avNx. Out of these last three, one could be fully sequenced after isolation and corresponded to H1avN2hu.

c) Genotyping and phylogenetic analysis

Full genome sequencing allowed the determination of the genotype for 60 isolates (Figure 2). The most common were genotypes D (31.7%) and A (18.3%). In the present study, 7 previously unreported genotypes (UG) were identified and designated as $UGx_{(1-7)}$. For two additional samples, two different H or N were detected in the sample (by PCR subtyping and NGS sequencing), while all other genes were consistent with a single origin. Since it was impossible to establish the genotype of the virus or viruses present, they were designated as undetermined genotype (UD_X). For simplification purposes, each segment

has been represented following the colour patterns used by Watson et al. (2015). In the cases where different lineages were found new colours have been proposed.
A great diversity was observed in the phylogenetic analysis. Regarding H (Figure 3) and N segments/genes (Figure 4), all isolates belonged to the expected lineages. However, it is worth noting that four H3 pig sequences clustered with human isolates from 2004-05 (Malaysia and Denmark) that were considered uncommon in pigs. This finding corresponds to isolates dating from the last two years and could possibly mean the disappearance of the classic H3 and its replacement by this new type.

Interestingly, for some genes, particularly M (Figure 5), local (Spanish) clustering was observed. Thus, of the 60 sequences obtained in the present study, 37 grouped in two clusters mainly composed of Spanish sequences. Similarly, for the NS gene, 39 Spanish sequences from the present study clustered together in two groups (Supplementary file 8). For PB1 and PB2, a local cluster within the pandemic group was observed; this clustering was not maintained for PA (Supplementary files 4, 5 and 6). Regarding the NP segment, two main clusters can be observed (Supplementary file 7).

4. Discussion

Influenza A virus is one of the main components of PRDC (Brockmeier, Halbur & Thacker, 2002). In the present study, about two thirds of the cases submitted because of a respiratory disease episode in the herd were positive for IAV by RT-qPCR. Although some selection bias may exist, this result indicates that IAV is a very common agent in those outbreaks in Spanish farms. Nevertheless, it is important to distinguish between a positive IAV result by RT-qPCR and attributing to IAV the role of primary agent in the outbreak. In the present study, we did not check for other respiratory pathogens that could have contributed to the clinical features observed in the farm. However, when we performed a random sampling in apparently subclinical farms, IAV was also commonly found in the nasal swabs examined. A deeper analysis of the data showed that while in subclinically infected herds the proportion of IAV positive samples rarely exceeded 50%, in most of the outbreaks the percentage of positive samples was above this value. Moreover, the average Cq values and the proportion of infected animals were significantly related. In addition, in subclinically infected animals, Cqs ≥30 were more frequent than in the outbreaks (not shown). Furthermore, in the outbreaks, sample size

was smaller as it was directed to animals showing clinical signs, on the contrary, for the random sampling, a larger sample size was needed and it proved to have a higher detection rate. Taken together, these facts suggest that the diagnosis of IAV as a primary agent of a respiratory outbreak requires a large targeted sampling of animals with compatible signs. Our results indicate that an influenza outbreak can be presumed when >50% of the samples are positive and average Cqs are below 30, as seen by the distribution of positive samples per case and their respective Cq (Figure 1). Nonetheless, there are other studies were, under subclinical circumstances, the percentage of IAV RT-PCR positive animals were over 70% (Ferreira et al., 2017; Simon-Grifé et al., 2012). Considering this, a better sampling approach should include an equivalent number of animals not showing clinical signs as control in order to confirm the diagnosis and properly evaluate the relative risks.

On the other hand, most positive animals were found in nurseries. This fact correlates with other published studies showing similar results (Ferreira et al., 2017; Simon-Grifé et al., 2012). This pattern of respiratory outbreaks is compatible with the presence of IAV endemic circulation, where in most cases the circulation of the virus and the increase of clinical outcome can be observed in the nurseries probably due to the declining of maternally derived antibodies (Gillespie, 1999; Rose et al., 2013; Torremorell et al., 2009).

IAV-positive cases were spread all over the territory. We were unable to detect any particular distribution of cases or lineages, except for the two H3huN1av isolates that were found in two adjacent farms in the same province. Although sampling was based on voluntary submissions and, therefore, the number and location of detected positive cases does not necessarily correlate with the real proportion and distribution of subtypes and lineages, it is clear that IAV is present in most farms.

The distribution of subtypes and lineages observed agrees with previous reports from Europe in the last years (Kyriakis et al., 2011; Simon et al., 2014; Van Reeth et al., 2008). In our case, just three lineages, H1avN2hu, H1avN1av and H1huN2hu, represented 75% of the cases. It is interesting to note that, as in other European countries H3N2 is becoming less common in the herds (Harder et al., 2013; Watson et al., 2015). In contrast, in a

serological study conducted in 2008-2009 in Spain, more than 90% of the examined pigs were seropositive to H3N2 (Simon-Grifé et al., 2011).

Watson et al. (2015) proposed a genotype classification for swine IAV. The classification was based on the composition and origin of the different viral genes. In the original description, 23 genotypes were reported, with an additional 4% of the strains being undetermined. Afterwards, other reports added new genotypes (Beato et al., 2016). In the present study we found 7 additional genotypes, and in our opinion, more genotypes are very likely to be found in the future. Accordingly, it is necessary to establish a widened scheme for the designation of the new variants that will most likely appear. It is worth noting that we found four isolates harbouring an infrequent H3 "human-like". This H3 was previously reported by Krog et al. (2017) in Denmark where a H3N2 virus with an internal pandemic cassette was detected. In our case, the H3 human-like isolates were harbouring an avian internal cassette either with the N2 like in Denmark or with an N1av. This is an indication of reassortment and spreading out of Denmark.

Moreover, when the phylogenetic analysis of the different viral genes was performed, it was evident that either strains of different genotypes could cluster together, or that strains from the same genotype may be included in different clusters. This suggested that the complexity of the generation of genetic diversity in swine IAV cannot be covered by just the use of subtype, lineage and genotype classifications.

The phylogenetic analysis indicated the existence of some local clusters, being this particularly noticeable for the M and NS genes. Although the development of local variants is expected, the fact that strains from very diverse subtypes, lineages and genotypes shared a variant of the M gene locally developed, suggests some degree of biological fitness or advantage for this variant. Moreover, some genotypes conserved their clusters in some of the genes, while other genotypes contained strains grouped into different clusters, disrupting any possible correlation between the genotype that can be assigned to a specific strain and its genetic or phylogenetic implications. Also, this result indicates a high level of intra and inter-genotype reassortment events.

From a methodological point of view, the present study also produced some interesting results. For example, subtyping by RT-qPCR worked out for most samples with low Cq

(<30) in the M gene PCR, but failed for higher Cqs. Interestingly, most of the samples yielding a Cq >30 could not be isolated neither. Primer mismatching could reduce the efficiency of the PCR and this could be the explanation for some of the samples that were not subtyped. However, 11 samples with very low Cq (17-20) could not be subtyped but were successfully isolated in cell culture. The design of new primers could have helped to mildly increase the efficiency of PCR subtyping in the present study. However, given the degree of diversity observed, it can be foreseen in the future, thata continuous update of primers will be necessary, including the problems associated with multiplexing at a large scale. Isolation and sequencing by NGS could be a suitable alternative, particularly, considering the decreasing costs of NGS technologies.

In this study we took the approach of isolating the virus and then deep sequenced it by using Illumina MiSeq. The reason for including the isolation step was the impossibility of performing direct sequencing on samples with Cqs lower than 24. By using this approach, maybe we were underestimating some of the diversity of Spanish IAV. This problem could be solved by PCR amplification instead of viral isolation (Nirmala et al., 2020). However, this step of PCR amplification could add some bias that would be especially difficult to assess when more than one virus is present in the sample. We first tested the fidelity of this approach by determining the divergence rate between direct sequencing and sequencing from a single passage isolate. The difference resulted to be in the range of 10^{-3} (namely 1 discrepancy per every 1,000 nucleotides). Obviously, some bias in the isolation can be produced by using MDCK cells and this could be in part responsible for the inability to sequence a second virus present in those samples where two H or N strains were initially found. However, the advantages of NGS for such diverse viruses like IAV balance the potential disadvantages of our approach.

5. Conclusion

IAV is widely present in most pig farms. The most common detected lineages were H1avN2hu and H1avN1av, representing the D and A genotypes, respectively. The genetic diversity of this virus is continuously expanding with clear changes in the predominant subtypes and lineages in relatively short periods of time. The current genotyping scheme has to be enlarged to include the new genotypes that can be found. As in any other case, for a good diagnosis of IAV, a differential diagnosis with the investigation of the

presence of other pathogens is needed. A large targeted sampling of clinically affected and healthy animals, considering the proportion of positive animals and viral loads in each group may help to discriminate cases where IAV is the primary agent of the outbreak.

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7. Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required.

8. Conflict of interest statement

No competing interests.

9. Data availability statement

All influenza sequences that have been generated on this article have been submitted to GenBank and will be available after article publication. Accession numbers are listed in the supplementary file.

References

1. Beato, M. S., Tassoni, L., Milani, A., Salviato, A., Di Martino, G., Mion, M., Bonfanti, L., Monne, I., Watson, S. J., & Fusaro, A. 2016. Circulation of multiple genotypes of H1N2 viruses in a swine farm in Italy over a two-month period. *Vet Microbiol*, 195: 25–29. https://doi.org/10.1016/j.vetmic.2016.08.015
2. Brockmeier, S. L., Halbur, P. G., & Thacker, E. L. 2002. Porcine respiratory disease complex. In: *Polymicrobial Diseases.* K. A. Brogden & J. M. Guthmiller (editors). ASM Press. Washington, DC, USA. 231–258 pp.
3. Busquets, N., Segalés, J., Córdoba, L., Mussá, T., Crisci, E., Martín-Valls, G. E., Simon-Grifé, M., Pérez-Simó, M., Pérez-Maíllo, M., Núñez, J. I., Abad, F. X., Fraile, L., Pina, S., Majó, N., Bensaid, A., Domingo, M., & Montoya, M. 2010. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. *Vet Res*, 41: 74. https://doi.org/10.1051/vetres/2010046
4. Eurostat. 2017. Pork production up in the EU. Retrieved from https://ec.europa.eu/eurostat/web/products-eurostat-news/-/DDN-20170919-1
5. Ferreira, J. B., Grgić, H., Friendship, R., Wideman, G., Nagy, É., & Poljak, Z. 2017. Longitudinal study of influenza A virus circulation in a nursery swine barn. *Vet Res,* 48: 1–16. https://doi.org/10.1186/s13567-017-0466-x
6. Gillespie, T. G. 1999. Diagnosing endemic swine influenza virus in nursery pigs using cross-sectional serologic profiling. *Swine Health Prod*, 7: 81–83.
7. Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser,* 41: 95-98.
8. Harder, T. C., grosse Beilage, E., Lange, E., Meiners, C., Dohring, S., Pesch, S., Noe, T., Grund, C., Beer, M., & Starick, E. 2013. Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. *J Virol*, 87: 10460–10476. https://doi.org/10.1128/jvi.00381-13
9. Henritzi, D., Hoffmann, B., Wacheck, S., Pesch, S., Herrler, G., Beer, M., & Harder, T. C. 2019. A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza Other Respir Viruses,* 13: 71–82. https://doi.org/10.1111/irv.12613
10. Henritzi, D., Zhao, N., Starick, E., Simon, G., Krog, J. S., Larsen, L. E., Reid, S. M., Brown, I. H., Chiapponi, C., Foni, E., Wacheck, S., Schmid, P., Beer, M., Hoffmann, B., & Harder, T. C. 2016. Rapid detection and subtyping of European swine influenza viruses in porcine clinical samples by haemagglutinin- and neuraminidase-specific tetra- and triplex real-time RT-PCRs. *Influenza Other Respir Viruses,* 10: 504–517. https://doi.org/10.1111/irv.12407
11. Howard, W. A., Essen, S. C., Strugnell, B. W., Russell, C., Barrass, L., Reid, S. M., & Brown, I. H. 2011. Reassortant pandemic (H1N1) 2009 virus in pigs, United Kingdom. *Emerg Infect Dis,* 17: 1049–1052.

https://doi.org/10.3201/eid1706.101886
12. Krog, J. S., Hjulsager, C. K., Larsen, M. A., & Larsen, L. E. 2017. Triple-reassortant influenza A virus with H3 of human seasonal origin, NA of swine origin, and internal A(H1N1) pandemic 2009 genes is established in Danish pigs. *Influenza Other Respir Viruses,* 11: 298–303. https://doi.org/10.1111/irv.12451
13. Kyriakis, C. S., Brown, I. H., Foni, E., Kuntz-Simon, G., Maldonado, J., Madec, F., Essen, S. C., Chiapponi, C., & Van Reeth, K. 2011. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. *Zoonoses Public Health,* 58: 93–101. https://doi.org/10.1111/j.1863-2378.2009.01301.x
14. Kyriakis, C. S., Gramer, M. R., Barbé, F., Van Doorsselaere, J., & Van Reeth, K. 2010. Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. *Vet Microbiol*, 144: 67–74. https://doi.org/10.1016/j.vetmic.2009.12.039
15. Kyriakis, C. S., Rose, N., Foni, E., Maldonado, J., Loeffen, W., Madec, F., Simon, G., & Van Reeth, K. 2013. Influenza A virus infection dynamics in swine farms in Belgium, France, Italy and Spain, 2006-2008. *Vet Microbiol*, 162: 543–550. https://doi.org/10.1016/j.vetmic.2012.11.014
16. Lange, J., Groth, M., Schlegel, M., Krumbholz, A., Wieczorek, K., Ulrich, R., Köppen, S., Schulz, K., Appl, D., Selbitz, H. J., Sauerbrei, A., Platzer, M., Zell, R., & Dürrwald, R. 2013. Reassortants of the pandemic (H1N1) 2009 virus and establishment of a novel porcine H1N2 influenza virus, lineage in Germany. *Vet Microbiol*, 167: 345–356. https://doi.org/10.1016/j.vetmic.2013.09.024
17. Martín-Valls, G. E., Simon-Grifé, M., van Boheemen, S., de Graaf, M., Bestebroer, T. M., Busquets, N., Martín, M., Casal, J., Fouchier, R. A. M., & Mateu, E. 2014. Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm. *Vet Microbiol*, 170: 266–277. https://doi.org/10.1016/j.vetmic.2014.02.031
18. Ministerio de Agricultura, Pesca y Alimentación. 2019. El sector de la carne de cerdo en cifras: principales indicadores económicos. Retrieved from https://www.mapa.gob.es/va/ganaderia/temas/produccion-y-mercados-ganaderos/indicadoreseconomicossectorporcinoano2018_tcm39-379728.pdf
19. Moreno, A., Barbieri, I., Sozzi, E., Luppi, A., Lelli, D., Lombardi, G., Zanoni, M. G., & Cordioli, P. 2009. Novel swine influenza virus subtype H3N1 in Italy. *Vet Microbiol*, 138: 361–367. https://doi.org/10.1016/j.vetmic.2009.04.007
20. Moreno, A., Di Trani, L., Faccini, S., Vaccari, G., Nigrelli, D., Boniotti, M. B., Falcone, E., Boni, A., Chiapponi, C., Sozzi, E., & Cordioli, P. 2011. Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. *Vet Microbiol*, 149: 472–477. https://doi.org/10.1016/j.vetmic.2010.12.011
21. Nirmala, J., Perez, A., Culhane, M. R., Allerson, M. W., Sreevatsan, S., & Torremorell, M. 2020. Genetic variability of influenza A virus in pigs at

weaning in Midwestern United States swine farms. *Transbound Emerg Dis*, 10.1111/tbed.13529. Advance online publication. https://doi.org/10.1111/tbed.13529
22. Rose, N., Hervé, S., Eveno, E., Barbier, N., Eono, F., Dorenlor, V., Andraud, M., Camsusou, C., Madec, F., & Simon, G. 2013. Dynamics of influenza A virus infections in permanently infected pig farms: evidence of recurrent infections, circulation of several swine influenza viruses and reassortment events. *Vet Res*, 44: 72. https://doi.org/10.1186/1297-9716-44-72
23. Ryt-Hansen, P., Larsen, I., Kristensen, C. S., Krog, J. S., Wacheck, S., & Larsen, L. E. 2019. Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies. *Vet Res*, 50: 1–10. https://doi.org/10.1186/s13567-019-0655-x
24. Simon, G., Larsen, L. E., Dürrwald, R., Foni, E., Harder, T., Van Reeth, K., Markowska-Daniel, I., Reid, S. M., Dan, A., Maldonado, J., Huovilainen, A., Billinis, C., Davidson, I., Agüero, M., Vila, T., Hervé, S., Breum, S. Ø., Chiapponi, C., Urbaniak, K., … Vincent, A. L. 2014. European surveillance network for influenza in pigs: surveillance programs, diagnostic tools and swine influenza virus subtypes identified in 14 European countries from 2010 to 2013. *PLoS One*, 9: 1–21. https://doi.org/10.1371/journal.pone.0115815
25. Simon-Grifé, M., Martín-Valls, G. E., Vilar, M. J., Busquets, N., Mora-Salvatierra, M., Bestebroer, T. M., Fouchier, R. A., 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. https://doi.org/10.1186/1297-9716-43-24
26. Simon-Grifé, M., Martín-Valls, G. E., Vilar, M. J., 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. https://doi.org/10.1016/j.vetmic.2010.10.015
27. Torremorell, M., Juarez, A., Chavez, E., Yescas, J., Doporto, J. M., & Gramer, M. 2009. Procedures to eliminate H3N2 swine influenza virus from a pig herd. *Vet Rec*, 165: 74–77. https://doi.org/10.1136/vetrec.165.3.74
28. Van Reeth, K., Brown, I. H., Dürrwald, R., Foni, E., Labarque, G., Lenihan, P., Maldonado, J., Markowska-Daniel, I., Pensaert, M., Pospisil, Z., & Koch, G. 2008. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003. *Influenza Other Respir Viruses,* 2: 99–105. https://doi.org/10.1111/j.1750-2659.2008.00043.x
29. Van Reeth, K., Labarque, G., & Pensaert, M. 2006. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. *Viral Immunol,* 19: 373–382. https://doi.org/10.1089/vim.2006.19.373
30. Watson, S. J., Langat, P., Reid, S. M., Lam, T. T., Cotten, M., Kelly, M., Van Reeth, K., Qiu, Y., Simon, G., Bonin, E., Foni, E., Chiapponi, C., Larsen, L., Hjulsager, C., Markowska-Daniel, I., Urbaniak, K., Dürrwald, R., Schlegel, M., Huovilainen, A., Davidson, I., … Kellam, P. 2015. Molecular epidemiology and

evolution of influenza viruses circulating within European swine between 2009 and 2013. *J Virol*, 89: 9920–9931. https://doi.org/10.1128/JVI.00840-15

Table 1. Distribution of case submissions by age group and proportion of IAV-positive cases (M gene RT-qPCR)

Age group	No. of submissions (%)	No. of positive submissions/Total submitted (%)
Suckling piglets	37 (17.5%)	26/37 (70.3%)
Nurseries	84 (39.8%)	53/84 (63.1%)
Fatteners	28 (13.3%)	22/28 (78.8%)
Sows*	15 (7.1%)	6/15 (40%) *
Other	47 (22.3%)	38/47 (80.9%)
Total	211 (100%)	145/211 (68.7%)

*$p<0.05$

Table 2. Distribution of influenza A positive pools per farm and their average Cq value in herds subclinically infected.

Positive farms (n=15)	Positive pools			Average Cq value
	Suckling piglets	Weaners	Fatteners	
Farm 1	4/5	0/5	0/5	31.3
Farm 2	5/5	5/5	1/5	27.6
Farm 3	1/5	4/5	N/A†	31.6
Farm 4	0/5	3/5	0/5	28.1
Farm 5	0/5	4/5	0/5	29.7
Farm 6	4/5	2/5	0/5	31.6
Farm 7	3/5	3/5	0/5	28.9
Farm 8	4/5	2/5	0/5	27.7
Farm 9	3/5	0/5	0/5	32.8
Farm 10	3/5	0/5	0/5	30.3
Farm 11	0/5	5/5	0/5	17.9
Farm 12	4/5	4/5	0/5	26.9
Farm 13	5/5	0/5	0/5	28.5
Farm 14	2/5	4/5	0/5	27.8
Farm 15	5/5	5/5	N/A†	27.5

† Farms without a fattening unit

Table 3. Distribution of detected lineages in influenza A positive pigs samples retrieved from cases of respiratory disease and one case from an apparently subclinical farm.

Lineages detected (n=95)	Nº isolates RT-qPCR	Nº isolates NGS	Nº isolates Total	Percentage
H1avN2hu	31	5†	36	33.6
H1avN1av	24	2	26	24.3
H1huN2hu	20	0	20	18.7
H3huN2hu	6	2	8	7.5
H1huN1av	6	0	6	5.6
H1pdmN1pdm	5	0	5	4.7
H3huN1av	1	2	3	2.8
H1pdmN1av/N1pdm	1	0	1	0.9
H1av/H1pdmN1av‡	0	1	1	0.9
H3huN1av/N2hu‡	1	0	1	0.9
Total	**95**	**12**	**107**	**100%**

†Includes one case retrieved from an apparent subclinical farm where the initial RT-qPCR assessment was H1avNx, and after successful isolation the sequence obtained was found to be H1avN2hu.

‡In two cases two H or two N were detected in the RT-qPCR.

Figure 1. Distribution and regression of positive animals per case (respiratory outbreaks) and the Cq values. The graph shows the Cq for positive samples distributed by the percentage of positive animals per case.

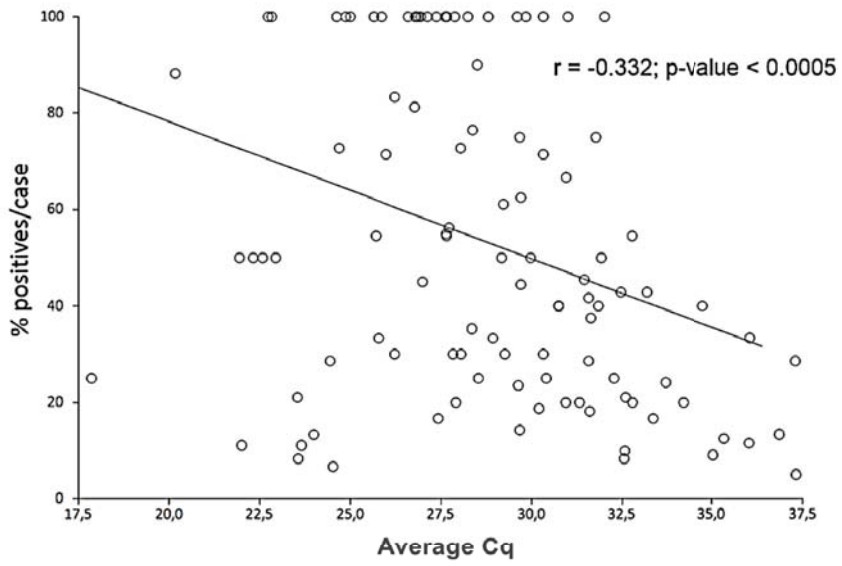
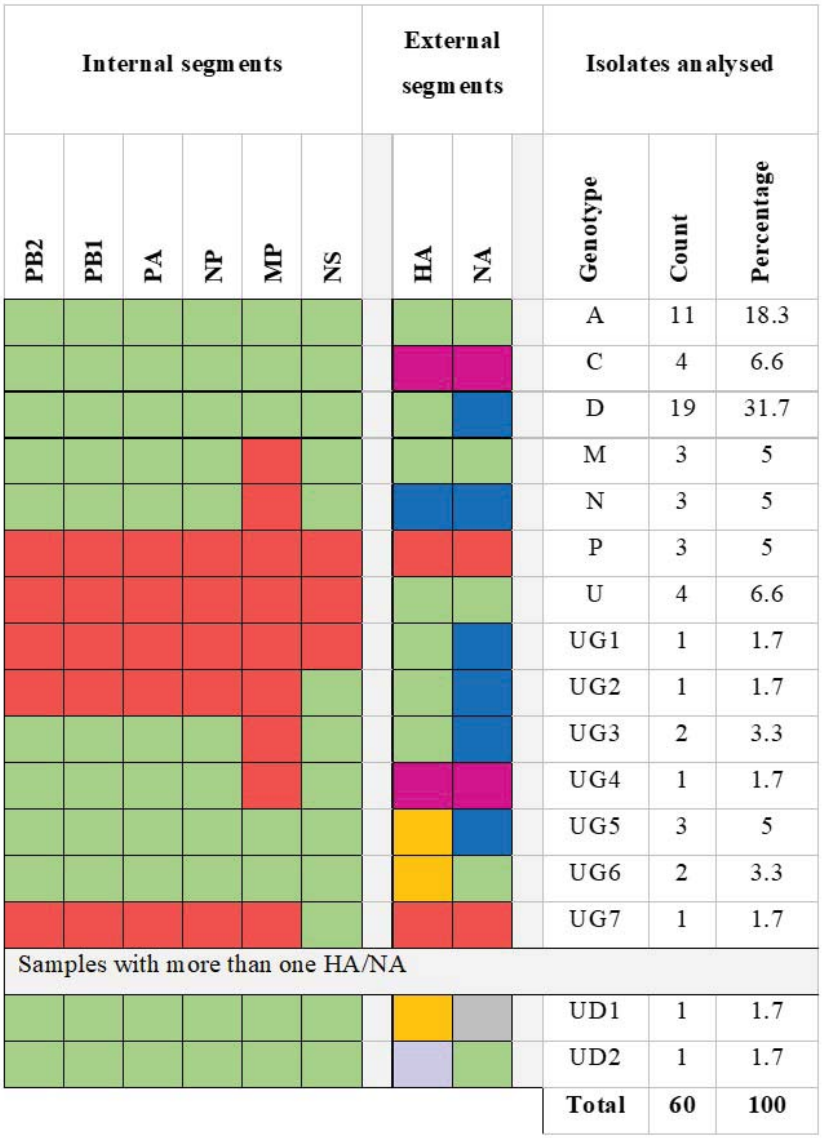


Figure 2. Distribution of genotypes in the 60 influenza A virus isolates sequenced. Designation of the genotypes followed the classification by Watson *et al.* (2015). For those genotypes previously unreported we used the designation "unreported genotype" (UG).



Internal segments						External segments		Isolates analysed		
PB2	PB1	PA	NP	MP	NS	HA	NA	Genotype	Count	Percentage
								A	11	18.3
								C	4	6.6
								D	19	31.7
								M	3	5
								N	3	5
								P	3	5
								U	4	6.6
								UG1	1	1.7
								UG2	1	1.7
								UG3	2	3.3
								UG4	1	1.7
								UG5	3	5
								UG6	2	3.3
								UG7	1	1.7
Samples with more than one HA/NA										
								UD1	1	1.7
								UD2	1	1.7
								Total	**60**	**100**

† Denominations and strains used as reference according to Watson *et al.* 2015.

‡Older closest relative strain found in GenBank.

§ In these cases two H or two N genes were detected but no differences for all other genes. In these cases we used the designation undetermined genotype (UD).

Figure 3. Maximum likelihood tree of the influenza A positive hemagglutinins H1 and H3 (1,000 iterations). Coloured boxes show the main lineages for Eurasian H1 and H3 as indicated in the figure. Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like H1), dark red (pandemic H1), purple (human like H1), orange (new seasonal human H3), and blue (classical human like swine H3) indicate a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).

