Animal influenza viruses - Impacts of influenza A virus in Danish swine herds

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PhD Thesis

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Cover picture:

Photo by Inge Larsen, 2017 (pig from one of the herds sampled in the project)

Table of contents

Preface and acknowledgements

This PhD thesis presents the work carried out in the Virology group at the National Veterinary Institute, Technical University of Denmark from October 2016 to September 2019. The project was co-funded by SEGES Danish Pig Research Center and IDT Biologika: thus, I have been very privileged to work at the interface between the University, the farmers and the medical industry. The PhD project has been a great experience for me since it offered an interesting mixture of fieldwork, laboratory work and research. Moreover, I have had the possibility to share my work at several conferences in Denmark, but also in other European countries as well as in China.
During the PhD project, I have had the pleasure to work with a great number of wonderful people - first of all my supervisors. The first and biggest acknowledgement goes to my main supervisor Lars Erik Larsen, who gave me the opportunity to work with him in his group with the most interesting of all viruses. He has always been there to support me, especially in "high-pressure" situations. He is a great inspiration to me as he is extremely knowledgeable, always very down to earth and easy to talk with. My co-supervisor Inge Larsen has likewise been a great support and a good friend throughout the project teaching me many practical things while working in the herds. I also want to thank my co-supervisor Charlotte Sonne Kristensen, who has also been a great contributor to my project and always been a great company at conferences, where she among other things introduced me to her network. Jesper Schak Krog, whose knowledge on molecular biology I deeply admire, has also been supporting me throughout the project as co-supervisor, helping me at see things from a different perspective. During the PhD project, I have also been lucky to work with several great people from IDT Biologika: special thanks go to Silke Wacheck for always being present to discuss my results and for showing a great interest in my project. I also want to thank Stefan Pesch, Jens Dam, Rikke Søgaard and Rikke Gry Nielsen. In addition, I also want to acknowledge my colleagues at DTU. First of all, I want to thank my fellow PhD students Nicole Bakkegård Goecke, Malene Rask Andersen, Nanett Kvist Nikolaisen and Sofie Maiken Riisgaard Starbæk who have helped making this PhD a fun and unforgettable experience. I want also to thank Charlotte Kristiane Hjulsager, Kerstin Skovgaard and Lise Kirstine Kviesgaard for their guidance and support. Likewise, I want to thank the laboratory technicians Hue Thi Thanh Tran, Tine Skotte Hammer, Nina Dam Grønnegaard, Sari Mia Dose and Carina Bøegh Folsing, who have been very helpful with the laboratory works but who have also been pivotal in many social arrangements for the group. A special thanks to the veterinarians Anders Elvstrøm, Sten Larsen, Gerben Hoornenborg and Gitte Drejer who have shown great interest in the PhD project and helped me identifying the participating herds. On the same note, I want to acknowledge all the participating herds for letting me perform my project in their herds,

and as well as the workers who helped with physically handling the pigs. Great thanks goes to the people who supervised me during my external research stay at Universitat Autònoma de Barcelona, including Gerard Eduard Martin Valls, Enrique María Mateu de Antonio, Marti Cortey Marques and Silvana Sosa Portugal.

I also want to thank my family and friends, who have supported me throughout the PhD project and have listened to a lot of pig-talks. I especially want to thank my sister for letting me stay at her place when visiting the herds, without complaining a single time on my smell or my swiny samples staying in her fridge. Last but not least, I want to thank my husband Antonio who has been my greatest support and always able to reassure me and encourage me in the process.

List of abbreviations

A(H1N1)pdm09 = influenza A virus of pandemic H1N1 subtype from 2009
AIV = avian influenza virus
Av = avian
BAL = bronchoalveolar lavage
cDNA = complementary DNA
cRNA = complementary RNA
Ct = cycle threshold
DPI = days post infection
ELISA = enzyme-linked immunosorbent assay
ER = endoplasmic reticulum
HA = hemagglutinin
HI = hemagglutinin inhibition
HPAI = high pathogenic avian influenza
Hu = human
IAV = influenza A virus
INF = interferon
Ig = immunoglobulin
Il = interleukin
LPAI = low pathogenic avian influenza
M = matrix
MDA/s = maternally derived antibody/antibodies
MDA+/- = maternally derived antibody positive/negative
MHC = major histocompatibility complex
mRNA = messenger RNA
NA = neuraminidase
NEP = nuclear export protein
NK = natural killer
NP = nucleoprotein
NS1 = non-structural protein 1
PA = polymerase acidic protein
PAMP = pathogen-associated molecular patterns
PB1 = polymerase basic protein 1
PB2 = polymerase basic protein 2
PCV2 = porcine circovirus 2
PRDC = porcine respiratory disease complex
PRCV = porcine respiratory coronavirus
PRR = pattern recognition receptor
PRRSv = porcine reproductive and respiratory syndrome virus
RT PCR = reverse transcriptase polymerase chain reaction
SPC = summary of product characteristics
Sw= swine
swIAV = swine influenza A virus
TNF = tumor necrosis factor
TRIG = triple reassortant internal gene cassette
VAERD = vaccine associated enhanced respiratory disease
vRNA = viral RNA
vRNP = viral ribonucleoprotein complex
WIV = whole inactivated virus

Summary

Since the filtration experiments performed by Richard E. Shope in 1931, swine influenza A virus (swIAV) has been linked to influenza-like symptoms in pigs, including fever, coughing, anorexia and apathy. Since then, several different subtypes and variants of swIAV have appeared and continue to pose clinical problems in affected herds globally. For a proper control of swIAV, it is crucial to understand the dynamics of the virus. However, several aspects of the infection remain unclear. It is uncertain how the virus spreads in and between herds, which age groups are infected and what the clinical impact of swIAV is. Several aspects of the immunity towards swIAV are also unknown e.g. how does it evolve and which responses are needed to protect the pigs. Other unsolved questions relate to the evolution of the virus, where the level and impact of antigenic drift needs investigations. Finally, there is a lack of studies providing field data on the efficacy of different control measures.

The main aim of this PhD project was to perform research activities in order to answer some of the above-mentioned questions. The thesis consists of four major parts. **Part 1** presents the background for the studies carried out during the PhD project and introduces the general aims of the project as well as the thesis outline. **Part 2** includes a literature review divided into two sections. The first section reviews the biology of influenza A virus (IAV), providing information on the taxonomy, virus lifecycle, subtypes, lineages and nomenclature, methods for IAV characterization, viral evolution and distribution of IAV in different species. The second section of the literature review focuses on swIAV and its pathogenesis, clinical signs, immunology, vaccines and maternally derived antibodies (MDAs). The epidemiology of swIAV is also reviewed both in regard to the distribution of swIAV globally and the transmission patterns within and between herds. Finally, a review of different control strategies is presented. **Part 3** includes the four manuscripts written during the PhD project. An intensive overall discussion and conclusion completes the thesis in **Part 4**, which also includes future perspectives for swIAV research.

Manuscript 1 describes and discusses the results of longitudinal field studies carried out in three Danish swine herds, with focus on the transmission dynamics and clinical impacts of swIAV. The results of the study revealed early presence of swIAV, which affected piglets as young as three days of age. The early infections were observed in all herds, despite the presence of IAV antibodies in the sows, indicating that the protection obtained by MDAs was incomplete. Moreover, a small number of sows tested positive for swIAV, indicating a possible role of the sows in the transmission dynamics. Interestingly, a number of pigs tested positive for swIAV over two consecutive or non-consecutive samplings, indicating a possibility for prolonged shedding and reinfection, respectively. Finally, the

clinical impacts of swIAV were documented by observing the correlations between swIAV, nasal discharge and increased coughing index.

Manuscript 2 describes a study performed in a single herd where it was decided to initiate vaccination of piglets at the time of castration with an inactivated swIAV vaccine. The results showed very limited effect of vaccination, as the total number of infected pigs and clinical signs were similar between the vaccinated- and the control group. Due to the higher frequency of sampling in the study, it was possible to investigate the presence of prolonged and recurrent shedding in details. Extensive genetic characterization of the swIAV sequences obtained from prolonged and recurrent shedders documented the presence of a single swIAV strain, and revealed evidence of antigenic drift in the recurrent shedders.

Manuscript 3 provided unique data on an acute swIAV outbreak in an enzootic infected herd, where it was subsequently decided to implement mass sow vaccination, thereby allowing for investigation of the effects of this vaccination strategy. Several interesting observations were made in this study. The acute outbreak with a new swIAV subtype caused early infection of piglets, as swIAV was almost exclusively identified in the one-week-old piglets. The early infection suggested that the piglets were not protected by MDAs. Interestingly, viral characterization revealed that the HA gene, of the enzootic swIAV strain and outbreak swIAV strain, belonged to the same lineage, but also showed major differences, specifically in the region encoding the globular head. Subsequent serological tests confirmed a lack of cross-protection between the two swIAV strains. After vaccination, the infection time was delayed and the viral load was reduced. However, an increase in numbers of prolonged shedders was observed, which along with the delayed infection time resulted in the spread of swIAV to all age groups of the herd. Moreover, evidence of an increased substitution rate with positive selection in the HA gene was observed after the implementation of mass sow vaccination.

Manuscript 4 was based on repeated cross-sectional studies with monthly sampling in a single herd over a one-year period. The enzootic nature of swIAV was confirmed as the virus persisted in the herds over the full project period. The results confirmed the observations made in Manuscript 1, as a large number of sows/gilts tested positive for swIAV during the study. Investigations into the viral evolution indicated that swIAV evolved in a similar manner to human seasonal influenza. These results provided a basis for explaining the vast diversity observed within the single swIAV lineages, and provoked a discussion of the possible implications of extensive antigenic drift on the control of swIAV.

Ultimately, this PhD project has contributed to a deeper understanding of the transmission dynamics and impacts of swIAV. The studies carried out in this PhD are the first to show extensive swIAV infection in newborn piglets, and emphasize the role of sows and gilts in the transmission dynamics. All four studies document the enzootic nature of swIAV within the herds, and provide evidence of prolonged and recurrent swIAV shedding, which could enhance the herd-level persistence of swIAV. The results suggest that the presence of pre-existing immunity is likely to play a role in the generation of prolonged and recurrent shedders, and potentially drive the evolution of swIAV. In addition, the results reveal a high level of genetic diversity and a fast evolution of swIAV, which potentially has consequences for the transmission and the control of the virus. Finally, results on vaccine efficacy have been provided, which in combination with the increased knowledge of the swIAV dynamics, will help the industry in the implementation of optimal control measures in the future.

Sammendrag (Danish summary)

Siden filtreringsforsøgene, udført af Richard E. Shope i 1931, er svine influenza A virus (swIAV) blevet associeret til influenzalignende symptomer hos svin såsom feber, hoste, anoreksi og apati. I de seneste år er utallige swIAV subtyper og varianter blevet identificeret, og bidrager til kliniske symptomer i de berørte besætninger globalt. For at kunne kontrollere swIAV er det yderst vigtigt at forstå virus dynamikken, men adskillige aspekter er på nuværende tidspunkt ikke blevet grundigt belyst. Det er således uklart hvordan swIAV spreder sig i og imellem besætningerne, hvilke aldersgrupper der rammes, og hvilke kliniske konsekvenser swIAV infektion har for grisen. Desuden er der også flere ukendte aspekter i forhold til immuniteten rettet mod swIAV, så som hvordan den udvikles og hvilket respons, der er nødvendigt for at opnå beskyttelse af grisene. Andre uklare aspekter vedrører virus evolution, herunder niveauet og betydningen af antigen drift. Ydermere er der mangel på feltstudier, som dokumentere effekten af forskellige kontrolstrategier.

Hovedformålet med dette ph.d. projekt var at udføre forskellige videnskabelige studier for at besvare nogle af de ovennævnte spørgsmål. Afhandlingen består af fire dele. **Del 1** indeholder en kort baggrund for de undersøgelser, der er udført i forbindelse med ph.d. projektet og introducere de overordnede formål, samt opbygningen af afhandlingen. **Del 2** indeholder en gennemgang af den eksisterende litteratur opdelt i to afsnit. Det første afsnit omhandler den generelle influenza A virus (IAV) biologi og giver bl.a. information om taksonomi, replikation, subtyper, stammer og nomenklatur, metoder til karakterisering, virus evolution og IAV i forskellige dyrearter. Det andet afsnit af er fokuseret på swIAV herunder patogenese, kliniske tegn, immunologi, vacciner og betydning af maternelle antistoffer (MDAs). Derefter gennemgås epidemiologien både i forhold til udviklingen og fordelingen af swIAV på et globalt plan, men også i forhold til transmissionsmønstrende i- og imellem besætningerne. Til slut gennemgås en række forskellige kontrolstrategier. Derefter følger **Del 3**, som inkluderer de fire manuskripter, genereret i løbet af ph.d. projektet. Slutteligt afrundes afhandlingen med en samlet diskussion med tilhørende konklusioner i **Del 4**, som også præsentere fremtidige perspektiver for forskning indenfor swIAV.

Manuskript 1 beskriver og diskuterer resultaterne af longitudinelle feltstudier udført i tre danske svinebesætninger med fokus på transmissionsdynamikken og den kliniske påvirkning af swIAV. Resultaterne afslørede en tidligt tilstedeværelse af swIAV, da smitte af tre dage gamle pattegrise blev påvist. Disse tidlige infektioner blev observeret i alle besætninger, og skete på trods af tilstedeværelsen af antistoffer rettet mod IAV hos søerne, hvilket indikerede at MDA ikke medførte

en tilstrækkelig beskyttelse af pattegrisene. Derudover blev swIAV fundet i et mindre antal søer, hvilket fremhævede en mulig rolle af søer i transmissionsdynamikken. En anden interessant observation var fund af grise, som testede positive for swIAV over fortløbende eller afbrudte prøvetagninger, hvilket indikerede at henholdsvis forlænget udskillelse og re-infektion var mulig. Til sidst blev den kliniske betydning af swIAV også understreget, da der blev fundet en korrelation mellem swIAV, næseflåd og et øget hoste-index.

Manuskript 2 bekriver et studie udført i en enkelt so-besætning, som besluttede at implementere vaccination af pattegrise ved kastrationstidspunktet med en inaktiveret swIAV-vaccine. Resultaterne viste meget begrænset effekt af vaccinationen, da både det totale antal inficerede grise og de kliniske tegn var ens blandt de vaccinerede og ikke-vaccinerede grise. Grundet den hyppige prøveudtagning udført i dette studie, var det muligt at udforske tilstedeværelsen af forlænget udskillelse og re-infektioner. Den genetiske karakterisering af swIAV fra grise defineret som forlængede udskillere eller re-inficerede dokumenterede forekomsten af en enkelt swIAV stamme i de enkelte grise, og afslørede forekomsten af antigen drift hos de re-inficerede grise.

Manuskript 3 beskriver et unikt dataset fra et akut swIAV udbrud i en enzootisk inficeret besætning, hvor det efterfølgende blev besluttet at implementere blitz vaccination af so-holdet, hvilket gjorde det muligt, at undersøge effekten af denne vaccinationsstrategi. Flere interessante observationer blev foretaget i dette studie. Det akutte udbrud med en ny subtype forårsagede tidlig infektion af pattegrisene, da swIAV næsten udelukkende blev identificeret en uge gamle pattegrise. Denne tidlige smitte antydede, at pattegrisene ikke var beskyttet af MDA. Viruskarakteriseringen afslørede at HA-genet fra den enzootiske stamme og udbrudsstammen begge var af samme afstamning, men udviste dog store forskelle i det globulære hoved af HA proteinet. De efterfølgende serologiske undersøgelser bekræftede den manglende krydsbeskyttelse mellem de to stammer. Blitz vaccination af so-holdet forsinkede infektionstidspunktet hos pattegrisene og reducerede virusudskillelsen. Imidlertid blev der observeret en stigning i antallet af forlængede udskillere, hvilket i kombination med det forsinkede infektionstidspunkt resulterede i spredning af swIAV til alle aldersgrupper i besætningen. Desuden blev der identificeret en forøget substitutionsrate og positiv selektion i HA genet, efter blitz-vaccination af so-holdet blev implementeret.

Manuskript 4 var baseret på gentagne tværsnitsstudier udført hver måned i en enkelt besætning over en periode på et år. Dette studie påviste den enzootiske tilstedeværelse af swIAV. Tilmed blev den antagne betydningen af søer og gylte i transmissionsdynamikken fra Manuskript 1 bekræftet, da et

stort antal søer og gylte testede positive i dette studie. Undersøgelser af virusevolutionen indikerede, at swIAV udviklede sig sammenligneligt med human sæson influenza. Resultaterne dannede derfor et grundlag for at forklare den voksende diversitet observeret inden for specifikke swIAV stammer, og medførte en diskussion af de mulige implikationer af antigen drift for kontrol af swIAV.

Tilsammen har aktiviteter gennemført i løbet af dette ph.d. projekt bidraget til en dybere forståelse af transmissionsdynamikken og de kliniske konsekvenser af swIAV. Det er de første studier, der klart dokumenterer den omfattende tilstedeværelse af swIAV i nyfødte pattegrise, og resultaterne understeger også søernes og gylternes rolle i transmissionsdynamikken. De fire studier dokumenterer alle den enzootiske tilstedeværelse af swIAV inden for besætningerne, og dokumenterede både forlænget udskillelse af swIAV og re-infektion hos den enkelte grise, hvilket kunne bidrage til den persisterende tilstedeværelse af swIAV på besætningsniveau. Resultaterne antyder, at eksisterende immunitet sandsynligvis spiller en rolle for den forlængede udskillelse og re-infektion, hvilket sætter et spørgsmålstegn ved værdien af de nuværende vaccinations programmer i mange danske besætninger. Derudover afslørede resultaterne en markant genetiske diversitet og hurtig evolution af swIAV, som potentielt kan have stor betydning for transmissions og kontrol af virusset. Endelig er der fremlagt resultater af vaccinationseffekt, som i kombination med den øgede viden om transmissionsdynamikken vil hjælpe industrien til at optimere de eksisterende kontrolforanstaltninger fremover.