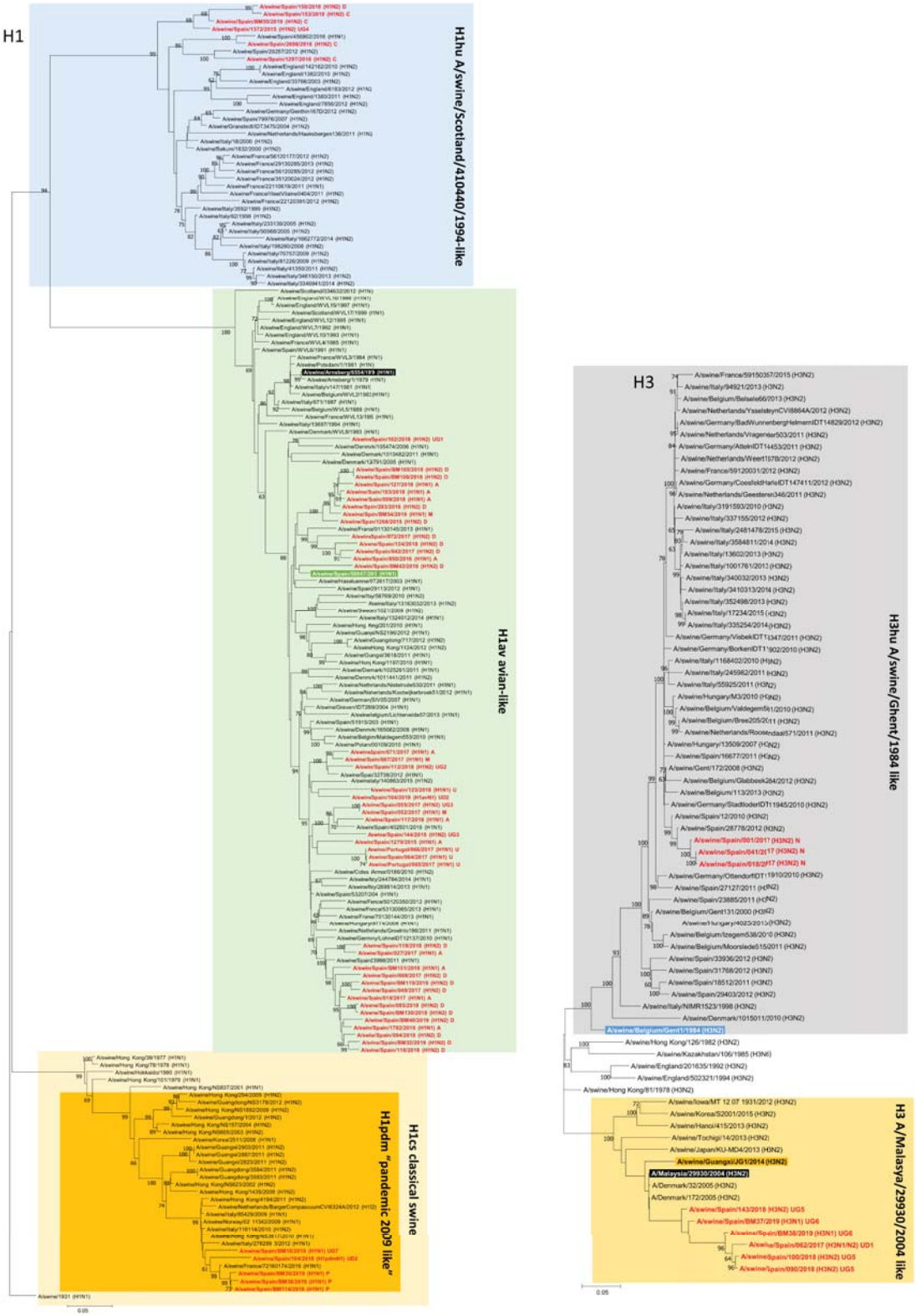


Figure 4. Maximum likelihood tree for influenza A positive N1 and N2 gene segments (1,000 iterations). Coloured boxes show the main lineages for Eurasian N1 and N2 as indicated in the figure. Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like N1), dark red (pandemic N1), and blue (human like swine N2), indicate a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).

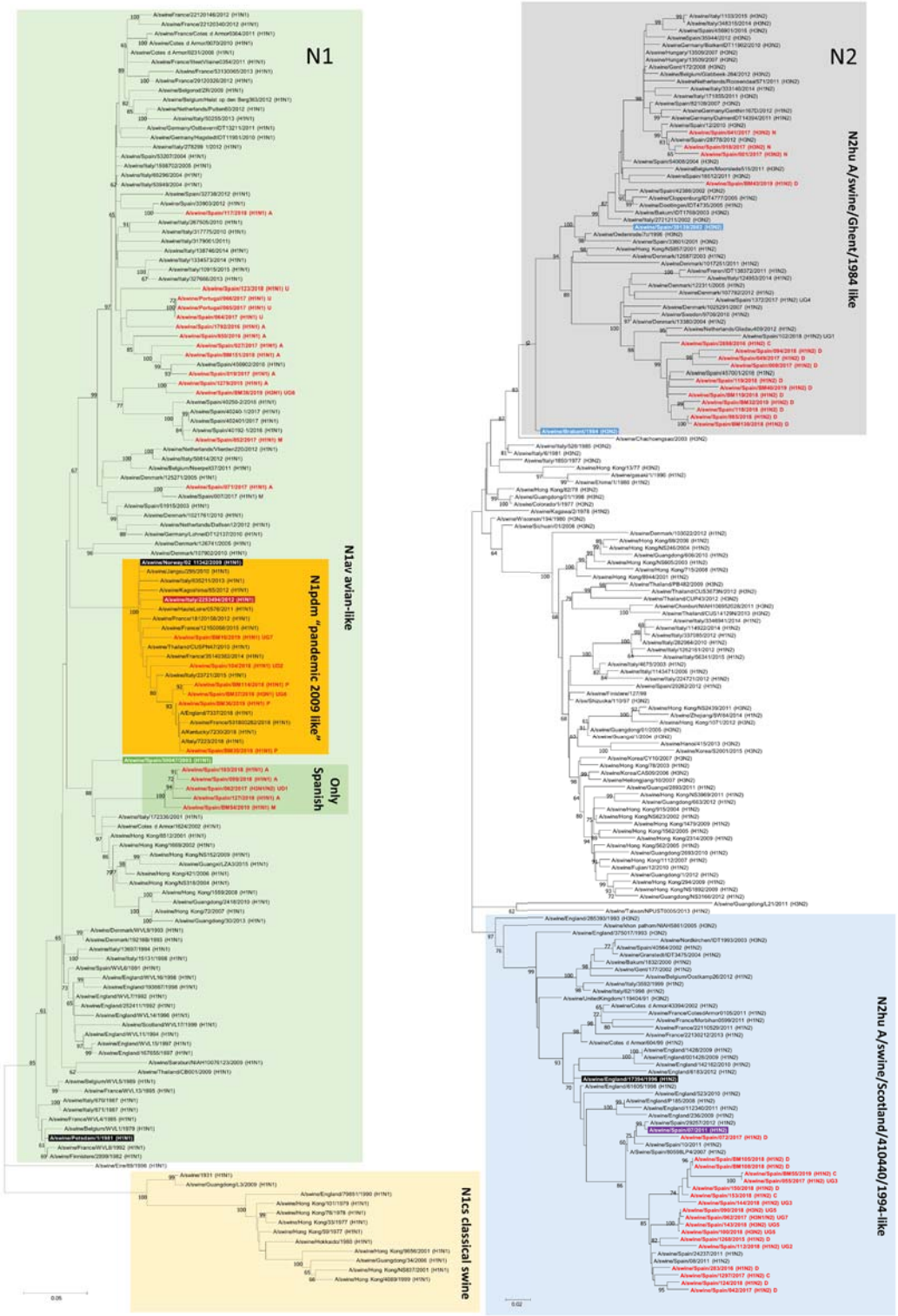
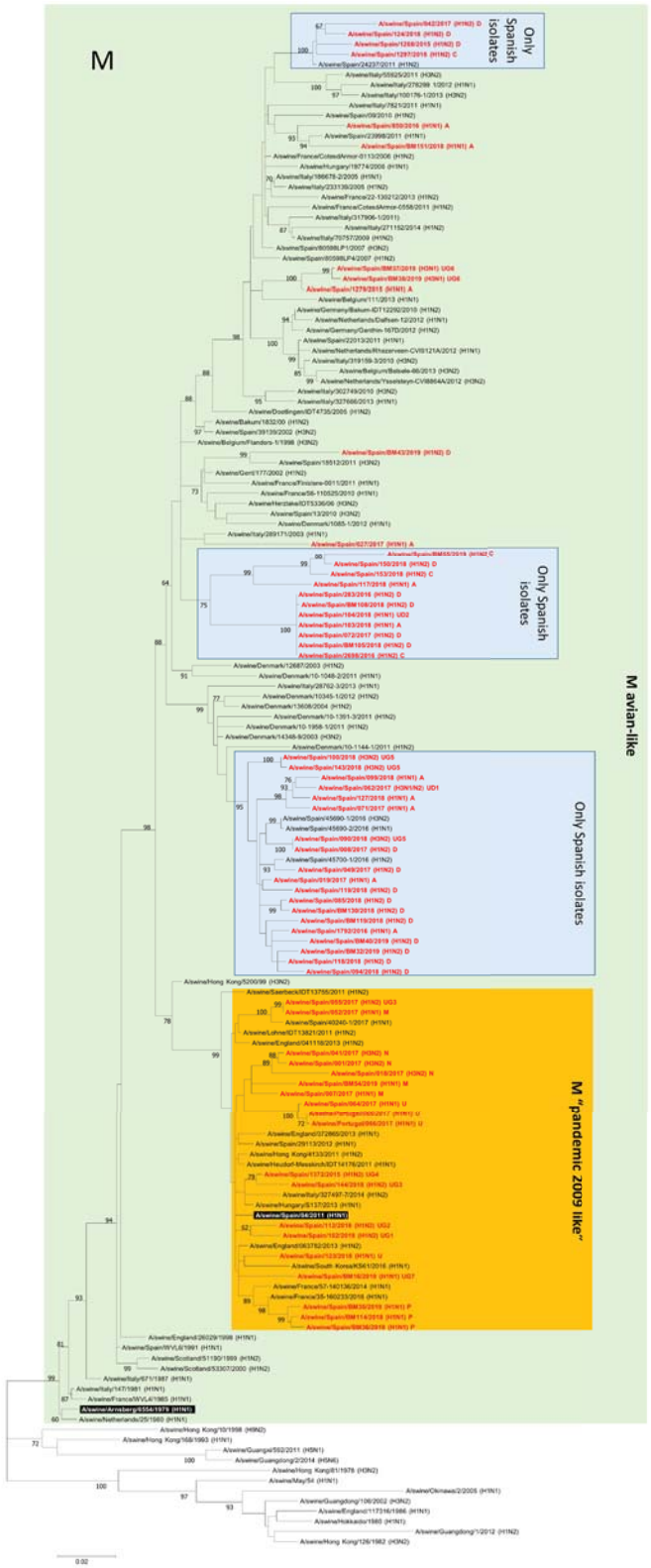


Figure 5. Maximum likelihood tree for influenza A positive Matrix gene segment (1,000 iterations). Coloured boxes show the main groups for Eurasian M genes. Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like N1), dark red (pandemic N1) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Clusters containing solely Spanish isolates are indicated. Values in the branches show the bootstrap values (only ≥60 are shown).



S1. IAV sequence references used for the filtering process for each genome segment.

Segment	Reference
H1av	CY010572.2 A/swine/Spain/51915/2003 (H1N1)
H1hu	KR700177.1 A/swine/Spain/29257/2012 (H1N2)
H1pdm	KT181106.1 A/Singapore/DMS30/2009 (H1N1)
H3hu	CY009380.1 A/swine/Spain/39139/2002 (H3N2)
Avian origin internal cassette	CY010579.2 A/swine/Spain/51915/2003 (H1N1)
Human origin internal cassette	JX908134.1 A/swine/Spain/09/2010 (H1N2)
N1av	CY010574.2 A/swine/Spain/51915/2003 (H1N1)
N1pdm	KT181534.1 A/Singapore/DMS30/2009 (H1N1)
N2hu	KR700179.1 A/swine/Spain/29257/2012 (H1N2)

S2. List of GenBank accession numbers for the 60 isolates that were fully sequenced. Following the segments order (PB2, PB1, PA, HA, NP, NA, M, NS1/NEP)

Whole genome strains		
Strain name	**Subtype**	**Accession numbers**
A/swine/Spain/001/2017	**H3N2**	MN934409, MN934053, MN933980, MN929029, MN933834, MN933435, MN933460, MN939537
A/swine/Spain/007/2017	**H1N1**	MN934408, MN934056, MN933979, MN932170, MN933831, MN932234, MN933483, MN939536
A/swine/Spain/008/2017	**H1N2**	MN934369, MN934031, MN933949, MN932188, MN933833, MN933406, MN933493, MN939494
A/swine/Spain/018/2017	**H3N2**	MN934410, MN934051, MN933990, MN929037, MN933848, MN933431, MN933454, MN939538
A/swine/Spain/019/2017	**H1N1**	MN934407, MN934052, MN933978, MN932171, MN933830, MN932235, MN933462, MN939535
A/swine/Spain/027/2017	**H1N1**	MN934400, MN934016, MN933964, MN932199, MN933808, MN932226, MN933468, MN939514
A/swine/Spain/041/2017	**H3N2**	MN934406, MN934050, MN933977, MN929030, MN933829, MN933434, MN933451, MN939534
A/swine/Spain/042/2017	**H1N2**	MN934404, MN934049, MN933976, MN932172, MN933828, MN933428, MN933461, MN939532
A/swine/Spain/049/2017	**H1N2**	MN934403, MN934048, MN933975, MN932173, MN933827, MN933422, MN933459, MN939531
A/swine/Spain/052/2017	**H1N1**	MN934402, MN934047, MN933974, MN932174, MN933826, MN932233, MN933458, MN939530
A/swine/Spain/055/2017	**H1N2**	MN934401, MN934046, MN933973, MN932178, MN933825, MN933433, MN933457, MN939529
A/swine/Spain/062/2017	**H3N1/N2**	MN934368, MN934032, MN933948, MN929032, MN933835, MN932219, MN933413,

		MN933492, MN939493
A/swine/Spain/064/2017	**H1N1**	MN934360, MN934028, MN933947, MN932197, MN933843, MN932221, MN933448, MN939499
A/swine/Portugal/065/2017	**H1N1**	MN934367, MN934033, MN933989, MN932189, MN933836, MN932218, MN933447, MN939492
A/swine/Portugal/066/2017	**H1N1**	MN934372, MN934034, MN933995, MN932190, MN933837, MN932217, MN933449, MN939491
A/swine/Spain/071/2017	**H1N1**	MN934405, MN934017, MN933961, MN932179, MN933824, MN932230, MN933483, MN939526
A/swine/Spain/072/2017	**H1N2**	MN934361, MN934027, MN933936, MN932175, MN933844, MN933416, MN933496, MN939498
A/swine/Spain/085/2018	**H1N2**	MN934399, MN934015, MN933965, MN932202, MN933807, MN933425, MN933467, MN939513
A/swine/Spain/090/2018	**H3N2**	MN934366, MN934035, MN933954, MN929033, MN933838, MN933412, MN933491, MN939490
A/swine/Spain/094/2018	**H1N2**	MN934395, MN934039, MN933960, MN932180, MN933823, MN933417, MN933490, MN939525
A/swine/Spain/099/2018	**H1N1**	MN934396, MN934040, MN933959, MN932181, MN933822, MN932231, MN933489, MN939524
A/swine/Spain/100/2018	**H3N2**	MN934389, MN934045, MN933991, MN929031, MN933817, MN933418, MN933476, MN939528
A/swine/Spain/102/2018	**H1N2**	MN934394, MN934041, MN933958, MN932182, MN933821, MN933419, MN933488, MN939523
A/swine/Spain/103/2018	**H1N1**	MN934371, MN934036, MN933958, MN932182, MN933839, MN932216, MN933495, MN939489

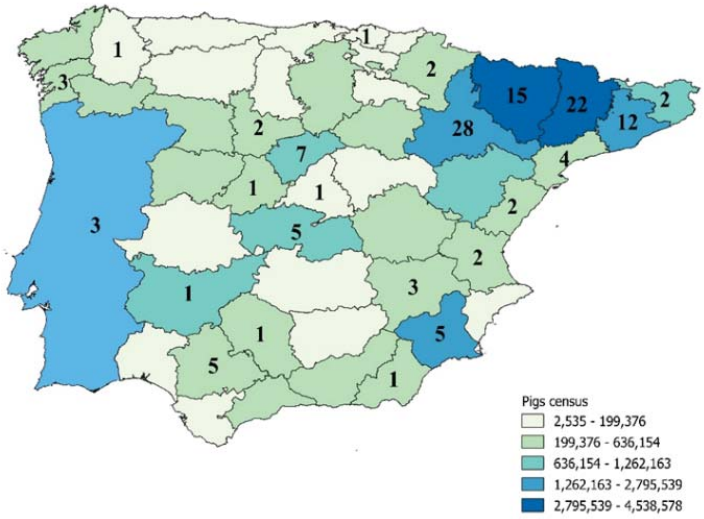
A/swine/Spain/104/2018	**H1N1**	MN934365, MN934037, MN933952, MN932192, MN932193, MN933840, MN932215, MN933487, MN939488
A/swine/Spain/112/2018	**H1N2**	MN934353, MN933999, MN933994, MN932183, MN933800, MN933420, MN933440, MN939481
A/swine/Spain/117/2018	**H1N1**	MN934370, MN934030, MN933993, MN932196, MN933832, MN932220, MN933450, MN939495
A/swine/Spain/118/2018	**H1N2**	MN934390, MN934012, MN933992, MN932184, MN933816, MN933427, MN933477, MN939527
A/swine/Spain/119/2018	**H1N2**	MN934397, MN934014, MN933966, MN932203, MN933806, MN933426, MN933466, MN939512
A/swine/Spain/123/2018	**H1N1**	MN934354, MN934000, MN933943, MN932205, MN933799, MN932237, MN933446, MN939480
A/swine/Spain/124/2018	**H1N2**	MN934398, MN934013, MN933962, MN932206, MN933805, MN933423, MN933465, MN939509
A/swine/Spain/127/2018	**H1N1**	MN934393, MN934042, MN933957, MN932185, MN933820, MN932232, MN933486, MN939522
A/swine/Spain/143/2018	**H3N2**	MN934392, MN934043, MN933956, MN929036, MN933818, MN933432, MN933485, MN939521
A/swine/Spain/144/2018	**H1N2**	MN934391, MN934044, MN933955, MN932187, MN933819, MN933421, MN933484, MN939517
A/swine/Spain/150/2018	**H1N2**	MN934352, MN934054, MN933946, MN932186, MN933845, MN933414, MN933456, MN939500
A/swine/Spain/153/2018	**H1N2**	MN934358, MN934003, MN933941, MN932213, MN933794, MN933408, MN933444, MN939485
A/swine/Spain/283/2016	**H1N2**	MN934374, MN934005, MN933981, MN932162,

		MN933790, MN933405, MN933453, MN939501
A/swine/Spain/850/2016	**H1N1**	MN934375, MN933997, MN933982, MN932163, MN933796, MN932222, MN933478, MN939502
A/swine/Spain/1268/2015	**H1N2**	MN934376, MN934006, MN933984, MN932164, MN933797, MN933402, MN933463, MN939505
A/swine/Spain/1279/2015	**H1N1**	MN934377, MN934007, MN933983, MN932167, MN933798, MN932223, MN933464, MN939506
A/swine/Spain/1297/2016	**H1N2**	MN934378, MN934008, MN933985, MN932169, MN933801, MN933401, MN933479, MN939503
A/swine/Spain/1372/2015	**H1N2**	MN934379, MN934009, MN933986, MN932168, MN933802, MN933404, MN933480, MN939504
A/swine/Spain/1792/2016	**H1N1**	MN934380, MN934011, MN933987, MN932166, MN933803, MN932224, MN933481, MN939507
A/swine/Spain/2698/2016	**H1N2**	MN934381, MN934010, MN933988, MN932165, MN933804, MN933403, MN933498, MN939508
A/swine/Spain/BM16/2019	**H1N1**	MN934384, MN934024, MN933971, MN932198, MN933814, MN932225, MN933474, MN939519
A/swine/Spain/BM32/2019	**H1N2**	MN934387, MN934023, MN933972, MN932200, MN933815, MN933424, MN933473, MN939516
A/swine/Spain/BM35/2019	**H1N1**	MN934411, MN934055, MN933937, MN932212, MN933849, MN932239, MN933439, MN939533
A/swine/Spain/BM36/2019	**H1N1**	MN934386, MN934022, MN933969, MN932201, MN933813, MN932227, MN933475, MN939510
A/swine/Spain/BM37/2019	**H3N1**	MN934385, MN934021, MN933970, MN929035, MN933812, MN932228, MN933470, MN939511

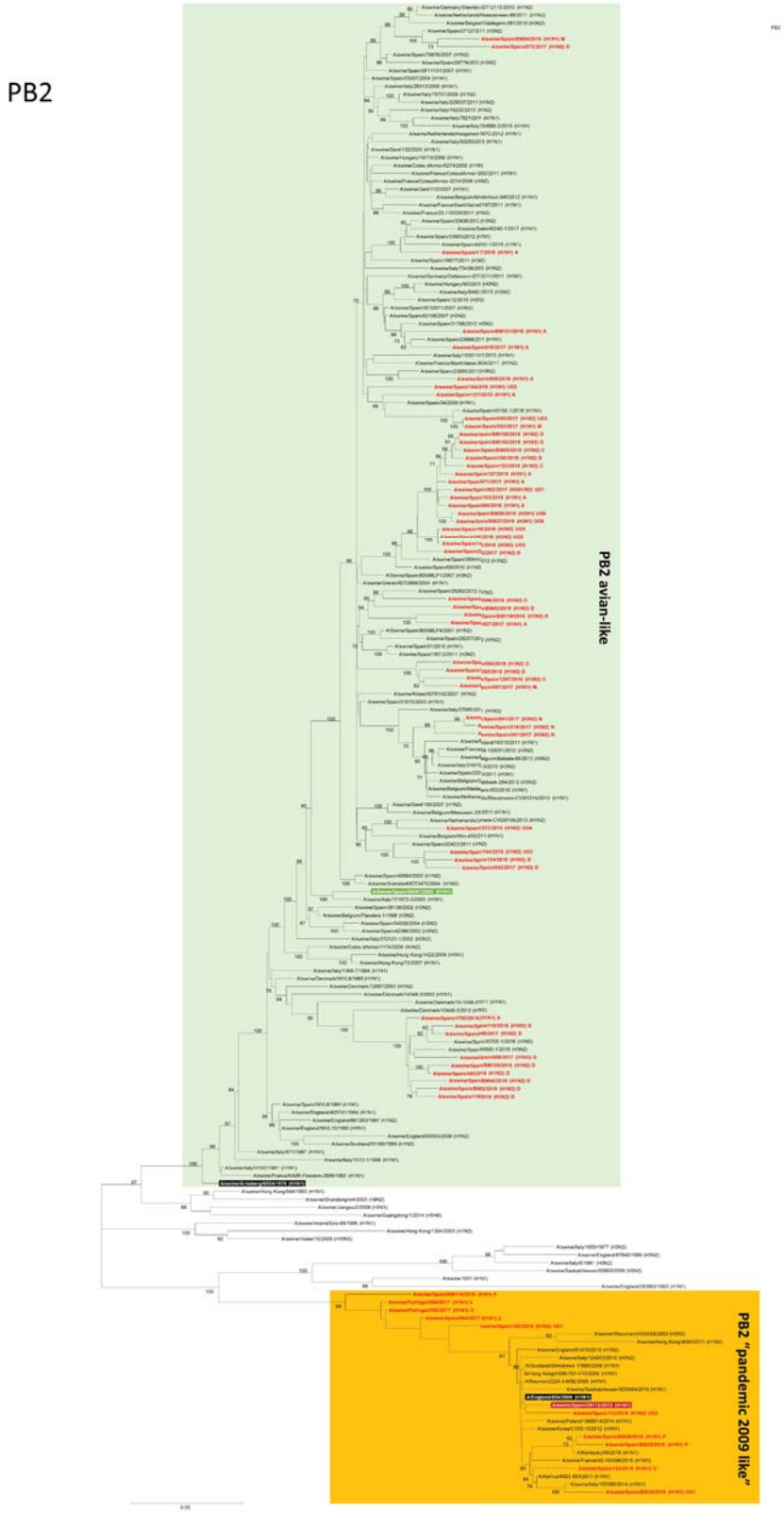
A/swine/Spain/BM38/2019	**H3N1**	MN934382, MN934020, MN933968, MN929034, MN933811, MN932229, MN933471, MN939520
A/swine/Spain/BM40/2019	**H1N2**	MN934355, MN933998, MN933938, MN932208, MN933791, MN933411, MN933441, MN939482
A/swine/Spain/BM43/2019	**H1N2**	MN934359, MN934004, MN933942, MN932209, MN933795, MN933409, MN933445, MN939486
A/swine/Spain/BM54/2019	**H1N1**	MN934356, MN934001, MN933939, MN932210, MN933792, MN932238, MN933442, MN939483
A/swine/Spain/BM55/2019	**H1N2**	MN934357, MN934002, MN933940, MN932211, MN933793, MN933410, MN933443, MN939484
A/swine/Spain/BM105/2018	**H1N2**	MN934364, MN934038, MN933951, MN932194, MN933841, MN933407, MN933497, MN939487
A/swine/Spain/BM108/2018	**H1N2**	MN934362, MN934026, MN933944, MN932176, MN933847, MN933415, MN933455, MN939497
A/swine/Spain/BM114/2018	**H1N1**	MN934373, MN934029, MN933950, MN932195, MN933842, MN932214, MN933452, MN939479
A/swine/Spain/BM119/2018	**H1N2**	MN934388, MN934019, MN933963, MN932204, MN933810, MN933430, MN933469, MN939518
A/swine/Spain/BM130/2018	**H1N2**	MN934363, MN934025, MN933945, MN932177, MN933846, MN933429, MN933494, MN939496
A/swine/Spain/BM151/2018	**H1N1**	MN934383, MN934018, MN933967, MN932207, MN933809, MN932236, MN933472, MN939515

S3. Geographic distribution of positive cases detected in Spain and Portugal.

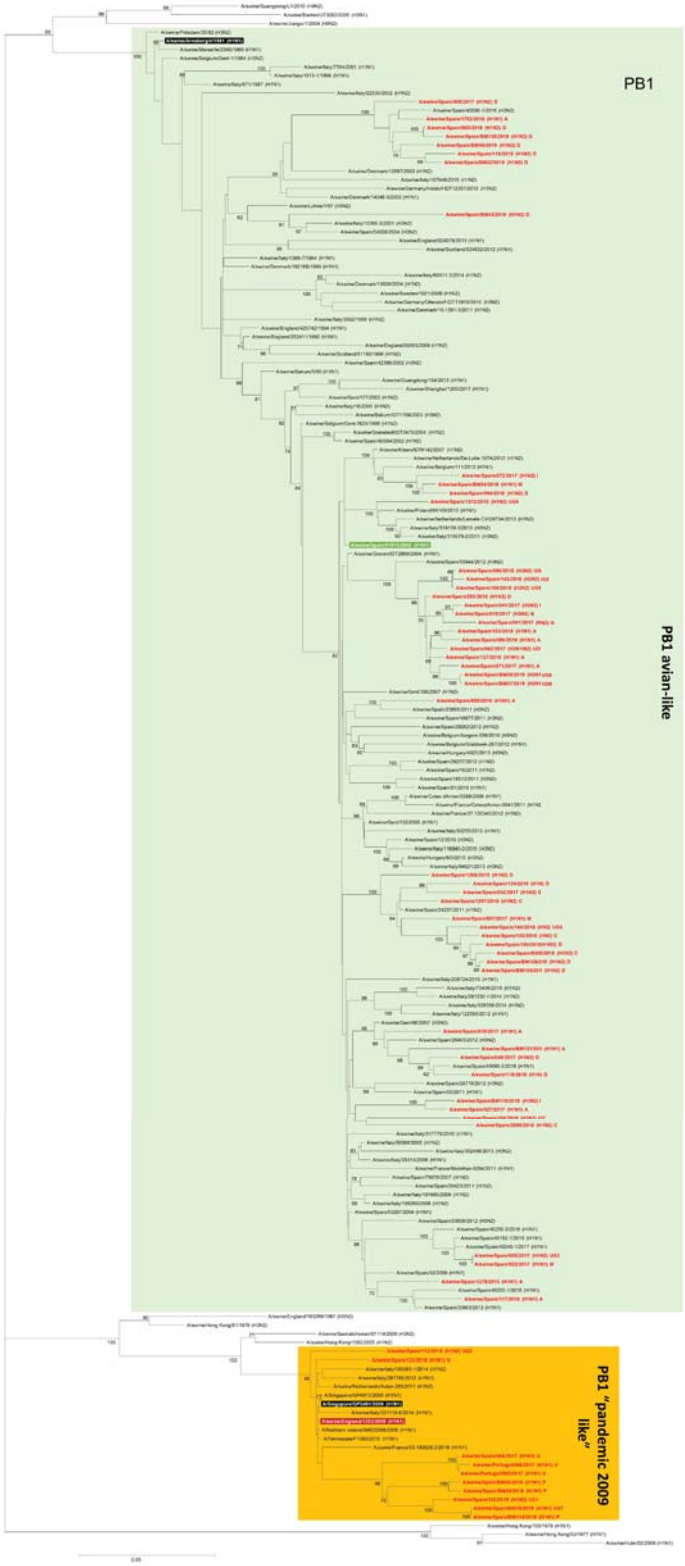
The map shows the localisation by province of IAV positive outbreaks for which the precise (130/145) localisation of the farm was provided.



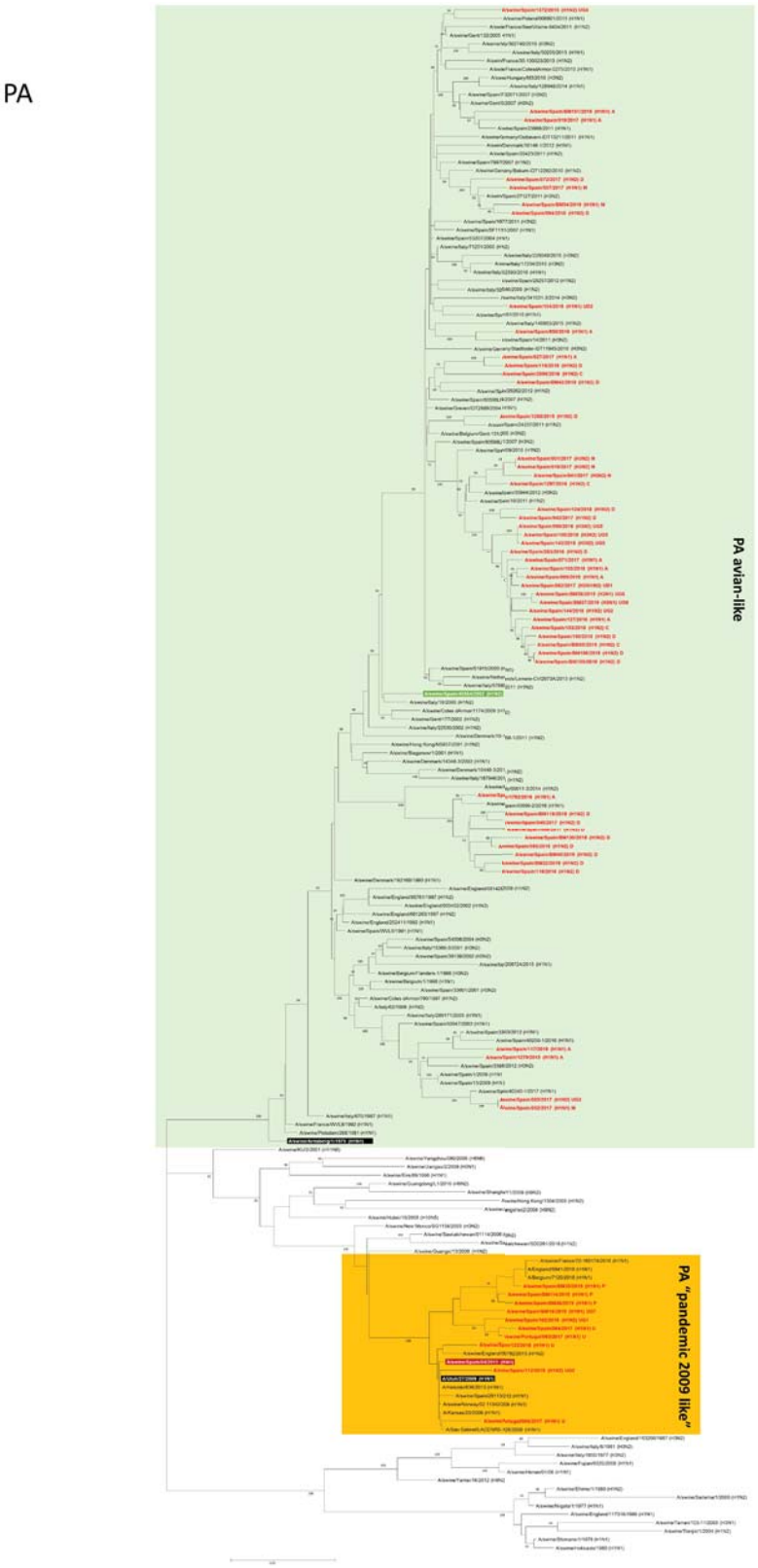
S4. Maximum likelihood tree for the PB2 gene segment (1,000 iterations). Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like), dark red (pandemic) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).



S5. Maximum likelihood tree for the PB1 gene segment (1,000 iterations). Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like), dark red (pandemic) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).

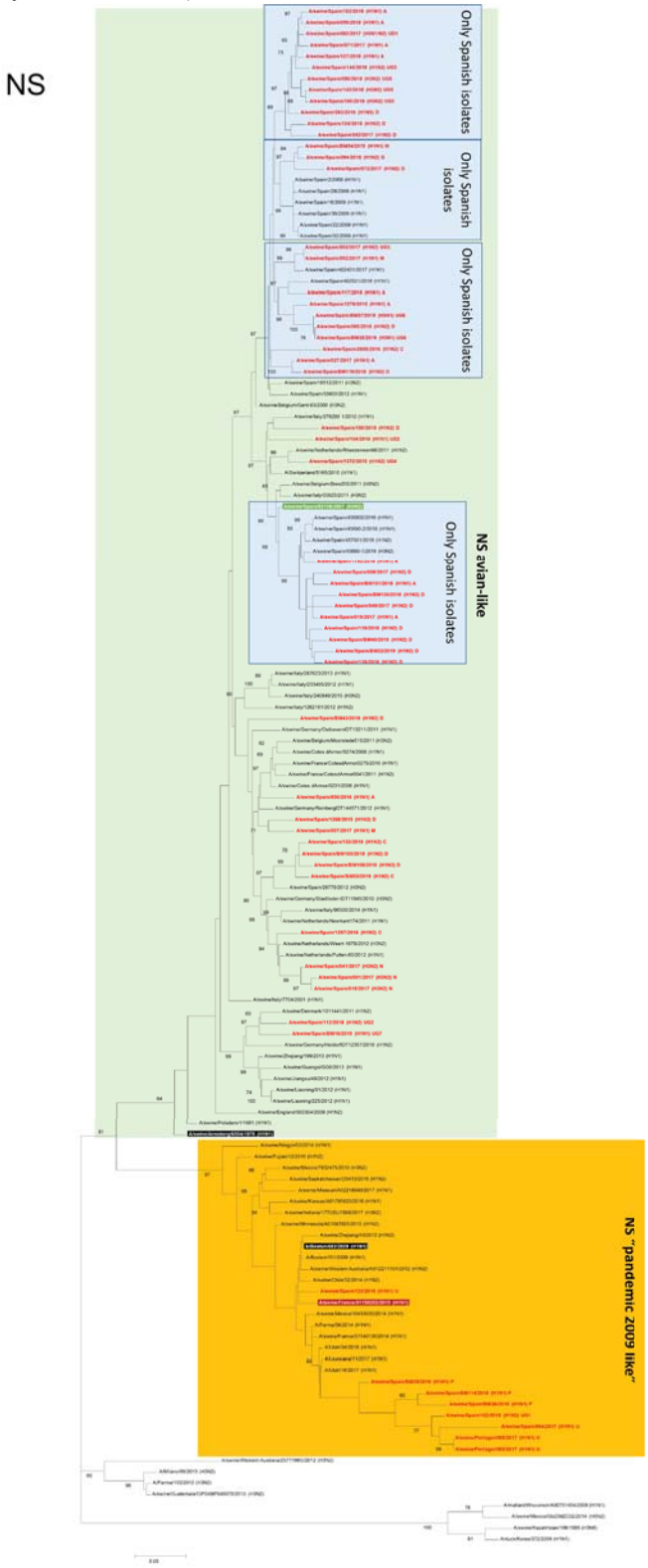


S6. Maximum likelihood tree for the PA gene segment (1,000 iterations). Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like), dark red (pandemic) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).



S7. Maximum likelihood tree for the NP gene segment (1,000 iterations). Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like), dark red (pandemic) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).

S8. Maximum likelihood tree for the NS gene segment (1,000 iterations). Coloured boxes show the main groups for the NS gene as indicated in the figure. Sequences highlighted in black and white letters represent a common ancestor for the whole lineage indicated by the box. Green (avian like), dark red (pandemic) a strain previously isolated in swine that could be used as a more recent reference of the lineage in swine. Sequences in red are those from the present study. Values in the branches show the bootstrap values (only ≥60 are shown).



Chapter 4.
Study 2

Title: Dynamics of influenza A virus transmission in endemic nurseries in a pig farm in Spain before and after vaccination of sows

Authors: Silvana Sosa Portugal, Martí Cortey, Carlos Casanovas, Susana Mesonero-Escuredo, Sergio Barrabés, Anna Vidal, Hepzibar Clilverd, Francesc Illas, Silke Wacheck, Enric Mateu & Gerard Martín-Valls.

Summary

Vaccination of sows is one of the most common strategies for controlling influenza A virus (IAV) in pig farms. The aim of the present study was to assess the dynamics of IAV transmission in nurseries of an endemic farm when using different vaccination schemes for the sows. For this, piglets in the nurseries were followed-up weekly from 3 to 9 weeks of age in three different periods. Firstly, the circulation of the virus was assessed by RT-qPCR before implementing any intervention by sampling the same 50 piglets in 4 consecutive batches (n=200 animals). Then, and based on the IAV strain circulating on the farm, a vaccination protocol was applied using a commercial H1avN1av+H1N2+H3N2 vaccine. All sows were blanket vaccinated and then, half of them received an extra dose three weeks prior to the farrowing date. Ten treatment (blanket + pre-farrowing vaccination) and 10 control sows (only blanket vaccination) were selected in 4 consecutive batches and five piglets/sow were followed weekly (n=400 animals). In a third longitudinal follow-up study, sows were vaccinated with a pandemic H1N1 vaccine with the same scheme as above and animals were followed similarly in 4 batches. In all three cases, nasal swabs were taken weekly and analysed by RT-qPCR and blood samples were collected at 3, 6 and 9 weeks of age to be examined by ELISA. Before the application of the first vaccination scheme, viral shedding was detected in piglets as early as of 4 weeks of age and the virus seemed to circulate all through the sampling period. The virus present in the farm was an H1avN1av. When the first vaccination program was applied, the onset of infection was delayed by two weeks (from the 4^{th} to the 6^{th} week of age) but the circulation of the virus was similar in treatment and control groups. Interestingly, weekly Beta-values increased after vaccination, particularly at six weeks of age, suggesting a faster transmission at that age. Testing of the farm strain with several monospecific antisera showed that anti H1N1pdm09 antiserum reacted stronger against the farm strain than the anti-H1avN1av antiserum against the vaccine. Thus, a H1N1pdm09 vaccine was applied. After the application of the second vaccination scheme, viral circulation started earlier, at the third week of age. Surprisingly, the circulating virus was classified as a H3huN2hu that was probably introduced in the farm after the second follow-up period. In all three follow-up periods, animals testing positive by RT-qPCR in two or more consecutive samplings were detected being more frequent (41.4% of the cases) after the vaccination with the H1avN1av ($p<0.05$). Also, in all periods between 1 and 5% of pigs apparently were re-infected by the same virus according to the RT-qPCR results. The prolonged shedding or the reinfection could not be related

to differences in the levels of the maternally-derived antibodies. In conclusion, blanket vaccination of sows resulted in the delay of the onset of viral circulation in nurseries, suggesting that maternally-derived antibodies were effective but once they disappeared all animals became infected. A considerable proportion of the animals shed virus for more than one week, in apparent contradiction with the canonical knowledge for IAV.

Keywords: Influenza A virus, transmission, vaccination, sows, piglets

1. Introduction

Influenza A virus (IAV) is a major cause of acute respiratory disease outbreaks in pigs. In its epidemic presentation -usually after the introduction of a new virus in a naïve population- an outbreak affecting most of the present pigs takes place. These outbreaks are frequently characterized by high fever and overt respiratory disease typical of flu and, occasionally, abortion in sows (Van Reeth & Vincent, 2019). Often the farm becomes endemically infected afterwards. In this case, piglets from successive batches will show signs of respiratory disease after weaning or in the maternities (Ferreira et al., 2017; Ryt-Hansen et al., 2019a). It is common that, in nurseries or fattening units, influenza happens in concurrence with other respiratory pathogens of the porcine respiratory disease complex having a high impact (Van Reeth & Vincent, 2019). Thus, many farms become infected almost permanently by IAV, either because of the establishment of an endemic cycle due to one or more strains, or due to a new introduction that causes an outbreak likely to become into an endemic situation with time (Pitzer et al., 2016).

Within the endemically infected farms, several studies have shown that a piglet may suffer consecutive infections by the same or very similar virus strains (Rose et al., 2013; Ryt-Hansen et al., 2019a; Simon-Grifé et al., 2012). The causes for this phenomenon are not fully known, but it has been suggested that infection in presence of maternally-derived antibodies (MDA), as it would happen in endemic farms, may result in an impaired development of immunity upon primary infection. This might facilitate a later re-infection (Deblanc et al., 2018; Loeffen et al., 2003). A recent report (Ryt-Hansen et al., 2019b) also suggested that some animals may act as prolonged shedders as revealed by positive RT-qPCR results in two or more consecutive weeks.

Vaccination of sows has shown to significantly reduce the clinical expression of IAV infection in young animals while not fully preventing IAV transmission as virus can still be detected in the respiratory tract of seropositive piglets (Kitikoon et al., 2006). As stated above, those MDA may result in prolonged IAV shedding if the animal got infected (Kitikoon et al., 2006; Loeffen et al., 2003; Rose et al., 2013). These circumstances may lead to a silent spread of the virus in the first weeks of life, which could partly explain the recurrence of epidemics after passive immunity waning (Loeffen et al., 2003).

Nevertheless, it has been shown that IAV infection and circulation in the nurseries may be decreased by using adequate sow vaccination protocols particularly when the vaccine matches very closely to the field strain (Chamba-Pardo et al., 2019). Considering the positive effect on the clinical expression of the infection and the potential for decreasing viral circulation, vaccines are still the most effective tools for controlling IAV. Unfortunately, very few commercial vaccines are available and matching antigenically the vaccine and the field strain is not always possible, particularly because of the constant expanding genetic diversity and the co-circulation of multiple lineages in a particular setting. The aim of the present study was to assess the impact on the virus transmission dynamics in the offspring of sows under two vaccination schemes using commercial vaccines.

2. Materials and Methods

2.1.Characteristics of the selected farm

The selected farm was located in the north-eastern part of Catalonia. It was an all-in/all-out farrow-to-wean farm with 2,500 Duroc sows. The farm operated in weekly batches with an average of 120 parturitions and 1,500 piglets flowing to nurseries every week. Gilts belonged to the same company but were shipped from a different site. The farm was historically PRRSV-free and respiratory signs compatible with IAV infection were recurrently observed in different batches of nursery piglets. The study started in February 2018 when nasal swabs were collected from the maternities and nurseries, and IAV was detected and confirmed by RT-PCR in both phases. After subtyping of positive samples (see the RT-qPCR section below), an H1avN1av was detected in all cases. This farm did not vaccinate against IAV.

In regards to the biosecurity measures followed in the farm, it had a clear differentiation between the clean and dirty areas, it had docks for loading and unloading, an area for visitors, and locker rooms with a separation between the clean and dirty areas. Visitors must fully change their clothes before going into the rooms, and boots must be changed as well when going between phases.

2.2.Study design

The study properly said took place from April 2018 to December 2019 and comprised three follow-up periods in the same endemically infected farm: a) period 1, before implementing any control measures; b) period 2, after implementing a vaccination program with a commercial trivalent vaccine (H1N1+H1N2+H3N2) and, c) period 3, after vaccination with a pandemic H1N1 vaccine. In each follow-up period nasal swabs were weekly taken from the piglets between 3 and 9 weeks of age, and blood samples were collected every three weeks (3-6-9 weeks of age).

2.3.First follow-up period

The purpose of the first longitudinal study was the assessment of the viral circulation of IAV in the nurseries before the implementation of any control measures. In this case 50 piglets/batch were followed in 4 consecutive batches (n=200 animals).

2.4.Second follow-up period

After completion of the first follow-up, a vaccination scheme was implemented in the sows using a trivalent whole virus inactivated vaccine including the Haselunne/IDT2617/2003 (H1N1), Bakum/IDT1769/2003 (H3N2) and Bakum/1832/2000 (H1N2) strains (commercial name Respiporc FLU3®). This vaccine is marketed for use in pigs from the age of 56 days onwards, including pregnant sows.
For this part, all sows were blanket vaccinated. Afterwards, half of them received an extra dose three weeks before farrowing (treatment group) while the other remained with the blanket vaccination only (control group). In this period, 4 consecutive farrowing batches were followed-up. In each batch five piglets of 10 treatment and 10 control sows were ear-tagged and weekly followed (n=400 animals).

2.5.Third follow-up period

Since vaccination with the first vaccine did not fully stop viral circulation in nurseries, the reactivity of the sera obtained from 9-week-old piglets that tested PCR positive at 5 weeks of age (n=2) was tested against the farm strain by the haemagglutination inhibition test (OIE, 2018). Also, the farm strain was tested against the monospecific H1avN1av vaccine antiserum and against a pdmH1N1 monospecific antiserum. Somewhat

surprisingly, the anti pdmH1N1 was the serum that produced the highest titre. Thus, it seemed appropriate to use a H1N1 pandemic vaccine for the third longitudinal study (a whole virus inactivated vaccine marketed under the name of Respiporc FLUpan™ H1N1, strain A/Jena/VI5258/2009(H1N1)pdm09. This vaccine is marketed to be used for the active immunisation of pigs from the age of 56 days onwards against.

For this last follow-up, vaccination was performed as before but with the pdmH1N1 vaccine. Ten sows per group and batch were selected and five piglets per sow were ear-tagged and weekly followed in 4 consecutive farrowing batches (n=400 animals).

2.6.Sampling

Nasal swabs were taken and kept at 4ºC in a virus transport medium (Virocult®, Sigma-Aldrich), being immediately processed upon arrival. Blood was collected from *vena cava cranialis* using blood collection tubes (BD Vacutainer®). Serum was frozen at −80°C until further analysis.

2.7.Processing of nasal swabs by RT-qPCR

Upon arrival to the laboratory, the swabs in the Virocult® transport media were vortexed and then tubes were centrifuged at 4,000 g for 5 min. The supernatant was recovered, aliquoted and frozen at -80°C for further processing. Initially, pools of two and three samples per litter (100 µl of supernatant) were made for the RNA extraction. Extraction was performed using the MagMAX™ CORE Nucleic Acid Purification Kit in a KingFisher Flex robot (ThermoFisher scientific). Detection of IAV in the pooled samples was performed by means of a RT-qPCR targeted to the IAV matrix gene (Busquets et al., 2010) using the AgPath-ID™ One-Step RT-PCR reagents (ThermoFisher scientific). Sampes from positive pools were recovered individually and RNA extraction and RT-PCR was performed as above. From these positive animals, samples that yielded a Cq value <30, were selected for a subtyping RT-qPCR identifying subtypes and European lineages including: H1av, H1hu, H1pdm, H3hu, N1av, N1pdm and N2hu (Henritzi et al., 2016, 2019).

2.8.Incidences and estimation of the transmission rates

The prevalence and the cumulative incidence (CI) of the infection were calculated based on the number of positive animals from each sampling time. CI was calculated as the

number of new infections observed at a given sampling point divided by the number of negative individuals present at the previous sampling week (susceptible pigs).
Beta values (β) were calculated based on the average number of individuals that were newly infected from one infectious individual per unit of time. Values were calculated separately for every group and pen. The duration of the infectious period for IAV was calculated in two different ways: a) an average of 6 days for all animals or, b) a weighed value considering 6 days for animals testing positive in just one sample, 10.5 when they tested positive in two consecutive samples (one week plus half a week) and 17.5 for animals testing positive in three consecutive samples (2 weeks plus half a week).

2.9.Viral isolation and next generation sequencing

Madin-Darby canine kidney (MDCK) cells (ATCC CRL-2936™) were used for the isolation of selected IAV-positive samples that yielded a Cq <30 after the RT-qPCR-positive (M gene). For this, 100 µl of the nasal swab supernatant was mixed with 900 µl of Minimum Essential Medium Eagle (MEM) supplemented with L-glutamine (200 mM) and penicillin-streptomycin (10,000 units and 10 mg/mL, respectively). Trypsin TPCK was added to treat the samples and obtain a final concentration of 10 µg/ml of trypsin. Trypsin-treated samples were inoculated in 25cm^2 cell culture flasks. After 1.5 h of incubation at 37ºC, the flasks were filled with 9 ml of medium without trypsin (final concentration of trypsin in the culture 1µg/ml) and cultures were incubated for up to 5 days. Cells were observed every 24 hours to check for the development of cytopathic effect (CPE). Once CPE was observed flasks were frozen at -80ºC, thawed and, after centrifugation, the cell culture supernatant was examined by qRT-PCR (M gene) to confirm the replication of the virus (lower Cq values compared to the initial inoculum). If CPE was not observed, blind passages were performed. Samples were discarded when CPE was not observed after a third passage.

Ten isolates (1 from the pre-vaccination period and 9 for the second follow-up; 7 from animals having prolonged shedding and two having short-one week-shedding) were fully sequenced using next generation sequencing (NGS) Illumina® MiSeq platform at Parc de Genòmica i Recerca-UAB. Extraction of viral RNA was done as explained above. The output reads in fastaq format (doubled paired) were checked for quality using Trimmomatic (matching of forward and reverse sequences and quality index > 20). Next,

high quality reads were filtered using IAV sequence references belonging to all known lineages for each of the eight genome segments. Consensus were generated using the Simple Consensus maker tool (https://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html) when a viral quasi-species was obtained. The segments were then manually aligned and trimmed using BioEdit sequence alignment editor for windows (Hall, 1999). When filtering did not yield a viral quasi-species, consensus sequence for that gene was forced using QuASAR software (https://github.com/piquelab/QuASAR). The resulting consensus sequence was blasted against available sequences in GenBank and the closest sequence was used to re-filter the original fastaq file.

The level of molecular differentiation per segment at the quasi-species level was inferred with an analysis of molecular variance (AMOVA, Excoffier et al. 1992), using the software Arlequin v.3.5 (Excoffier & Lischer 2010). The model estimated the differentitation for every nucleotide position in every segment at three levels, among groups, among quasi-species within groups and within quasi-species. The tested model considered all samples separately, one sample per group.

2.10. ELISA

Antibody detection and quantification was done through indirect ELISA using the commercial kit Civtest® Suis influenza (Laboratorios Hipra S.A.). To evaluate the declining of maternally derived antibodies, these determinations were performed on all negative animals of the basal circulation. Also, to determine the effect of the vaccination protocol, a selection of no less than 50 animals at three weeks of age for both control and treatment groups from the two follow-ups after vaccination were evaluated. Finally, a selection of 210 and 50 animals infected in presence or absence of MDA respectively, 83 animals with prolonged shedding and 20 animals with repeated infection were ELISA evaluated at the moment of infection and in the next sampling for evaluating if there was an effect of the serological status at the moment of the infection in terms of duration of the shedding and on seroconversion.

3. Results

3.1.Incidence of IAV infections and beta-values in the three follow-up periods.

Figure 1 shows the evolution of cumulative incidence in each of the follow-up periods as determined in each pen. In the first follow-up period before the implementation of any control program, circulation of the virus was very uneven in some pens where circulation started at 3 weeks of age and others where it started between the 5th and the 7th week of age. Similarly, the slope of the epidemic curve was uneven. In general, when infection started earlier, the slope was smoother compared to that of pens where animals were firstly detected as infected at 7 weeks of age. Sequencing of one isolate indicated that the virus circulating in the farm was an H1avN1av lineage genotype A (sequences submitted to Genbank as MN934396, MN434040, MN433959, MN932181, MN933822, MN932231, MN933489, MN939524). The similarity (amino acid) of this virus to the H1avN1av vaccine used in the second follow-up period was 92.5% for the HA, 90.0% for NA, 86.6% for NS and ranged from 95 to 97% for all other genes.

After the identification of the IAV circulating strain, the vaccine program and the second follow-up period started. The analysis of the cumulative incidence per pen in that second period showed that after the implementation of the program, infections before the 5th week of age were uncommon. Interestingly in the control group (only blanket vaccination of sows) the behaviour of the incidence in different pens was a little less homogeneous than in the treatment group, where infection had the same behaviour in all but one pen (Figure 1B).

Interestingly the examination of beta values per week (Figure 2), clearly showed the changes in the epidemiologic patterns before and after the implementation of the vaccination program. While in the pre-vaccination follow-up period all but two beta values were below 7.5, and most of them were below 5.0. After vaccination, and regardless of the group, about one third of the beta values were above 7.5. Most of the higher beta values concentrated at the 6th week of age. These results indicated that the infection was being displaced to older ages but the speed transmission increased at that moment.

Given the results of the first vaccination program where viral circulation could not be fully stopped, we decided to re-evaluate the antigenic correspondence of the circulating IAV strain with the vaccine. For that purpose, monospecific antisera against the vaccine H1avN1av and a monospecific antisera against A/Jena/VI5258/2009(H1N1)pdm09 strain were tested against the farm strain. Unexpectedly, the anti-AH1N1pdm09 reacted stronger (1:320 with the farm strain than the anti H1avN1av, 1:40) and, accordingly, it was decided to change the vaccine in use in the farm (third follow-up study).

The examination of the cumulative incidence in that third period very soon revealed a different pattern compared to the previous batches. In this case, in many pens, incidences of 40-100% were reached as soon as at the 4^{th}-5^{th} week of age. To gain understanding in the situation, 74 RT-qPCR positive samples were subtyped and all the examined ones corresponded to a virus that, when sequenced (NGS) was derived from a seasonal H3huN2hu (GenBank accession nº KR701344) different than the common swine A/Swine/Ghent/1984/H3huN2hu.

This finding gave us the opportunity to examine the epidemiologic pattern of a newly introduced strain. The analysis of beta values showed a significant difference between control and treated groups. While in pens containing treatment animals 3/32 (9.4%) of the calculated Beta-values were above 7.5, in the control pens 13/35 (37.1%) of the calculated Beta-values were above 7.5 (p=0.02) (Figure 2C).