Part 1 - Background, aims and thesis outline

Background, aims and thesis outline

Swine influenza A virus (swIAV) is an enzootic disease on a global level [1]. SwIAV causes respiratory disease, which poses health and welfare issues [2–5]. In addition, infection with swIAV can impair the growth rate and lead to secondary infections, which impacts the production economy and increase the antibiotics usage [6,7]. In Denmark, swIAV is the most prevalent pathogen found in diagnostic cases with an anamnesis of respiratory disease in swine, emphasizing its clinical importance in the herds [8]. Recently, several studies have provided evidence of swIAV persistence within single herds, which underlines how difficult swIAV is to control once introduced [9–14]. Furthermore, the increased surveillance of swIAV implemented in many countries after the human pandemic in 2009, has revealed that a high number of novel swIAV subtypes and strains are circulating nationally but also within single herds [10,12,13,15–24]. These aspects of swIAV emphasize the importance of understanding the infection dynamics of swIAV in- and between swine herds, highlight the challenge for veterinarians and farmers to control swIAV and last, but not least, stress the need for optimal control measures. From a human health point-of-view, it is also a great concern that numerous swIAV subtypes and strains are circulating, as swIAV previously has contributed to the generation of human pandemics [1,25,26].

Despite the obvious importance of swIAV, basic knowledge on the transmission dynamics, impact, genetic variability and the effect of control strategies of swIAV are still lacking. The overall aim of this thesis was therefore to investigate the transmission dynamics of swIAV in Danish swine herds, examine the genetic variability of swIAV, and to examine the clinical impact of the infection. A secondary aim was to evaluate the effects of the different vaccine strategies used in Danish swine herds to control swIAV. To fulfil these aims a series of longitudinal field studies was performed.

The aim of the first study performed during the PhD project was to obtain basic knowledge on the transmission dynamics and clinical impact of swIAV in three Danish farrow-to-grower herds. In this study, four batches of pigs were included in each herd and sampled continuously over three months.

The second study aimed at obtaining data on the efficacy of early piglet vaccination with an inactivated swIAV vaccine, at the time of castration, in a swIAV positive farrow-to-grower herd. In this study, 160 piglets from 11 litters were included. Every second piglet of the litters was vaccinated at castration and all piglets were sampled weekly until six weeks of age.

The third study aimed at investigating the viral dynamics, genetic and antigenic variability and the effect of maternally derived antibodies and vaccination in a herd that experienced an acute outbreak

with swIAV and subsequently decided to implement mass sow vaccination. The study design was similar to that of the first study. However, two sampling rounds were performed; before and after the implementation of mass sow vaccination.

The fourth study was initiated based on the genetic data obtained during the first three studies, and aimed at documenting the enzootic nature of swIAV, as well as, investigating the viral evolution over a one-year period in a single farrow-to-grower herd. Monthly samplings of sows and pigs were performed in this herd and generated swIAV sequences for evolutionary analyses.

The thesis is divided into four main parts. The first part presents a brief background, gives the overall aims of the PhD project and provides an overview of the four studies included in the thesis. Part 2 comprises the introduction of the thesis, which is divided into two sections. The first section provides the overall background knowledge on the biology of influenza A virus (IAV) and the second section contains a review on swIAV, with focus on the pathogenesis, clinical signs, immunology, vaccines, epidemiology and transmission dynamics. Part 3 includes the four manuscripts generated during the PhD project, and Part 4 provides an overall discussion of the outcome of PhD project, along with conclusions and perspectives on future research on swIAV.

Part 2 – Introduction

Influenza A virus biology

Taxonomy

Influenza A virus (IAV) belongs to the family *Orthomyxoviridae*. All members of the family have a segmented negative sense single stranded RNA genome. The family is further divided into seven genera including *Influenza A virus*, *Influenza B virus*, *Influenza C virus*, *Influenza D*, *Isavirus*, *Quaranjavirus* and *Thogotovirus*. Influenza A, B, C and D viruses share a high degree of conserved nucleotides at the 3′ and 5′ end of the genome, which suggests that they have evolved from a common ancestor [27–30]. Influenza A, B, C and D viruses are different based on the number-, length- and diversity of the RNA segments and in regards to pathogenicity and host specificity. Influenza A viruses infect both birds and mammals including humans, pigs, mink, dogs, cats, horses, seals, bats and whales, whereas influenza B and C viruses primarily infect humans and influenza D virus mainly infects cattle. However, sporadically cases of influenza B, C, D infections have been documented in pigs, small ruminants, ferrets and seals [28,31–33].

Virus structure

IAV is pleomorphic and spheroidal in structure. The size of the virion is between 80-120nm. The outer envelope of the virion consists of a lipid bilayer with glycoprotein spikes protruding through the membrane. The spikes includes both homotrimers of the hemagglutinin (HA) protein and homotetramers of the neuraminidase (NA) protein, and together these two proteins play a key role in receptor binding, receptor cleavage and membrane fusion (Figure 1) [34]. The HA protein is the most abundant protein on the viral particle, occupying approx. 80 % of the viral surface, whereas the NA protein occupies approx. 17 %. The remaining surface is occupied by the M2 ion channel protein [35,36]. The precursor for the HA protein is termed HA0. Upon infection, HA0 is cleaved by cellular proteases into two subunits termed HA1 and HA2. The HA1 subunit mainly comprises the globular domain of the HA protein, which contains the receptor binding site and is responsible for viral attachment through binding to the receptor sialic acid α-2.3 (α2.3-SA) or 2.6 (α2.6-SA) of the host cell [34]. The globular head of the HA protein is a key target for neutralizing antibodies and several antigenic sites have been identified in this part of the protein [37–41]. Both the HA1 and HA2 subunits contribute to the stalk domain of the HA protein, however, HA2 constitutes the major part (Figure 2). The stalk of the HA protein, includes both an ectodomain and a helical chain that is anchored in viral envelope [42]. The NA protein has important enzymatic activity, which cleaves the sialic acid from the HA protein. The NA protein is thereby vital for vital for release of progeny virus [43].

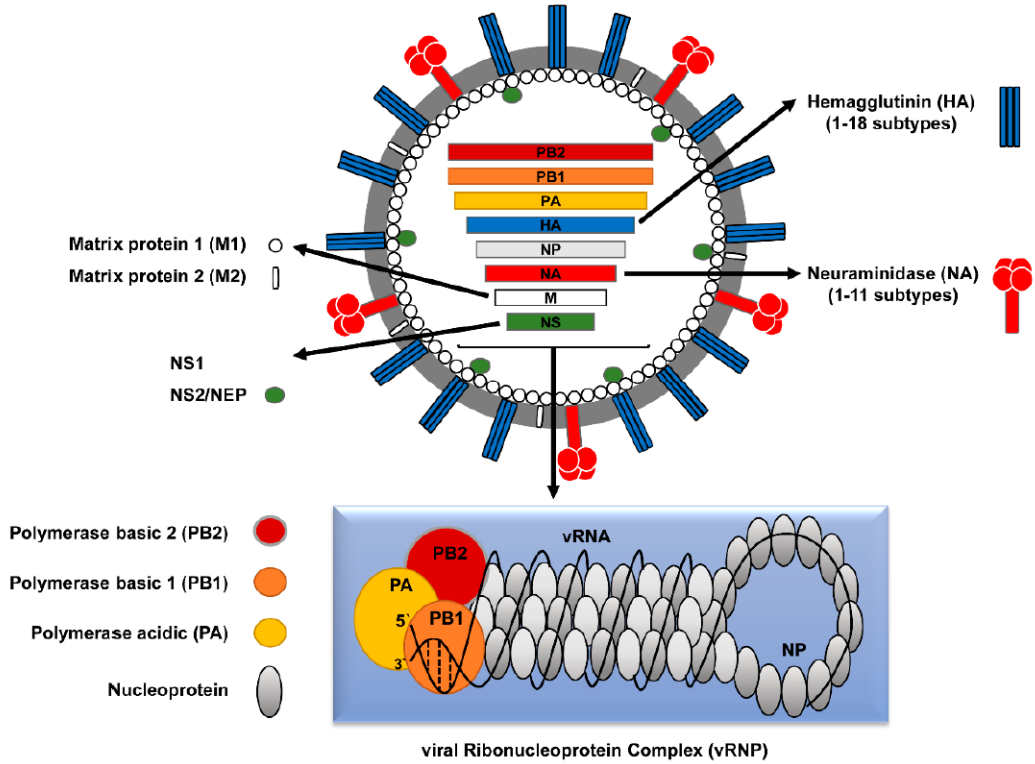

Figure 1. Schematic structure of an IAV particle and the viral ribonucleoprotein complexes. The hemagglutinin (HA), the neuraminidase (NA) and the ion channel Matrix protein 2 (M2) are transmembrane proteins penetrating the host derived viral envelope. The matrix protein 1 (M1) lines the inner surface of the lipid membrane in association with the NEP and the viral ribonucleoprotein complexes (vRNPs). The viral genome contains eight vRNPs (PB2, PB1, PA, HA, NP, NA, M and NS), which are comprised of negative sense RNA segments in association with the nucleoprotein (NP) protein and the three polymerase subunits (PA, PB1 and PB2) [25].

The inner layer of the viral envelope is lined with, and supported by, the matrix protein 1 (M1), whereas the matrix 2 (M2) protein penetrates the viral envelope creating small ion channels. The viral envelope surrounds the viral genome, which consist of eight RNA segments each folded into a rod-shaped, double-helical ribonucleoprotein complex (vRNP). The vRNPs contain the viral RNA, the viral polymerase, consisting of the polymerase acidic protein (PA), the polymerase basic 1 and 2 proteins (PB1 and PB2) and multiple copies of the nucleoprotein (NP) (Figure 1) [44]. The eight segments vary in size between 890-2341 nucleotides and encode several different structural and nonstructural proteins. The 3´- and 5´-end of each segment has a conserved sequence of nucleotides [45]. Segments 1, 2 and 3 encode the three polymerase proteins PB2, PB1 and PA, which in combination constitute the 3P polymerase complex. In addition, segment 2 encodes the pro-apoptotic protein PB1-F2 and segment 3 encodes the PA-X protein, which modulates the host response. Segments 4 and 6 encode the major surface glycoproteins HA and NA, respectively, whereas Segment 5 encodes the NP protein. The M1 and M2 proteins are encoded by Segment 7, and finally Segment 8 encodes nonstructural protein 1 (NS1) and the nuclear export protein (NEP) [25].

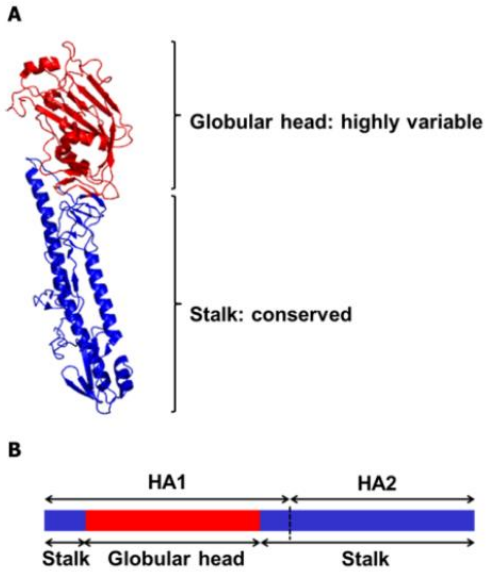

Figure 2. Schematic presentation of the HA protein and the HA gene. A) presents a ribbon diagram of the HA protein consisting of two domains; the highly variable globular head domain (red), including the receptor binding site and antigenic sites and the more conserved stalk domain (blue), which is partly anchored in the viral envelope. B) represents the HA gene including the HA1 and H2 subunit. HA1 mainly encodes the HA1 domain, but also a minor part of the stalk domain, whereas HA2 only encodes the stalk domain [42].

Virus lifecycle

Entry

In order to enter the host cell, the HA1 globular domain of the HA surface protein attaches to the receptor sialic (N-acetylneuraminic) acid on the host cell surface [46]. Sialic acids are acidic monosaccharides commonly found on the surface of many different cells types in a wide range of animals. Two sialic acids are important receptors for IAV, and are termed α-2.3- and α-2.6, dependent of the linkage to either carbon-2 or carbon-3 galactose. Different IAVs have different receptor preferences to one of the two linkages (α-2.3- or α-2.6). Human and swine IAVs generally bind to α-2.6-linked sialic acid, which is found more abundantly throughout the respiratory epithelium of humans and swine compared to the α-2.3 linked sialic acid. Moreover, the α-2.3 linked sialic acids of humans and swine are primary located in the lower respiratory tract, while α-2.6-linked sialic acids are distributed in both the upper and lower respiratory tract. Conversely, avian IAV typically binds preferentially to α-2.3 linked sialic acid, which is the most common sialic acid found in avian species, and which is also distributed in the intestine [47–49]. After attachment to the cell surface, the virus enters the host cell by endocytosis through clathrin-coated pits [50] or by micropinocytosis [51]. The acidity of the endosome triggers a conformational change in the HA molecule, leading to the exposure of the fusion peptide at the N-terminus of HA2, which mediates

the fusion of the viral envelope and the endosome membrane [52]. This fusion results in a pore through which the vRNPs can be released. The release of vRNPs into the cytoplasm is mediated by the action of the M2 ion channel, which initiates pumping of hydrogen ions from the endosome into the viral particle. This leads to un-coating of the virus, which results in release of the vRNPs into the cellular cytoplasm (Figure 3) [53,54].

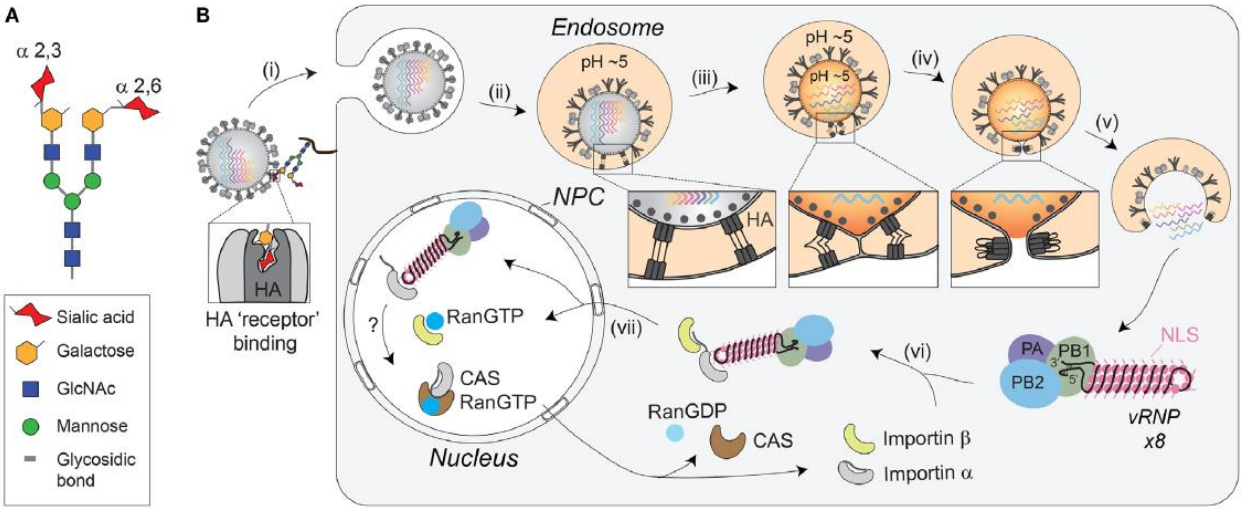

Figure 3. Illustration of IAV host cell entry and replication. A) Diagram of the host cell receptor containing sialic acid as either α 2.3 or α 2.6 linked. B) Schematic presentation of IAV attachment, entry and replication cycle. i) Binding of the globular domain of the HA protein to the sialic acid on the host cell surface, which triggers virus internalization into the endosome. ii) The acidic environment of the endosome facilitates a conformational change in the HA, which results in exposure of the fusion peptide that initiates fusion with the endosomal membrane. iii) The HA conformation collapses and enhance fusion of the viral envelope and the endosomal membrane. v) The M2 ion channel pumps hydrogen ions into the viral particle stimulating release of the vRNPs into the cytosol. vi) The nuclear localization signal on the vRNPs are recognized by host cell importin α and β. vii) the vRNPs are transported by importin α and β into the cell nucleus where the transcription and replication of the viral RNA takes place [55].