3.2.Viral shedding and serology

3.2.1. RT-qPCR

In all follow-up periods, some animals tested positive to IAV by RT-PCR in nasal swabs in two or more consecutive sampling times (from now on designated as “prolonged shedders”). During the first follow-up (pre-vaccination scheme), 30.6% of the infected animals tested positive in at least two consecutive weeks (30/98, $CI_{95\%}$: 21.9-40.9%). This proportion was 41.4% in the second follow-up (145/350, $CI_{95\%}$: 36.2-46.8%, no significant differences between treatments and controls). In the third follow-up period, when the H3N2 strain was introduced in the farm, the pattern changed. The overall proportion of prolonged shedders for that third period was 11.0% (29/264, $CI_{95\%}$: 7.6-

15.6%) with no significant differences between treatments and controls. Comparison of the three periods showed significant differences between all three periods ($p<0.001$). When the weighed excretion period was calculated, taking into account the results above, it resulted in 7.4 days (1.06 weeks) in the first follow-up, 7.8 days (1.11 weeks) in the second follow-up period (first vaccination trial) and 6.5 days (0.92) for the third vaccination trial.

Next, we examined the viral loads of nasal swabs with regards to the period, group and considering if the sample was the first positive test, the second or the third positive sample for a given animal. Figure 3 summarises the Ct values obtained from nasal swabs in first, second and third positive samples. Briefly, before applying any vaccination, first positive samples averaged a Ct of 23.4 and second positive samples had an average Ct of 27.7 ($p<0.05$). In the second follow-up period (first vaccination trial), first positive samples averaged a Ct of 27.5 vs. 30.0 for second positive samples (non-significant) while in the third period of study average values were 28.5 for the first positive samples and 30.7 for the second positive samples (non-significant). The viral load was significantly higher ($p<0.05$) in the first period compared to the other two. No differences were observed between treatments and controls in none of the two vaccination trials. Although it was not significant, it is worth to note that for the few animals that tested positive in three consecutive samplings in the first period or for the controls in the second, the third sample usually yield a low Ct comparable to the first one (Figure 3).

Sequencing of nine isolates retrieved after the implementation of the H1avN1av vaccination revealed some changes (supplementary material S1) with regards to the pre-vaccination isolate but no significant different were observed between samples from treatments and controls or from short term or prolonged shedders.

Furthermore, some pigs tested positive for IAV at non-consecutive sampling times separated by two or three weeks. This category of animals accounted for 1.5%, 2.3% and 5.8% in the first, second and third follow-up studies (non-significant).

3.2.2. Serology

Then, we examined the presence of antibodies in a sample of the followed animals. At three weeks of age, most animals were seropositive with high S/P ratios. Figure 4 shows the distribution of S/P values in the different follow-up periods. Interestingly, the average S/P at three weeks of ages before implementing any vaccination program was higher than that of piglets in follow-up periods 2 and 3 when vaccination of sows was applied (92.5 vs. 71.3 vs. 50.7, $p<0.05$). To note, when the trivalent vaccine was applied no difference was seen between antibody levels in treatment or control animals while when the H1N1pdm vaccine was used, the average S/P ratio of treatment animals was higher than that of controls (62.6 vs. 32.4, $p<0.05$). No significant differences were found for the proportion of seropositive and seronegative animals in ELISA or the S/P values with regards to the fact that a given animal was a prolonged shedder or was re-infected later in life.

4. Discussion

Control of IAV in pig farms is difficult. At present, and in spite of its limitations, vaccination is still the main tool for controlling the infection whether applied to breeders or to weaners/fatteners. Vaccination of sows is aimed to limit the potential impact of an outbreak on the reproductive performance of the herd, as well as with the intention to promote transfer of passive immunity to piglets. In endemic farms, where transmission in nurseries is particularly important, that transfer of MDA might be of interest. The aim of the present study was to test the effect of vaccination of sows on the dynamics of the IAV infection in nurseries.

Our study farm had a historical record of influenza with circulation of the virus in the nurseries. The study started with the assessment of the circulation of the virus before the intervention. The results showed that an H1avN1av virus (genotype A) was present in all examined animals. This lineage and genotype is the second most common in Spain (Sosa Portugal et al., 2020). The pattern of viral circulation in nurseries before intervention was very diverse. In some pens, circulation started at 4 weeks of age, in others at any other moment until the 7th week of age. This probably reflected both, the diverse immune status of the sows and the stochastic components of transmission. The assessment by ELISA of the MDA levels in 3-week-old piglets showed that most piglets had high S/P ratios

(consistent with the transfer of antibodies from sows that had been infected at some moment) with a proportion of animals with low S/P ratios (because inadequate colostrum intake or because they were the offspring of uninfected sows). Weekly beta values rarely surpassed 7.5 and mostly were below 5 in agreement with previous observations in endemic farms (Pileri et al., 2017).

Introduction of the vaccination program with the commercial inactivated trivalent vaccine containing an H1avN1av strain produced an evident change in the transmission pattern. The vaccination program consisted in a first blanket vaccination (all sows) followed by a pre-farrowing vaccination in the treatment group. This program resulted in a practical cease of viral circulation before 5-6 weeks of age, regardless of whether the sows received blanket and pre-farrowing doses or just a blanket vaccination. This result could suggest that circulation of the H1avN1av virus was reduced or even abolished in the sows' stock. In that scenario, piglets would be mostly infected from the environment or from infected pen mates in nurseries and little, or nothing, from the sows or in maternities.

The examination of the antibody levels at 3 weeks of age showed that both control and treatment piglets had similar levels of MDA and that these levels were lower than those of pre-vaccination batches. This was somewhat surprising since pre-farrowing vaccination would have been expected to increase MDA transfer. This would be consistent with the hypothesis that the blanket vaccination of sows reduced transmission of the H1avN1av in breeders. A decreasing rate of infection in sows would result in that most of the sows' immunity would be produced only by vaccination but not by infection as before. Considering that since the start of the trial to the end of the second follow-up period several months passed, a large proportion of sows would have been replaced (replacement rate in the farm was close to 50% per year). As a consequence, the sow population would be more homogeneous (blanket vaccination) but with lower immunity than if acquired by infection.

In this comparison between the first and second follow-up periods beta values were also different. In both treatments and controls, the calculation of beta values frequently surpassed 7.5 or 10. In other words, while before vaccination the spread of the infection was fast, but in many farms persisted for some weeks, after vaccination the behaviour became more homogeneous and explosive with most of the infections taking place in just

one week. This would suggest a role of MDA in delaying the onset of the infection but also, since MDA were more homogeneous and lower on average than before, passive protection would probably disappear in all animals more or less synchronically, favouring an explosive transmission either from undetected infected animals or by infection from the premises.

Given the limited impact of the first vaccination program, we decided to test the reactivity of the antiserum raised against the vaccine H1avN1av in front of the farm H1avN1av. The result showed that an anti-vaccine hyperimmune serum with a titre of 1:320 only reacted to 1:40 with the farm isolate in the haemagglutination inhibition test in spite of the close similarity in the HA genes. In a second step, we retested the farm strain with an anti-H1N1pdm09 serum and, surprisingly the pdm09 antiserum produced similar results against the farm strain than against itself. This pdm09 antiserum was shown to have no significant reactivity with the vaccine H1avN1av. This result was surprising but there are several explanations characteristic of avian-like and pandemic H1 that could explain this result. On one hand, cross-reaction and also cross-protection between H1av and H1pdm have been previously described (Busquets et al., 2010; Kyriakis et al., 2010). On the other hand, there is a high antigenic heterogenicity within H1av (Lewis et al., 2016), sometimes related with the farm of origin (de Jong et al., 2001), and in some cases complete lack of cross-reactivity (Martin-Valls et al., 2014).

According to the hemagglutination inhibition results it was decided to start a second vaccination trial using a H1N1pdm09 strain (third follow-up period). Very soon after the implementation of this second vaccination scheme it was evident that the pattern of viral circulation in the nurseries changed again showing a very early viral circulation. In some cases, 40-60% of the three-week old piglets were already positive by RT-qPCR. This was an indication of a likely infection of sows. Analysis of the circulating virus showed that it was now related to a seasonal H3huN2hu. This was a proof of either an introduction of a new strain or of the emergence of a virus that was hidden in the background. Considering the results of the second follow-up period, if the circulation of the original H1avN1av virus in sows would have been stopped by the vaccination, this would have left a void for a new IAV. We believe that this is a likely hypothesis. The new H3huN2hu has been detected circulating in Europe in the last three years (Krog et al., 2017).

When levels of MDA were examined, they were lower in this third period compared to the previous ones. Also, there were differences between the offspring of control and treatment sows. This result is difficult to interpret but could be related to the test used. As a matter of fact, it has been described that ELISAs, in spite of using a nucleocapsid antigen, may preferentially detect antibodies against certain subtypes or lineages.

In all three follow-up periods, a large proportion were positive to the virus for two or three consecutive weeks. This prolonged and recurrent shedding has been reported in other recent studies, as well as the possibility of re-infection with the same strain (Chamba-Pardo et al., 2019; Ferreira et al., 2017; Ryt-Hansen et al.., 2019a). The causes for this are not clear but several studies (Allerson et al., 2013; Loeffen et al., 2003; Rose et al., 2013) indicated that infection in the presence of MDA may increase the duration of the shedding period and may impair the development the active immunity. This could be an explanation; however, in our case when S/P ratios at either 3 weeks of age or at the moment of infection (only for 6-week-old animals) were compared, significant differences were not found. Certainly, the use of ELISA may not be adequate enough to make a prediction of protection or interference with the development of active immunity but, in principle, it should suffice to show the level of MDA. Beyond this, it has to be taken into account, that IAV never leaves the respiratory tract and, therefore, assessment of the IgA levels in the respiratory tract could be more meaningful than results of blood serology. On the other hand, based on sequencing results, virus found in nasal swabs of prolonged shedders could not be identified as escape mutants based on changes in the HA or NA genes.

So, a clear correlation with MDA and prolonged shedding cannot be raised from the present study and, in our opinion, this needs to be clarified in further studies. Firstly, if presence of MDA may result in prolonged shedding, this can be detrimental for the control of the infection, resulting in higher R values. Secondly, the fact that the Ct values of the second positive samples were consistently lower (>30) before the implementation of any vaccination, open the question on the biological significance of this shedding. In a previous study (Sosa Portugal et al., 2020), isolation of IAV from samples with Ct >30 was difficult suggesting a low amount of viable virus in such samples. Third, the fact that vaccination of sows reduced the viral load (in both first and second positive samples in

the first vaccination trial and for second positive samples in the second vaccination trial), suggest that antibodies may have a role in controlling this phenomenon.

Interestingly, the proportion of prolonged shedders was also different in the different periods being higher when the first vaccination was applied and lower in the third follow-up period when the H3N2 virus was introduced. This is difficult to interpret but could not be directly related to the levels of MDA antibodies at 3-weeks of age, the earliest determination. However, because ELISA determines anti-nucleocapsid antibodies the role of neutralizing MDA cannot be fully discarded. It is also possible that this phenomenon could be dependent on the strain.

Noteworthy, animals testing positive for a third time often had again a low Ct. This, together with the fact that a proportion of animals (1-5%) tested positive by RT-qPCR in two separated weeks suggest that it is possible that some animals got re-infected shortly after the first infection or that the shedding period can be for even longer than 2 weeks.

5. Conclusion

The present study highlights the complex epidemiology of IAV in endemic pig farms and the little understood role of MDA in protection but also in the potential interference with the development of active immunity. Also, it suggests that vaccination of sows may displace infection from sows to nurseries; if so, the role of environment as a source of infection could be critical. Continuous monitoring of the farm is needed since new strains can be introduced with some frequency. Understanding of the epidemiology of swine IAV is of relevance for swine and public health.

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7. Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required.

8. Conflict of interest statement

No competing interests.

References

1. Allerson, M., Deen, J., Detmer, S. E., Gramer, M. R., Joo, H. S., Romagosa, A., & Torremorell, M. 2013. The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations. *Vaccine*, 31:500–505. https://doi.org/10.1016/j.vaccine.2012.11.023
2. Busquets, N., Segalés, J., Córdoba, L., Mussá, T., Crisci, E., Martín-Valls, G. E., Simon-Grifé, M., Pérez-Simó, M., Pérez-Maíllo, M., Núñez, J. I., Abad, F. X., Fraile, L., Pina, S., Majó, N., Bensaid, A., Domingo, M., & Montoya, M. 2010. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. *Vet Res*, 41: 74. https://doi.org/10.1051/vetres/2010046
3. Chamba Pardo, F. O., Wayne, S., Culhane, M. R., Perez, A., Allerson, M., & Torremorell, M. 2019. Effect of strain-specific maternally-derived antibodies on influenza A virus infection dynamics in nursery pigs. *PLoS One*, 14: 1–15. https://doi.org/10.1371/journal.pone.0210700
4. de Jong, J. C., Heinen, P. P., Loeffen, W. L. A., Van Nieuwstadt, A. P., Claas, E. C. J., Bestebroer, T. M., Bijlsma, K., Verweij, C., Osterhaus, A. D. M. E., Rimmelzwaan, G. F., Fouchier, R. A. M., & Kimman, T. G. 2001. Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. *Vaccine*, 19: 4452–4464. https://doi.org/10.1016/S0264-410X(01)00190-6
5. Deblanc, C., Hervé, S., Gorin, S., Cador, C., Andraud, M., Quéguiner, S., Barbier, N., Paboeuf, F., Rose, N., & Simon, G. 2018. Maternally-derived antibodies do not inhibit swine influenza virus replication in piglets but decrease excreted virus infectivity and impair post-infectious immune responses. *Vet Microbiol*, 216: 142–152. https://doi.org/10.1016/j.vetmic.2018.01.019
6. Excoffier, L., & Lischer, H.E. L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour,* 10: 564-567.

7. Excoffier, L., Smouse, P. E., & Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics,* 131: 479-91.
8. Ferreira, J. B., Grgić, H., Friendship, R., Wideman, G., Nagy, É., & Poljak, Z. 2017. Longitudinal study of influenza A virus circulation in a nursery swine barn. *Vet Res,* 48: 1–16. https://doi.org/10.1186/s13567-017-0466-x
9. Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser,* 41: 95-98.
10. Henritzi, D., Hoffmann, B., Wacheck, S., Pesch, S., Herrler, G., Beer, M., & Harder, T. C. 2019. A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza Other Respir Viruses,* 13: 71–82. https://doi.org/10.1111/irv.12613
11. Henritzi, D., Zhao, N., Starick, E., Simon, G., Krog, J. S., Larsen, L. E., Reid, S. M., Brown, I. H., Chiapponi, C., Foni, E., Wacheck, S., Schmid, P., Beer, M., Hoffmann, B., & Harder, T. C. 2016. Rapid detection and subtyping of European swine influenza viruses in porcine clinical samples by haemagglutinin- and neuraminidase-specific tetra- and triplex real-time RT-PCRs. *Influenza Other Respir Viruses,* 10: 504–517. https://doi.org/10.1111/irv.12407
12. Kitikoon, P., Nilubol, D., Erickson, B. J., Janke, B. H., Hoover, T. C., Sornsen, S. A., & Thacker, E. L. 2006. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. *Vet Immunol Immunopathol,* 112: 117–128. https://doi.org/10.1016/j.vetimm.2006.02.008
13. Krog, J. S., Hjulsager, C. K., Larsen, M. A., & Larsen, L. E. 2017. Triple-reassortant influenza A virus with H3 of human seasonal origin, NA of swine origin, and internal A(H1N1) pandemic 2009 genes is established in Danish pigs. *Influenza Other Respir Viruses,* 11: 298–303. https://doi.org/10.1111/irv.12451
14. Kyriakis, C. S., Olsen, C. W., Carman, S., Brown, I. H., Brookes, S. M., Doorsselaere, J. V., & Reeth, K. V. 2010. Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. *Emerg Infect Dis*, 16: 96–99. https://doi.org/10.3201/eid1601.091190
15. Lewis, N. S., Russell, C. A., Langat, P., Anderson, T. K., Berger, K., Bielejec, F., Burke, D. F., Dudas, G., Fonville, J. M., Fouchier, R. A., Kellam, P., Koel, B. F., Lemey, P., Nguyen, T., Nuansrichy, B., Peiris, J. M., Saito, T., Simon, G., Skepner,

E., Takemae, N., … Vincent, A. L. 2016. The global antigenic diversity of swine influenza A viruses. *Elife*, 5: e12217. https://doi.org/10.7554/eLife.12217

16. Loeffen, W. L. A., Heinen, P. P., Bianchi, A. T. J., Hunneman, W. A., & Verheijden, J. H. M. 2003. Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. *Vet Immunol Immunopathol,* 92: 23–35. https://doi.org/10.1016/S0165-2427(03)00019-9
17. Martín-Valls, G. E., Simon-Grifé, M., van Boheemen, S., de Graaf, M., Bestebroer, T. M., Busquets, N., Martín, M., Casal, J., Fouchier, R. A. M., & Mateu, E. 2014. Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm. *Vet Microbiol*, 170: 266–277. https://doi.org/10.1016/j.vetmic.2014.02.031
18. OIE (World Organization for Animal Health). 2018. Manual for diagnostic tests and vaccines for terrestrial animals. Available at: https://www.oie.int/en/standard-setting/terrestrial-manual/access-online/
19. Pileri, E., Martín-Valls, G. E., Díaz, I., Allepuz, A., Simon-Grifé, M., García-Saenz, A., Casal, J., & Mateu, E. 2017. Estimation of the transmission parameters for swine influenza and porcine reproductive and respiratory syndrome viruses in pigs from weaning to slaughter under natural conditions. *Prev Vet Med,* 138: 147-155. doi:10.1016/j.prevetmed.2017.01.008
20. Pitzer, V. E., Aguas, R., Riley, S., Loeffen, W. L., Wood, J. L., & Grenfell, B. T. 2016. High turnover drives prolonged persistence of influenza in managed pig herds. *J R Soc Interface,* 13: 20160138. https://doi.org/10.1098/rsif.2016.0138
21. Rose, N., Hervé, S., Eveno, E., Barbier, N., Eono, F., Dorenlor, V., Andraud, M., Camsusou, C., Madec, F., & Simon, G. 2013. Dynamics of influenza A virus infections in permanently infected pig farms: evidence of recurrent infections, circulation of several swine influenza viruses and reassortment events. *Vet Res*, 44: 72. https://doi.org/10.1186/1297-9716-44-72
22. Ryt-Hansen, P., Larsen, I., Kristensen, C. S., Krog, J. S., Wacheck, S., & Larsen, L. E. 2019a. Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies. *Vet Res*, 50: 1–10. https://doi.org/10.1186/s13567-019-0655-x
23. Ryt-Hansen, P., Pedersen, A. G., Larsen, I., Krog, J. S., Kristensen, C. S., & Larsen, L. E. 2019b. Acute Influenza A virus outbreak in an enzootic infected sow herd:

Impact on viral dynamics, genetic and antigenic variability and effect of maternally derived antibodies and vaccination. *PLoS One*, 14: 1–23. https://doi.org/10.1371/journal.pone.0224854

24. Simon-Grifé, M., Martín-Valls, G. E., Vilar, M. J., Busquets, N., Mora-Salvatierra, M., Bestebroer, T. M., Fouchier, R. A., 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. https://doi.org/10.1186/1297-9716-43-24
25. Sosa Portugal, S., Cortey, M., Tello, M., Casanovas, C., Mesonero-Escuredo, S., Barrabés, S., Pineda, P., Wacheck, S., Martín-Valls, G., & Mateu, E. 2020. Diversity of influenza A viruses retrieved from respiratory disease outbreaks and subclinically infected herds in Spain (2017-2019). *Transbound Emerg Dis*, 10.1111/tbed.13709. Advance online publication. https://doi.org/10.1111/tbed.13709
26. Van Reeth, K., & Vincent, A. 2019. Influenza viruses. In: Diseases of Swine. Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, Zhang J (editors). Wiley and Sons Inc. Hoboken, NJ, USA. 576-593 pp.

Figure 1. Cumulative incidence (CI) shown per pen over a period of seven weeks. A) Basal values prior to the application of any vaccination scheme. B) CI after the application of the first vaccination scheme; Group that received an extra dose (left); group that only received blanket vaccination (right). C) CI after the application of the second vaccination scheme. Group that received an extra dose (left); group that only received blanket vaccination (right).

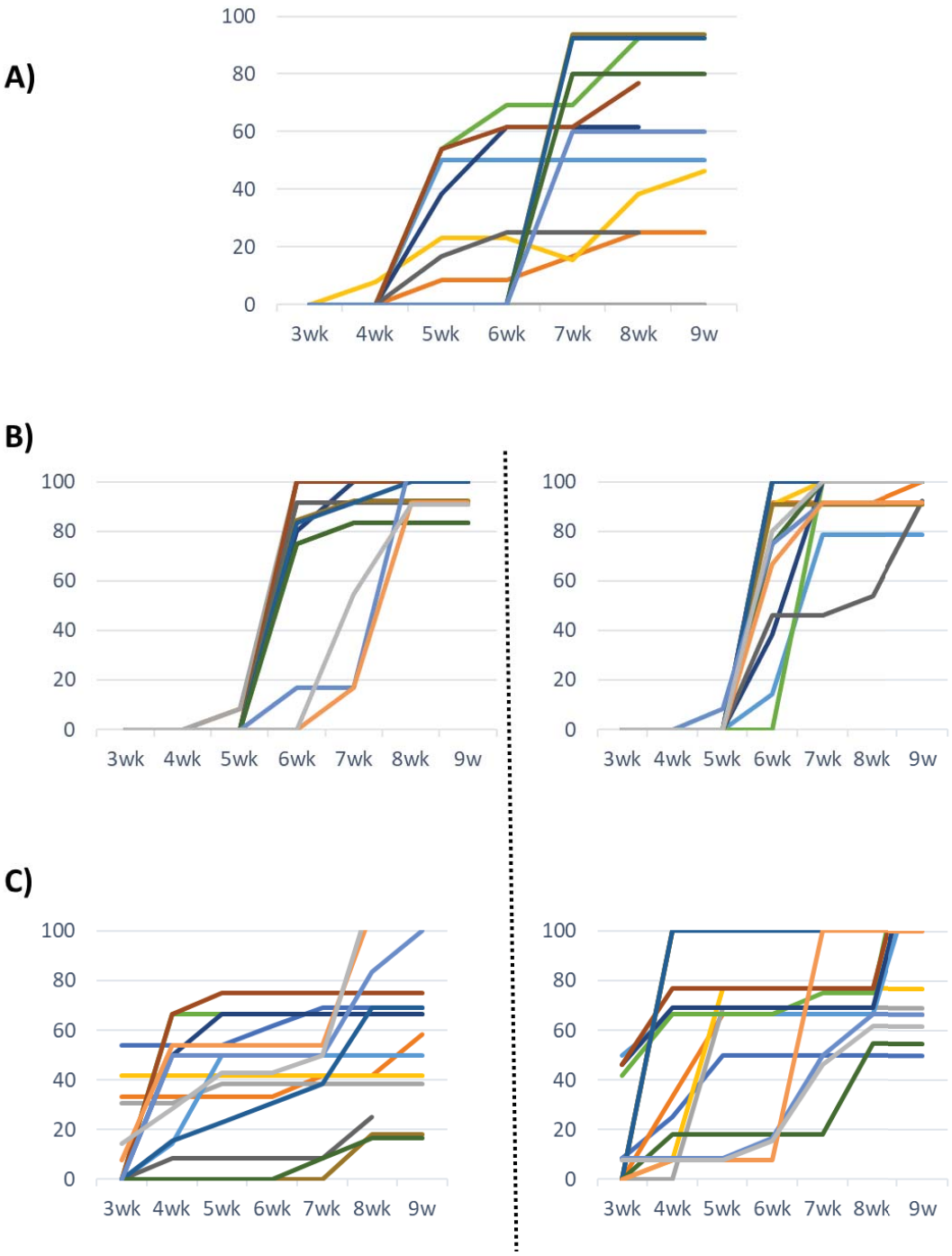

Figure 2. Distribution of beta values calculated per pen over a period of seven weeks A) Prior to the application of any vaccination. B) After the application of the first vaccination scheme; Treatment group: top, green circles; control group: bottom, red circles. C) After the application of the second vaccination scheme. Treatment group: top, green circles; control group: bottom, red circles.

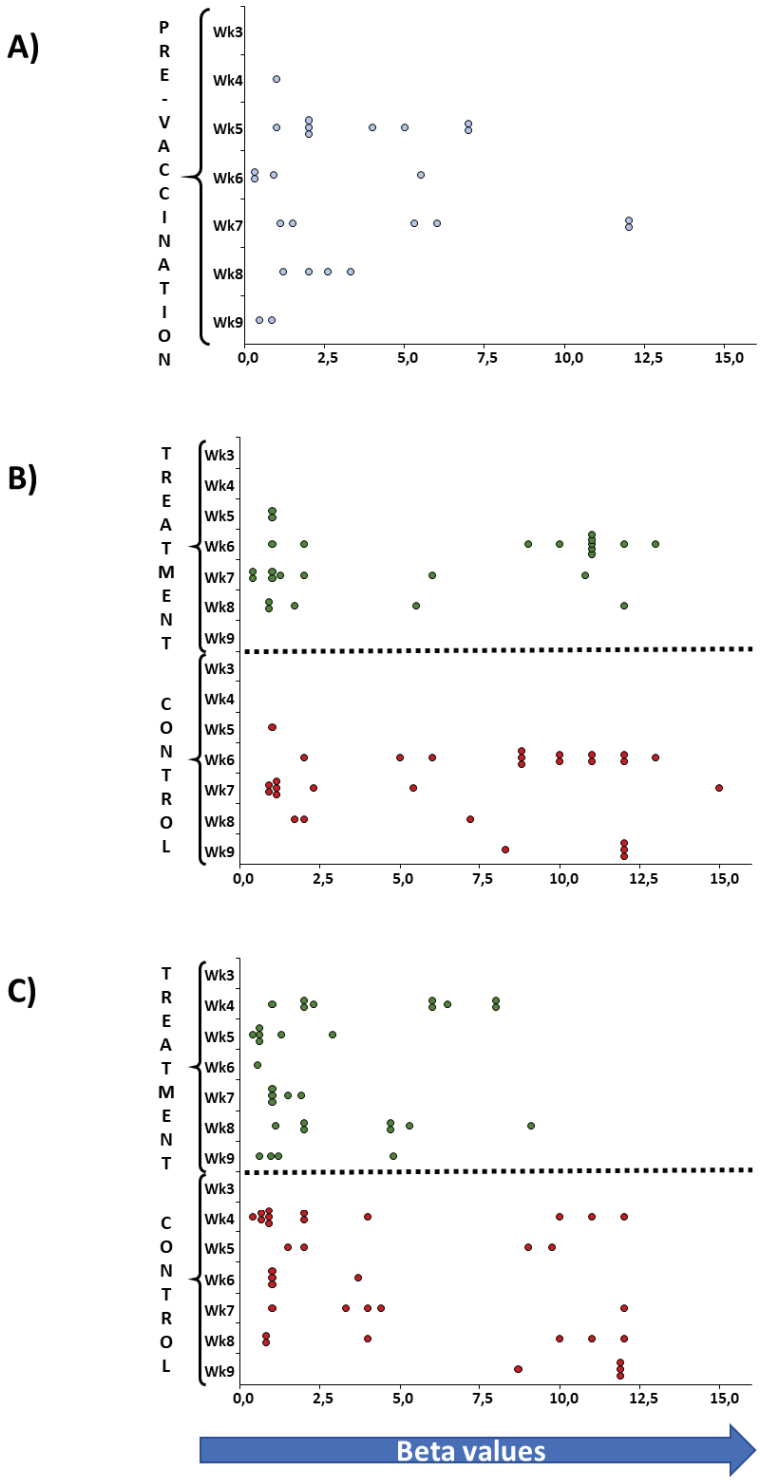

Figure 3. Ct values for different positive sampling times in prolonged shedders.
The panel shows box and whisker plots of the Ct values (minimum, 25% quartile, median, 75% quartile and maximum) for nasal swabs of animals that tested positive by RT-qPCR more than once (up to three times). A) Prior to the any vaccination protocol; B) After the first vaccination scheme; C) After the second vaccination scheme. For B and C, coloured boxes show the results of the offspring of the sows that received an extra dose before farrowing (treatment), and non-coloured boxes of those who did not (control).

A
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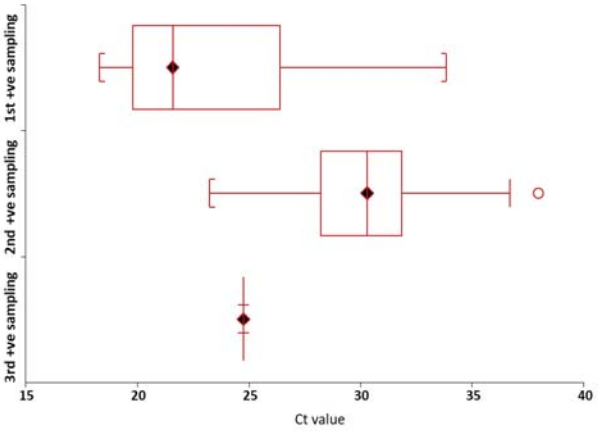

B
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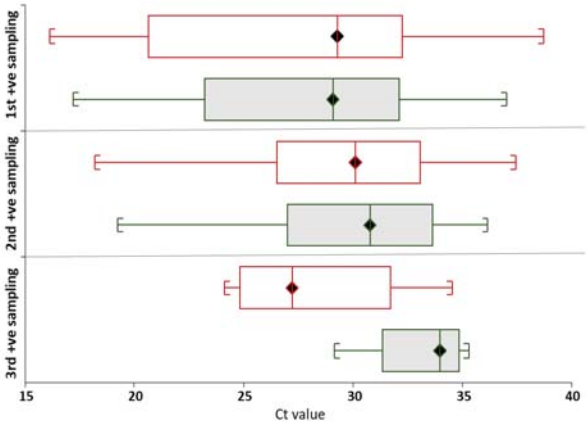

C
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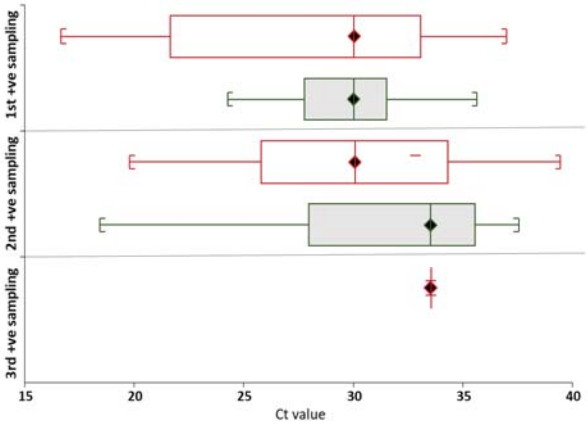
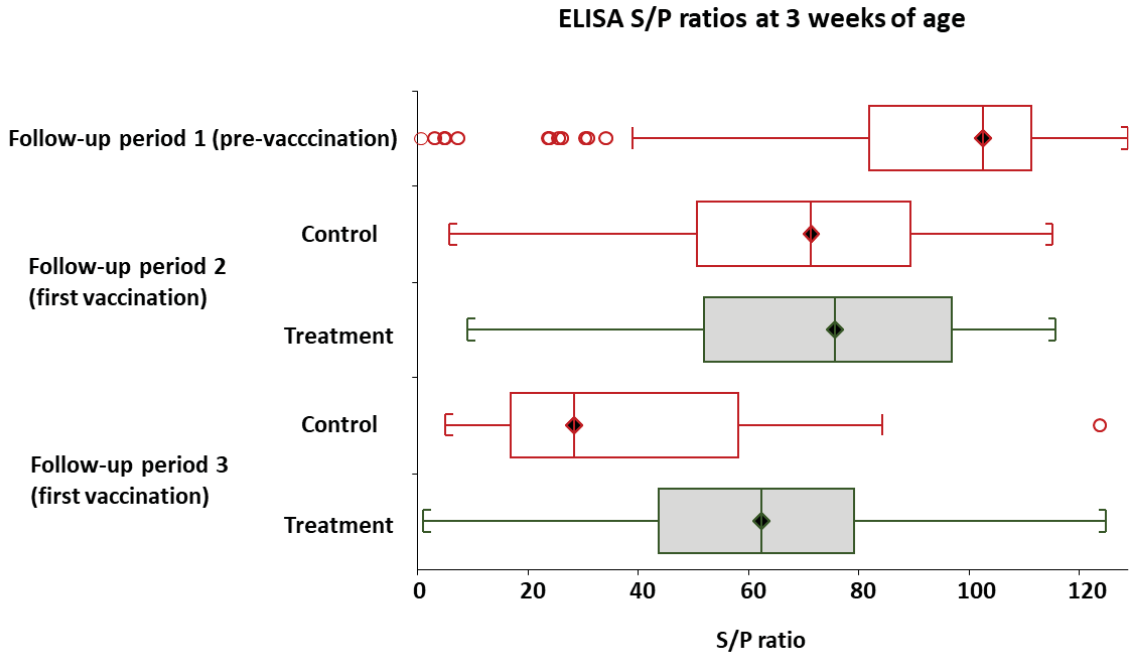


Figure 4. S/P ratios in ELISA at three weeks of ages in the different follow-up periods.

The panel shows box and whisker plots of the values/P ratios (minimum, 25% quartile, median, 75% quartile and maximum) at three weeks of age. A) First follow-up period, prior to the any vaccination protocol; B) Second follow up period, after the first vaccination scheme; C) Third follow-up period, after the second vaccination scheme. For B and C, coloured boxes show the results of the offspring of the sows that received an extra dose before farrowing (treatment), and non-coloured boxes of those who did not (control).



Supplementary material 1. Analysis of molecular variance for the set of H1avN1av isolates recovered from the second follow-up period after vaccination.

The tables indicate the significant changes with regards to the original sequence of the H1avN1av circulating before vaccination. Post-vaccination isolates differed from the pre-vaccination sequence but were similar among them. Non-synonymous mutations are indicated with an asterisk. Upper haemagglutinin; lower, neuraminidase.

Haemagglutinin		
Position in gene	**Fixation index**	**P-value**
849*	0.99	0.06452
1053	0.87127	0
90	0.81249	0
822	0.77628	0
627	0.76178	0
1374	0.75711	0.00391
1362	0.74759	0
651	0.72682	0
851	0.70729	0
1370	0.68961	0
219	0.43478	0
838	0.33378	0
460	0.13837	0
681	0.13171	0.00196
639	0.12725	0
1377	0.08907	0
635	0.07431	0
1101	0.06818	0
459*	0.06143	0.0479
683	0.06042	0

Neuraminidase		
Position in gene	**Fixation index**	**P-value**
35	0.95278	0
1392	0.93651	0
327	0.91481	0
379	0.83802	0
1038	0.75915	0
378	0.72535	0
1191	0.72018	0.00196
958	0.12157	0
189	0.10305	0
1246	0.08403	0
1120	0.08	0.04985

Basic Polymerase 2		
Position in gene	**Fixation Index**	**P-value**
925*	0.9598	0
1531	0.9347	0.0010
372	0.9282	0.0029
513	0.9148	0
810	0.9050	0
2070	0.8664	0
252	0.8230	0
1020	0.7807	0
1765	0.6778	0
375	0.2591	0.0029
204	0.2500	0.0557
498	0.1401	0
1880	0.0703	0

Basic Polymerase 1		
Position in gene	**Fixation Index**	**P-value**
2156	0.9438	0
2192	0.9169	0.0010
1523	0.9101	0.0029
719	0.9100	0.0606
1124	0.8996	0.0029
686	0.8941	0
152	0.8920	0.0039
257	0.8920	0.0010
1679	0.8593	0.0039
860	0.8301	0.0029
1274	0.7675	0
1787	0.7177	0
2134	0.5100	0.0567
2207	0.2011	0
1147	0.1300	0.0587
2268	0.0949	0
160*	0.0670	0
620	0.0609	0

Acid Polymerase		
Position in gene	**Fixation Index**	**P-value**
56	0.9601	0.0059
806	0.9536	0
1595	0.9200	0.0577
854	0.8901	0.0010
47	0.8834	0
905	0.8818	0
404	0.8701	0.0010
494	0.8405	0
575	0.8257	0
1710	0.8183	0
638	0.7643	0
1349	0.7412	0
260	0.6421	0
1625	0.1179	0
1706	0.0615	0

Nucleoprotein		
Position in Gener	**Fixation Index**	**P-value**
125	0.8115	0.0029
707	0.7088	0
77	0.2821	0.0020
387	0.2700	0.0420
312	0.2345	0
770	0.1100	0.0538
1068	0.1035	0
349	0.0600	0.0401
218	0.0577	0
62	0.0511	0.0020

Matrix		
Position in Gene	**Fixation Index**	**P-value**
56	0.7447	0
221	0.7007	0
47	0.0819	0

Non-structural protein 1		
Position in Gene	**Fixation Index**	**P-value**
81	0.9300	0.0616
616	0.8901	0
625	0.7840	0.0020
89	0.7309	0
415	0.5538	0
302	0.0802	0
508	0.0672	0

Part III:
General discussion and conclusions

Chapter 5.
General discussion

Influenza is one of the most studied diseases in the history of humankind. Nevertheless, many aspects of the epidemiology of this disease remain far from being fully understood. Influenza infections in pigs have many features in common with human influenza and humans have been an important source of influenza viruses for pigs. Pigs have contributed to infection in humans as well. Therefore, increasing our understanding of the epidemiology of influenza in pigs may help to understand some features of human influenza.

However, influenza is also an important swine disease that may have a significant impact on pig production. Influenza A viruses are a major component of the porcine respiratory disease complex that often are superimposed to other infections or are complicated by bacterial pathogens. Although the precise burden of influenza is not known, some estimations indicate a relevant impact (Haden et al., 2012; Stika et al., 2018).

The present thesis was aimed to increase the understanding of two key elements: the genetic diversity of influenza A viruses and the potential efficacy of vaccination to control endemic infections in nurseries.

The first study of the thesis was focused on: a) the characterisation of IAV in outbreaks of respiratory disease compatible with influenza and, b) the presence of the virus in farms with no apparent clinical disease. Results from this study showed that IAV is present in a large proportion of respiratory disease outbreaks, particularly in nurseries and suckling piglets. Interestingly, the proportion of the detected subtypes and lineages has varied compared to the ones reported in previous studies (Martín-Valls et al., 2014; Simon et al., 2014; Simon-Grifé et al., 2011; Van Reeth et al., 2008; Watson et al., 2015). While in the past decade, H1avN1av was the most prevalent lineage, now H1avN2hu seems to be the predominant one. Similarly, the previously common H3huN2hu has almost disappeared. In contrast, a H3 “human-like” with N2hu or N1av has emerged (Krog et al., 2017). This changing pattern of predominant lineages emphasises the plasticity of influenza viruses and shows the dynamic nature of the influenza epidemiology.

Examination of IAV genotypes further reinforced this notion since the genotyping scheme of Watson et al. (2015) proved to be insufficient to represent the existing diversity. In just four years after the publication of such scheme, we detected seven

unreported genotypes, five of them harbouring one or more internal genes of the 2009 pandemic virus. Since no differences in virulence among genotypes were noticed or reported by the submitting veterinarians, those observations lead to the questioning of the practical usefulness of the genotyping scheme beyond reflecting the dynamic flow of viral genes. The phylogenetic analysis of the fully sequenced isolates also showed the existence of local clusters for a given gene within a genotype. This is a rather interesting pattern. On one hand, there is a high flow of viral genes that create a continuously expanding diversity of genotypes and, on the other hand, the generation of local variants would be compatible with the relative isolation of each farm, which establishes an endemic cycle of infection. This needs deeper exploration to gain understanding on how farms are infected, how the virus is maintained in them and, eventually, how often there are lateral introductions. The investigation of factors affecting these interactions could be the subject of another study.

During the present study, we found ourselves somehow limited by the primers and probes meant for subtyping, as they failed in a proportion of cases. In some instances, failure was probably related to mismatches between the primers or the probe with a given isolate (for example with the new H3 human like). In other cases, the main problem was the low viral load in the sample. Actually, subtyping by RT-qPCR was not possible in most cases were Ct was over 30. Moreover, isolation in MDCK was not possible in many cases where samples had a Ct $\geq$ 30. These observations pose two relevant questions, one from a methodological point of view, the other from a biological one. Methodologically, it is obvious that updating of primers and probes is a constant necessity with influenza but, since diversity is continuously expanding, this creates a problem with the standardization of highly multiplexed PCRs. This problem can be overcome by using NGS technologies. Biologically, the impossibility to isolate or subtype viruses that are present in the farm create doubts about what is the role of such isolates. Are they circulating at a low level in subclinical animals? Are they just less transmissible variants? At present we do not have an answer but we think this is worth to be studied further.

In the second part of the thesis, the focus was put on controlling the infection in nurseries by applying different vaccination strategies in the sows. The initial hypothesis was that pre-farrowing vaccination would boost the transfer of colostrum antibodies and, through this, it would increase either the duration or the strength of the maternally-derived

immunity. We selected an endemic farm and assessed the viral circulation patterns at the beginning of the study. The farm was certainly endemic in the nurseries, where viral circulation was detected as early as at 4 weeks of age. In those pre-treatment batches, the pattern of transmission was uneven and the peak of incidence could be earlier or later. In the treatment phase, we decided to perform an initial blanket vaccination to produce a more homogeneous immunity in the herd and then to treat the selected sows with the pre-farrowing vaccination. After assessing the basal viral circulation, it was clear that the initial blanket vaccination had a bigger and more efficacious impact on the onset of infection than the implementation of a pre-farrowing dose. Just by introducing the blanket vaccination there was a delay of the beginning of the viral circulation of about 2 – 3 weeks. No significant circulation was detected before 6 weeks of age but when the infection appeared it rapidly spread and, in one or two weeks close to 100% of the piglets were infected regardless of the treatment of the sows. The explanation for this was not obvious since, at the same time, the offspring of both treatment and control sows had on average lower S/P antibody ratios compared to pre-vaccination piglets. A hypothesis that could fit all these facts is that blanket vaccination contributed to decrease (or even abolished) viral circulation among breeders. As a result, no transmission to suckling piglets would have happened in maternities, and piglets would have only become infected once they were moved to the nurseries (maybe contracting the infection from the contaminated premises). In that scenario, as soon as MDA stop protecting the animals, infection should spread rapidly. This would again be consistent with the higher beta-values for the transmission between the offspring of vaccinated sows.

Testing of the reactivity of the farm H1avN1av strain showed that an anti-pandemic antiserum had a stronger reactivity than the anti-vaccine H1avN1av antiserum. This was surprising and although some reactivity could have been expected, this result remains largely unexplained. Anyway, based on this result a vaccination trial with an H1N1pdm09 was attempted. Noteworthy, after the application of the pdm09 vaccine, the pattern of infection in piglets changed with a high incidence at three weeks of age, earlier than in any previous batch. When analysing the virus present at that moment it resulted to be a H3 “human-like” N2hu seasonal IAV. This change of virus indicated a lateral introduction or the emergence of a virus hidden in the background. In any case, the pattern of early circulation suggested that sows were not immune and that transmission happened

in maternities. Both facts would favour the hypothesis of a lateral introduction. Moreover, this introduction emphasises the need for continuing monitoring of the farms.

The patterns of infection and shedding that we detected in our studies have similarity to those recently reported in a similar study conducted in Denmark (Ryt-Hansen et al., 2019a,b). The presence of some animals acting as prolonged shedders (virus detected for two and even three consecutive sampling times), as well as of some animals testing positive after being negative for a space of 2-4 weeks, question the canonical idea of influenza as a short-lived infection with a very short period of shedding. In many of the two times positive animals, one of the samples produced a low Ct (<25) while the second resulted in a high Ct (>30). These cases need careful epidemiologic interpretation. The second result with a high Ct might represent remnants of viral RNA without real significance for the transmission. However, in some animals the two samples had low Ct values and this should be taken as possibly real shedding of infectious virus. Neither the prolonged shedding status, nor the animals that were apparently re-infected later in live could be related to the levels of MDA as measured by ELISA.

Certainly, the antibodies measured in the ELISA are directed to the viral nucleocapsid and therefore, they are not a correlate of protection or neutralization of the virus. However, it would be expectable that animals with higher levels of MDA against the nucleocapsid would have higher levels of antibodies against HA or M. Here several questions remain open: are these prolonged shedders born with some innate predisposition? Is the prolonged shedding the result of a defective immune response? Were those animals infected whilst still having some maternally-derived immunity at the respiratory mucosae? Analysis of the sequences of the viruses retrieved form prolonged shedders showed that they did not differ substantially from the sequences of short-term shedders and do not support the hypothesis that virus in prolonged shedders are escape mutants. The understanding of the role of those prolonged shedders, the development of immunity in animals that got infected in the presence of MDA and on the general drivers of the dynamics of the endemic infection in pig farms are much needed.

One of the main questions behind the results of the present thesis is if vaccination was effective in practical terms. This is difficult to answer without an economic evaluation,

particularly between the pre-vaccination period and the first vaccination trial. This could be a continuation of the present study.

Influenza viruses never stop evolving and this could have a potential impact in the future of public health. For a virus like this, it is pivotal to continue with surveillance programmes and full genome sequencing can be a powerful and affordable tool in the near future. Many questions remain still open in the epidemiology of swine influenza and its control. To be studied in the future.

References (Introduction and General discussion)

1. Abente, E. J., Rajao, D. S., Santos, J., Kaplan, B. S., Nicholson, T. L., Brockmeier, S. L., Gauger, P. C., Perez, D. R., & Vincent, A. L. 2018. Comparison of adjuvanted-whole inactivated virus and live-attenuated virus vaccines against challenge with contemporary, antigenically distinct H3N2 influenza A viruses. *J Virol*, 92: e01323-18. https://doi.org/10.1128/JVI.01323-18
2. Adiego Sancho, B., Omenaca Teres, M., Martinez Cuenca, S., Rodrigo Val, P., Sanchez Villanueva, P., Casas, I., Pozo, F., & Perez Brena, P. 2009. Human case of swine influenza A (H1N1), Aragon, Spain, November 2008. *Euro Surveill*, 14: 19120.
3. Ahlquist, P. 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science*, 296:1270-1273. https://doi.org/10.1126/science.1069132
4. Allerson, M. W., Cardona, C. J., & Torremorell, M. 2013. Indirect transmission of influenza A virus between pig populations under two different biosecurity settings. *PloS One*, 8: e67293. https://doi.org/10.1371/journal.pone.0067293.
5. Allerson, M., Deen, J., Detmer, S. E., Gramer, M. R., Joo, H. S., Romagosa, A., & Torremorell, M. 2013. The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations. *Vaccine*, 31:500–505. https://doi.org/10.1016/j.vaccine.2012.11.023
6. Alvarez, J., Sarradell, J., Kerkaert, B., Bandyopadhyay, D., Torremorell, M., Morrison, R., & Perez, A. 2015. Association of the presence of influenza A virus and porcine reproductive and respiratory syndrome virus in sow farms with post-weaning mortality. *Prev Vet Med*, 121: 240–245. https://doi.org/10.1016/j.prevetmed.2015.07.003
7. Anderson, T. K., Campbell, B. A., Nelson, M. I., Lewis, N. S., Janas-Martindale, A., Killian, M. L., & Vincent, A. L. 2015. Characterization of co-circulating swine influenza A viruses in North America and the identification of a novel H1 genetic clade with antigenic significance. *Virus Res*, 201: 24–31. https://doi.org/10.1016/j.virusres.2015.02.009
8. Anderson, T. K., Chang, J., Arendsee, Z. W., Venkatesh, D., Souza, C. K., Kimble, J. B., Lewis, N. S., Davis, C. T., & Vincent, A. L. 2020. Swine influenza A viruses and the tangled relationship with humans. *Cold Spring Harb Perspect Med*, a038737. Advance online publication. https://doi.org/10.1101/cshperspect.a038737
9. Anderson, T. K., Macken, C. A., Lewis, N. S., Scheuermann, R. H., Van Reeth, K., Brown, I. H., Swenson, S. L., Simon, G., Saito, T., Berhane, Y., Ciacci-Zanella, J., Pereda, A., Davis, C. T., Donis, R. O., Webby, R. J., & Vincent, A. L. 2016. A phylogeny-based global nomenclature system and automated annotation tool for h1 hemagglutinin genes from swine influenza A viruses. *mSphere*, 1: e00275-16. https://doi.org/10.1128/mSphere.00275-16
10. Anderson, T. K., Nelson, M. I., Kitikoon, P., Swenson, S. L., Korslund, J. A., &

Vincent, A. L. 2013. Population dynamics of cocirculating swine influenza A viruses in the United States from 2009 to 2012. *Influenza Other Respir Viruses*, 7: 42–51. https://doi.org/10.1111/irv.12193

11. Bailey, E. S., Fieldhouse, J. K., Alarja, N. A., Chen, D. D., Kovalik, M. E., Zemke, J. N., Choi, J. Y., Borkenhagen, L. K., Toh, T. H., Lee, J., Chong, K. S., & Gray, G. C. 2020. First sequence of influenza D virus identified in poultry farm bioaerosols in Sarawak, Malaysia. *Trop Dis Travel Med Vaccines,* 6:5. https://doi.org/10.1186/s40794-020-0105-9
12. Bancroft, C. T., & Parslow, T. G. 2002. Evidence for segment-nonspecific packaging of the influenza a virus genome. *J Virol*, 76: 7133–7139. https://doi.org/10.1128/jvi.76.14.7133-7139.2002
13. Bányai, K., Kovács, E., Tóth, Á., Biksi, I., Szentpáli-Gavallér, K., Bálint, Á., Dencso, L., & Dán, Á. 2012. Genome sequence of a monoreassortant H1N1 swine influenza virus isolated from a pig in Hungary. *J Virol*, 86: 13133. https://doi.org/10.1128/JVI.02503-12
14. Barbé, F., Labarque, G., Pensaert, M., & Van Reeth, K. 2009. Performance of a commercial swine influenza virus H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with European influenza viruses. *J Vet Diagn Invest,* 21: 88–96. https://doi.org/10.1177/104063870902100113
15. Basak, S., Tomana, M., & Compans, R. W. 1985. Sialic acid is incorporated into influenza hemagglutinin glycoproteins in the absence of viral neuraminidase. *Virus Res*, 2: 61–68. https://doi.org/10.1016/0168-1702(85)90060-7
16. Bean, B., Moore, B. M., Sterner, B., Peterson, L. R., Gerding, D. N., & Balfour, H. H., Jr. 1982. Survival of influenza viruses on environmental surfaces. *J Infect Dis,* 146: 47–51. https://doi.org/10.1093/infdis/146.1.47
17. Beato, M. S., Tassoni, L., Milani, A., Salviato, A., Di Martino, G., Mion, M., Bonfanti, L., Monne, I., Watson, S. J., & Fusaro, A. 2016. Circulation of multiple genotypes of H1N2 viruses in a swine farm in Italy over a two-month period. *Vet Microbiol*, 195: 25–29. https://doi.org/10.1016/j.vetmic.2016.08.015
18. Belongia, E. A., Kieke, B. A., Donahue, J. G., Greenlee, R. T., Balish, A., Foust, A., Lindstrom, S., Shay, D. K., & Marshfield Influenza Study Group. 2009. Effectiveness of inactivated influenza vaccines varied substantially with antigenic match from the 2004-2005 season to the 2006-2007 season. *J Infect Dis,* 199: 159–167. https://doi.org/10.1086/595861
19. Bizebard, T., Gigant, B., Rigolet, P., Rasmussen, B., Diat, O., Böseckei, P., Wharton, S. A., Skehel, J. J., & Knossow, M. 1995. Structure of influenza virus haemagglutinin complexed with a neutralizing antibody. *Nature*, 376: 92–94. https://doi.org/10.1038/376092a0
20. Boulo, S., Akarsu, H., Ruigrok, R. W., & Baudin, F. 2007. Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Res*, 124: 12–21. https://doi.org/10.1016/j.virusres.2006.09.013
21. Bowman, A. S., Sreevatsan, S., Killian, M. L., Page, S. L., Nelson, S. W.,

Nolting, J. M., Cardona, C., & Slemons, R. D. 2012. Molecular evidence for interspecies transmission of H3N2pM/H3N2v influenza A viruses at an Ohio agricultural fair, July 2012. *Emerg Microbes Infect,* 1: e33. https://doi.org/10.1038/emi.2012.33

22. Bowman, A. S., Workman, J. D., Nolting, J. M., Nelson, S. W., & Slemons, R. D. 2014. Exploration of risk factors contributing to the presence of influenza A virus in swine at agricultural fairs. *Emerg Microbes Infect,* 3: e5. https://doi.org/10.1038/emi.2014.5
23. Breum, S. Ø., Hjulsager, C. K., Trebbien, R., & Larsen, L. E. 2013. Influenza A virus with a human-like N2 gene is circulating in pigs. *Genome Announc*, 1: e00712-13. https://doi.org/10.1128/genomeA.00712-13
24. Brockmeier, S. L., Halbur, P. G., & Thacker, E. L. 2002. Porcine respiratory disease complex. In: *Polymicrobial Diseases.* K. A. Brogden & J. M. Guthmiller (editors). ASM Press. Washington, DC, USA. 231–258 pp.
25. Brown I. H. 2000. The epidemiology and evolution of influenza viruses in pigs. *Vet Microbiol*, 74: 29–46. https://doi.org/10.1016/s0378-1135(00)00164-4
26. Brown, I. H., Harris, P. A., & Alexander, D. J. 1995. Serological studies of influenza viruses in pigs in Great Britain 1991-2. *Epidemiol Infect,* 114: 511–520. https://doi.org/10.1017/s0950268800052225
27. Brown, I. H., Harris, P. A., McCauley, J. W., & Alexander, D. J. 1998. Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. *J Gen Virol,* 79: 2947–2955. https://doi.org/10.1099/0022-1317-79-12-2947
28. Brown, I. H., Ludwig, S., Olsen, C. W., Hannoun, C., Scholtissek, C., Hinshaw, V. S., Harris, P. A., McCauley, J. W., Strong, I., & Alexander, D. J. 1997. Antigenic and genetic analyses of H1N1 influenza A viruses from European pigs. *J Gen Virol,* 78: 553–562. https://doi.org/10.1099/0022-1317-78-3-553
29. Busquets, N., Segalés, J., Córdoba, L., Mussá, T., Crisci, E., Martín-Valls, G. E., Simon-Grifé, M., Pérez-Simó, M., Pérez-Maíllo, M., Núñez, J. I., Abad, F. X., Fraile, L., Pina, S., Majó, N., Bensaid, A., Domingo, M., & Montoya, M. 2010. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. *Vet Res*, 41: 74. https://doi.org/10.1051/vetres/2010046
30. Cador, C., Andraud, M., Willem, L., & Rose, N. 2017. Control of endemic swine flu persistence in farrow-to-finish pig farms: a stochastic metapopulation modeling assessment. *Vet Res*, 48: 58. https://doi.org/10.1186/s13567-017-0462-1
31. Cador, C., Hervé, S., Andraud, M., Gorin, S., Paboeuf, F., Barbier, N., Quéguiner, S., Deblanc, C., Simon, G., & Rose, N. 2016a. Maternally-derived antibodies do not prevent transmission of swine influenza A virus between pigs. *Vet Res*, 47: 86. https://doi.org/10.1186/s13567-016-0365-6
32. Cador, C., Rose, N., Willem, L., & Andraud, M. 2016b. Maternally derived immunity extends swine influenza a virus persistence within farrow-to-finish pig farms: insights from a stochastic event-driven metapopulation model. *PloS*