Replication

After release of the vRNPs into the cytoplasm, the nuclear localization signal of the vRNPs viral stimulates cellular proteins (importin α and β) to transport the vRNPs through nuclear pores into the host cell nucleus, where all viral RNA (vRNA) synthesis take place [55,56]. As the genome of IAV consists of negative sense RNA the virus brings its own RNA-dependent RNA polymerase (consisting of PA, PB1 and PB2), which use the negative sense vRNA as template for production of either mRNA, which codes for the viral proteins, or complementary RNA (cRNA), which is an intermediate for subsequent transcription of genomic negative sense vRNA [53]. For IAV to be able to use host cell machinery for protein synthesis, the viral mRNA needs to resemble host cell mRNA

and influenza viruses have two unique features to ensure this. First, the vRNA contains five to seven uracils, which becomes transcribed into adenosines and thereby creates a poly(A) tail [57]. Second, capping of viral mRNA occurs through a process called cap-snatching. In this process different subunits of the 3P polymerase complex works together to bind (PB2), cleave (PA) and insert the cap of cellular mRNAs into the catalytic center of PB1 for extension of the vRNA [55,58–60]. The results is capped mRNAs with a poly(A) tail, which can leave the nucleus and become translated alongside host mRNAs in the ribosomes of the cytoplasm. Following translation, newly synthesized NP, PB1, PB2 and PA proteins are transported to the nucleus, where the NP encapsidates the newly synthesized vRNAs and new vRNPs are formed. Subsequently the M1 and NEP proteins mediate the nuclear export of the newly synthesized vRNPs, which are then ready for assembly of the virion [61]. The surface proteins HA, NA and M2 are synthesized on endoplasmic reticulum (ER) -associated ribosomes and are subsequently transported to the Golgi apparatus for further modifications. The three proteins contain signals that, following final modifications, ensure transportation to the cell membrane, where they are incorperated into virions [55]. The NS1 protein is of great importance in the inhibition of the antiviral defenses of the host cell, as it antagonize the interferon response. Moreover, the NS protein enhances the translation of viral mRNAs [62].

Assembly and release

When the structural proteins have been anchored in the cellular membrane and the vRNPs have been transported to the cell membrane, new virions are formed by viral budding, which occurs through a conformational change in the plasma membrane close to the vRNPs. The viral envelope thereby consist of a host cell derived lipid bilayer. The budding is initiated by an accumulation of HA, NA and M1 protein at the membrane, however, the exact details of the budding process remain unclear. As mentioned earlier, the HA protein has the ability to bind sialic acid, so for complete release of the virion, a cleavage of the sialic acid, through the sialidase activity of NA protein, is needed. In addition, NA protein removes sialic acid from the newly formed viral envelope to avoid aggregation of virus particles [53,55]. Newly formed IAV particles need to contain all eight vRNP segments to be infectious, and several studies has documented that the majority of released virions do indeed contain eight different segments. Therefore, it has been proposed that packaging is somehow controlled, even though the exact mechanisms behind this remains to be identified [63,64].

IAV subtypes

The majority of the identified combinations of HA and NA (subtypes) have been isolated from Aquatic birds, which are therefore considered to be the natural reservoir for IAV. Overall, 16 HA

(H1-H16) and nine NA (N1-N9) subtypes have been identified in aquatic birds. The different subtypes of HAs and NAs can be divided into two main groups based on genetic and antigenic similarities. The HA group 1 consists of H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, whereas group 2 includes H3, H4, H7, H10, H14 and H15. NA group 1 includes N1, N4, N5 and N8, whereas group 2 includes N2, N3, N6, N7 and N9 [65]. In 2013, two new influenza A virus subtypes were discovered in bats, which extended the number of HA and NA subtypes with H17, H18, N10 and N11. The two new HA subtypes belong to group 1, whereas the NA subtypes are genetically distinct from the above mentioned NA groups [66]. Interestingly, analysis of the two bat influenza A subtypes revealed that the sialic acid receptor was not utilized for host cell entry in contrast to all the other known IAV subtypes [67].

SwIAV subtypes and nomenclature

Three influenza A subtypes circulate in the pig population; H1N1, H1N2 and H3N2 [1]. The HA and NA subtype can be further divided into lineages based on genetic traits of either human (hu), swine (sw) or avian (av) origin, although all influenza A viruses are believed to originate from an avian source [68]. In addition, the HA and NA genes can be further divided into clades or stains based on phylogenetic analysis of sequence data. A global phylogeny-based nomenclature system and annotation tool has been developed for certain HA subtypes [69–71], which defines the lineage and specific clade of a given sequence. However, the tool has only recently become available, does not include all subtypes and is not used by all researchers. Therefore, the swIAV nomenclature can be rather confusing. For example the first enzootic swine influenza lineage, originating from the human pandemic caused by the "Spanish flu" in 1918, was classified as the "classical swine H1N1" lineage [72]. Over time new variants with diverse sequences and different combinations of gene segments appeared and led to different clades within the classical swine H1N1 lineage, which now also includes the 2009 influenza A pandemic strain (A(H1N1)pdm09) [73–75]. The abbreviation "pdm" is often used by researchers to indicate that one or several genes are from the A(H1N1)pdm09 origin. A newly proposed nomenclature of swIAV H1 subtypes, terms the classical swine lineage "1.A lineage", whereas swIAV of a human seasonal lineage are termed "2.A lineage" and the swIAV of the Eurasian avian-like lineage are termed the "3.A lineage" [69]. A detailed description of the lineages and clades circulating within America, Asia and Europe is presented later. Phylogenetic analysis has determined a shared amino acid identity of 40 to 70% among different IAV subtypes and 80-100 % within subtypes [76]. The Center for Disease Control and Prevention (CDC) has provided a guideline for naming influenza virus sequences for uploading in e.g. GenBank [77]. The

name should include; the antigenic type (e.g. influenza A, B, C or D), the host of origin, geographical origin, strain number, year of isolation and the subtype of the hemagglutinin and neuraminidase.

Methods for IAV subtyping and characterization

There are different methods for identifying the subtype of a given IAV. Hemagglutinin inhibition (HI) test is a widely used method that is used to determine the HA subtype [78]. HI tests are based on a specific panel of subtype-specific antisera, which limits the sensitivity- and the specificity of subtypes that can be determined. Furthermore, a high degree of cross-reaction between subtypes and strains/are observed in HI tests and the interpretation of the results are somewhat subjective [79,80]. A similar method is available for NA subtyping, and is termed neuraminidase inhibition test (NI-test) [81]. Currently, reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing have become popular tools for subtyping and the results can be interpreted in a more objective manner. Real time reverse transcription (RT) PCRs have been developed for many of the different IAV subtypes, clades and strains and can be combined in multiplex PCR assays resulting in a very fast, cheap and less-laborious testing compared to the HI test [82–84]. In addition to determining the subtypes, the assays can also include primers targeting the internal cassette of genes. However, the primers used in real time RT PCRs are designed specifically for different IAV subtypes, strains and clades, thereby limiting the possibility of discovering new or non-expected IAV. This risk can be eliminated by using sequencing either of amplified PCR products (with un-specific primers) or directly on viral RNA. Sanger sequencing [85] or next generation sequencing (NGS) [86] can be exploited to obtain sequence data. The sequencing can either include just the surface genes (HA and NA), revealing the subtype, or full genome sequencing, which includes also the internal gene cassette, thereby allowing for variant determination. The generation of the databases NCBI Genbank and GISAID that contains a large number of IAV sequences increases the likelihood of finding a close sequence match and is thereby an important aid in subtype and strain/clade determination. If one sample e.g. a nasal swab contains two different subtypes, the sequencing data can be difficult to interpret, as it is not known how the different genes were combined inside the single virion. To limit this risk, IAV is often grown in cells before full genome sequencing [87].

Modes of IAV evolution

Influenza virus has the ability to evolve through three different mechanisms; genetic and antigenic drift, viral reassortment and viral recombination [31,88,89].

Genetic and antigenic drift

Genetic drift is caused by a lack of proof-reading by the viral polymerase during replication, which results in the accumulation of a number of errors/point mutations over time. This process is common for all RNA viruses in contrast to most DNA viruses, that have a polymerase with a 3´-5´ proofreading capability [89–91]. Genetic drift occurs in all of the IAV gene segments, but a higher degree of changes is observed in the HA and NA genes as a result of the host humoral immunity that mainly targets the surface proteins. Genetic drift can potentially lead to antigenic drift, if the virus obtains mutations in antigenic sites, affecting antibody binding, thereby creating escape variants. As neutralizing antibodies mainly targets the globular head of the HA protein, a higher genetic variability is often seen in this domain [46,88,89,92–94]. A study from 1983 investigated the genetic drift of the human IAV H3N2 subtype from 1968-1980 and showed that the vast majority of the amino acid changes occurred in the globular head of the HA protein and clustered in antigenic sites, whereas the stalk domain of the HA protein was highly conserved. The same pattern had at that time been observed for other HA subtypes, and the authors therefore suggested that the overall conformation of antigenic regions of the HA was similar among subtypes though specific residues might differ [95]. A later study focusing on the H1 and H3 subtypes of human IAV, estimated the level of genetic drift to be 0.0037 nucleotide substitutions per site per year for H3 and 0.0018 for H1, and thereby provided evidence for different evolutionary rates between subtypes. The study likewise described that a relatively small subset of the HA1 codons was more prone to mutations compared to the overall HA1 gene [96]. The general perception has been that the level of antigenic drift was much lower for swIAV than for human adapted viruses, due to the short lifespan of pigs, which limits the level of preexisting immunity [31,72]. One of the first studies that estimated the difference in mutation rate between swine and human H1N1 was conducted in 1991. In this study it was determined that the rate of synonymous mutations were similar, but the rate of non-synonymous mutations were three times higher in humans (0.00041 vs 0.00125 nucleotide substitutions per site per year) and were more often present in antigenic sites [97]. This tendency was later confirmed in other studies and thereby indicated that while the level of genetic drift was similar among human and swine IAV, the antigenic drift was higher in humans [97–100]. However, other studies have provided evidence of pronounced antigenic drift in swIAV [101–107], which in one case prompted an update of the swine IAV vaccine strain [108]. In humans, antigenic drift has a high impact on the efficacy of human influenza vaccines, which is why WHO created a Global Influenza Network who meets every six months for making a recommendation for the specific strains to be used in the subsequent vaccine [109].

Viral reassortment

Another mechanism by which IAV can evolve is through "viral reassortment". Viral reassortment can arise when the host cell is infected with two genetically different IAV subtypes/strains, and different segments from the two subtypes/strains become mixed in the new virion during packaging (Figure 4). Due to the segmented nature of the genome, IAV is highly prone to reassortment events, and several novel influenza subtypes have been created in this way [92].

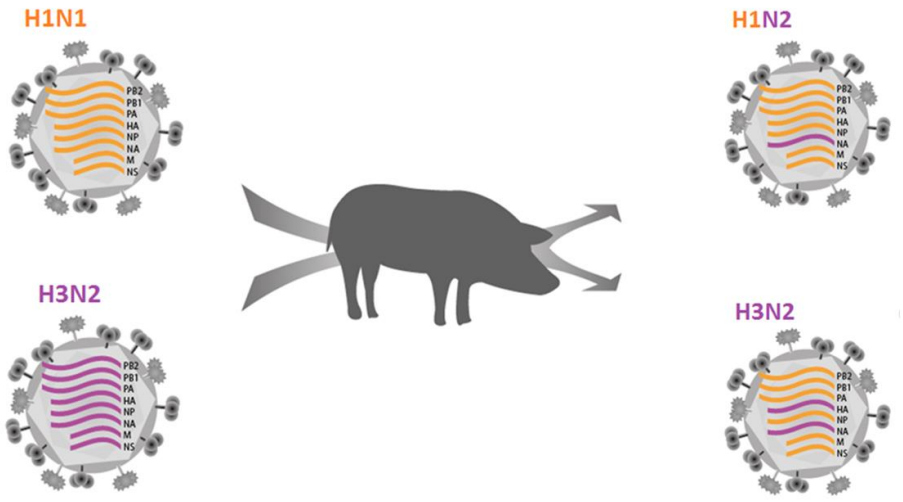

Figure 4. Illustration of the viral reassortment between two IAV subtypes. The generation of an H1N2 reflects the event leading to the "Danish swine H1N2", whereas the generation of an H3N2 reflects the adaption of the human Hong Kong H3N2 virus to swine by acquisition of the internal cassette of H1N1 avian origin. The illustration was made by Jesper Schak Krog.

The term "antigenic shift" is used to highlight the generation of a novel combination of gene segments to which the population is immunologically naïve, and more specifically in the case of an introduction of gene segments of an animal e.g. swine or avian origin into the human population. Two major human pandemics have been caused by antigenic shift in the years 1957 (H2N2) [110] and 1968 (H3N2) [111]. While the 2009 pandemic (H1N1) was also a result of reassortment events, it was different from the two above-mentioned pandemics, in that there was no involvement of a human seasonal IAV strains. Conversely, the A(H1N1)pdm09 is believed to be the result of reassortment events between different swIAV strains occurring in the swine population, with a subsequent transmission to humans [112]. Pigs have been proposed to act as the sole "mixing vessel" for the creation of new reassortant IAVs as they were found to have sialic acid receptors specific to both human and avian IAVs in the upper respiratory tract, and as both avian and human IAV had previously adapted to swine [113–115]. However, recent studies have shown that the distribution of sialic acid receptors in pigs actually resembles that of humans [48,116]. Therefore, it is probably not

the receptor distribution of pigs that makes them unique for the generation of new IAV but, more likely, their presence at the human-avian interface.

Viral recombination

The third and more rare mechanism by which influenza viruses can evolve is by non-homologous recombination between two different RNA segments. This has e.g. been observed between the HA gene and the NP gene both in vitro [117] and in a field case of avian H7N3 where the recombination led to an increase in virulence [118]. Evidence of homologous recombination in IAV of other species is still controversial and no clear evidence has been provided so far [31,119].

IAV in different species

Avian influenza

The IAV subtypes H1-16 and N1-N9 are, as previously mentioned, believed to originate from an avian source, as all these subtypes have been isolated from wild aquatic birds. The majority of IAV subtypes only circulate among birds as avian influenza virus (AIV) and only a few of these subtypes have adapted to a mammalian host (Figure 5). Similar to other IAVs, AIV subtypes are classified based on the HA and NA proteins. However, unlike other IAVs, AIV of the H5 and H7 subtypes are further categorized by their pathogenicity as either low pathogenic avian influenza (LPAI) or high pathogenic avian influenza (HPAI). LPAI are mainly asymptomatic in wild aquatic birds, while LPAI in domestic birds can lead to mild respiratory and/or reproductive diseases. The transfer of the LPAI to domestic birds or mammals can induce a rapid evolution of the LPAI into a HPAI with high mortality [25]. HPAI are characterized by their ability to infect the host systemically, a trait they acquire by substitutions in the HA cleavage sites leading to the presence of multiple basic amino acids. The basic amino acids makes the HA cleavable by ubiquitous proteases, which are present in a broad range of host cells, whereas proteases capable of cleaving the LPAI HA are only present in the respiratory tract and in the intestine [120–122]. The HPAI phenotype is so far restricted to the subtypes: H5Nx and H7Nx [123]. Infection of humans with AIV happens rarely, as the appropriate receptors are not easily accessible. AIV has a receptor preference for the α2.3-SA receptor, which is widespread in the human lungs but sparsely present in the upper respiratory airway of humans. Most cases of humans infected with AIV have been linked to intensive direct contact to an infected animal. However, when humans are infected with AIV the mortality is significantly higher than for human IAV infections. Fortunately, several changes in AIV are needed for AIV to become transmissible in humans, including a change in receptor preference, the ability of viral replication under different

temperatures [124] and a change from a fecal-oral transmission to airborne transmission. A concern is that human infection with AIV could lead to the adaption of the virus to the mammalian host, and thereby fulfill the above mentioned criteria [25]. Infection studies have shown that serial passage of AIV in ferrets can lead to the needed changes in receptor preference and airborne transmission [125–127].

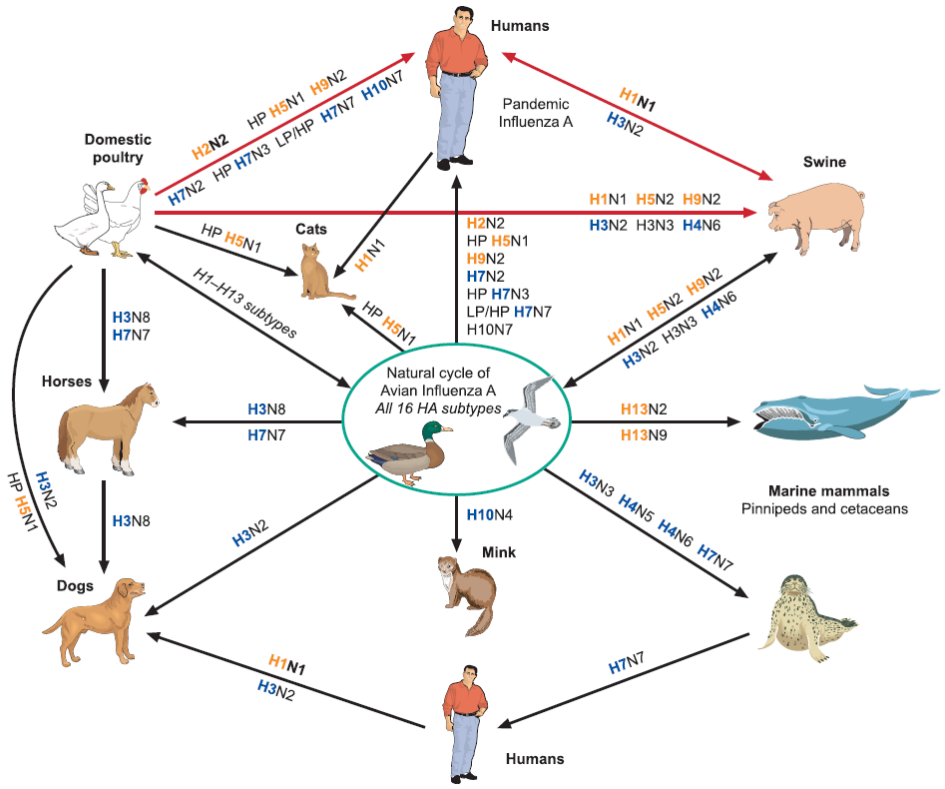

Figure 5. Illustration presenting an overview of the different IAV subtypes identified in different species and their origin [31].