One, 11: e0163672. https://doi.org/10.1371/journal.pone.0163672
33. Campitelli, L., Donatelli, I., Foni, E., Castrucci, M. R., Fabiani, C., Kawaoka, Y., Krauss, S., & Webster, R. G. 1997. Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. *Virology*, 232: 310–318. https://doi.org/10.1006/viro.1997.8514
34. Caton, A. J., Brownlee, G. G., Yewdell, J. W., & Gerhard, W. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell*, 31: 417–427. https://doi.org/10.1016/0092-8674(82)90135-0
35. Chamba Pardo, F. O., Alba-Casals, A., Nerem, J., Morrison, R. B., Puig, P., & Torremorell, M. 2017. Influenza herd-level prevalence and seasonality in breed-to-wean pig farms in the Midwestern United States. *Front Vet Sci,* 4: 167. https://doi.org/10.3389/fvets.2017.00167
36. Chamba Pardo, F. O., Schelkopf, A., Allerson, M., Morrison, R., Culhane, M., Perez, A., & Torremorell, M. 2018. Breed-to-wean farm factors associated with influenza A virus infection in piglets at weaning. *Prev Vet Med*, 161: 33–40. https://doi.org/10.1016/j.prevetmed.2018.10.008
37. Chamba Pardo, F. O., Wayne, S., Culhane, M. R., Perez, A., Allerson, M., & Torremorell, M. 2019. Effect of strain-specific maternally-derived antibodies on influenza A virus infection dynamics in nursery pigs. *PLoS One*, 14: 1–15. https://doi.org/10.1371/journal.pone.0210700
38. Chambers, B. S., Parkhouse, K., Ross, T. M., Alby, K., & Hensley, S. E. 2015. Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014-2015 Influenza Season. *Cell Rep*, 12: 1–6. https://doi.org/10.1016/j.celrep.2015.06.005
39. Chang, H. K., Park, J. H., Song, M. S., Oh, T. K., Kim, S. Y., Kim, C. J., Kim, H., Sung, M. H., Han, H. S., Hahn, Y. S., & Choi, Y. K. 2008. Development of multiplex rt-PCR assays for rapid detection and subtyping of influenza type A viruses from clinical specimens. *J Microbiol Biotechnol,* 18: 1164–1169.
40. Charley, B., Riffault, S., & Van Reeth, K. 2006. Porcine innate and adaptative immune responses to influenza and coronavirus infections. *Ann N Y Acad Sci,* 1081: 130–136. https://doi.org/10.1196/annals.1373.014
41. Chastagner, A., Bonin, E., Fablet, C., Quéguiner, S., Hirchaud, E., Lucas, P., Gorin, S., Barbier, N., Béven, V., Garin, E., Blanchard, Y., Rose, N., Hervé, S., & Simon, G. 2019. Virus persistence in pig herds led to successive reassortment events between swine and human influenza A viruses, resulting in the emergence of a novel triple-reassortant swine influenza virus. *Vet Res*, 50: 77. https://doi.org/10.1186/s13567-019-0699-y
42. Chen, C., & Zhuang, X. 2008. Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. *Proc Natl Acad Sci U S A,* 105: 11790–11795. https://doi.org/10.1073/pnas.0803711105
43. Chen, H., Alvarez, J., Ng, S. H., Nielsen, R., & Zhai, W. 2019. Passage adaptation correlates with the reduced efficacy of the influenza vaccine. *Clin Infect Dis,* 69: 1198–1204. https://doi.org/10.1093/cid/ciy1065
44. Chen, R., & Holmes, E. C. 2006. Avian influenza virus exhibits rapid

evolutionary dynamics. *Mol Biol Evol,* 23: 2336–2341. https://doi.org/10.1093/molbev/msl102
45. Chiapponi, C., Baioni, L., Luppi, A., Moreno, A., Castellan, A., & Foni, E. 2013. Full-genome sequence of a reassortant H1N1 swine influenza virus isolated from pigs in Italy. *Genome Announc,* 1: e00778-13. https://doi.org/10.1128/genomeA.00778-13
46. Choi, M. J., Torremorell, M., Bender, J. B., Smith, K., Boxrud, D., Ertl, J. R., Yang, M., Suwannakarn, K., Her, D., Nguyen, J., Uyeki, T. M., Levine, M., Lindstrom, S., Katz, J. M., Jhung, M., Vetter, S., Wong, K. K., Sreevatsan, S., & Lynfield, R. 2015. Live animal markets in Minnesota: A potential source for emergence of novel influenza A viruses and interspecies transmission. *Clin Infect Dis,* 61: 1355–1362. https://doi.org/10.1093/cid/civ618
47. Choi, Y. K., Pascua, P. N., & Song, M. S. 2013. Swine influenza viruses: an Asian perspective. *Curr Top Microbiol Immunol,* 370: 147–172. https://doi.org/10.1007/82_2011_195
48. Ciacci-Zanella, J. R., Vincent, A. L., Prickett, J. R., Zimmerman, S. M., & Zimmerman, J. J. 2010. Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species. *J Vet Diagn Invest,* 22: 3–9. https://doi.org/10.1177/104063871002200102
49. Clavijo, A., Tresnan, D. B., Jolie, R., & Zhou, E. M. 2002. Comparison of embryonated chicken eggs with MDCK cell culture for the isolation of swine influenza virus. *Can J Vet Res,* 66: 117–121.
50. Copeland, C. S., Doms, R. W., Bolzau, E. M., Webster, R. G., & Helenius, A. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J Cell Biol,* 103: 1179–1191. https://doi.org/10.1083/jcb.103.4.1179
51. Copeland, C. S., Zimmer, K. P., Wagner, K. R., Healey, G. A., Mellman, I., & Helenius, A. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell*, 53: 197–209. https://doi.org/10.1016/0092-8674 (88)90381-9
52. Corzo, C. A., Allerson, M., Gramer, M., Morrison, R. B., & Torremorell, M. 2014. Detection of airborne influenza a virus in experimentally infected pigs with maternally derived antibodies. *Transbound Emerg Dis,* 61: 28–36. https://doi.org/10.1111/j.1865-1682.2012.01367.x
53. Corzo, Cesar A., Gramer, M., Kuhn, M., Mohr, M., & Morrison, R. 2012. Observations regarding influenza A virus shedding in a swine breeding farm after mass vaccination. *J Swine Health Prod,* 20: 283–289.
54. Culhane, M. R., & Detmer, S. E. 2014. Sample types, collection, and transport for influenza A viruses of swine. *Methods Mol Biol,* 1161: 259–263. https://doi.org/10.1007/978-1-4939-0758-8_21
55. Daly, P., Gustafson, R., & Kendall, P. 2007. Introduction to pandemic influenza. *BCMJ,* 49: 240–244. https://doi.org/10.1079/9781845936259.0000
56. Dawood, F. S., Iuliano, A. D., Reed, C., Meltzer, M. I., Shay, D. K., Cheng, P. Y., Bandaranayake, D., Breiman, R. F., Brooks, W. A., Buchy, P., Feikin, D. R.,

Fowler, K. B., Gordon, A., Hien, N. T., Horby, P., Huang, Q. S., Katz, M. A., Krishnan, A., Lal, R., Montgomery, J. M., … Widdowson, M. A. 2012. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *Lancet Infect Dis,* 12: 687–695. https://doi.org/10.1016/S1473-3099(12)70121-4

57. Dawood, F. S., Jain, S., Finelli, L., Shaw, M. W., Lindstrom, S., Garten, R. J., Gubareva, L. V, Xu, X., Bridges, C. B., & Uyeki, T. M. 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med,* 360: 2605–2615. https://doi.org/10.1056/NEJMoa0903810
58. de Jong, J. C., Heinen, P. P., Loeffen, W. L. A., Van Nieuwstadt, A. P., Claas, E. C. J., Bestebroer, T. M., Bijlsma, K., Verweij, C., Osterhaus, A. D. M. E., Rimmelzwaan, G. F., Fouchier, R. A. M., & Kimman, T. G. 2001. Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. *Vaccine*, 19: 4452–4464. https://doi.org/10.1016/S0264-410X(01)00190-6
59. de Jong, J. C., Smith, D. J., Lapedes, A. S., Donatelli, I., Campitelli, L., Barigazzi, G., Van Reeth, K., Jones, T. C., Rimmelzwaan, G. F., Osterhaus, A. D. M. E., & Fouchier, R. A. M. 2007. Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. *J Virol,* 81: 4315–4322. https://doi.org/10.1128/JVI.02458-06
60. De Vleeschauwer, A. R., Van Poucke, S. G., Karasin, A. I., Olsen, C. W., & Van Reeth, K. 2011. Cross-protection between antigenically distinct H1N1 swine influenza viruses from Europe and North America. *Influenza Other Respir Viruses,* 5: 115–122. https://doi.org/10.1111/j.1750-2659.2010.00164.x
61. de Vries, E., Tscherne, D. M., Wienholts, M. J., Cobos-Jiménez, V., Scholte, F., García-Sastre, A., Rottier, P. J., & de Haan, C. A. 2011. Dissection of the influenza A virus endocytic routes reveals macropinocytosis as an alternative entry pathway. *PLoS Pathog*, 7: e1001329. https://doi.org/10.1371/journal.ppat.1001329
62. Deblanc, C., Hervé, S., Gorin, S., Cador, C., Andraud, M., Quéguiner, S., Barbier, N., Paboeuf, F., Rose, N., & Simon, G. 2018. Maternally-derived antibodies do not inhibit swine influenza virus replication in piglets but decrease excreted virus infectivity and impair post-infectious immune responses. *Vet Microbiol*, 216: 142–152. https://doi.org/10.1016/j.vetmic.2018.01.019
63. Detmer, S. E., Patnayak, D. P., Jiang, Y., Gramer, M. R., & Goyal, S. M. 2011. Detection of influenza A virus in porcine oral fluid samples. *J Vet Diagn Invest,* 23: 241–247. https://doi.org/10.1177/104063871102300207
64. Diaz, A., Allerson, M., Culhane, M., Sreevatsan, S., & Torremorell, M. 2013. Antigenic drift of H1N1 influenza A virus in pigs with and without passive immunity. *Influenza Other Respir Viruses,* 7: 52–60. https://doi.org/10.1111/irv.12190
65. Diaz, A., Marthaler, D., Culhane, M., & Sreevatsan, S. 2017. Complete genome sequencing of influenza A viruses within swine farrow- persistence, and subsidence of diverse. *J Virol*, 91: 1–16.

66. Diaz, A., Perez, A., Sreevatsan, S., Davies, P., Culhane, M., & Torremorell, M. 2015. Association between influenza A virus infection and pigs subpopulations in endemically infected breeding herds. *PLoS One*, 10: 1–11. https://doi.org/10.1371/journal.pone.0129213
67. Dibárbora, M., Cappuccio, J., Olivera, V., Quiroga, M., Machuca, M., Perfumo, C., Pérez, D., & Pereda, A. 2013. Swine influenza: clinical, serological, pathological, and virological cross-sectional studies in nine farms in Argentina. *Influenza Other Respir Viruses,* 7*:* 10–15. https://doi.org/10.1111/irv.12200
68. Diekmann, O., Heesterbeek, J. A. P., & Metz, J. A. J. 1990. On the definition and the computation of the basic reproduction ratio R0 in models for infectious diseases in heterogeneous populations. *J Math Biol*, 28: 365–382. https://doi.org/10.1007/BF00178324
69. Dietz, K. 1993. The estimation of the basic reproduction number for infectious diseases. *Stat Methods Med Res,* 2: 23–41. https://doi.org/10.1177/096228029300200103
70. Dorjee, S., Revie, C. W., Poljak, Z., Mcnab, W. B., Mcclure, J. T., & Sanchez, J. 2016. One-health simulation modelling: assessment of control strategies against the spread of influenza between swine and human populations using NAADSM. *Transbound Emerg Dis*, 63: e229–e244. https://doi.org/10.1111/tbed.12260
71. Dou, D., Revol, R., Östbye, H., Wang, H., & Daniels, R. 2018. Influenza A virus cell entry, replication, virion assembly and movement. *Front Immunol, 9*: 1-17. https://doi.org/10.3389/fimmu.2018.01581
72. Ducatez, M. F., Awoume, F., & Webby, R. J. 2015. Influenza A(H1N1)pdm09 virus in pigs, Togo, 2013. *Vet Microbiol*, 177: 201–205. https://doi.org/10.1016/j.vetmic.2015.02.028
73. Easterday, B. C. 1980. The epidemiology and ecology of swine influenza as a zoonotic disease. *Comp Immunol Microbiol Infect Dis*, 3: 105–109. https://doi.org/10.1016/0147-9571(80)90045-4
74. Edwards, J. L., Nelson, S. W., Workman, J. D., Slemons, R. D., Szablewski, C. M., Nolting, J. M., & Bowman, A. S. 2014. Utility of snout wipe samples for influenza A virus surveillance in exhibition swine populations. *Influenza Other Respir Viruses,* 8: 574–579. https://doi.org/10.1111/irv.12270
75. Eickhoff, T. C., & Meiklejohn, G. 1969. Protection against Hong Kong influneza by adjuvant vaccine containing A2-Ann Arbor-67. *Bull World Health Organ* 41: 562–563.
76. Eurostat. 2017. Pork production up in the EU. Retrieved from https://ec.europa.eu/eurostat/web/products-eurostat-news/-/DDN-20170919-1
77. Fablet, C., Simon, G., Dorenlor, V., Eono, F., Eveno, E., Gorin, S., Quéguiner, S., Madec, F., & Rose, N. 2013. Different herd level factors associated with H1N1 or H1N2 influenza virus infections in fattening pigs. *Prev Vet Med*, 112: 257–265. https://doi.org/10.1016/j.prevetmed.2013.07.006
78. Farrington, C. P. 2003. On vaccine efficacy and reproduction numbers. *Math Biosci,* 185: 89–109. https://doi.org/10.1016/S0025-5564(03)00061-0

79. Feng, Z., Gomez, J., Bowman, A. S., Ye, J., Long, L.-P., Nelson, S. W., Yang, J., Martin, B., Jia, K., Nolting, J. M., Cunningham, F., Cardona, C., Zhang, J., Yoon, K.-J., Slemons, R. D., & Wan, X.-F. 2013. Antigenic characterization of H3N2 influenza a viruses from Ohio agricultural fairs. *J Virol*, 87: 7655–7667. https://doi.org/10.1128/jvi.00804-13
80. Ferreira, J. B., Grgić, H., Friendship, R., Wideman, G., Nagy, É., & Poljak, Z. 2017. Longitudinal study of influenza A virus circulation in a nursery swine barn. *Vet Res,* 48: 1–16. https://doi.org/10.1186/s13567-017-0466-x
81. Fouchier, R. A. M., Bestebroer, T. M., Herfst, S., Van der Kemp, L., Rimmelzwaan, G. F., & Osterhaus, A. D. M. E. 2000. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J Clin Microbiol,* 38: 4096–4101. https://doi.org/10.1128/jcm.38.11.4096-4101.2000
82. Frank, A. L., Couch, R. B., Griffis, C. A., & Baxter, B. D. 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. *J Clin Microbiol,* 10: 32–36. https://doi.org/10.1128/jcm.10.1.32-36.1979
83. Gambaryan, A. S., Robertson, J. S., & Matrosovich, M. N. 1999. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. *Virology*, 258: 232–239. https://doi.org/10.1006/viro.1999.9732
84. Gambaryan, A. S., Tuzikov, A. B., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., Robertson, J. S., Bovin, N. V., & Matrosovich, M. N. 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6'-sialyl (N-acetyllactosamine). *Virology*, 232: 345–350. https://doi.org/10.1006/viro.1997.8572
85. Garrido-Mantilla, J., Alvarez, J., Culhane, M., Nirmala, J., Cano, J. P., & Torremorell, M. 2019. Comparison of individual, group and environmental sampling strategies to conduct influenza surveillance in pigs. *BMC Vet Res*, 15: 1–10. https://doi.org/10.1186/s12917-019-1805-0
86. Gauger, P. C., Vincent, A. L., Loving, C. L., Henningson, J. N., Lager, K. M., Janke, B. H., Kehrli, J. E., & Roth, J. A. 2012. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. *Vet Pathol,* 49: 900–912. https://doi.org/10.1177/0300985812439724
87. Gauger, Phillip C, & Vincent, A. L. 2014. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine. *Methods Mol Biol,* 1161: 313–324. https://doi.org/10.1007/978-1-4939-0758-8_26
88. Gauger, Phillip C, Loving, C. L., & Vincent, A. L. 2014. Enzyme-linked immunosorbent assay for detection of serum or mucosal isotype-specific IgG and IgA whole-virus antibody to influenza A virus in swine. *Methods Mol Biol,*

1161: 303–312. https://doi.org/10.1007/978-1-4939-0758-8_25
89. Gauger, Phillip C., Loving, C. L., Lager, K. M., Janke, B. H., Kehrli, M. E., Roth, J. A., & Vincent, A. L. 2013. Vaccine-associated enhanced respiratory disease does not interfere with the adaptive immune response following challenge with pandemic A/H1N1 2009. *Viral Immunol*, 26: 314–321. https://doi.org/10.1089/vim.2013.0018
90. Gauger, Phillip C., Vincent, A. L., Loving, C. L., Lager, K. M., Janke, B. H., Kehrli, M. E., & Roth, J. A. 2011. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (δ-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. *Vaccine*, 29: 2712–2719. https://doi.org/10.1016/j.vaccine.2011.01.082
91. Gaush, C. R., & Smith, T. F. 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. *Appl Microbiol,* 16: 588–594. https://doi.org/10.1128/AEM.16.4.588-594.1968
92. Genzow, M., Goodell, C., Kaiser, T. J., Johnson, W., & Eichmeyer, M. 2018. Live attenuated influenza virus vaccine reduces virus shedding of newborn piglets in the presence of maternal antibody. *Influenza Other Respir Viruses,* 12: 353–359. https://doi.org/10.1111/irv.12531
93. Gillespie, T. G. 1999. Diagnosing endemic swine influenza virus in nursery pigs using cross-sectional serologic profiling. *Swine Health Prod*, 7: 81–83.
94. Goecke, N. B., Krog, J. S., Hjulsager, C. K., Skovgaard, K., Harder, T. C., Breum, S. Ø., & Larsen, L. E. 2018. Subtyping of swine influenza viruses using a high-throughput real-time pcr platform. *Front Cell Infect Microbiol,* 8: 165. https://doi.org/10.3389/fcimb.2018.00165
95. Goodell, C. K., Prickett, J., Kittawornrat, A., Zhou, F., Rauh, R., Nelson, W., O'Connell, C., Burrell, A., Wang, C., Yoon, K. J., & Zimmerman, J. J. 2013. Probability of detecting influenza A virus subtypes H1N1 and H3N2 in individual pig nasal swabs and pen-based oral fluid specimens over time. *Vet Microbiol*, 166: 450–460. https://doi.org/10.1016/j.vetmic.2013.06.029
96. Gorin, S., Fablet, C., Quéguiner, S., Barbier, N., Paboeuf, F., Hervé, S., Rose, N., & Simon, G. 2019. Assessment of influenza D virus in domestic pigs and wild boars in France: apparent limited spread within swine populations despite serological evidence of breeding sow exposure. *Viruses*, 12: 1–11. https://doi.org/10.3390/v12010025
97. Grgić, H., Gallant, J., & Poljak, Z. 2017. Virological surveillance of influenza A subtypes isolated in 2014 from clinical outbreaks in Canadian swine. *Viruses*, 9: 55. https://doi.org/10.3390/v9030055
98. Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R. W. H., Ortin, J., Hart, D. J., & Cusack, S. 2008. The structural basis for cap binding by influenza virus polymerase subunit PB2. *Nat Struct Mol Biol,* 15: 500–506. https://doi.org/10.1038/nsmb.1421
99. Gumbert, S., Froehlich, S., Rieger, A., Stadler, J., Ritzmann, M., & Zoels, S. 2020. Reproductive performance of pandemic influenza A virus infected sow herds before and after implementation of a vaccine against the influenza A

(H1N1)pdm09 virus. *Porcine Health Manag,* 6: 4. https://doi.org/10.1186/s40813-019-0141-x

100. Haden C, Painter T, Fangman T, Holtkamp D. 2012. Assessing production parameters and economic impact of swine in influenza, PRRS and Mycoplasma hyopeumoniae on finishing pigs in large production system. *43rd AASV Annual Meeting*, Denver, March 10th–13th, p. 75.

101. Harder, T. C., grosse Beilage, E., Lange, E., Meiners, C., Dohring, S., Pesch, S., Noe, T., Grund, C., Beer, M., & Starick, E. 2013. Expanded cocirculation of stable subtypes, emerging lineages, and new sporadic reassortants of porcine influenza viruses in swine populations in Northwest Germany. *J Virol*, 87: 10460–10476. https://doi.org/10.1128/jvi.00381-13

102. Heinen, P. P., De Boer-Luijtze, E. A., & Bianchi, T. J. 2001. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. *J Gen Virol,* 82: 2697–2707. https://doi.org/10.1099/0022-1317-82-11-2697

103. Heinen, Paul P., Rijsewijks, F. A., de Boer-Luitjtze, E. A., & Bianchi, A. T. J. 2002. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. *J Gen Virol,* 83: 1851–1859. https://doi.org/10.1099/0022-1317-83-8-1851

104. Henritzi, D., Hoffmann, B., Wacheck, S., Pesch, S., Herrler, G., Beer, M., & Harder, T. C. 2019. A newly developed tetraplex real-time RT-PCR for simultaneous screening of influenza virus types A, B, C and D. *Influenza Other Respir Viruses,* 13: 71–82. https://doi.org/10.1111/irv.12613

105. Henritzi, D., Zhao, N., Starick, E., Simon, G., Krog, J. S., Larsen, L. E., Reid, S. M., Brown, I. H., Chiapponi, C., Foni, E., Wacheck, S., Schmid, P., Beer, M., Hoffmann, B., & Harder, T. C. 2016. Rapid detection and subtyping of European swine influenza viruses in porcine clinical samples by haemagglutinin- and neuraminidase-specific tetra- and triplex real-time RT-PCRs. *Influenza Other Respir Viruses,* 10: 504–517. https://doi.org/10.1111/irv.12407

106. Hernandez, L. A., Miller, C. L., & Vaughn, E. M. 2016. Particle and subunit-based hemagglutinin vaccines provide protective efficacy against H1N1 influenza in pigs. *Vet Microbiol*, 191: 35–43. https://doi.org/10.1016/j.vetmic.2016.05.011

107. Hervé, S., Garin, E., Calavas, D., Lecarpentier, L., Ngwa-Mbot, D., Poliak, S., Wendling, S., Rose, N., & Simon, G. 2019. Virological and epidemiological patterns of swine influenza A virus infections in France: Cumulative data from the RESAVIP surveillance network, 2011-2018. *Vet Microbiol*, 239*:* 108477. https://doi.org/10.1016/j.vetmic.2019.108477

108. Hinshaw, V. S., Webster, R. G., Easterday, B. C., & Bean, W. J. 1981. Replication of avian influenza A viruses in mammals. *Infect Immun,* 34: 354–361. https://doi.org/10.1128/iai.34.2.354-361.1981

109. Hirst, G. K. 1941. The agglutination of red cells by allantoic fluid of

chick embryos infected with influenza virus. *Science,* 94: 22–23. https://doi.org/10.1126/science.94.2427.22
110. Holzer, B., Martini, V., Edmans, M., & Tchilian, E. 2019. T and B cell immune responses to influenza viruses in pigs. *Front Immunol,* 10: 98. https://doi.org/10.3389/fimmu.2019.00098
111. Howard, W. A., Essen, S. C., Strugnell, B. W., Russell, C., Barrass, L., Reid, S. M., & Brown, I. H. 2011. Reassortant pandemic (H1N1) 2009 virus in pigs, United Kingdom. *Emerg Infect Dis,* 17: 1049–1052. https://doi.org/10.3201/eid1706.101886
112. Howden, K. J., Brockhoff, E. J., Caya, F. D., McLeod, L. J., Lavoie, M., Ing, J. D., Bystrom, J. M., Alexandersen, S., Pasick, J. M., Berhane, Y., Morrison, M. E., Keenliside, J. M., Laurendeau, S., & Rohonczy, E. B. 2009. Special report rapport spécial: An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. *Can Vet J,* 50: 1153–1161.
113. Huet, S., Avilov, S. V., Ferbitz, L., Daigle, N., Cusack, S., & Ellenberg, J. 2010. Nuclear import and assembly of influenza A virus RNA polymerase studied in live cells by fluorescence cross-correlation spectroscopy. *J Virol*, 84: 1254–1264. https://doi.org/10.1128/jvi.01533-09
114. Ito, T., Couceiro, J. N., Kelm, S., Baum, L. G., Krauss, S., Castrucci, M. R., Donatelli, I., Kida, H., Paulson, J. C., Webster, R. G., & Kawaoka, Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol*, 72: 7367–7373.
115. Jagger, B. W., Wise, H. M., Kash, J. C., Walters, K. A., Wills, N. M., Xiao, Y. L., Dunfee, R. L., Schwartzman, L. M., Ozinsky, A., Bell, G. L., Dalton, R. M., Lo, A., Efstathiou, S., Atkins, J. F., Firth, A. E., Taubenberger, J. K., & Digard, P. 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science,* 337: 199–204. https://doi.org/10.1126/science.1222213
116. Janke, B. H. 2000. Diagnosis of swine influenza. *Swine Health Prod*, 8: 79–84.
117. Janke, B. H. 2014. Influenza A virus infections in swine: Pathogenesis and diagnosis. *Vet Pathol*, 51: 410–426. https://doi.org/10.1177/0300985813513043
118. Joseph, U., Su, Y. C. F., Vijaykrishna, D., & Smith, G. J. D. 2017. The ecology and adaptive evolution of influenza A interspecies transmission. *Influenza Other Respir Viruses,* 11: 74–84. https://doi.org/10.1111/irv.12412
119. Kaiser, T. J., Smiley, R. A., Fergen, B., Eichmeyer, M., & Genzow, M. 2019. Influenza A virus shedding reduction observed at 12 weeks post-vaccination when newborn pigs are administered live-attenuated influenza virus vaccine. *Influenza Other Respir Viruses,* 13: 274–278. https://doi.org/10.1111/irv.12630
120. Karasin, A I, Schutten, M. M., Cooper, L. A., Smith, C. B., Subbarao, K., Anderson, G. A., Carman, S., & Olsen, C. W. 2000. Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999:

evidence for wholly human and reassortant virus genotypes. *Virus Res*, 68: 71–85. https://doi.org/10.1016/s0168-1702(00)00154-4

121. Karasin, Alexander I, Carman, S., & Olsen, C. W. 2006. Identification of human H1N2 and human-swine reassortant H1N2 and H1N1 influenza A viruses among pigs in Ontario, Canada (2003 to 2005). *J Clin Microbiol,* 44: 1123–1126. https://doi.org/10.1128/JCM.44.3.1123-1126.2006

122. Karasin, Alexander I, West, K., Carman, S., & Olsen, C. W. 2004. Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. *J Clin Microbiol,* 42: 4349–4354. https://doi.org/10.1128/JCM.42.9.4349-4354.2004

123. Katz, J. M., & Webster, R. G. 1992. Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. *J Gen Virol*, 73*:* 1159–1165. https://doi.org/10.1099/0022-1317-73-5-1159

124. Kevin Dobesh; Keith Bretey DVM, MS, D. D. H. 2013. Utilizing snout wiping for PCR detection and virus isolation of SIV in nursery pigs. *45th AASV Annual Meeting*, Poster presentation.