Human IAV and zoonotic aspects

Avian influenza viruses of both high and low pathogenic types have several times crossed the species barrier and infected humans and other mammals (Figure 5). Of greatest concern is the transmission of H5/H7 AI to humans, as they generally have caused high mortality. The mortality of H5/H7 AI infections in humans has been estimated to be 52 % for all H5N1cases from 2003-2019 and 39 % for all H7N9 cases from 2013-2018 [128]. However, none of the H5/H7 viruses has become established as human seasonal flu, probably due to the limited airborne transmission.

Historically, three different HA subtypes (H1, H2 and H3) in combination with two different NA types (N1 and N2) have become established human lineages, and all human seasonal IAV strains,

started with a human IAV pandemic (Figure 6). In the 20th century, four major IAV pandemics have occurred. The first documented IAV pandemic occurred in 1918 and was named "the Spanish Flu". The H1N1 IAV subtype was later identified as the causative agent, and was believed to have adapted to mammals from an IAV of avian origin. However, the IAV identified did not possess the HA cleavage site substitutions which characterize HPAI [72,129]. The Spanish Flu was devastating and led to approx. 50 million deaths worldwide [130]. The next pandemic arose in 1957 and was termed "the Asian Flu". The virus initially spread throughout Asia, but later disseminated to the United States (US) and United Kingdom (UK). An H2N2 subtype was identified as the cause of the pandemic, and genetic investigations revealed that the virus was a reassortant virus, with genes of both avian and human origin; the HA, NA and PB1 genes were of avian origin, whereas the remaining five genes were of Spanish Flu H1N1 origin [111,131]. In 1968, a new pandemic strain, H3N2, evolved and was named "the Hong Kong flu". The Hong Kong flu H3N2 virus arose by a reassortment event between an avian H3Nx subtype and the human Asian flu H2N2 subtype. The HA gene and the PB1 gene were of avian origin, whereas the remaining genes were of Asian flu H2N2 origin, which retained five gene segments (NS, M, NP, PB2 and PA) of Spanish Flu origin [111,131]. Re-introduction of the Spanish Flu occurred in 1977-1978 and was named "the Russian Flu". The IAV isolated from the outbreak was an H1N1 and genetic analysis revealed a major resemblance to the Spanish Flu and was therefore believed to have escaped from a laboratory, probably in Russia [132]. The re-introduction of the Spanish Flu did not replace the already circulating Hong Kong flu H3N2 IAV and since then both subtypes have been circulating as humans seasonal influenza [133]. In 2009, the first pandemic of the 21st century was introduced. This virus was of an H1N1 subtype and was first recorded in Mexico in February 2009. In May 2009, the virus had spread globally, and 41 countries had registered outbreaks. The genetic analysis suggested that the virus was again generated by a reassortment event, but this time without participation of the circulating human seasonal strains. Instead, the strain was a reassortant between North American triple reassortant swIAV and Eurasian avian-like H1N1 (swIAV subtypes are reviewed in detail later). The resulting H1N1 virus consisted of the HA, NP, and NS genes of classical swine H1N1 origin, the NA and M of Eurasian avian-like origin and the remaining genes of the American TRIG origin [134]. The virus was first termed the "swine flu", as it was believed to have its origin in the swine population. This name had major economic consequences for the pork industry as the public questioned the safety of pork meat [135]. However, OIE, WHO and FAO later made an official statement that the virus could not transmit through the meat, and named the virus "pandemic H1N1 2009" (A(H1N1)pdm09), as an alternative name [136]. A(H1N1)pdm09 quickly replaced the above-mentioned human seasonal H1N1 and is now circulating together with H3N2 as seasonal IAVs in

humans [137]. A(H1N1)pdm09 was not the first "swine" influenza virus to infect humans. The first reported case of swIAV infection in humans was in 1958 and involved a woman who had close contact to pigs and later transmitted the virus to five other family members [138,139]. Since then several cases have been reported in USA, Asia and Europe, and the majority of the cases report contact to pigs [140]. Recently, several cases of H3N2 infection of humans from North American swine fairs have been documented and one paper estimated that 468 cases had occurred between 2005-2017 [141].

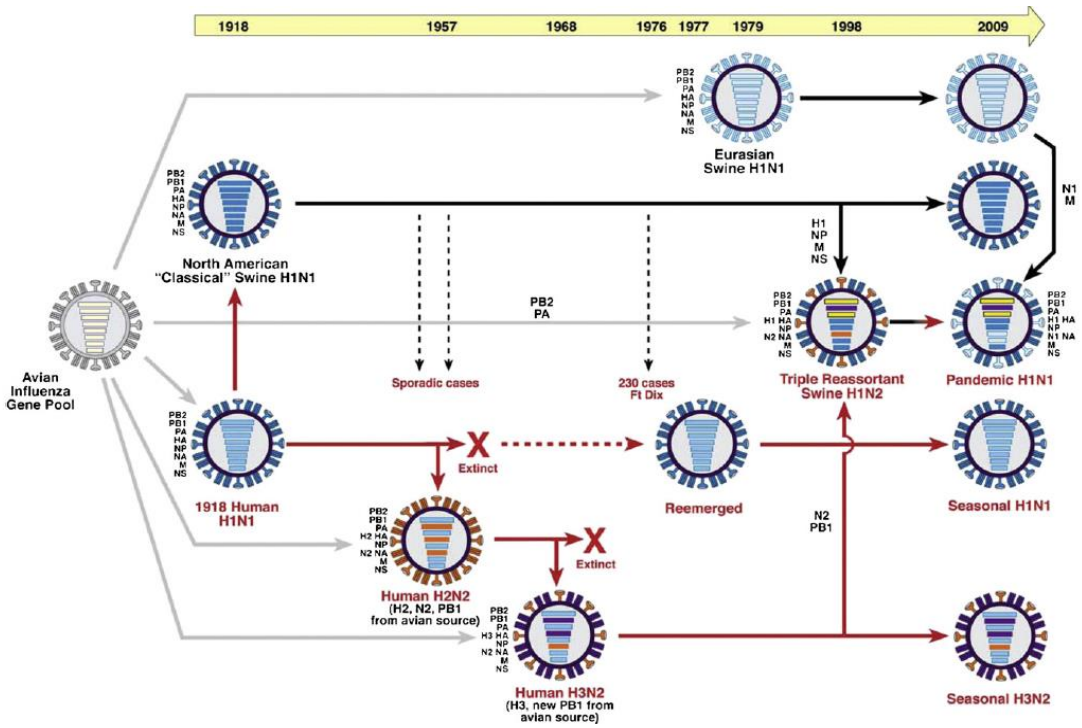

Figure 6. Illustration of the origin and different subtypes circulating in the human population from 1918 till 2009 [142]. The grey arrows represents the delivery of genes with an avian origin. The red arrows represents the evolution of human IAV and the dashed red arrows indicates a period without the circulation of H1N1 in humans. The black arrows represents the evolution of swIAV and the black dashed arrows represents zoonotic infections with swIAV.

In summary, two seasonal influenza A subtypes are currently circulating in humans; H3N2 and A(H1N1)pdm09 [143]. In the northern hemisphere, the temperate temperature results in a certain seasonality of IAV and most IAV cases occur from late November onwards. In other parts of the world where no winter season is present, the circulation of IAV is different. In the tropics, IAV infections have been documented in all seasons, but still with some peaks for example in relation to the rainy season. Infections generally lead to clinical signs of respiratory disease, fever, fatigue and headaches but can also lead to more serious disease dependent of the immune status, age and co-

morbidities. An increase in mortality due to IAV infections is observed in pregnant women, babies and elderly (>64 years) [144,145].

IAV in other mammals

IAV has a wide host range and has been isolated from humans, birds, pigs, ferrets, minks, marine mammals, horses, bats, dogs and cats [31,67]. Figure 5 provides an overview of the subtypes isolated from different animal species, however, erroneously, the figure only included one IAV subtype in mink. However, natural infection with A(H1N1)pdm09 [146], H3N2 [147], H9N2 [148], H10N4 and H10N7 [149] subtypes have been documented in mink in several countries and the infections were related to clinical signs of respiratory disease and pneumonia. In cats and other felids, avian influenza of the H5N1 subtype have been documented to cause illness [150] and horizontal transmission of this subtype has also been documented in cats [151,152]. In addition, several severe cases of A(H1N1)pdm09 have been described in cats [153–156], and serological tests have also suggested infection of cats with other human seasonal IAV [157]. Several different IAV subtypes including A(H1N1)pdm09 [158–161], H3N1 [162], H3N2 [163], H5N1 [164] and H9N2[165] have been identified in dogs and transmission of H3N8 from horses to dogs has also been reported [166]. The first documented case of H3N8 in horses was 1963 [167], and this subtype has since been endemic in horses. Around the eighties, genetic and antigenic data revealed a spilt into an European- and an American lineage [168]. An H7N7 subtype had previously been circulating in horses, however, this subtype has not been documented in horses since 1980 [169].

Swine influenza A virus

Pathogenesis

The disease observed in pigs infected with swIAV is very similar to that of humans infected with seasonal IAV, and is defined as an acute respiratory disease with a high morbidity and low mortality. The initial infection occurs in the cells of the upper respiratory tract including the nasal, tracheal and bronchial epithelium. The cells of the lungs can also become infected. The spread of swIAV to the lungs can lead to development of intestinal pneumonia and/or bronchiolitis or bronchitis. The lesions are primary restricted to the cranial and apical lung lobes. SwIAV itself causes damage to the mucosa, which leads to necrosis, consolidation and hyperplasia of bronchial lymphoid tissue and epithelial cells. The pig will usually develop fever within the first four days of infection, and virus can be detected in nasal secretions one to three days after infection. The shedding of virus usually last around one week and can be isolated from nasal and oral secretions [3,170–175]. The initial

immune response to infection is dominated by the innate immune system, where several pro-inflammatory cytokines are excreted and NK-cells are recruited for cell lysis. Moreover, neutrophilic granulocytes and mononuclear cells can be found in the lungs and in lung debris. Several studies have found a correlation between the level of some cytokines and disease development, and the immune response to the infection is therefore considered a contributing factor to the disease [5,48,174,176,177]. Subsequently the adaptive immune response will be stimulated and production of antibodies can be detected already three to seven days after infection [170,173,178].

Clinical signs

One of the first published descriptions of clinical signs in relation to swIAV dates back to 1927 [2]. The paper describes a field case of swIAV, where the entire herd was affected within a few days, and the first symptoms were loss of appetite, lethargy and fever. In addition, when the pigs were forced to move, coughing and troubled respiration were observed. Conjunctivitis and nasal discharge were also present in several pigs. These signs still constitute the hallmarks of influenza infection in pigs, and many of the observations are more or less in agreement with the clinical findings of experimental swIAV infection studies. Several experimental swIAV infections studies have revealed that either intranasal, intratracheal or aerogenic inoculation of pigs with swIAV result in infection of the epithelial cells of the upper respiratory tract and lungs, which subsequently induce clinical signs [3,170,173,174,179,180]. In summary, the studies described that the pigs developed fever within one to three days after inoculation, and clinical signs such as dyspnea, coughing, sneezing, nasal discharge, anorexia and depression were observed. However, other experimental inoculation studies have also shown subclinical disease without fever or the above-mentioned clinical signs [3,171,178,181].

In the field, fever, sneezing and coughing are correlated with PCR detection of swIAV [10]. In addition, swIAV has been correlated with decreased feed intake, reduced weight gain and reduced feed conversion efficiency [7,182,183]. However, subclinical infection with swIAV in the field has also been reported [9,184,185].

SwIAV infections in sows have been associated with reproduction problems [185–190] and in some cases lead to the death of pregnant sows. A field case reporting an outbreak with an H3N2 subtype caused an increase in abortions and sudden deaths of pregnant sows [191]. Another experimental study, also documented reproduction problems caused by swIAV infections [192]. In the study, three seropositive gilts were challenged with an H3N2 subtype at the beginning of the third trimester. The results revealed that the challenged sows had two to three stillborn piglets per litter, whereas the

three non-challenged sows had no stillborn piglets. Blood samples obtained from the stillborn piglets were negative in HI tests, indicating that no swIAV had crossed the placenta. Pyrexia caused by the swIAV infection is believed to be the main cause of abortions and stillbirths [187].

SwIAV and PRDC

An even greater impact of swIAV can be expected in the presence of other respiratory pathogens. swIAV is recognized as part of the porcine respiratory disease complex (PRDC) along with pathogens such as *porcine reproductive and respiratory syndrome virus* (PRRSv), *porcine circovirus type 2* (PCV2), *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* and *Bordetella bronchiseptica*. The different pathogens exert different functions to contribute to PRDC (Figure 7). SwIAV infection damages the epithelia and affects the cytokine response, which can lead to co-infections and an enhanced disease outcome [6,193–195].

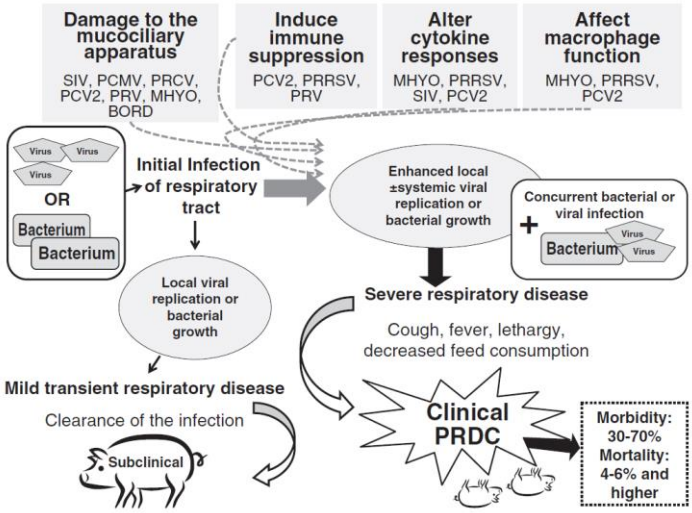

Figure 7. Illustrations of the different pathogens, mechanisms and clinical outcome of PRDC [6]

In a specific case, 700 feeder pigs experienced an acute onset of respiratory disease [196]. Pathological examination revealed multifocal suppurative bronchopneumonia and SwIAV, PCV2 and *Pasteurella multocida* were diagnosed. The mortality of the affected groups in the herd (10 %) was much higher than that expected of a swIAV infection alone. The impact of co-infections with swIAV has also been investigated in experimental studies. One experimental study examined the disease severity in pigs infected with a single pathogen including PRRSv, porcine respiratory coronavirus (PRCV) and swIAV with pigs co-infected with either PRRSv-PRCV or PRRSv-SIV. In all cases, the dual infection resulted in more severe clinical signs, longer duration of fever and reduced weight gain compared to the single infections [182]. Another experimental study, examined

the interaction between infection with *Mycoplasma hyopneumoniae* and swIAV. The pigs were initially inoculated with *Mycoplasma hyopneumoniae* and 21 days later inoculated with swIAV. The disease outcome was thereafter compared with two groups of pigs, being challenged with one of the two pathogens, respectively. The results revealed that dual infected pigs coughed for significantly more days than any single infected pigs. However, no difference was identified in regards to microscopic lung lesions and the severity of macroscopic lesions was only significantly higher in the dual infection group at 14DPI [197].

Immunology

The innate immune response

The first line of defense against swIAV is the innate immune response, which responds un-specifically to infections. The initial defense mechanism/barrier that swIAV encounters upon entry into the host is the intrinsic respiratory mucus layer of the upper respiratory tract. The mucus consists of cells, cellular debris and polypeptides, which are connected by the glycoprotein "mucin" secreted by goblet cells and submucosal glands of the respiratory tract. The mucus of pigs has been found to contain decoy sialic acids (SP-D) which aid in obstructing swIAV entry in host cells. The mucus with entrapped pathogens is continuously cleared from the respiratory tract by the ciliated epithelial cells [116]. If swIAV manages to cross the mucus layer and infect host cells, the receptor-mediated innate response is initiated. First, pathogen-associated molecular patterns (PAMPs) on the IAV are recognized by different pattern recognition receptors (PRRs) of the host cell, which further stimulate the secretion of type 1 interferons (INFs), pro-inflammatory cytokines, chemokines and eicosanoids [198]. Several pro-inflammatory cytokines have been found to be elevated upon recognition of swIAV in pigs. These include IL-1, IL-4, IL-6, IL-8, IL-10, INFγ, INFα and TNFα [173,199,200]. Moreover, an increase in the acute phase proteins, termed C-reactive protein and haptoglobin, has been observed [199,200]. The pro-inflammatory cytokines and the acute phase proteins are indicative of a local and systemic acute phase response, which induce inflammation, and stimulates cells of the adaptive immune system. An experimental study aimed at identifying the correlation between the level of different pro-inflammatory cytokines (IFNα, TNFα, IL-1, IL-6 and IL-8), clinical signs and lung viral titer [174]. The results of the study showed a direct relationship between the virus titer of the lungs and the IFNα and IL-6 levels. A clear correlation between elevated body temperatures and the production of TNFα and IL-6 was also documented. Moreover, a strong association between clinical signs of respiratory disease and IFNα, TNFα and IL-6 titers were discovered. The majority of the inoculated pigs showed fever, depression and breathing difficulties, whereas a minority of the

pigs only experienced respiratory symptoms. The affected cytokines identified in this study, had all previously been correlated with local responses such as increased mucus production and acquisition of inflammatory cells, including neutrophils and NK-cells to the lungs as well as to systemic responses such as fever, anorexia and depression. In addition to contributing to the development of respiratory disease, the innate response towards swIAV infection, has also been proposed to cause abortions and stillbirths in sows [186,188,192,201,202]. In conclusion, swIAV infections stimulate a pronounced innate response, which greatly contributes to the key clinical signs related to influenza infections.

The adaptive immune response

If IAV still manages to maintain the infection after battling the innate immune response, the adaptive immune system is needed for final viral clearance. The adaptive immune system is capable of responding to IAV in a specific manner both through a humoral- and through a cellular response, mediated by B- and T-lymphocytes, respectively. There are many bridges between the innate and the adaptive immunity as several innate mechanisms functions to stimulate adaptive responses. Antigen-presenting cells (APCs), e.g. macrophages and dendritic cells are key components in the activation of the adaptive response. The function of APCs is to present peptides, derived from either exogenous phagocytized antigens or intracellular synthesized viral proteins, on the major histocompatibility complex (MHC) class I or II located on the cell surface. Subsequently, the peptides bound to the MHC complex are recognized by $CD8^+$ T-lymphocytes and $CD4^+$ T-lymphocytes, which can lead to killing of the infected cell and/or secretion of cytokines to stimulate an elaborate adaptive response including B lymphocyte activation with production of antibodies [173,203–205]. Antibodies directed against IAV target the HA, NA, M and NP proteins. However, the majority of neutralizing antibodies are those directed against the globular head of the HA protein, which can block viral attachment and uptake by the host cells. Antibodies directed against the NA protein can, on the other hand, prevent the release of progeny IAV from the host cell, whereas antibodies against the M and NP gene do not have a direct impact on the viral lifecycle but act to enhance the elimination of infected cells [87,205,206].

In pigs, IgA, IgG and IgM can be detected as early as three to four days post swIAV infection PI [207], whereas the peak concentration of IgA in bronchoalveolar lavage (BAL) and nasal washes and IgG in the blood had been shown to occur at approx. 14 DPI [180,208]. IgA and IgG are especially important in the defense against swIAV [183,208,209]. The mucosal-associated lymphoid tissue (MALT) is the location for initial antibody release, but dendritic cells and macrophages also travel to the regional lymph nodes and to the spleen to stimulate B lymphocytes that release antibodies to the

systemic circulation and to the mucosa of the respiratory tract [203]. A difference in distribution of IgA and IgG has been identified in swine [208,210]. IgA is mainly present in the mucosa of the upper airway and is very important in limiting or preventing the infection in the upper airways. This correlates with the fact that IgA is, to a greater extent than IgG, locally produced in the upper respiratory tract. In contrast, IgG reaches much higher serum titers compared to IgA. High titers of both IgA and IgG in the lungs are observed upon swIAV challenge, and play an important role in preventing lower respiratory tract infections. After a primary swIAV infection, the host will be primed for a faster response to secondary swIAV infection mediated by memory B- and T-lymphocytes [211]. However, full protection within variants of the same HA subtype cannot be expected and the likelihood of cross protection is even smaller between different HA subtypes [87,108,203,205,208,212]. In conclusion, even though the cell mediated immune system plays an important role in fighting IAV infection, the neutralizing antibodies are the key effector molecules to clear the infection and therefore swIAV vaccines have mainly been designed to stimulate a strong humoral response.

Vaccines

Effect of maternally derived antibodies

Pigs have an epitheliochorial placenta, which is impermeable to immunoglobulins. This, along with fact that the neonate piglet does not have a fully developed immune system until the time of weaning, emphasizes the importance of colostrum delivery to the piglets. The dominating antibody isotype of the colostrum is IgG. IgA and IgM will however later be delivered from sow to piglet through the milk. Along with antibodies, cells are also delivered through the colostrum and it is estimated that approx. 26 % of the cells are lymphocytes with a favor of T cells rather than B cells [213]

One of the most important components in fighting swIAV is neutralizing antibodies, which mainly target the HA protein. To stimulate a protective antibody response whole inactivated virus (WIV) vaccines are frequently applied in swine herds. One of the most widespread vaccine strategies used, is sow vaccination, which aims at stimulating subsequent MDA uptake in piglets trough the colostrum [87]. Several studies have investigated the effect of MDA, and shown different levels of protection [10,11,178,183,214,215].

In an experimental study [214], transmission of swIAV was evaluated in two groups of either MDA positive (MDA+) or MDA negative (MDA-) pigs. Reproduction numbers (R_0) of 5.8 for the MDA+

group and 14.8 for the MDA- group were obtained. All contact pigs (direct and indirect) became infected in both groups. However, the MDA+ group showed a delayed onset of viral shedding compared to the MDA- group. These results indicated that while the presence of MDA was correlated with a lower R_0, the infection could still be maintained as the number was higher than one [216]. Interestingly, the authors observed that the delayed start of viral shedding in the MDA+ group, extended the overall period with viral shedding within the batch compared to the MDA- group. Therefore, they speculated that the presence of MDAs on a population scale could contribute to the persistence of swIAV in the herd. Furthermore, the analyses of the data from the MDA+ group revealed that the quantity of MDA also had an impact, since pigs with low levels of antibodies ($MDA+_{Low}$) were the first to become infected in the MDA+ group and showed a serological response to swIAV infection, contrary to the pigs with high levels of MDA ($MDA+_{High}$). However, no difference in the average total amount of virus shed and overall average shedding time was observed between any of the three groups. Based on the results of this study a stochastic event-driven metapopulation model was built [217]. This model confirmed that the presence of MDA in pigs extended the duration of the swIAV circulation within batches and thereby increased the chance of efficient batch-to-batch transmission, which overall results in a prolonged swIAV persistence in the herd.

Two studies provided more detailed data on the immune response and clinical signs during swIAV infection in pigs with or without MDAs. Moreover, both studies tried to re-infect the same pigs after MDA decline. The first study was conducted by a Dutch group [183]. In this study, two groups of pigs (MDA+ or MDA-) were included based on the antibody status of the sows from which they were born. The results of this study suggested that MDA provided greater clinical protection against primary swIAV infection compared to the MDA- group. However, an increased shedding time was observed in the MDA+ group and the results suggested that the presence of MDA at primary infection weakened the development of immunity, and possibly resulted in three of the MDA+ pigs being susceptible to re-infection with the same swIAV strain after MDA decline. The second study, also investigating the details of primary and secondary swIAV infection in relation to MDA, was conducted by a French group [178]. In this study, the MDA+ pigs were born from vaccinated sows. The first experiment of the study included two groups of pigs (MDA+ or MDA-), which were inoculated at five weeks of age and again at nine weeks of age. The study similarly showed that MDA did not prevent swIAV infection but did provide some level of clinical protection, dependent on the age of the pig and the level of MDAs. In addition, the results suggested that the presence of MDA during primary infection might result in shedding of fewer infectious particles. However, the

study also confirmed a weakened development of immunity in the presence of MDA, but in contrast to the Dutch study, no re-infection was possible.

Interestingly, the immune suppression experienced in the presence of MDA was already been described for swIAV infections in pigs in the seventies [218]. In this study, MDA- pigs showed clinical signs upon swIAV inoculation and developed a primary immune response to swIAV measured by HI-tests and the presence of 2-mercaptoethanol (ME) resistant or sensitive antibodies. The same pigs were protected against infection during the subsequent swIAV inoculation. Conversely, swIAV inoculation of MDA+ pigs, revealed that, in the presence of high levels of MDA, the pigs were protected against clinical signs of disease, shed swIAV, but did not produce measurable antibodies. Furthermore, secondary inoculation induced clinical disease resembling that of primary inoculation of the MDA- pigs. On the other hand, pigs with low levels of MDA at the primary infection developed clinical disease resembling that of a MDA negative pig at primary exposure. However, these pigs were believed to be immunologically stimulated even though no antibody production was observed, as only mild clinical signs were observed at the secondary inoculation.

Although the above-mentioned studies did not show protection against swIAV infection in the presence of MDA, other studies have shown more encouraging results. One of these studies obtained piglets from vaccinated sows originating from a swIAV negative herd. At weaning, the piglets were transported to an experimental facility and challenged with two different swIAV strains, resulting in one homologous vaccine-challenge group and one heterologous-challenge group. The results revealed that all the pig of the heterologous-challenge group became infected. Conversely, only 1/20 homologous challenged pigs was infected, indicating that the presence of homologous MDAs in piglets reduced the reproduction number (R_0) of swIAV significantly. Interestingly, the reproduction number (R_0) was not significantly different between the heterologous-challenge group and the MDA- control group [219]. Two field studies also showed an effect of sow vaccination in reducing and delaying the shedding of swIAV in piglets and weaners [220,221]. It should be noted that the HA sequence identity between the vaccine and challenge strain was ranging from 94.8-100 % in all three studies [219–221].

In conclusion, the above-mentioned studies have documented that the effect of MDAs is dependent on the level achieved in the piglets. In addition, the homology between vaccine strain and the subsequent challenge strain potentially has an impact on the efficacy. Sterile immunity towards swIAV based on MDA is rare, but several of the studies have shown full or partial clinical

protection. The presence of MDAs during primary swIAV infection can delay the time of infection and increase the individual shedding time, in turn contributing to the herd-level swIAV persistence. Moreover, there is compelling evidence that MDA presence at the time of primary swIAV infection can weaken the development of immunity against swIAV. However, only one study has been able to re-infect pigs with the same strain after MDA-waning, which emphasize the need for additional studies investigating the role of MDA in development of immunity.

Adverse effects of antibodies and VAERD

Even though MDAs can provide a certain degree of protection in piglets it is also important to consider possible "adverse" or "unwarranted" effects, which can be related to the presence of MDA at the time of infection. As mentioned above, several studies have reported a weakened immune response towards swIAV in the presence of MDAs at the time of infection, and it has been speculated if this weakened response might cause the pigs to be susceptible for re-infection with the same strain [178,183,218]. Some of the same mechanisms observed with regard to interference with MDA during vaccination can possibly aid in explaining the weakened immune response observed in MDA+ pigs exposed to swIAV. Overall, there are two different hypotheses to explain the lack of seroconversion observed in MDA+ individuals after vaccination. The first hypothesis is based on the idea that B-cells are inhibited by epitope masking, meaning that MDA will bind to the vaccine antigen and thereby cover the epitopes that would normally stimulate the B-cells in creating antibodies. Another mechanism to explain the inhibition of B-cells in the presence of MDA, is that binding of MDA (IgG) to the Fcγ-receptor IIB, has a role in down-regulating B cell responses. The down regulating effect is stimulated when an antigen binds to the B cell receptor (BCR) and a MDA binds to the Fcγ-receptor IIB in another region of the same B cell. Subsequently a cross-linkage between the two receptors occurs resulting in the two receptors coming in close proximity. This close proximity leads to an inhibitory motif on Fcγ-receptor IIB affecting the tyrosine-based activation motif of the BCR, resulting in inhibition of antigen-specific B cell activation and decreased B cell proliferation and antibody secretion. Two additional mechanisms have also been proposed. One is based on the idea that macrophages will remove antigen-antibody complexes from the blood, which will minimize the actual immune response to the vaccine. The other mechanism is based on the idea that the MDA will neutralize the vaccine virus [222].

Another aspect worth considering in regard to control strategies for swIAV in the herds is vaccine associated enhanced respiratory disease (VAERD), which has been documented in relation to swIAV infections in vaccinated pigs [223]. VAERD has been observed in pigs vaccinated with commercial

WIV vaccines and subsequently challenged with a heterologous swIAV strain, to which there was a lack of cross-reaction in HI-tests. This unfortunate outcome of vaccination has both been documented in vaccinated pigs, that were later challenged with heterologous swIAV [224,225] but also in piglets receiving heterologous MDA from vaccinated sows and then challenged [226,227]. One of the first studies that suggested VAERD in relation of swIAV infection in pigs was conducted in 2008 by Vincent et al. [224]. The pigs in this study were vaccinated twice with an inactivated human-like H1N1 strain. The vaccination was applied at three weeks of age and six weeks of age, and subsequently the pigs were challenged with a swIAV of the H1N2 subtype at eight weeks of age. No cross-protection was observed between the two strains and in addition, one third of the pigs showed enhanced pneumonia compared to a non-vaccinated challenged group. Interestingly, the study also revealed that pigs being challenged with the human-like H1N1 and then subsequently exposed to the reassortant H1N2 experienced some level of protection against the second challenge strain, as only mild lung lesions and no measurable viral shedding in nasal swabs and lungs were detected. This indicated that natural exposure to swIAV provided a broader protection than the inactivated vaccine, even between heterologous strains. Using a similar study design, but other vaccine and challenge strains, another study showed similar results as the vaccinated group showed elevated clinical signs and enhanced pneumonia compared to the unvaccinated pigs [225]. Subsequently, a study was conducted to investigate the mechanism behind VAERD [228], using the same vaccine-challenges model as described above [225]. This study revealed that the lack of cross-reacting neutralizing antibodies and the presence of the HA2 antibodies were part of the mechanism behind VAERD. VAERD has also been documented in relation to sow vaccination, as heterologous vaccination of sows leading to heterologous MDA uptake in piglets caused enhanced pneumonia following challenge [226,227].

In conclusion, the results of the above-mentioned studies suggest that there is a risk of VAERD if using a vaccine containing a heterologous strain compared to the herd strain. However, the level of homology needed between the vaccine strain and the challenge/herd strain to avoid VAERD remains unclear. Furthermore, there is a lack of studies reporting VAERD under field settings. Therefore, more studies are crucial to elucidate the impact and importance of VAERD under field conditions.

Original antigenic sin:

The original antigenic sin in relation to IAV infections of humans is a widely known phenomenon and was first described in 1953 [229]. The phenomenon is based on the idea that the initial influenza infection obtained during the childhood will generate lifelong immunity (memory cells and

neutralizing antibodies) and during IAV infections with novel strains, antibodies towards the old "childhood strain" will be boosted at the expense of antibodies targeting the novel IAV strain. The rationale behind this is, that when an individual is exposed to numerous different influenza strains, antibodies towards the shared epitopes will be generated, but antibodies towards the varying epitopes will be either not be produced or be produced to a lesser extent, resulting in antigenic sin [230]. No studies have investigated if this phenomenon also occurs in pigs.

Current use of vaccines

Currently, the only commercial vaccine types available for controlling swIAV in Europe are WIV vaccines. These include mono-, di- or trivalent vaccines, containing the different swIAV subtypes [206]. WIV vaccines are administered as intra muscular prime-boost injections, and stimulate mainly a humoral systemic antibody response in the host dominated by the production of IgG, which can reduce the spread of swIAV to the lungs and the excretion [231–237]. The antibodies can be neutralizing if they match the challenge virus [87,231,238].

In Denmark, as well as in many other countries, the main strategy utilized for control of swIAV is sow vaccination programs [239]. Overall, there are two main strategies for sow vaccination. The first is termed "mass/blitz/blanket" vaccination and the second is termed "pre-farrow/rolling" vaccination [240]. Mass vaccination is a vaccination strategy where the farmer vaccinates all sows present in the herd at defined time points during the year, normally including between one to four vaccinations every year. The farmer may choose to include the gilts in this strategy or have a separate strategy for gilts dependent on the level of import and quarantine facilities in the herd. Pre-farrow vaccination, on the other hand, is a strategy where the different batches of sows are vaccinated based on the reproduction cycle. The sows will in this case normally have a basic-vaccination, including either one or two vaccinations, and then receive a booster vaccination approx. two weeks before farrowing to ensure a production of colostrum MDA for the piglets, and provide clinical protection [241]. In addition to sow vaccination, most commercial vaccines are also available for use in pigs after MDA decline. However, vaccination for this age group is not as common as sow vaccination [242].

Two commercial vaccines are available in Denmark. Respiporc FLU3 [232] is the most widely used vaccine and contains three different strains: Bakum/IDT1769/2003 (H3N2), Haselünne/IDT2617/2003 (H1N1) and Bakum/1832/2000 (H1N2). These strains cover the most prevalent subtypes in Denmark including the Danish H1N2, which has the avian HA gene resembling that of the Haselünne/IDT2617/2003 (H1N1) strain and the N2 resembling that of the Bakum/IDT1769/2003 (H3N2) strain. The vaccine is licensed for sows and pigs above eight weeks

of age and claims to reduce clinical signs and spread of the virus to the lungs. Moreover, if sows vaccinated with two doses at a three-week interval, receive booster vaccination two weeks before farrowing, the vaccine claims clinical protection of piglets until 33 days-of-age through MDA. The second vaccine on the Danish market is Respiporc FLUpan [233], which includes the pandemic strain A/Jena/VI5258/2009(H1N1)pdm09. The vaccine is approved for use in pigs above eight weeks and claims reduction of virus excretion and spreading to the lungs. Both vaccines use Carbomer as an adjuvant, and require at least two doses for development of immunity.

Several experimental studies have shown either full protection, reduced viral shedding and/or clinical effect in MDA negative pigs when vaccinated twice with a whole virus inactivated vaccine and challenges with a homologous influenza strain [234,243–247]. However, only few studies have investigated the effect of the current vaccines in field studies and they have mainly focused on the protection achieved through MDAs [220,221,248].