125. Khuntirat, B. P., Yoon, I. K., Blair, P. J., Krueger, W. S., Chittaganpitch, M., Putnam, S. D., Supawat, K., Gibbons, R. V., Pattamadilok, S., Sawanpanyalert, P., Heil, G. L., Friary, J. A., Capuano, A. W., & Gray, G. C. 2011. Evidence for subclinical avian influenza virus infections among rural thai villagers. *Clin Infect Dis,* 53: 107–116. https://doi.org/10.1093/cid/cir525

126. Kilbourne, E. D. 2006. Influenza pandemics of the 20th century. Emerg Infect Dis, 12: 9–14. https://doi.org/10.3201/eid1201.051254

127. Killian, M. L. 2014. Hemagglutination assay for influenza virus. *Methods Mol Biol,* 1161: 3–9. https://doi.org/10.1007/978-1-4939-0758-8_1

128. Kitikoon, P., Gauger, P. C., & Vincent, A. L. 2014. Hemagglutinin inhibition assay with swine sera. *Methods Mol Biol,* 1161: 295–301. https://doi.org/10.1007/978-1-4939-0758-8_24

129. Kitikoon, P., Nilubol, D., Erickson, B. J., Janke, B. H., Hoover, T. C., Sornsen, S. A., & Thacker, E. L. 2006. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. *Vet Immunol Immunopathol,* 112: 117–128. https://doi.org/10.1016/j.vetimm.2006.02.008

130. Klenk, H. D., & Garten, W. 1994. Host cell proteases controlling virus pathogenicity. *Trends Microbiol,* 2: 39–43. https://doi.org/10.1016/0966-842x(94)90123-6

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

132. Krammer, F., Weir, J. P., Engelhardt, O., Katz, J. M., & Cox, R. J. 2020. Meeting report and review: Immunological assays and correlates of protection for next-generation influenza vaccines. *Influenza Other Respir Viruses,* 14: 237–243. https://doi.org/10.1111/irv.12706

133. Krog, J. S., Hjulsager, C. K., Larsen, M. A., & Larsen, L. E. 2017.

Triple-reassortant influenza A virus with H3 of human seasonal origin, NA of swine origin, and internal A(H1N1) pandemic 2009 genes is established in Danish pigs. *Influenza Other Respir Viruses,* 11: 298–303. https://doi.org/10.1111/irv.12451

134. Krueger, W. S., & Gray, G. C. 2013. Swine influenza virus infections in man. *Curr Top Microbiol Immunol*, 370: 201–225. https://doi.org/10.1007/82_2012_268

135. Kumlin, U., Olofsson, S., Dimock, K., & Arnberg, N. 2008. Sialic acid tissue distribution and influenza virus tropism. *Influenza Other Respir Viruses,* 2: 147–154. https://doi.org/10.1111/j.1750-2659.2008.00051.x

136. Kwit, K., Pomorska-Mól, M., & Markowska-Daniel, I. 2014. The influence of experimental infection of gilts with swine H1N2 influenza A virus during the second month of gestation on the course of pregnancy, reproduction parameters and clinical status. *BMC Vet Res*, 10: 1–12. https://doi.org/10.1186/1746-6148-10-123

137. Kyriakis, C. S., Brown, I. H., Foni, E., Kuntz-Simon, G., Maldonado, J., Madec, F., Essen, S. C., Chiapponi, C., & Van Reeth, K. 2011. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. *Zoonoses Public Health,* 58: 93–101. https://doi.org/10.1111/j.1863-2378.2009.01301.x

138. Kyriakis, C. S., Gramer, M. R., Barbé, F., Van Doorsselaere, J., & Van Reeth, K. 2010. Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. *Vet Microbiol*, 144: 67–74. https://doi.org/10.1016/j.vetmic.2009.12.039

139. Kyriakis, C. S., Olsen, C. W., Carman, S., Brown, I. H., Brookes, S. M., Doorsselaere, J. V., & Reeth, K. V. 2010. Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe. *Emerg Infect Dis*, 16: 96–99. https://doi.org/10.3201/eid1601.091190

140. Landolt, G. A., Karasin, A. I., Hofer, C., Mahaney, J., Svaren, J., & Olsen, C. W. 2005. Use of real-time reverse transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses. *Am J Vet Res,* 66: 119–124. https://doi.org/10.2460/ajvr.2005.66.119

141. Lange, J., Groth, M., Schlegel, M., Krumbholz, A., Wieczorek, K., Ulrich, R., Köppen, S., Schulz, K., Appl, D., Selbitz, H. J., Sauerbrei, A., Platzer, M., Zell, R., & Dürrwald, R. 2013. Reassortants of the pandemic (H1N1) 2009 virus and establishment of a novel porcine H1N2 influenza virus, lineage in Germany. *Vet Microbiol*, 167: 345–356. https://doi.org/10.1016/j.vetmic.2013.09.024

142. Larsen, D. L., Karasin, A., Zuckermann, F., & Olsen, C. W. 2000. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. *Vet Microbiol*, 74: 117–131. https://doi.org/10.1016/S0378-1135(00)00172-3

143. Larsen, L. E., Nielsen, C. K., Aakerblom, S., Hjulsager, C. K., Nielsen, J. P., Stege, H. H., Kristensen, C. S., Elvstrom, A., & Lau, L. 2010. Dynamics of

swine influenza infections in the farrowing unit of a Danish sow herd. *21st International Pig Veterinary Society Congress*, Vancouver, p. 80.

144. Laver, W. G., Air, G. M., & Webster, R. G. 1981. Mechanism of antigenic drift in influenza virus. Amino acid sequence changes in an antigenically active region of Hong Kong (H3N2) influenza virus hemagglutinin. *J Mol Biol,* 145: 339–361. https://doi.org/10.1016/0022-2836(81)90209-6

145. Lee, B. W., Bey, R. F., Baarsch, M. J., & Larson, M. E. 1995. Class specific antibody response to influenza A H1N1 infection in swine. *Vet Microbiol*, 43: 241–250. https://doi.org/10.1016/0378-1135(94)00099-i

146. Lee, J., Wang, L., Palinski, R., Walsh, T., He, D., Li, Y., Wu, R., Lang, Y., Sunwoo, S. Y., Richt, J. A., & Ma, W. 2019. Comparison of pathogenicity and transmissibility of influenza B and D viruses in pigs. *Viruses*, 11: 905. https://doi.org/10.3390/v11100905

147. Leuwerke, B., Kitikoon, P., Evans, R., & Thacker, E. 2008. Comparison of three serological assays to determine the cross-reactivity of antibodies from eight genetically diverse U.S. swine influenza viruses. *J Vet Diagn Invest,* 20: 426–432. https://doi.org/10.1177/104063870802000403

148. Lewis, N. S., Russell, C. A., Langat, P., Anderson, T. K., Berger, K., Bielejec, F., Burke, D. F., Dudas, G., Fonville, J. M., Fouchier, R. A., Kellam, P., Koel, B. F., Lemey, P., Nguyen, T., Nuansrichy, B., Peiris, J. M., Saito, T., Simon, G., Skepner, E., Takemae, N., … Vincent, A. L. 2016. The global antigenic diversity of swine influenza A viruses. *Elife*, 5: e12217. https://doi.org/10.7554/eLife.12217

149. Lewis, P. A., & Shope, R. E. 1931. Swine influenza: II. A hemophilic bacillus from the respiratory tract of infected swine. *J Exp Med*, 54: 361–371. https://doi.org/10.1084/jem.54.3.361

150. Littauer, E. Q., Esser, E. S., Antao, O. Q., Vassilieva, E. V, Compans, R. W., & Skountzou, I. 2017. H1N1 influenza virus infection results in adverse pregnancy outcomes by disrupting tissue-specific hormonal regulation. *PLoS Pathog*, 13: e1006757. https://doi.org/10.1371/journal.ppat.1006757

151. Loeffen, W. L. A., Heinen, P. P., Bianchi, A. T. J., Hunneman, W. A., & Verheijden, J. H. M. 2003. Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. *Vet Immunol Immunopathol,* 92: 23–35. https://doi.org/10.1016/S0165-2427(03)00019-9

152. Loeffen, W. L., Kamp, E. M., Stockhofe-Zurwieden, N., van Nieuwstadt, A. P., Bongers, J. H., Hunneman, W. A., Elbers, A. R., Baars, J., Nell, T., & van Zijderveld, F. G. 1999. Survey of infectious agents involved in acute respiratory disease in finishing pigs. *Vet Rec*, 145: 123–129. https://doi.org/10.1136/vr.145.5.123

153. López-Soria, S., Maldonado, J., Riera, P., Nofrarías, M., Espinal, A., Valero, O., Blanchard, P., Jestin, A., Casal, J., Domingo, M., Artigas, C., & Segalés, J. 2010. Selected swine viral pathogens in indoor pigs in Spain.

Seroprevalence and farm-level characteristics. *Transbound Emerg Dis, 57*: 171–179. https://doi.org/10.1111/j.1865-1682.2010.01135.x

154. Lorusso, A., Vincent, A. L., Gramer, M. E., Lager, K. M., & Ciacci-Zanella, J. R. 2013. Contemporary epidemiology of North American lineage triple reassortant influenza A viruses in pigs. *Curr Top Microbiol Immunol,* 370: 113–132. https://doi.org/10.1007/82_2011_196

155. Lorusso, A., Vincent, A. L., Harland, M. L., Alt, D., Bayles, D. O., Swenson, S. L., Gramer, M. R., Russell, C. A., Smith, D. J., Lager, K. M., & Lewis, N. S. 2011. Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. *J Gen Virol*, 92: 919–930. https://doi.org/10.1099/vir.0.027557-0

156. Loving, C. L., Vincent, A. L., Pena, L., & Perez, D. R. 2012. Heightened adaptive immune responses following vaccination with a temperature-sensitive, live-attenuated influenza virus compared to adjuvanted, whole-inactivated virus in pigs. *Vaccine*, 30: 5830–5838. https://doi.org/10.1016/j.vaccine.2012.07.033

157. Ma, W., Lager, K. M., Vincent, A. L., Janke, B. H., Gramer, M. R., & Richt, J. A. 2009. The role of swine in the generation of novel influenza viruses. *Zoonoses Public Health,* 56: 326–337. https://doi.org/10.1111/j.1863-2378.2008.01217.x

158. Ma, Wenjun, Vincent, A. L., Gramer, M. R., Brockwell, C. B., Lager, K. M., Janke, B. H., Gauger, P. C., Patnayak, D. P., Webby, R. J., & Richt, J. A. 2007. Identification of H2N3 influenza A viruses from swine in the United States. *Proc Natl Acad Sci U S A*, 104: 20949–20954. https://doi.org/10.1073/pnas.0710286104

159. Madec, F., Gourreau, J. M., Kaiser, C., & Aymard, M. 1984. Apparition de manifestations grippales chez les porcs en association avec un virus A/H3 N2. *Bull Acad Vet Fr,* 4: 513. https://doi.org/10.4267/2042/65007

160. Maeda, T., Kawasaki, K., & Ohnishi, S. I. 1981. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. *Proc Natl Acad Sci U S A*, 78: 4133–4137. https://doi.org/10.1073/pnas.78.7.4133

161. Marston, D. A., McElhinney, L. M., Ellis, R. J., Horton, D. L., Wise, E. L., Leech, S. L., David, D., de Lamballerie, X., & Fooks, A. R. 2013. Next generation sequencing of viral RNA genomes. *BMC Genomics*, 14:444 https://doi.org/10.1186/1471-2164-14-444

162. Martín-Valls, G. E., Simon-Grifé, M., van Boheemen, S., de Graaf, M., Bestebroer, T. M., Busquets, N., Martín, M., Casal, J., Fouchier, R. A. M., & Mateu, E. 2014. Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm. *Vet Microbiol*, 170: 266–277. https://doi.org/10.1016/j.vetmic.2014.02.031

163. Matrosovich, M. N., Gambaryan, A. S., Teneberg, S., Piskarev, V. E., Yamnikova, S. S., Lvov, D. K., Robertson, J. S., & Karlsson, K. A. 1997. Avian influenza A viruses differ from human viruses by recognition of

sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology*, 233: 224–234. https://doi.org/10.1006/viro.1997.8580

164. Matrosovich, M. N., Matrosovich, T. Y., Gray, T., Roberts, N. A., & Klenk, H. D. 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A*, 101: 4620–4624. https://doi.org/10.1073/pnas.0308001101

165. Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M. R., Donatelli, I., & Kawaoka, Y. 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol*, 74: 8502–8512. https://doi.org/10.1128/jvi.74.18.8502-8512.2000

166. McCune, S., Arriola, C. S., Gilman, R. H., Romero, M. A., Ayvar, V., Cama, V. A., Montgomery, J. M., Gonzales, A. E., & Bayer, A. M. 2012. Interspecies interactions and potential Influenza A virus risk in small swine farms in Peru. *BMC Infect Dis*, 12: 58. https://doi.org/10.1186/1471-2334-12-58

167. McGill, J., Heusel, J. W., & Legge, K. L. 2009. Innate immune control and regulation of influenza virus infections. *J Leukoc Biol,* 86: 803–812. https://doi.org/10.1189/jlb.0509368

168. McGinnis, J., Laplante, J., Shudt, M., & George, K. S. 2016. Next generation sequencing for whole genome analysis and surveillance of influenza A viruses. *J Clin Virol,* 79*:* 44–50. https://doi.org/10.1016/j.jcv.2016.03.005

169. Meguro, H., Bryant, J. D., Torrence, A. E., & Wright, P. F. 1979. Canine kidney cell line for isolation of respiratory viruses. *J Clin Microbiol, 9:* 175–179.

170. Meseko, C. A., Heidari, A., Odaibo, G. N., & Olaleye, D. O. 2019. Complete genome sequencing of H1N1pdm09 swine influenza isolates from Nigeria reveals likely reverse zoonotic transmission at the human-animal interface in intensive piggery. *Infect Ecol Epidemiol,* 9: 1–10. https://doi.org/10.1080/20008686.2019.1696632

171. Moreno, A., Di Trani, L., Faccini, S., Vaccari, G., Nigrelli, D., Boniotti, M. B., Falcone, E., Boni, A., Chiapponi, C., Sozzi, E., & Cordioli, P. 2011. Novel H1N2 swine influenza reassortant strain in pigs derived from the pandemic H1N1/2009 virus. *Vet Microbiol*, 149: 472–477. https://doi.org/10.1016/j.vetmic.2010.12.011

172. Moreno, A., Gabanelli, E., Sozzi, E., Lelli, D., Chiapponi, C., Ciccozzi, M., Zehender, G., & Cordioli, P. 2013. Different evolutionary trends of swine H1N2 influenza viruses in Italy compared to European viruses. *Vet Res*, 44: 1–14. https://doi.org/10.1186/1297-9716-44-112

173. Morens, D. M., Taubenberger, J. K., Folkers, G. K., & Fauci, A. S. 2010. Pandemic influenza's 500th anniversary. *Clin Infect Dis,* 51: 1442–1444. https://doi.org/10.1086/657429

174. Nachbagauer, R., & Krammer, F. 2017. Universal influenza virus vaccines and therapeutic antibodies. *Clin Microbiol Infect,* 23: 222–228.

https://doi.org/10.1016/j.cmi.2017.02.009
175. Naeve, C. W., Hinshaw, V. S., & Webster, R. G. 1984. Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. *J Virol*, 51: 567–569. https://doi.org/10.1128/jvi.51.2.567-569.1984
176. Nardelli, L., Pascucci, S., Gualandi, G. L., & Loda, P. 1978. Outbreaks of Classical swine influenza in Italy in 1976. *Zentralbl Veterinarmed B,* 25: 853–857. https://doi.org/10.1111/j.1439-0450.1978.tb01062.x
177. Neira, V., Rabinowitz, P., Rendahl, A., Paccha, B., Gibbs, S. G., & Torremorell, M. 2016. Characterization of viral load, viability and persistence of influenza A virus in air and on surfaces of swine production facilities. *PloS One*, 11: e0146616. https://doi.org/10.1371/journal.pone.0146616
178. Nelson, M. I., & Vincent, A. L. 2015. Reverse zoonosis of influenza to swine: New perspectives on the human-animal interface. *Trends Microbiol*, 23: 142–153. https://doi.org/10.1016/j.tim.2014.12.002
179. Nelson, M. I., Detmer, S. E., Wentworth, D. E., Tan, Y., Schwartzbard, A., Halpin, R. A., Stockwell, T. B., Lin, X., Vincent, A. L., Gramer, M. R., & Holmes, E. C. 2012. Genomic reassortment of influenza A virus in North American swine, 1998-2011. *J Gen Virol*, 93: 2584–2589. https://doi.org/10.1099/vir.0.045930-0
180. Nelson, M. I., Lemey, P., Tan, Y., Vincent, A., LamTommy, T. T. Y., Detmer, S., Viboud, C., Suchard, M. A., Rambaut, A., Holmes, E. C., & Gramer, M. 2011. Spatial dynamics of human-origin H1 influenza a virus in North American swine. *PLoS Pathog*, 7: 1–11. https://doi.org/10.1371/journal.ppat.1002077
181. Nelson, M. I., Stucker, K. M., Schobel, S. A., Trovão, N. S., Das, S. R., Dugan, V. G., Nelson, S. W., Sreevatsan, S., Killian, M. L., Nolting, J. M., Wentworth, D. E., & Bowman, A. S. 2016. Introduction, evolution, and dissemination of influenza A viruses in exhibition swine in the United States during 2009 to 2013. *J Virol*, 90: 10963–10971. https://doi.org/10.1128/jvi.01457-16
182. Nguyen, D. C., Uyeki, T. M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H., Xu, X., Balish, A., Klimov, A., Tumpey, T. M., Swayne, D. E., Huynh, L. P. T., Nghiem, H. K., Nguyen, H. H. T., Hoang, L. T., … Katz, J. M. 2005. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J Virol*, 79: 4201–4212. https://doi.org/10.1128/JVI.79.7.4201-4212.2005
183. Nidom, C. A., Takano, R., Yamada, S., Sakai-Tagawa, Y., Daulay, S., Aswadi, D., Suzuki, T., Suzuki, Y., Shinya, K., Iwatsuki-Horimoto, K., Muramoto, Y., & Kawaoka, Y. 2010. Influenza A (H5N1) viruses from pigs, Indonesia. *Emerg Infect Dis,* 16: 1515–1523. https://doi.org/10.3201/eid1610.100508
184. Njabo, K. Y., Fuller, T. L., Chasar, A., Pollinger, J. P., Cattoli, G.,

Terregino, C., Monne, I., Reynes, J.-M., Njouom, R., & Smith, T. B. 2012. Pandemic A/H1N1/2009 influenza virus in swine, Cameroon, 2010. *Vet Microbiol*, 156: 189–192. https://doi.org/10.1016/j.vetmic.2011.09.003

185. Nonthabenjawan, N., Chanvatik, S., Chaiyawong, S., Jairak, W., Boonyapisusopha, S., Tuanudom, R., Thontiravong, A., Bunpapong, N., & Amonsin, A. 2015. Genetic diversity of swine influenza viruses in Thai swine farms, 2011–2014. *Virus Genes*, 50: 221–230. https://doi.org/10.1007/s11262-014-1153-x

186. OIE (World Organization for Animal Health). 2018. Manual for diagnostic tests and vaccines for terrestrial animals. Available at: https://www.oie.int/en/standard-setting/terrestrial-manual/access-online/

187. Okuya, K., Matsuu, A., Kawabata, T., Koike, F., Ito, M., Furuya, T., Taneno, A., Akimoto, S., Deguchi, E., & Ozawa, M. 2018. Distribution of gene segments of the pandemic A(H1N1) 2009 virus lineage in pig populations. *Transbound Emerg Dis*, 65: 1502–1513. https://doi.org/10.1111/tbed.12887

188. Olitsky, P. K., & Gates, F. L. 1921. Experimental studies of the nasopharyngeal secretions from influenza patients: II. Filterability and resistance to glycerol. *J Exp Med,* 33: 361–372. https://doi.org/10.1084/jem.33.3.361

189. Olsen, C. W., Karasin, A. I., Carman, S., Li, Y., Bastien, N., Ojkic, D., Alves, D., Charbonneau, G., Henning, B. M., Low, D. E., Burton, L., & Broukhanski, G. 2006. Triple reassortant H3N2 influenza A viruses, Canada, 2005. *Emerg Infect Dis,* 12: 1132–1135. https://doi.org/10.3201/eid1207.060268

190. Ottis, K., & Bachmann, P. A. 1980. Occurrence of Hsw1N1 subtype influenza A viruses in wild ducks in Europe. *Arch Virol,* 63: 185–190. https://doi.org/10.1007/BF01315025

191. Palese, P., Tobita, K., Ueda, M., & Compans, R. W. 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology*, 61: 397–410. https://doi.org/https://doi.org/10.1016/0042-6822(74)90276-1

192. Panyasing, Y., Goodell, C. K., Wang, C., Kittawornrat, A., Prickett, J. R., Schwartz, K. J., Ballagi, A., Lizano, S., & Zimmerman, J. J. 2014. Detection of influenza A virus nucleoprotein antibodies in oral fluid specimens from pigs infected under experimental conditions using a blocking ELISA. *Transbound Emerg Dis*, 61: 177–184. https://doi.org/10.1111/tbed.12019

193. Pedersen, J. C. 2014. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. *Methods Mol Biol,* 1161: 11–25. https://doi.org/10.1007/978-1-4939-0758-8_2

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

195. Pfeiffer, R. 1892. I.-Preliminary Communication on the Exciting causes of Influenza. *Br Med J,* 1:128. https://doi.org/10.1136/bmj.1.1620.128

196. Pileri, E., Martín-Valls, G. E., Díaz, I., Allepuz, A., Simon-Grifé, M., García-Saenz, A., Casal, J., & Mateu, E. 2017. Estimation of the transmission parameters for swine influenza and porcine reproductive and respiratory syndrome viruses in pigs from weaning to slaughter under natural conditions. *Prev Vet Med*, 138: 147–155. https://doi.org/10.1016/j.prevetmed.2017.01.008
197. Pitzer, V. E., Aguas, R., Riley, S., Loeffen, W. L., Wood, J. L., & Grenfell, B. T. 2016. High turnover drives prolonged persistence of influenza in managed pig herds. *J R Soc Interface,* 13: 20160138. https://doi.org/10.1098/rsif.2016.0138
198. Plotch, S. J., Bouloy, M., Ulmanen, I., & Krug, R. M. 1981. A unique cap(m^7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell*, *23*: 847–858. https://doi.org/10.1016/0092-8674(81)90449-9
199. Potter C. W. 2008. A history of influenza. *J Appl Microbiol*, 91: 572–579. https://doi.org/10.1046/j.1365-2672.2001.01492.x
200. Quiñones-Mateu, M. E., Avila, S., Reyes-Teran, G., & Martinez, M. A. 2014. Deep sequencing: becoming a critical tool in clinical virology. *J Clin Virol*, 61: 9–19. https://doi.org/https://doi.org/10.1016/j.jcv.2014.06.013
201. Radford, A. D., Chapman, D., Dixon, L., Chantrey, J., Darby, A. C., & Hall, N. 2012. Application of next-generation sequencing technologies in virology. *J Gen Virol,* 93: 1853–1868. https://doi.org/10.1099/vir.0.043182-0
202. Rajão, D. S., & Vincent, A. L. 2015. Swine as a model for influenza avirus infection and immunity. *ILAR J*, 56: 44–52. https://doi.org/10.1093/ilar/ilv002
203. Rajão, D. S., Loving, C. L., Gauger, P. C., Kitikoon, P., & Vincent, A. L. 2014. Influenza A virus hemagglutinin protein subunit vaccine elicits vaccine-associated enhanced respiratory disease in pigs. *Vaccine*, 32: 5170–5176. https://doi.org/10.1016/j.vaccine.2014.07.059
204. Rajão, D. S., Vincent, A. L., & Perez, D. R. 2018. Adaptation of human influenza viruses to swine. *Front Vet Sci,* 5: 347. https://doi.org/10.3389/fvets.2018.00347
205. Rajão, D. S., Walia, R. R., Campbell, B., Gauger, P. C., Janas-Martindale, A., Killian, M. L., & Vincent, A. L. 2017. Reassortment between swine H3N2 and 2009 Pandemic H1N1 in the United states resulted in influenza A viruses with diverse genetic constellations with variable virulence in pigs. *J Virol*, 91: 1–12. https://doi.org/10.1128/jvi.01763-16
206. Ramirez, A., Wang, C., Prickett, J. R., Pogranichniy, R., Yoon, K. J., Main, R., Johnson, J. K., Rademacher, C., Hoogland, M., Hoffmann, P., Kurtz, A., Kurtz, E., & Zimmerman, J. 2012. Efficient surveillance of pig populations using oral fluids. *Prev Vet Med*, 104: 292–300. https://doi.org/10.1016/j.prevetmed.2011.11.008
207. Reeth, K. V., Brown, I., Essen, S., & Pensaert, M. 2004. Genetic relationships, serological cross-reaction and cross-protection between H1N2 and

other influenza A virus subtypes endemic in European pigs. *Virus Res*, 103: 115–124. https://doi.org/10.1016/j.virusres.2004.02.023

208. Reich, S., Guilligay, D., Pflug, A., Malet, H., Berger, I., Crepin, T., Hart, D., Lunardi, T., Nanao, M., Ruigrok, R. W. H., & Cusack, S. 2014. Structural insight into cap-snatching and RNA synthesis by influenza polymerase. *Nature*, 516: 361–366. https://doi.org/10.1038/nature14009

209. Reina, J., Fernandez-Baca, V., Blanco, I., & Munar, M. 1997. Comparison of Madin-Darby canine kidney cells (MDCK) with a green monkey continuous cell line (Vero) and human lung embryonated cells (MRC-5) in the isolation of influenza a virus from nasopharyngeal aspirates by shell vial culture. *J Clin Microbiol,* 35: 1900–1901. https://doi.org/10.1128/jcm.35.7.1900-1901.1997

210. Reynolds, J. J., Torremorell, M., & Craft, M. E. 2014. Mathematical modeling of influenza A virus dynamics within swine farms and the effects of vaccination. *PloS One*, 9: e106177. https://doi.org/10.1371/journal.pone.0106177

211. Robertson, J. S., Schubert, M., & Lazzarini, R. A. 1981. Polyadenylation sites for influenza virus mRNA. *J Virol*, 38: 157–163. https://doi.org/10.1128/jvi.38.1.157-163.1981

212. Rogers, G. N., Pritchett, T. J., Lane, J. L., & Paulson, J. C. 1983. Differential sensitivity of human, avian, and equine influenza a viruses to a glycoprotein inhibitor of infection: selection of receptor specific variants. *Virology*, 131: 394–408. https://doi.org/https://doi.org/10.1016/0042-6822(83)90507-X

213. Röhm, C., Süss, J., Pohle, V., & Webster, R. G. 1996. Different hemagglutinin cleavage site variants of H7N7 in an influenza outbreak in chickens in Leipzig, Germany. *Virology*, 218: 253–257. https://doi.org/10.1006/viro.1996.0187

214. Romagosa, A., Allerson, M., Gramer, M., Joo, H., Deen, J., Detmer, S., & Torremorell, M. 2011. Vaccination of influenza a virus decreases transmission rates in pigs. *Vet Res*, 42: 120. https://doi.org/10.1186/1297-9716-42-120

215. Romagosa, A., Gramer, M., Joo, H. S., & Torremorell, M. 2012. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. *Influenza Other Respir Viruses,* 6: 110–118. https://doi.org/10.1111/j.1750-2659.2011.00276.x

216. Rose, N., & Andraud, M. 2017. The use of vaccines to control pathogen spread in pig populations. *Porcine Health Manag,* 3: 1–8. https://doi.org/10.1186/s40813-017-0053-6

217. Rose, N., Hervé, S., Eveno, E., Barbier, N., Eono, F., Dorenlor, V., Andraud, M., Camsusou, C., Madec, F., & Simon, G. 2013. Dynamics of influenza A virus infections in permanently infected pig farms: evidence of recurrent infections, circulation of several swine influenza viruses and reassortment events. *Vet Res*, 44: 72. https://doi.org/10.1186/1297-9716-44-72