The array of vaccines available in North America stands in great contrast to the European selection. First of all the licensing requirements were changed in 2007, which gave the manufactures the option to update the strain in currently licensed vaccines solely based on serology tests and not challenge studies. Furthermore, American manufacturers are not obliged to share the name or sequence of their vaccine strain only the given lineage [249]. In addition, autogenous swIAV vaccines are legal for use in the United States, but can only be used in the specific herd in which the strain was diagnosed. Autogenous vaccines in the U.S. do not require extensive safety testing compared to the licensed vaccines, but most go through safety testing in laboratory animals and purity testing to prove the absence of fungi and bacteria [249]. In 2008, autogenous vaccines represented 50 % of the swIAV vaccine sales in the U.S. [250]. In 2017, a live-attenuated intra nasal vaccine became available for swine on the U.S. market. The vaccine includes both a classical swine H1N1 and the American H3N2 containing the TRIG internal cassette with a truncated NS1 gene [251,252]. The vaccine is approved for use in piglets from one day of age and studies have shown that the vaccine can reduce viral shedding, even in the presence of MDAs [251,253,254]. In addition, the vaccine has shown partial protection against heterologous challenge without inducing VAERD [255]. However, the studies only provided limited data on the clinical protection, and no field studies are available.

Novel vaccine candidates

An example of an alternative IAV vaccine candidate is the live vector vaccine. The replication-defective human adenovirus 5 has been used as a vector for carrying IAV genes. In pigs, a vaccine carrying the HA gene of an A(H1N1)pdm09 subtype has been created and tested in an experimental

trial, where vaccinated pigs were challenged with homologous or heterologous virus [256]. The results showed full protection against homologous challenge, and partial protection against heterologous challenge observed by a lower viral load and reduced shedding time. Another method employed for production of IAV vaccines is the use of alphavirus replicon particles. These particles are single cycle vectors that are replication defective and can express an antigen of choice in the host cell e.g. the HA gene of IAV. An experimental trial was performed using two different types of particles, either carrying an HA gene of a human H3N2 origin or of swine H3N2 origin [257]. Despite lack of a challenge group in this study, the results revealed very high HI-titers 21 days after vaccination. Later, the same vector, carrying the HA gene of an A(H1N1)pdm09 subtype, was tested in an experimental trial with subsequent homologous challenge [258]. Again, a high specific antibody response was observed subsequent to vaccination, and the vaccinated pigs showed reduced viral shedding and lung pathology compared to the control group. Another virus utilized as a vector for expressing the HA gene of IAV is the pseudorabies virus (PrV). A study presented the results of using the attenuated PrV, which is used as a modified live vaccine against Aujeszky's disease, to express the HA gene of an A(H1N1)pdm09 [259]. The results revealed that vaccinated pigs subjected to homologous challenge, showed fewer clinical signs and had significantly lower virus shedding, compared to the non-vaccinated control pigs. Attempts have also been made to create a plasmid based DNA-vaccine to be used for IAV vaccination in swine. One of these studies tested the effect of vaccination in pigs with plasmids containing either the NP gene or the HA gene of two human H1N1 subtypes [260]. The pigs were vaccinated by transferring the plasmids into the epidermis or the tongue using a "gene gun". The results showed that the vaccinated pigs obtained a great humoral response towards both the NP gene and the HA gene. However, only the vaccine based on plasmids containing the HA gene showed a decrease in viral load and shedding time in a subsequent homologous challenge. A more recent study from 2018, tested a similar polyvalent DNA vaccine containing six different IAV genes including the NP and M gene of the Spanish Flu, the HA and NA gene of the Hong Kong Flu and HA and NA gene of the A(H1N1)pdm09 [261]. Pigs were vaccinated with a low or high dose using needle free intradermal injections, and subsequently challenged with an A(H1N1)pdm09 subtype. All vaccinated pigs developed antibodies to the different IAV proteins expressed by the vaccine. Moreover, none of the pigs receiving the high dose vaccination showed any viral shedding and lung lesions indicating full protection. However, it should be noted not all pigs of the control group showed virus shedding after challenge. The pigs receiving the low dose vaccination were only partially protected as lung lesions were observed. Another way to induce immunity is to use virus-like particles (VLPs). These VLPs only contain matrix/capsid and surface proteins and no viral RNA, and are thereby non-infectious. Baculoviruses expressing the M1,

HA and NA gene of an A(H1N1)pdm09 strain, have been used to generate VLPs, which were subsequently tested in a vaccine-challenge trial with a homologous IAV strain [262]. The results revealed that the vaccine provided complete protection of the lungs. However, nasal shedding of IAV was still observed and the effects on clinical signs were unclear.

Epidemiology – global distribution

North American swine influenza virus surveillance, prevalence and subtype diversity

Influenza in swine was retrospectively documented in North America in 1918-1919 at the same time as the Spanish Flu H1N1 subtype was causing a major pandemic in humans [179]. The virus isolated from pigs was later discovered to be closely related to the human strain, and was termed "classical swine H1N1" [129,179]. This virus became established in the North American swine population and remained the only documented subtype until 1980, when a serological study revealed that while classical swine H1N1 was the most prevalent subtype, a small percentage of human-like H3N2 was also present [263]. Indeed, in 1991 an H3N2 closely related to human H3N2 was isolated from a swine herd in Canada [264].

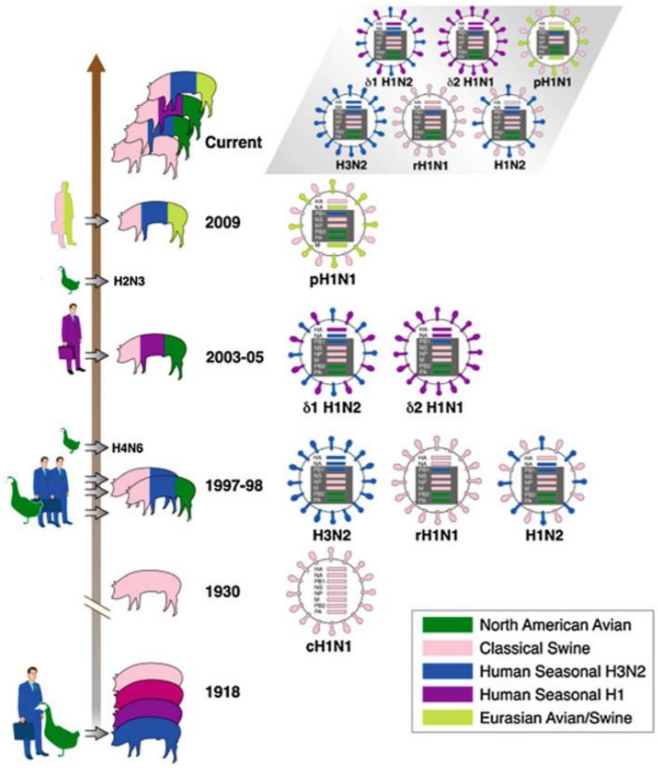

Figure 8. Illustration of the different subtypes and origin of swIAV circulating in the North American swine population from 1918 until 2012. SwIAV lineages are colored pink, Eurasian avian-like lineages are colored green and human lineages are colored purple or blue. The vertical arrow represent the timeline of the epidemiology of the different swIAV strains, whereas the horizontal arrows represents donations of viral gene segments from human or avian origin into the swine population [21].

The diversity of swIAV in North America changed dramatically in 1998, as two new subtypes were discovered in relation to four outbreaks in three different states (Figure 8). The virus isolated from the first outbreak in North Carolina was a double-reassortant with the HA, NA and PB1 originating from human H3N2, and the remaining genes originating from the classical swine H1N1. The virus isolated from the three other outbreaks was a triple-reassortant, which contained the HA, NA and PB1 genes from the human H3N2 mentioned above, the NP, NS and M genes from classical swine H1N1 and the PA and PB2 genes of North American avian origin [73]. The new subtype thereby contained genes of human, swine and avian origin, and the internal gene cassette was later termed triple reassortant internal gene (TRIG) cassette [242]. Two years later, the new triple reassortant H3N2 had become widely established in the US pig population [265]. Soon thereafter, this subtype reassorted with the classical swine H1N1, resulting in two new viruses. One of the two virus was an H1N1 subtype and contained the HA and NA genes of the classical swine H1N1, but TRIG internal cassette. This subtype was termed "rH1N1" [74]. The other virus was an H1N2 subtype, containing the HA gene of the classical swine H1N1 origin the remaining genes from the triple reassortant H3N2 [75]. In 2005, human seasonal IAV entered the North American pig population, and by reassortment events gained the TRIG cassette. This event gave rise to a new human H1 gene, which was genetically distinct from the H1 gene of the classical swine H1N1, and also introduce NA genes of human origin [266]. Based on phylogenetic results of the HA genes of the different H1 circulating in the US, a new nomenclature was proposed, dividing the classical swine H1N1 HA gene in-to three clusters (α, β, and γ) [266]. Furthermore, a separate cluster (δ) of the human seasonal H1 was introduced and later divided into two subclusters (δ1 and δ2) [267]. As mentioned earlier, a major human pandemic occurred in 2009 with the A(H1N1)pdm09 virus. The virus quickly spread worldwide and also entered the pig population, where countries such as Australia and Norway that have had an swIAV free pig population were infected [268–270]. The A(H1N1)pdm09 is, in itself, a reassortant which includes the HA, NP, NS of classical swine H1N1 origin, the NA and M from Eurasian avian-like H1N1 and the polymerase genes of the TRIG cassette. This was the first time that Eurasian swIAV genes had been detected in the U.S. The phylogenetic results indicated that while the ancestor of the pandemic HA genes was classical swine H1N1, the HA genes made a separate cluster within the classical swine HA lineage [271,272]. In 2016, a retrospective study was performed on swIAVs isolated from diagnostic samples collected in relation to respiratory disease in swine from 2010-2014 in Mexico. The phylogenetic analysis revealed that the Eurasian IAV had been circulating for many years in Mexico and had reassorted to create several different genotypes, and that the emergence of the A(H1N1)pdm09 was likely to have occurred in swine in Mexico [112]. The genetic differences from previous documented swIAVs clearly revealed a lack of surveillance of

swine influenza worldwide. Currently the A(H1N1)pdm09, the above-mentioned human reasortant H1N2 and H1N1 circulate together with the original triple reassortant H3N2 plus the reassortants with the classical swine H1N1; rH1N1 and H1N2 (Figure 8). Furthermore, several new reassortants keeps evolving where internal genes and surface genes of all the subtypes are exchanged [1,273,274].

Asian swine influenza virus surveillance, prevalence and subtype diversity

Introductions of both swIAV of American and European lineages have occurred over the years in Asia, and also introductions of human seasonal IAV have contributed to the diversity of subtypes isolated. China has the largest pork production in the world [275]. Therefore, the following review mainly contains data from China. In 1974, the classical swine H1N1 was detected for the first time in China. Three years later, this subtype was also found in Japan. The subtype subsequently reassorted to obtain the NA gene resembling the old Hong Kong flu H3N2 subtype, and both the classical H1N1 and the reassortant H1N2 became enzootic in China, while mainly the reassortant H1N2 dominated in Japan [276,277]. Moreover, other studies provided serological evidence of both the Hong Kong flu H3N2 and human seasonal H3N2 circulating in Chinese swine[278]. In 1993, an avian like H1N1 was first detected in China. However, it was later discovered that this subtype appeared to be a separate introduction from birds to pigs in Asia, and not an introduction of the European avian-like H1N1 circulating in swine [279]. In 2001, a paper was published on the surveillance data obtained in China from 1998-2000 and again provided evidence that human seasonal H3N2 had been circulating and was established in the Chinese swine population, but also revealed that an avian H9N2 subtype was circulating in Chinese swine [280]. Subsequently, several other cases of avian IAV in swine have been documented in Asia, including H5N1 [281,282] H6N6 [283] H4N8 [284]. In 2001, the Eurasian avian-like H1N1 was detected in Asia and one year later the American H3N2 containing the TRIG internal cassette was detected. Several different reasortants appeared following these introductions, and the most dominating variant of Eurasian H1N1 contained a NS gene derived from the American TRIG cassette [285]. In October 2009, A(H1N1)pdm09 was detected in China and Japan for the first time. The United states and Europe had reported the isolation of A(H1N1) earlier than this date and thereby the speculations that the virus originated from Asia was not supported [271,286,287]. A later study investigating the swIAV subtypes isolates in China between 2013-2015, revealed ten novel reassortants, all containing one or more genes of A(H1N1)pdm09 origin [288]. Furthermore, a recent study confirmed the presence of several reassortants in China and documented a new H1N2 subtype containing the HA gene of Eurasian H1N1 origin, the NA gene of human N2 origin and an A(H1N1)pdm09 M gene. Moreover, the study revealed that the finding of internal genes of A(H1N1)pdm09 origin was more and more

frequent. However, the swIAV sequenced in this study still maintained the NS gene of the American TRIG origin, and additionally the Eurasian M gene was frequently found [41].

European swine influenza virus surveillance, prevalence and subtype diversity

The 1918 descendent classical swine H1N1 eventually spread to Europe, where it was documented in the middle of the 20th century [289,290]. Thereafter, the virus was not observed until 1976, where it caused major outbreaks in Italy following an introduction of pigs from the U.S. From there, the virus slowly spread to many other European countries and became enzootic [291–297]. However, in 1979 a new H1N1 began to dominate the circulation of swIAV in Europe. This new strain was proposed to be a whole avian IAV strain that had jumped from birds to pigs, since all the genes resembled genes found in AIV and was therefore termed "Eurasian avian-like H1N1" [298–300]. Within a few years, the Eurasian avian-like H1N1 became the dominant strain, and the classical swine H1N1 disappeared from Europe. Today, the Eurasian avian-like H1N1 still dominate, and has participated in a number of reassortment events [18,19]. However, H1N1 was not the only subtype that was introduced into Europe in the 70's. The Hong Kong flu H3N2, which caused the human pandemic in 1968, was subsequently transmitted into the swine population. One of the first studies presenting serological evidence for the Hong Kong flu H3N2 in swine in Europe was conducted in Great Britain in 1972 [301], and was later confirmed in other parts of Europe [302,303]. In the early eighties, the Hong Kong flu H3N2 acquired the Eurasian avian-like H1N1 internal gene cassette and created a new virus (swine-adapted Hong Kong H3N2) that soon became the dominating H3N2 virus in Europe [304]. In 1985, isolation of swine-adapted Hong Kong H3N2 was for the first time linked to clinical outbreaks of respiratory disease in swine [305]. Since then, H3N2 has circulated in most European countries, and has drifted quite far from the original Hong Kong flu H3N2 [108]. In addition, in 1994 a new reassortant including an H1 gene most identical to a human IAV strain circulating in the early eighties [306,307] and an N2 gene similar to that of the swine-adapted Hong Kong H3N2 was identified in Great Britain [102]. This virus - termed "human like" H1N2 - subsequently became established in continental Europe [102,308]. Another H1N2 reassortant, which contains the HA gene of the Eurasian avian-like H1N1 and the NA gene of the swine-adapted Hong Kong H3N2 virus was initially detected in Denmark and has subsequently spread to other European countries [18,19,181,309,310]. As mentioned above, in April 2009, a new major pandemic occurred in the human population involving a swine-derived virus termed "A(H1N1)pdm09". This virus had not been identified in pigs prior to the 2009 human outbreak [311]. In September 2009, the first case of A(H1N1)pdm09 was documented in pigs in Ireland in relation to mild respiratory disease in growing and finishing pigs [312], and subsequently the virus was found in most European countries [17–

19,269,313]. A(H1N1)pdm09 replaced the human seasonal H1N1 [314] and is thereby a seasonal source of transmission to pigs. Meanwhile, evidence of a swine specific genogroup (swD) of the A(H1N1)pdm09 virus has been presented by a French research group [104] and was confirmed by the Danish swine influenza surveillance data (unpublished data). The study suggested that A(H1N1)pdm09 has become well established in the pig population and has started to drift/adapt to pigs. The French group still identified cross-reactivity between the swine genogroup and human A(H1N1)pdm09, but also speculated that over time the drift might lead to new antigenic variants. The introduction of A(H1N1)pdm09 virus with genes of swine influenza origin emphasized the role of pigs as a possible source of new human pandemics [271], and therefore lead to an intensified surveillance of swIAV. This increased surveillance, together with concurrent developments in next generation sequencing tools [86] has led to the discovery of a wide range of new reassortants between the A(H1N1)pdm09 and the previous enzootic subtypes circulating in pigs in Europe [18,19,190,315–317].

Danish swine influenza virus surveillance, prevalence and subtype diversity

The introduction of different subtypes in Danish pigs resembles to some extent the same pattern as observed in the rest of Europe. In 1981, the Eurasian avian-like H1N1 was fist isolated in Denmark and subsequently the swine-adapted Hong Kong H3N2 virus containing the Eurasian avian-like internal gene cassette was identified in 1990. In 2003, a reassortant between these two viruses became established and contained the HA gene and the internal gene cassette of Eurasian avian-like H1N1 origin, and the NA gene from the swine-adapted Hong Kong H3N2 virus. This subtype was termed “Danish swine H1N2” [18,181]. In 2010, the A(H1N1)pdm09 subtype was identified in Danish pigs. A passive annual swIAV surveillance was initiated in 2012 by request of the National Danish food administration [8]. The subtypes identified from 2013-2018 are shown in Figure 9. In 2013, the Eurasian avian-like H1N1 represented a relatively high percentage (31 %) of the subtypes identified. However, this subtype was gradually replaced by the Danish swine H1N2 resulting in the Eurasian avian-like H1N1 only making up 5 % of all subtypes identified in 2018, whereas the Danish swine H1N2 made up 69 %. The Danish swine H1N2 has been the dominating strain for many years and the prevalence still increases. The number of subtypes containing the HA gene of A(H1N1)pdm09 origin has ranged between 20-30 % since 2013, and the number of different subtypes including a HA or NA gene of A(H1N1)pdm09 origin has been increasing over the last couple of years. In addition, an increasing prevalence of Danish swine H1N2 containing internal genes of the A(H1N1)pdm09 origin has been observed since the surveillance program was initiated. The swine-adapted Hong Kong H3N2 has not been diagnosed in Denmark since 2014. However, a

triple-reassortant “H3hu05N2sw” swIAV was identified in 2013-2016. This subtype contained a HA gene of human 2005 seasonal H3N2 origin, the NA gene of the Danish swine H1N2 origin and the internal gene cassette of A(H1N1)pdm09 origin [190]. In addition, the Eurasian avian-like H1 gene in constellation with a human seasonal N2 gene has been identified at a low prevalence during the last seven years. The Eurasian avian-like gene cassette of this subtype has also been replaced by an internal gene cassette of A(H1N1)pdm09 origin. The “human like” H1N2, has to this date not been identified in Denmark.

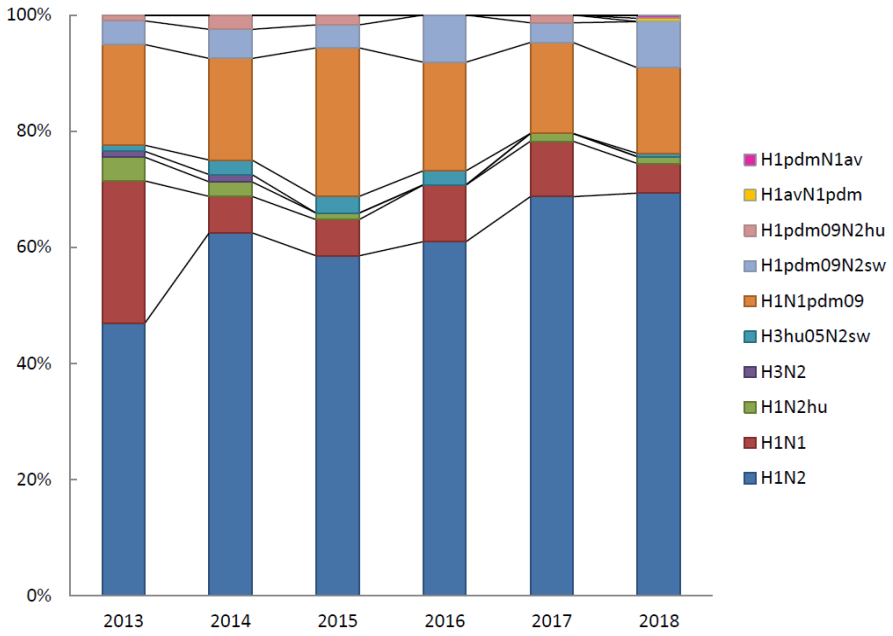

Figure 9. Illustration on the distribution of different swIAV subtypes registered in the Danish swine influenza program from 2013-2018. The origin of the HA and NA genes are specified in the subtype. “pdm” indicates A(H1N1)pdm09 origin, “av” for Eurasian avian-like H1Nx origin, “sw” indicates swine-adapted Hong Kong H3N2 origin. and “hu” indicates human seasonal flu origin, specifically “hu05” indicates human 2005 seasonal flu origin [8].

Epidemiology - transmission dynamics

Within the herd

In Europe, only a few longitudinal studies have been conducted to study the transmission- and disease dynamics within individual swineherds. One of the studies was performed in three French swine herds [10]. Within each herd, three consecutive batches of pigs were included in the study and followed from birth until slaughter. The result showed that the clinical outbreaks registered by the farmers correlated with the presence of swIAV. Interestingly, the clinical outbreaks occurred at approx. the same time in all batches of all three herds. Multiple subtypes were circulating within each herd, and in one herd, reassortant swIAV was discovered. The reproduction number R_0 was

estimated to 2.5-6.9, and the shedding period was estimated to be between six to 10.4 days. The results furthermore, revealed that the presence of MDA likely protected the pigs clinically when infected before 35-40 days of age. In addition, a correlation was observed between the shedding time and the presence of MDA, indicating a longer shedding time when infected in the presence of MDA. Moreover, the pigs infected in the presence of MDA were also less likely to seroconvert after infection. Finally, early swIAV infections were more likely to occur in the piglets originating from sows receiving a large number of cross-fostered piglets, and having low antibody titres one week post farrowing.

Another longitudinal study was conducted in two Spanish swine herds [11]. From each herd, one whole batch of pigs was included and followed from three weeks of age until slaughter. The two herds showed completely different dynamics, as Farm 1 had swIAV circulating continuously over the whole sampling period representing an enzootic infected herd. Conversely, Farm 2 experienced an epizootic outbreak of swIAV. The results from Farm 1 revealed that swIAV was present already at three to four weeks of age despite the pig originating from sows with IAV antibodies. Moreover, the presence of swIAV in all sections of the herd suggested that swIAV was introduced both by movement of infected pigs, but also as a consequence of poor biosecurity procedures. Overall, 51 % of the included pigs tested positive for swIAV at least once during the study period, while 7.4 % of the infected pigs tested positive on two non-consecutive sampling times. Only one subtype was identified in the herd, and the results therefore suggested that some pigs were re-infected with the same subtype. The authors proposed that this could be due to antigenic drift producing escape variants. The drawback of this study was that lack of sampling in pigs below three weeks of age.

A number of American studies have also described the within-herd swIAV transmission dynamics. One study described the swIAV transmission dynamics in a two-site swine herd, which had a farrow-to-wean site (Site 1) on one location and wean-to-finish site (Site 2) on another location [9]. The results of the study demonstrated that swIAV was mainly present in piglets >11 days of age at Site 1 and was not able to identify swIAV in gilts and sows. The same subtype detected in piglets at Site 1 was identified in the weaned pigs at Site 2. The study thereby emphasized the importance of considering piglets as a reservoir for swIAV maintenance in the farrow-to-wean site and highlighted the risk of swIAV introduction when buying weaned pigs. A large-scale study was subsequently conducted in five farrow-to-wean herds over one year period with cross-sectional monthly samplings of new gilts (present on a farm for less than four weeks), gilts (present on a farm for more than 4 weeks), sows and three-week-old piglets [12]. The results revealed that all herds had swIAV positive piglets, whereas the level of positive new gilts and gilts varied among herds. However, in one herd

up to 26 % of the new gilts were found positive for swIAV. In all herds, both an H1N1 and an H3N2 subtype were circulating and a great number of different reassortant strains were discovered. Another study, also investigated the presence of swIAV in three different subpopulation (new gilts, resident gilts and piglets) of five breeding herds [14]. The study discovered that the odds of testing positive for swIAV were 7.9 and 4.4 times higher in the new gilt subpopulation and in piglets compared to resident gilts, respectively.

In 2017, a longitudinal study investigating the swIAV dynamics within a single wean-to-finish herd was performed [13]. In this study, 132 three-week-old piglets were sampled weekly for 15 weeks. The results showed that the pigs experienced two epizootic events during the 15 weeks, with two different swIAV strains. The first event occurred shortly after arrival and the second epizootic occurred approx. three weeks later, with a new swIAV strain. Moreover, a third swIAV strain was also detected at low levels during the epizootics. In this study, recurrent infections, defined as the same pig testing positive twice in non-consecutive weeks, occurred in 24 pigs. Nineteen of the pigs were positive for two different swIAV strains at the different sampling times, whereas five of the pigs were positive for the same swIAV strain differing only one-two amino acids in the HA protein. The authors listed a number of explanations for these possible re-infections including environmental contamination, antigenic drift, host factors including immunity and MDA. Moreover, consecutive shedding for more than one week with the same swIAV strain was observed in five pigs. Four of the five pigs showed no amino acid differences in the HA protein between samplings.

The impact of seasonality of swIAV circulation has long been up for debate. Only one of the above-mentioned studies observed a level of seasonality in the occurrence of swIAV [14], whereas the other studies were able to identify swIAV throughout the year in accordance with previous findings [8,17,19]. However, a number of studies have provided data suggesting that swIAV occurrence might be influenced by the seasons [15,23,311,318,319]. Generally, the studies observed an increase in swIAV occurrence over around October-December and around March-April. Underlying reasons for seasonality having an impact on swIAV circulation, were related to changes in outdoor temperatures and the absolute humidity [15,23,320].

In summary, the transmission dynamics of swIAV is rather complex and is dependent on the specific herd and its structure, immune status, biosecurity procedures and handling of pigs, which is in accordance with what is known about the general routes of swIAV transmission in swine. Transmission through direct contact between pigs remains the main route of transmission. However swIAV is also transmitted by aerosols, large droplets and contaminated fomites carried by pigs,

equipment or personnel [321,322]. The above mentioned studies demonstrate that swIAV can be found in all levels of the production cycle and again emphasize that full protection of piglets through MDA cannot always be expected. In addition, two of the studies provided evidence of re-infections with the same subtype. Both epizootic and enzootic infections were described, and often a complex reservoir of different swIAV strains was found within a single herd. Finally, swIAV can be found in swine herds all year-around. However, some studies suggest that peaks in infections can be explained by seasonal changes in outdoor temperature and absolute humidity.

Between herds

A study conducted in 2012 aimed at detecting and quantifying swIAV in air samples collected in- and around swIAV infected herds [323]. The inside-herd air samples were positive for swIAV by PCR in 3/4 herds and viral isolation based on the air samples was possible in two of the herds. The exhaust air samples were also positive for swIAV by PCR in 3/4 herds, however viral isolation was only possible from one of the herds. In addition, swIAV was detected by PCR down-wind from two of the herds at distances up to 2.1 km. Even though the level of swIAV in these samples was low, sequencing proved that the virus was similar to the strain detected in the herd of expected origin. The study thereby highlighted the potential risk of airborne transmission within and between herds. The possible spread of swIAV between herds through the air, has also been suggested by two other studies that discovered the within-herd seroprevalence of swIAV to be higher in herds located in densely pig populated areas [324,325].

Transportation/movement of pigs between herds is another route of transmission. Many herds contain several different production sites, which sometimes are spread over large areas. One of the above mentioned studies describing the swIAV dynamics in a two-site swine herd, clearly showed that the swIAV strain found in the farrow-to-wean site, could subsequently be found in the wean-to-finish site [9]. In addition to transport between sites, an increasing number of herds are specialized in one specific part of the production cycle e.g. farrow-to-wean or wean-to-finish, and therefore an increasing number of pigs are moved between herds. Some of the herds receive pigs from several different suppliers, and will also change supplier quite often due to supply and demand. In the U.S., the movement of swine has been clearly linked to the introduction of specific swIAV subtypes [22]. Moreover, export of live-pigs between countries has increased. Denmark is a good example, as 14.2 million live weaners were exported in 2017, without any testing for swIAV [326]. The fact that transport of pigs between herds increases the risk for swIAV transmission is further emphasized by a

number of studies which have proposed introduction of gilts as a possible risk factor for swIAV introductions [9,12,14,327].

In summary, the structural dynamics of the global and national swine production contributes to a significant transportation of pigs between herds, which increases the risk of swIAV dissemination.

Humans are an additional route of IAV introductions in swine herds. As reviewed earlier, swIAV can be transmitted from pigs to humans, but human IAV can also be transmitted from humans to pigs (reverse zoonosis). This has been especially evident for the A(H1N1)pdm09 subtype [270,328]. Humans thereby pose a risk for IAV introduction into the swine herds, and it is therefore recommendable for herd personal to be vaccinated against IAV, and be banned from work when showing influenza like-illness [1].

Control of swIAV – vaccination, management and biosecurity

When swIAV has first entered a swine herd, there are several different methods to reduce the transmission and clinical signs. The most frequently used method is vaccination as reviewed above. However, improvement of management and biosecurity are also important, and can aid in reducing both the within-herd and between-herd transmission. Mathematical modelling of the effect of different herd interventions on the reduction of swIAV transmission was performed by an American group [240]. The results of this study showed that after swIAV had spread between different units of the herd, extinction was very unlikely. Moreover, swIAV persistence subsequent to swIAV introduction occurred in herd sizes as small as 500 animals. The overall best combination of interventions was the use of mass-sow vaccination with a homologous strain every two months, early weaning (< one week of age), gilt separation, homologous gilt vaccination and six months between gilt introductions. This combination strategy reduced the overall swIAV prevalence by 51 % and the prevalence in piglets by 74 %. However, swIAV was only eliminated in 23 % of the simulations. A field case-study in a farrow-to-feeder herd experiencing an acute swIAV outbreak, also reported a significant reduction in the swIAV prevalence in piglets after establishing sow vaccination and improving management and the internal biosecurity program. Specifically, double vaccination of sows and gilts was performed with the first dose administered before fecundation and the second dose administered three weeks before farrowing. Moreover, the time until weaning was prolonged one week to reduce turnover and the cross fostering of piglets between sows in different farrowing areas was banned. Moreover, the use of disinfectant between breeding cycles and the use of overshoes for different areas of the herd was also enforced. However, due to the study design it was not possible to determine if the reduction of disease was caused by the interventions practised or due

to the natural course of the disease [248]. Another effective but costly method to eliminate swIAV from an infected herds, has been presented in one study, where herd closure and partial depopulation was implemented and swIAV was eliminated [327]. As mentioned earlier, swIAV can be transmitted by fomites, and therefore biosecurity measures to eliminate indirect transmission of swIAV are important for the control of swIAV [322]. Finally the results of the within herd swIAV transmission dynamics, reviewed above, emphasize that the access to susceptible individuals is a driver of swIAV persistence at the herd level. Thereby, all measures to minimize the availability of susceptible individuals must be considered highly relevant in controlling swIAV. These measures are already widely used for controlling PRRSv, and include all in/all out principles, no transfer of pigs "back" in the production system, no movements between rooms, minimization of cross fostering and use of nursing sows [329].

Part 3 – Manuscripts

Manuscript 1

Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies

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Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies

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Abstract

A longitudinal study was performed in three Danish farrow to grower (30 kilos) herds over a 4-month period to investigate the dynamics and clinical impacts of influenza A virus (IAV) infections. In each herd, four batches consisting of four sows each with five ear-tagged piglets were included. Nasal swabs and/or blood were sampled from the sows and/or the piglets prior to farrowing and at weeks 1, 3, and 5 and at the end of the nursery period. Clinical examinations were performed at each sampling time. The sows and piglets were tested for IAV and IAV antibodies in nasal swabs and blood samples, respectively. The results revealed three enzootically infected herds, where the majority of the pigs were infected during the first 5 weeks after birth. Infected piglets of only 3 days of age were detected in the farrowing unit, where the sows were also shedding virus. In all herds, low to moderate numbers of infected pigs (ranging from 3.6 to 20.7%) were found to be virus positive in nasal swabs at two consecutive sampling times. Furthermore, clinical signs of respiratory disease were associated with IAV detection. The findings of this study documented that IAV can persist in herds and that piglets as young as 3 days can be infected despite the presence of maternally derived antibodies.

Introduction

Influenza A virus (IAV) is one of the most important viral pathogens in swine herds globally and is considered a significant cofactor in the porcine respiratory disease complex (PRDC) [1, 2]. IAV was first detected in European pigs in the 1970s [3] and has since been related to acute outbreaks of respiratory disease in swine herds that typically resolved within a few weeks [4, 5]. However, in recent years, a number of studies have shown that the dynamics of IAV infections have changed and that IAV can persist in herds. The change is probably a result of the increased herd size that ensures a weekly flow of naive individuals who can maintain the infection [6–12].

IAV is highly prevalent in Danish swine herds, and the results of the national passive surveillance program have revealed that the prevalence of IAV exceeds 45% in the diagnostic samples submitted from pigs with a history of respiratory disease. This makes IAV the most prevalent pathogen found in relation to PRDC in Denmark [13]. H1N1, H1N2 and H3N2 constitute the majority of the circulating IAV subtypes, and each subtype has a significant variety of different lineages with different genetic traits of avian (av), human (hu) or swine (sw) origin [14]. The most prevalent subtype in Denmark is the H1avN2sw, which has the avian-like hemagglutinin (HA) gene and the neuraminidase (NA) gene from the human-like reassortant swine H3N2sw [15]. In 2010, pandemic A(H1N1)pdm09 appeared in Denmark and is now the second most prevalent subtype, constituting 20% of the strains. Furthermore, the internal genes of this strain have been incorporated into more than 80% of the most

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prevalent strain H1avN2sw [13]. In addition to these dominating enzootic strains, a number of reassortants have been detected, including strains harboring the HA and NA genes from human seasonal flu strains, indicating that human-to-pig transmission takes place [13, 16].

The change in viral dynamics and the increased complexity of the circulating variants pose a challenge for farmers and veterinarians when determining control methods [17]. Thus, there is a great need for studies designed to increase our knowledge of the transmission dynamics and impacts of IAV under field conditions. Few studies have focused on the transmission of IAV early in the farrowing unit [6], as most studies have initiated sampling at an age close to weaning [11, 12] and have been performed as cross-sectional studies [18, 19]. The primary aim of the present study was to determine the prevalence of influenza-positive pigs over time by conducting an observational longitudinal cohort study in three Danish swine herds. A secondary aim was to investigate the association between virus-positive pigs and clinical signs. It is important to investigate the transmission dynamics and the clinical impact in pigs of this age because the pigs are highly susceptible and because this period includes the time when the pigs go from relying on passive immunity to having an active immune response towards IAV. Furthermore, infected piglets at weaning may be the source of the infection in the nursery unit and further downstream.