218. Rott, R., Klenk, H. D., Nagai, Y., & Tashiro, M. 1995. Influenza viruses, cell enzymes, and pathogenicity. *Am J Respir Crit Care Med,* 152: S16–S19. https://doi.org/10.1164/ajrccm/152.4_Pt_2.S16
219. Russel, R. J. 2008. Orthomyxoviruses: Structure of Antigens. In: *Encyclopedia of Virology*. B. W. J. Mahy & M. H. V. Van Regenmortel, (editors). Academic Press Inc. San Diego, CA, USA. 489–494 pp.
220. Ryt-Hansen, P., Larsen, I., Kristensen, C. S., Krog, J. S., & Larsen, L. E. 2019a. Limited impact of influenza A virus vaccination of piglets in an enzootic infected sow herd. *Res Vet Sci*, 127: 47–56. https://doi.org/10.1016/j.rvsc.2019.10.015
221. Ryt-Hansen, P., Larsen, I., Kristensen, C. S., Krog, J. S., Wacheck, S., & Larsen, L. E. 2019b. Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies. *Vet Res*, 50: 1–10. https://doi.org/10.1186/s13567-019-0655-x
222. Ryt-Hansen, P., Pedersen, A. G., Larsen, I., Krog, J. S., Kristensen, C. S., & Larsen, L. E. 2019c. Acute Influenza A virus outbreak in an enzootic infected sow herd: Impact on viral dynamics, genetic and antigenic variability and effect of maternally derived antibodies and vaccination. *PLoS One*, 14: 1–23. https://doi.org/10.1371/journal.pone.0224854
223. Samji, T. 2009. Influenza A: understanding the viral life cycle. *Yale J Biol Med,* 82: 153–159.
224. Sandbulte, M., Spickler, A., Zaabel, P., & Roth, J. 2015. Optimal use of vaccines for control of influenza A virus in swine. *Vaccines*, 3: 22–73. https://doi.org/10.3390/vaccines3010022
225. Schirmer, M., Ijaz, U. Z., D'Amore, R., Hall, N., Sloan, W. T., & Quince, C. 2015. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nucleic Acids Res,* 43:e37 https://doi.org/10.1093/nar/gku1341
226. Schulze, I. T. 1997. Effects of Glycosylation on the Properties and Functions of Influenza Virus Hemagglutinin. *J Infect Dis*, 176: S24–S28. https://doi.org/10.1086/514170
227. Sencer, D. J., & Millar, J. D. 2006. Reflections on the 1976 swine flu vaccination program. *Emerg Infect Dis,* 12: 29–33. https://doi.org/10.3201/eid1201.051007
228. Serafini Poeta Silva, A. P., de Freitas Costa, E., Sousa E Silva, G., Souza, C. K., Schaefer, R., da Silva Vaz, I., Jr, & Corbellini, L. G. 2019. Biosecurity practices associated with influenza A virus seroprevalence in sows from southern Brazilian breeding herds. *Prev Vet Med*, 166: 1–7. https://doi.org/10.1016/j.prevetmed.2019.02.013
229. Shapiro, G. I., Gurney, T., & Krug, R. M. 1987. Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. *J Virol*, 61: 764–773. https://doi.org/10.1128/jvi.61.3.764-773.1987

230. Sharma, A., Zeller, M. A., Li, G., Harmon, K. M., Zhang, J., Hoang, H., Anderson, T. K., Vincent, A. L., & Gauger, P. C. 2020. Detection of live attenuated influenza vaccine virus and evidence of reassortment in the U.S. swine population. *J Vet Diagn Invest,* 32: 301–311. https://doi.org/10.1177/1040638720907918
231. Shaw, M. L., & Palese, P. 2008. Orthomyxoviruses: Molecular Biology. In: *Encyclopedia of Virology.* B. W. J. Mahy, & M. H. V. Van Regenmortel, (editors). Academic Press Inc. San Diego, CA, USA. 483–489 pp.
232. Sheerar, M. G., Easterday, B. C., & Hinshaw, V. S. 1989. Antigenic conservation of H1N1 swine influenza viruses. *J Gen Virol*, 70: 3297–3303. https://doi.org/10.1099/0022-1317-70-12-3297
233. Shope, R. E. 1931. Swine influenza: III. Filtration experiments and etiology. *J Exp Med,* 54: 373–385. https://doi.org/10.1084/jem.54.3.373
234. Shope, R. E. 1936. Immunization experiments with swine influenza virus. *J Exp Med,* 64: 47–62. https://doi.org/10.1084/jem.64.1.47
235. Simon, G., Larsen, L. E., Dürrwald, R., Foni, E., Harder, T., Van Reeth, K., Markowska-Daniel, I., Reid, S. M., Dan, A., Maldonado, J., Huovilainen, A., Billinis, C., Davidson, I., Agüero, M., Vila, T., Hervé, S., Breum, S. Ø., Chiapponi, C., Urbaniak, K., … Vincent, A. L. 2014. European surveillance network for influenza in pigs: surveillance programs, diagnostic tools and swine influenza virus subtypes identified in 14 European countries from 2010 to 2013. *PLoS One*, 9: 1–21. https://doi.org/10.1371/journal.pone.0115815
236. Simon-Grifé, M., Martín-Valls, G. E., Vilar, M. J., Busquets, N., Mora-Salvatierra, M., Bestebroer, T. M., Fouchier, R. A., 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. https://doi.org/10.1186/1297-9716-43-24
237. Simon-Grifé, M., Martín-Valls, G. E., Vilar, M. J., 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. https://doi.org/10.1016/j.vetmic.2010.10.015
238. Sisteré-Oró, M., López-Serrano, S., Veljkovic, V., Pina-Pedrero, S., Vergara-Alert, J., Córdoba, L., Pérez-Maillo, M., Pleguezuelos, P., Vidal, E., Segalés, J., Nielsen, J., Fomsgaard, A., & Darji, A. 2019. DNA vaccine based on conserved HA-peptides induces strong immune response and rapidly clears influenza virus infection from vaccinated pigs. *PLoS One*, 14: 1–21. https://doi.org/10.1371/journal.pone.0222201
239. Skehel, J. J., & Wiley, D. C. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem,* 69: 531–569. https://doi.org/10.1146/annurev.biochem.69.1.531
240. Skibbe, D., Zhou, E. M., & Janke, B. H. 2004. Comparison of a commercial enzyme-linked immunosorbent assay with hemagglutination inhibition assay for serodiagnosis of swine influenza virus (H1N1) infection. *J Vet Diagn Invest,* 16: 86–89. https://doi.org/10.1177/104063870401600116

241. Skowronski, D. M., Janjua, N. Z., De Serres, G., Sabaiduc, S., Eshaghi, A., Dickinson, J. A., Fonseca, K., Winter, A. L., Gubbay, J. B., Krajden, M., Petric, M., Charest, H., Bastien, N., Kwindt, T. L., Mahmud, S. M., Van Caeseele, P., & Li, Y. 2014. Low 2012-13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses. *PloS One*, 9: e92153. https://doi.org/10.1371/journal.pone.0092153

242. Smith, G. J. D., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghwani, J., Bhatt, S., Peiris, J. S. M., Guan, Y., & Rambaut, A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza a epidemic. *Nature*, 459: 1122–1125. https://doi.org/10.1038/nature08182

243. Smith, G. L., & Hay, A. J. 1982. Replication of the influenza virus genome. *Virology*, 118: 96–108. https://doi.org/10.1016/0042-6822(82)90323-3

244. Smith, W., Andrewes, C. H., & Laidlaw, P. P. 1935. Influenza: experiments on the immunization of ferrets and mice. *Br J Exp Pathol,* 16: 291–302.

245. Snoeck, C. J., Abiola, O. J., Sausy, A., Okwen, M. P., Olubayo, A. G., Owoade, A. A., & Muller, C. P. 2015. Serological evidence of pandemic (H1N1) 2009 virus in pigs, West and Central Africa. *Vet Microbiol*, 176: 165–171. https://doi.org/10.1016/j.vetmic.2014.12.022

246. Souza, C. K., Oldiges, D. P., Poeta, A. P. S., Vaz, I. da S., Schaefer, R., Gava, D., Ciacci-Zanella, J. R., Canal, C. W., & Corbellini, L. G. 2019. Serological surveillance and factors associated with influenza A virus in backyard pigs in Southern Brazil. *Zoonoses Public Health,* 66: 125–132. https://doi.org/10.1111/zph.12542

247. Spackman, E. 2014. A brief introduction to avian influenza virus. *Methods Mol Biol,* 1161: 61–68. https://doi.org/10.1007/978-1-4939-0758-8_6

248. Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T., & Suarez, D. L. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol,* 40: 3256–3260. https://doi.org/10.1128/jcm.40.9.3256-3260.2002

249. Starick, E., Lange, E., Fereidouni, S., Bunzenthal, C., Hoveler, R., Kuczka, A., Grosse Beilage, E., Hamann, H.-P., Klingelhofer, I., Steinhauer, D., Vahlenkamp, T., Beer, M., & Harder, T. 2011. Reassorted pandemic (H1N1) 2009 influenza A virus discovered from pigs in Germany. *J Gen Virol*, 92: 1184–1188. https://doi.org/10.1099/vir.0.028662-0

250. Stika R, Holtkamp D, Goodell C, et al. 2018. Assessment of the association between respiratory pathogen burden and the productivity of growing pigs. *49th AASV Annual Meeting*, Poster presentation.

251. Swenson, S. L., Vincent, L. L., Lute, B. M., Janke, B. H., Lechtenberg, K. F., Landgraf, J. G., Schmitt, B. J., Kinker, D. R., & McMillen, J. K. 2001. A comparison of diagnostic assays for the detection of type A swine influenza

virus from nasal swabs and lungs. *J Vet Diagn Invest,* 13: 36–42. https://doi.org/10.1177/104063870101300108

252. Taubenberger, J. K., & Morens, D. M. 2006. 1918 Influenza: the mother of all pandemics. *Emerg Infect Dis,* 12: 15–22. https://doi.org/10.3201/eid1209.05-0979

253. Tellier, R. 2006. Review of aerosol transmission of influenza A virus. *Emerg Infect Dis,* 12: 1657–1662. https://doi.org/10.3201/eid1211.060426

254. Thacker, E., & Janke, B. 2008. Swine influenza virus: zoonotic potential and vaccination strategies for the control of avian and swine influenzas. *J Infect Dis,* 197: S19–S24. https://doi.org/10.1086/524988

255. Tobita, K. 1975. Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. *Med Microbiol Immunol,* 162: 23–27. https://doi.org/10.1007/BF02123574

256. Tong, S., Li, Y., Rivailler, P., Conrardy, C., Alvarez Castillo, D. A., Chen, L. M., Recuenco, S., Ellison, J. A., Davis, C. T., York, I. A., Turmelle, A. S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M. R., Tang, K., Rowe, L. A., Sammons, S., … Donis, R. O. 2012. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A*, *109*: 4269–4274. https://doi.org/10.1073/pnas.1116200109

257. Torremorell, M., Allerson, M., Corzo, C., Diaz, A., & Gramer, M. 2012. Transmission of influenza A virus in pigs. *Transbound Emerg Dis*, 59*:* 68–84. https://doi.org/10.1111/j.1865-1682.2011.01300.x

258. Torremorell, M., Juarez, A., Chavez, E., Yescas, J., Doporto, J. M., & Gramer, M. 2009. Procedures to eliminate H3N2 swine influenza virus from a pig herd. *Vet Rec*, 165: 74–77. https://doi.org/10.1136/vetrec.165.3.74

259. Trebbien, R., Bragstad, K., Larsen, L. E., Nielsen, J., Bøtner, A., Heegaard, P. M., Fomsgaard, A., Viuff, B., & Hjulsager, C. K. 2013. Genetic and biological characterisation of an avian-like H1N2 swine influenza virus generated by reassortment of circulating avian-like H1N1 and H3N2 subtypes in Denmark. *Virol J*, 10: 290. https://doi.org/10.1186/1743-422X-10-290

260. Tsai, C. P., & Tsai, H. J. 2019. Influenza B viruses in pigs, Taiwan. *Influenza Other Respir Viruses*, 13: 91–105. https://doi.org/10.1111/irv.12588

261. Underwood, P. A., Skehel, J. J., & Wiley, D. C. 1987. Receptor-binding characteristics of monoclonal antibody-selected antigenic variants of influenza virus. *J Virol*, 61: 206–208. https://doi.org/10.1128/jvi.61.1.206-208.1987

262. Van Reeth, K, & Pensaert, M. 1994. Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. *Vet Rec*, 135: 594–597.

263. Van Reeth, K, Labarque, G., Nauwynck, H., & Pensaert, M. 1999. Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. *Res Vet Sci,* 67: 47–52. https://doi.org/10.1053/rvsc.1998.0277

264. Van Reeth, K., & Vincent, A. 2019. Influenza viruses. In: Diseases of Swine. Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW,

Zhang J (editors). Wiley and Sons Inc. Hoboken, NJ, USA. 576-593 pp.
265. Van Reeth, K. 2007. Avian and swine influenza viruses: our current understanding of the zoonotic risk. *Vet Res*, 38: 243–260. https://doi.org/10.1051/vetres:2006062
266. Van Reeth, K., & Ma, W. 2013. Swine influenza virus vaccines: to change or not to change-that's the question. *Curr Top Microbiol Immunol,* 370: 173–200. https://doi.org/10.1007/82_2012_266
267. Van Reeth, K., Brown, I. H., Dürrwald, R., Foni, E., Labarque, G., Lenihan, P., Maldonado, J., Markowska-Daniel, I., Pensaert, M., Pospisil, Z., & Koch, G. 2008. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003. *Influenza Other Respir Viruses,* 2: 99–105. https://doi.org/10.1111/j.1750-2659.2008.00043.x
268. Van Reeth, K., Gregory, V., Hay, A., & Pensaert, M. 2003. Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. *Vaccine*, 21: 1375–1381. https://doi.org/10.1016/s0264-410x(02)00688-6
269. Van Reeth, K., Labarque, G., & Pensaert, M. 2006. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. *Viral Immunol,* 19: 373–382. https://doi.org/10.1089/vim.2006.19.373
270. Van Reeth, K., Van Gucht, S., & Pensaert, M. 2002. Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs. *Viral Immunol*, 15: 583–594. https://doi.org/10.1089/088282402320914520
271. Velthuis, A. G. J., Bouma, A., Katsma, W. E. A., Nodelijk, G., & De Jong, M. C. M. 2007. Design and analysis of small-scale transmission experiments with animals. *Epidemiol Infect,* 135: 202–217. https://doi.org/10.1017/S095026880600673X
272. Vijaykrishna, D, Poon, L. L. M., Zhu, H. C., Ma, S. K., Li, O. T. W., Cheung, C. L., Smith, G. J. D., Peiris, J. S. M., & Guan, Y. 2010. Reassortment of pandemic H1N1/2009 influenza A virus in swine. *Science,* 328: 1529. https://doi.org/10.1126/science.1189132
273. Vijaykrishna, Dhanasekaran, Smith, G. J. D., Pybus, O. G., Zhu, H., Bhatt, S., Poon, L. L. M., Riley, S., Bahl, J., Ma, S. K., Cheung, C. L., Perera, R. A. P. M., Chen, H., Shortridge, K. F., Webby, R. J., Webster, R. G., Guan, Y., & Peiris, J. S. M. 2011. Long-term evolution and transmission dynamics of swine influenza A virus. *Nature*, 473: 519–522. https://doi.org/10.1038/nature10004
274. Vincent, A. L., Ma, W., Lager, K. M., Janke, B. H., & Richt, J. A. 2008. Swine influenza viruses a North American perspective. *Adv Virus Res,* 72: 127–154. https://doi.org/10.1016/S0065-3527(08)00403-X
275. Vincent, A. L., Ma, W., Lager, K. M., Richt, J. A., Janke, B. H., Sandbulte, M. R., Gauger, P. C., Loving, C. L., Webby, R. J., & Garcia-Sastre, A. 2012. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing

vaccine-associated enhanced respiratory disease. *J Virol*, 86: 10597–10605. https://doi.org/10.1128/jvi.01439-12

276. Vincent, A. L., Perez, D. R., Rajão, D., Anderson, T. K., Abente, E. J., Walia, R. R., & Lewis, N. S. 2017. Influenza A virus vaccines for swine. *Vet Microbiol*, 206: 35–44. https://doi.org/10.1016/j.vetmic.2016.11.026

277. Vincent, L. L., Janke, B. H., Paul, P. S., & Halbur, P. G. 1997. A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. *J Vet Diagn Invest,* 9: 191–195. https://doi.org/10.1177/104063879700900214

278. Wagner, R., Matrosovich, M., & Klenk, H. D. 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol,* 12: 159–166. https://doi.org/10.1002/rmv.352

279. Walia, R. R., Anderson, T. K., & Vincent, A. L. 2019. Regional patterns of genetic diversity in swine influenza A viruses in the United States from 2010 to 2016. *Influenza Other Respir Viruses,* 13: 262–273. https://doi.org/10.1111/irv.12559

280. Ward, B. J., Pillet, S., Charland, N., Trepanier, S., Couillard, J., & Landry, N. 2018. The establishment of surrogates and correlates of protection: useful tools for the licensure of effective influenza vaccines? *Hum Vaccin Immunother*, 14: 647–656. https://doi.org/10.1080/21645515.2017.1413518

281. Watson, S. J., Langat, P., Reid, S. M., Lam, T. T., Cotten, M., Kelly, M., Van Reeth, K., Qiu, Y., Simon, G., Bonin, E., Foni, E., Chiapponi, C., Larsen, L., Hjulsager, C., Markowska-Daniel, I., Urbaniak, K., Dürrwald, R., Schlegel, M., Huovilainen, A., Davidson, I., … Kellam, P. 2015. Molecular epidemiology and evolution of influenza viruses circulating within European swine between 2009 and 2013. *J Virol*, 89: 9920–9931. https://doi.org/10.1128/JVI.00840-15

282. Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., & Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev,* 56: 152–179. https://doi.org/10.1128/mmbr.56.1.152-179.1992

283. Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., & Wiley, D. C. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature*, 333: 426–431. https://doi.org/10.1038/333426a0

284. Welsh, M. D., Baird, P. M., Guelbenzu-Gonzalo, M. P., Hanna, A., Reid, S. M., Essen, S., Russell, C., Thomas, S., Barrass, L., McNeilly, F., McKillen, J., Todd, D., Harkin, V., McDowell, S., Choudhury, B., Irvine, R. M., Borobia, J., Grant, J., & Brown, I. H. 2010. Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs. *Vet Rec*, 166: 642–645. https://doi.org/10.1136/vr.4851

285. Wesley, R. D., Tang, M., & Lager, K. M. 2004. Protection of weaned pigs by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of H3N2 swine influenza virus. *Vaccine*, 22: 3427–3434. https://doi.org/https://doi.org/10.1016/j.vaccine.2004.02.040

286. White, L. A., Torremorell, M., & Craft, M. E. 2017. Influenza A virus in swine breeding herds: combination of vaccination and biosecurity practices can reduce likelihood of endemic piglet reservoir. *Prev Vet Med*, 138: 55–69. https://doi.org/10.1016/j.prevetmed.2016.12.013
287. Wiley, D. C., & Skehel, J. J. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem,* 56: 365–394. https://doi.org/10.1146/annurev.bi.56.070187.002053
288. Wiley, D. C., Wilson, I. A., & Skehel, J. J. 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature*, 289: 373–378. https://doi.org/10.1038/289373a0
289. Wilson, I. A., Skehel, J. J., & Wiley, D. C. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. *Nature*, 289: 366–373. https://doi.org/10.1038/289366a0
290. Wise, H. M., Foeglein, A., Sun, J., Dalton, R. M., Patel, S., Howard, W., Anderson, E. C., Barclay, W. S., & Digard, P. 2009. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol*, 83: 8021–8031. https://doi.org/10.1128/JVI.00826-09
291. Wong, K. K., Gambhir, M., Finelli, L., Swerdlow, D. L., Ostroff, S., & Reed, C. 2013. Transmissibility of variant influenza from Swine to humans: a modeling approach. *Clin Infect Dis,* 57*:* S16–S22. https://doi.org/10.1093/cid/cit303
292. World Health Organization (WHO). 2009. Epidemiological summary of pandemic influenza A (H1N1) 2009 virus - Ontario, Canada, June 2009. *REH,* 84: 485–491.
293. World Health Organization (WHO). 2010, August 6. Pandemic (H1N1) 2009 – update 112. Retrieved from: https://www.who.int/csr/don/2010_08_06/en/
294. World Health Organization (WHO). 2011. Manual for the laboratory diagnosis and virological surveillance of influenza. World Health Organization.
295. Yilmaz, A., Umar, S., Turan, N., Aydin, O., Tali, H. E., Oguzoglu, T. C., Yilmaz, H., Richt, J. A., & Ducatez, M. F. 2020. First report of influenza D virus infection in Turkish cattle with respiratory disease. *Res Vet Sci,* 130: 98–102. https://doi.org/10.1016/j.rvsc.2020.02.017
296. Yoon, K.-J., & Janke, B. H. 2002. Swine Influenza: Etiology, Epidemiology, and Diagnosis. In: *Trends in Emerging Viral Infections of Swine.* A. Morilla, K. –J. Yoon, & J. J. Zimmerman (editors). Iowa State Press. Iowa, USA. 23–28 pp. https://doi.org/doi:10.1002/9780470376812.ch2a
297. Yu, H., Zhou, Y.-J., Li, G.-X., Ma, J.-H., Yan, L.-P., Wang, B., Yang, F.-R., Huang, M., & Tong, G.-Z. 2011. Genetic diversity of H9N2 influenza viruses from pigs in China: a potential threat to human health? *Vet Microbiol*, 149: 254–261. https://doi.org/10.1016/j.vetmic.2010.11.008
298. Zell, R., Groth, M., Krumbholz, A., Lange, J., Philipps, A., & Dürrwald,

R. 2020. Displacement of the Gent/1999 human-like swine H1N2 influenza A virus lineage by novel H1N2 reassortants in Germany. *Arch Virol,* 165: 55–67. https://doi.org/10.1007/s00705-019-04457-w

299. Zhang, J., & Gauger, P. C. 2014. Isolation of swine influenza virus in cell cultures and embryonated chicken eggs. *Methods Mol Biol,* 1161: 265–276. https://doi.org/10.1007/978-1-4939-0758-8_22

300. Zhou, N. N., Senne, D. A., Landgraf, J. S., Swenson, S. L., Erickson, G., Rossow, K., Liu, L., Yoon, K., Krauss, S., & Webster, R. G. 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. *J Virol*, 73: 8851–8856. https://doi.org/10.1128/jvi.73.10.8851-8856.1999

301. Zhu, H., Webby, R., Lam, T. T. Y., Smith, D. K., Peiris, J. S. M., & Guan, Y. 2013. History of swine influenza viruses in Asia. *Curr Top Microbiol Immunol,* 370: 57–68. https://doi.org/10.1007/82_2011_179

302. Zimmer, S. M., & Burke, D. S. 2009. Historical perspective - Emergence of influenza a (H1N1) viruses. *N Engl J Med*, 61: 279. https://doi.org/10.1056/NEJMra0904322

Conclusions

1. The present study reports for the first time an H1avN2hu in pigs in Spain that has become the most prevalent lineage, overpowering the formerly predominant H1avN1av. This would be consistent with the introduction of a fit strain in a susceptible population.
2. Similarly, to France and Denmark, in Spain, the H3huN2hu has declined significantly compared to previous published reports. The causes for such a decline are unknown and cannot be foreseen from our results.
3. The H3huN1av detected in our study can be classified as a local subtype since no other similar isolates are known in Europe. This H3hu is related to human isolates from the early 2000s and to other swine isolates in Denmark but the phylogenetic analysis does not permit to establish with precision the origin and the time of such introduction.
4. We detected seven previously unreported genotypes of IAV. Considering that the genotype classification is only five years old and that was elaborated based on isolates of all over Europe. These new findings suggest a rapid evolution of swine influenza viruses and the urgent need for an update of the classification scheme.
5. The introduction of the first vaccination scheme modified the dynamics of IAV infection in the nurseries regardless of the group, resulting in a later onset of transmission, higher beta values and a more homogeneous behaviour of the infection between pens. This coincided with lower levels of maternally-derived antibodies. Taking together these results, they suggest that viral circulation ceased in sows, and piglets became infected only when entered the nursery premises.
6. In the first vaccination trial, pre-farrowing vaccination did not result in a decreased incidence or a major change in the dynamics of infection in the nurseries and no major differences were seen between treatment and control animals. This suggests that the impact of homogenising the immune status of sows by using a blanket vaccination was stronger than that of the pre-farrowing vaccination.
7. A high percentage of the infected animals (between 11% and 41%) yielded positive RT-qPCR results in two or more consecutive samplings. This fact could not be related to the presence or the levels of maternally derived antibodies or to the emergence of escape mutants, but was significantly higher when vaccine and field strain were lineage-matched. It is important to elucidate in further studies the

contribution of the passive immunity, the idiosyncrasy of the pig and the strain in this phenomenon.

8. The lateral introduction of a H3huN2hu strain in the trial farm emphasises the need to continue the monitoring of IAV in pig farms, particularly when a change in the transmission patterns are noticed.

Appendix

A. Poster presentations in congresses

1. Sosa Portugal, S., Martí, C., Casanovas, C., Barrabés, S., Mesonero-Escuredo, S., Tello, M., Soto, J., Mateu, E., & Martín-Valls, G. 2018. Epidemiological surveillance and characterization of influenza A viruses (IAV) in Spanish and Portuguese pig farms. 10th ESPHM (Spain). 9^{th} –11^{th} May 2018.
2. Sosa Portugal, S., Martí, C., Casanovas, C., Barrabés, S., Mesonero-Escuredo, S., Tello, M., Mateu, E., & Martín-Valls, G. 2019. Epidemiological surveillance and characterization of influenza A viruses (IAV) in Spanish and Portuguese pig farms. 11th ESPHM (Netherlands). 22^{nd} – 24^{th} May 2019.
3. Sosa Portugal, S., Cortey, M., Casanovas, C., Mesonero-Escuredo, S., Barrabés, S., Vidal, A., Clilverd, H., Illas, F., Wacheck, S., Mateu, E., & Martín-Valls, G. 2020. Dynamics of influenza A virus transmission in endemic nurseries in a pig farm in Spain before and after vaccination of sows. 12th ESPHM (Switzerland) *Postponed to April 14^{th} – 16^{th} 2021 due to COVID-19.*
4. Li, Y., Sosa, S., Casanovas, C., Barrabés, S., Mesonero-Escuredo, S., Tello, M., Cano, E., Navarro, N., Díaz, I., Mateu, E., & Martín-Valls, G. 2020. Assessment of the nasal virome from respiratory outbreaks with or without the participation of swine influenza virus and randomly sampled herds in Spain. IPVS (Brasil) *Postponed to November 3^{rd} – 6^{th} 2020 due to COVID-19.*

B. Oral presentations in congresses

1. Diversidad de los virus de la influenza porcina tipo A provenientes de brotes de enfermedad respiratoria y de granjas subclínicamente infectadas en la Península Ibérica (2017 – 2019). 2020. XXII Jornadas de Porcino de la UAB y AVPC. 31 de Enero 2020.
2. Sosa Portugal, S., Martí, C., Tello, M., Casanovas, C., Barrabés, S., Mesonero-Escuredo, S., Pineda, P., Wacheck, S., Mateu, E., & Martín-Valls, G. 2020. Diversity of influenza A viruses retrieved from respiratory disease outbreaks and subclinically infected herds in Spain (2017-2019). 12th ESPHM (Switzerland) *Postponed to April 14^{th} – 16^{th} 2021 due to COVID-19.*

C. Other publications

1. Sosa Portugal, S., Casanovas, C., Barrabés, S., Mesonero-Escuredo, S., Mateu, E., & Martín-Valls, G. 2019. Clasificación de los virus de la gripe porcina ¿qué

significado tiene? *Suis*, 156: 26-28.