Materials and methods

Ethical statement

This study was carried out in strict accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union. All experimental procedures were conducted in accordance with the recommendations given by the National Veterinary Institute of Denmark.

Selection of target herds

All herds should fulfill the following criteria: Minimum 300 sows, production from farrowing-30 kilos, weekly production system, history of respiratory disease or laboratory confirmation of IAV, no litter equalization of the ear-tagged piglets, no vaccination against IAV in the past year and no startup of vaccination of either sows or piglets against IAV during the study period.

Screening for IAV in the target herds

Before a herd was included in the study, a screening for IAV was performed by testing nasal swabs from 5 1-week-old piglets, 5 3-week-old piglets, 10 5-week-old weaners and 10 8-week-old weaners by reverse transcription real-time PCR (RT-rtPCR).

Description of the included herds

Herd 1

This herd had approximately 900 sows and a farrowing area divided into six units with no clear sectioning between age groups. The piglets were weaned at 4 weeks of age. When the piglets reached approximately 15 kilos, they were moved to a separate stable until they were sold at 30 kilos. The herd was not included in the Danish SPF-system [20] but was declared free of porcine reproductive and respiratory syndrome virus (PRRSv). The herd had previously tested positive for H1avN2sw and had recurrent problems with respiratory disease. The herd did not use a strict all-in/all-out strategy in any of the units, and no quarantine stability was used for incoming gilts. In the herd, a high degree of litter equalization was used along with nursing sows. Stables were washed between production rounds in the farrowing unit and disinfected using calcium hydroxide.

Herd 2

This herd had approximately 900 sows and a farrowing area divided into four units with no clear sectioning between age groups. The piglets were weaned at 4 weeks of age. When the piglets reached approximately 20 kilos, they were moved to a separate stable until they were sold at 30 kilos. The herd had an SPF herd health status, indicating that the herd tested free of infections annually, including *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* serotype, 2, 6, and 12, PRRSv type 1 and 2, *Brachyspira hyodysenteriae*, *Pasteurella multocida*, *Sarcoptes Scabiei* var. *Suis* and *Haematopinus suis*. The herd had recurrent problems with respiratory disease but had never been tested for the presence of IAV. The herd did not use a strict all-in/all-out strategy in any of the units, but a quarantine stable was used for incoming gilts. In the herd, a high degree of litter equalization was used along with nursing sows. Stables were washed between batches and disinfected using calcium hydroxide.

Herd 3

This herd had approximately 450 sows and a farrowing area divided into two units with no clear sectioning between age groups. The piglets were weaned at 4 weeks of age and kept in the same grower unit until they were sold at 30 kilos. This herd was known to be IAV-positive and had recurrent clinical signs of respiratory disease. Similar to Herd 2, the herd had a blue SPF status, indicating that it was declared free of the same diseases as Herd 2. The herd only performed minimal litter smoothing and limited the use of nursing sows.

Gilts were recruited from the same herd. Stables were cleaned only once a year, without disinfectants.

Study design

This investigation was designed as an observational longitudinal cohort study in 3 Danish sow herds. In each herd, four batches of four conveniently selected sows were included with farrowing dates 1 week apart. Five piglets from each sow were randomly chosen by ear tagging of every third piglet in the litter at birth. The ear-tagged piglets were sampled with nasal swabs during weeks 1, 3, and 5 and again before being sold at approximately 30 kilos (at 10–12 weeks of age). As the piglets were not born on the same day, the actual sampling date differed up to 4 days between pigs. Furthermore, the ear-tagged pigs were blood sampled during week 3 and at approximately 30 kilos (weeks 10–12). From sows, blood was sampled 2 weeks before farrowing, and a nasal swab was taken 1 week after farrowing (Table 1). A total of 16 sows and 80 piglets were selected for sampling at weeks 1, 3, 5 and 10–12 over a total period of 4 months in each herd. Ear-tagged piglets stayed with their own mother sow until weaning. The sampling size was initially defined based on assumptions on body weight gains and production results, but these indicators were excluded from the final assessment due to inadequate quality of data from the herds.

Sampling

Nasal swabs were collected with a small or large sterile cotton swab (Medical Wire, UK) depending on the age of the animal. The swab was inserted and turned 360° in both nostrils of each pig. Afterwards, the swabs were immersed in Sigma Virocult media (Medical Wire, UK) and kept at 2–8 °C for a maximum of 2 days until RNA extraction.

Blood was sampled from *vena jugularis* of the sows and from *vena cava cranialis* of the piglets and stored in vacutainer serum tubes (Becton–Dickinson, Denmark) at 5 °C for a maximum of 2 days until they were centrifuged at 3000 rpm for 10 min, and the serum was frozen at −20° until further analysis.

Clinical observations

Each individual ear-tagged pig was examined for the presence of the following clinical signs at each sampling time: dyspnea, lacrimation, nasal discharge (s = serous, m = mucous and p = purulent), conjunctivitis, diarrhea and lameness. Additionally, the pigs had a body condition score specified ranging from 1 to 4. Every pen with an ear-tagged piglet had a coughing index (CI) calculated at every sampling time using a method based on a previous study on *Mycoplasma hyopneumoniae* [21]. The CI was calculated based on the number of coughs and sneezes over 3 min divided by the number of pigs in the pen.

Pooling of the samples and RNA extraction

The nasal swabs were pooled per litter with five samples in one pool corresponding to the five ear-tagged piglets from each sow. Four sows of each batch were also pooled. The Sigma Virocult media containing the cotton swab were vortexed and poured into a 1.5 mL tube (Eppendorf), wherefrom 100 µL was withdrawn for the pool. The pool was vortexed and centrifuged, and 200 µL was withdrawn and mixed with 400 µL RLT-buffer (QIAGEN, Copenhagen, Denmark) containing 2-mercaptoethanol (Merck, Darmstadt, Germany). The RNA was extracted from the sample using the RNeasy mini kit (QIAGEN) automated on the QIAcube (QIAGEN) according to instructions from the supplier.

Reverse transcription real-time RT-PCR

A previously published RT-rtPCR assay targeting the matrix gene of IAV [22] was used to determine if a pool was IAV positive. The OneStep RT-PCR Kit (QIAGEN) was used with the published primers. All PCRs were run on the Rotor-Gene Q (QIAGEN) using the following program: 50 °C, 30 min; 95 °C, 15 min; and cycling 45× (95 °C for 10 s, 60 °C for 20 s, 64 °C for 1 s, 68 °C for 1 s, 72 °C for 30 s). A pool was considered positive if it had a ct value < 36. If a pool tested positive, the RNA was extracted from the individual samples of the pool using the same method as described above. The RNA was then again subjected to the RT-rtPCR assay described above to determine which individual pigs were positive for IAV.

All positive individual samples with a ct value < 31 were then retested using a multiplex RT-rtPCR assay to determine the influenza A subtype. The QuantiTect Rev

Table 1 Sampling program for sows and piglets

	Two weeks before farrowing	Week 1	Week 3	Week 5	Weeks 10–12
Sows	Blood samples	Nasal swabs			
Piglets		Nasal swabs	Blood sample + nasal swabs	Nasal swabs	Blood sample + nasal swabs

Table 2 List of adjusted primers and probes used for the RT-PCR multiplex for subtyping

Assay	Primer/Probe	Sequence (5′-3′)
H1pdm	H1fw2sw-2	GAA GTT CAA GCC GGA AAT AGC A
H1av	H1-av-P	*ROX*-TCT GGT TAC GCA GCW GAT CAG AAA AGC AC-*BHQ2*
H3hu	H3-hu_mink-F	GAT GAT GGA GAA AAC TGC ACA CTA
N2sw	N2-F	GAG TAT GGT GGA CBT CAA AYA G
	N2-R	TTG CGA AAG CTT ATA TAG GCA TGA
	N2-P	*AF532*-CCA TCA GGC CAT GAG CCT GAV CCA TA-*BHQ1*
N2hu	N2hu-P	*AF532*-T[+C]A [+A]CT CYA CAT AAA AGC ACC [+G]-*BHQ1*

"F" indicates the forward primer, "R" indicates the reverse primer, and "P" indicates the probe. Letters in "[ ]" indicate a locked nucleic acid (LNA). The letters in italics indicate the reporter and quencher.

transcription kit (QIAGEN) was used with the primers and probes from a previous study [23] with a few primer adjustments, as listed in Table 2. PCR was run on the Rotor-Gene Q (QIAGEN) using the following program: 50 °C for 20 min, 95 °C for 15 min, and cycling 40× (94 °C for 60 s and 60 °C for 90 s).

Serology

All blood samples were tested for antibodies against the NP gene of IAV, which is highly conserved between subtypes, using a commercially available blocking ELISA (IDEXX; Influenza A Ab Test; IDEXX Laboratories, Inc.).

Descriptive and statistical analysis

The prevalence of influenza was determined at the litter, individual pigs and batch levels. The prevalence of the IAV in the litters of each herd was calculated based on the number of litters that were positive at each sampling time from the total number of litters present at each sampling time. For the individual pigs, the prevalence was also based on the number of individual nasal swabs testing positive for IAV subtracted from the total amount of pigs present at each sampling time. In addition, "total prevalence" was estimated based on the total number of individual pigs testing positive at minimum one sampling time during the entire study period compared to the initial number of pigs at the beginning of the study. Finally, the batch level prevalence of IAV was calculated based on the number of individuals testing positive for IAV compared to the total number of pigs included in the batch.

For each herd, a statistical analysis was performed comparing IAV-positive and IAV-negative individuals at a given age (week 1, week 3, week 5 and weeks 10–12) with the presence of one of the clinical signs registered at the individual level using the Chi-square test. This analysis was also performed on the total clinical data from all three herds to evaluate an overall association. To reveal a possible significant difference between being a litter/pen with at least one IAV-positive animal and being a negative litter/pen in relation to the coughing index, a Fisher's exact test was performed on the means of the CIs. This test was performed both herdwise and on the total data of all three herds. A *P*-value below 0.05 was considered statistically significant. The statistics and graphs were completed using GraphPad Software [24] and Microsoft Excel.

Results

IAV subtypes

At the time of screening, Herd 1 tested positive for H1avN2sw, and this subtype was also found throughout the study period in all positive pigs except one 5-week-old pig, which was infected with an H1avN1 subtype in the nursery. Herd 2 had A(H1N1)pdm09 detected at the time of screening and was later detected in both the farrowing unit and the nursery stable. H1avN2sw was identified at the time of the screening and was the only subtype circulating in Herd 3 in both the farrowing and nursery unit.

IAV—at the litter level

The results of the tests of the pooled samples, each including a single litter, indicated that the majority of the litters encountered IAV during the study period (Table 3). At week 1, each of the three herds had IAV-positive litters, with Herd 3 standing out, in that half of the tested

Table 3 Percentage of influenza A virus-positive pools from nasal swabs collected from pigs and tested by RT-PCR

	Herd 1	Herd 2	Herd 3
Positive pools			
Week 1	(4/16) 25%	(3/15) 20%	(8/16) 50%
Week 3	(8/16) 50%	(5/15) 33.3%	(6/16) 37.5%
Week 5	(3/16) 18.7%	(7/15) 46.7%	(10/16) 62.5%
Weeks 10–12	(0/16) 0%	(1/15) 6.6%	(0/16) 0%
Total	(11/16) 69%	(12/15) 80%	(14/16) 87.5%

Table 4 Percentage of influenza A virus-positive pigs from nasal swabs collected from pigs and tested by RT-PCR

	Herd 1	Herd 2	Herd 3
Week 1	17.3% (13/77[a])	9.2% (6/65[a])	34.6% (27/78[a])
Week 3	16.2% (11/68)	15.9% (10/63)	29.5% (23/78)
Week 5	4.8% (3/62)	20.3% (12/59)	36% (28/78)
Week 10–12	0% (0/61)	2% (1/45)	0% (0/76)
Total	34% (26/77)	41.5% (27/65)	69% (54/78)

[a] The total number of piglets present at the beginning of the study deviates from 80 due to mortality between birth and first sampling. Pigs that have been infected twice only count once in the total prevalence.

litters were positive. In week 3, 33–50% of the litters were positive, and at week 5, the percentage of positive litters was 19, 47 and 63% in Herds 1, 2 and 3, respectively. In contrast, only one litter in total was positive at weeks 10–12 (in Herd 2).

IAV—at the individual level

From all the positive pools, the individual samples from the five ear-tagged pigs were tested. The total % of IAV-positive individuals was estimated by summing the number of infected individuals at each sampling time (weeks 1, 3, 5 and 10–12), regardless of the batch (Table 4).

Due to mortality during the study, the total number of pigs included was less than planned and varied between herds and sampling times (Table 4).

Herd 1 showed the highest prevalence of infected individuals in the farrowing unit, with ~17% of the individuals being infected at weeks 1 and 3. After the piglets were transferred to the nursery unit, a much lower prevalence (4.8%) was observed at week 5, and none of the pigs tested positive at weeks 10–12.

Herd 2 had a relatively low prevalence of IAV at week 1, with only 9.2% of the piglets being infected; however, at week 3, the prevalence increased to 15.9% and peaked in the nursery, with 20.3% of the pigs being infected at week 5. At weeks 10–12, only one pig tested positive for IAV.

Herd 3 had a more constant but high prevalence of IAV over the first three sampling times. Approximately 30% of the individuals were infected at each sampling time, and the highest prevalence (36%) was observed at week 5 after transfer to the nursery unit. Consistent with the finding in Herd 1, all pigs were negative at weeks 10–12.

IAV—at the batch level

A difference in the time of infection was observed between the different batches, and no clear pattern was observed overall when comparing the three herds (Figure 1). However, in Herd 1 and Herd 3, the batches in which an IAV-positive sow was present, a high number

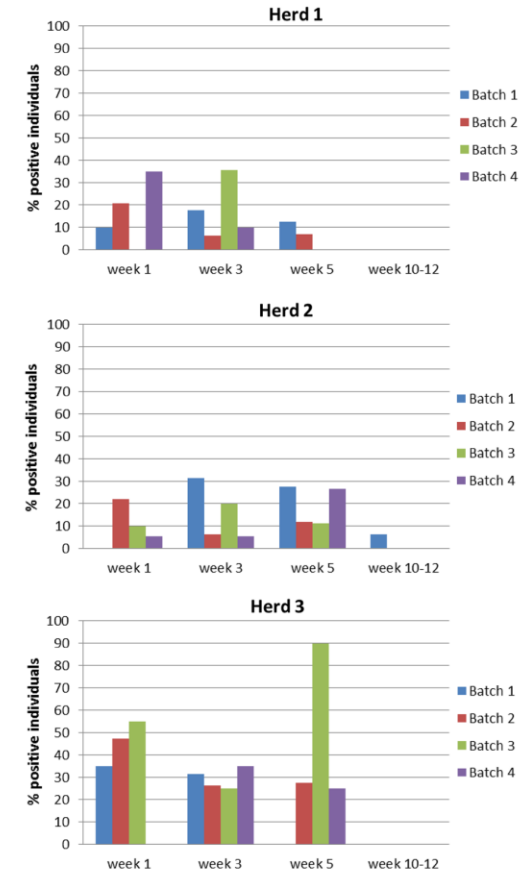

Figure 1 Percentage of IAV-positive litters in the four batches of the three herds. The columns present the percentage of positive individuals in each batch at each sampling time (week 1, week 3, week 5 and weeks 10–12) for each of the three herds.

of infected piglets at week 1 were observed (IAV results of the sows are shown below). In Herd 1, the prevalence of infected piglets at week 1 ranged between 21 and 35% in the two batches that had an IAV-positive sow (Batch 2 and Batch 4), whereas the number was even higher in Herd 3, ranging from 47 to 55% of piglets in the two batches including an IAV-positive sow (Batch 2 and Batch 3). In Herd 3, all the piglets of the two IAV-positive sows were infected at week 1, whereas this was the case for one of the IAV-positive sows in Herd 1 (Additional file 1).

Shedding period and viral load

In all herds, several pigs tested positive for IAV at two consecutive sampling times. In Herd 1, two pigs were positive for IAV in weeks 1 and 3, and in Herd 2, one pig

was positive for IAV at both week 3 and week 5. In Herd 3, a much higher prevalence was observed, with eleven pigs testing positive at two consecutive sampling times, and one pig even tested positive over three consecutive samplings (week 1 to week 5). Out of the ten remaining pigs in Herd 3, four were positive for IAV in weeks 1 and 3, while six were positive in weeks 3 and 5. The overall prevalence of infected pigs that were positive over two consecutive samplings ranged from 3.6 to 20.7% of the total number of infected pigs in each herd.

In addition to pigs that were positive at two consecutive samplings, one and 13 piglets were infected at two nonconsecutive sampling times (week 1 and week 5) in Herds 1 and 3, respectively. Detailed results are shown in Additional file 1.

The average ct value of the IAV-positive pigs in Herd 1 increased with age. In contrast, in Herds 2 and 3, the lowest average ct values were detected at week 5, which for both herds coincided with the peak of infected individuals.

IAV antibodies—ear-tagged pigs

The prevalence of antibodies at weeks 3 and 10–12 of the ear-tagged pigs is shown in Figure 2. Herds 1 and 3 showed a similar pattern, where 68–78% of the piglets were positive for IAV antibodies at week 3, whereas a significant decline was observed at weeks 10–12, where only 9–24% of the piglets were positive. Herd 2 showed a different pattern, with 31–36% of the pigs IAV antibody positive at both sampling times. No clear relationship between virus positivity and serological status was detected, as both piglets originating from antibody-positive sows and pigs from antibody-negative sows became infected during the first 5 weeks after farrowing (Additional file 1).

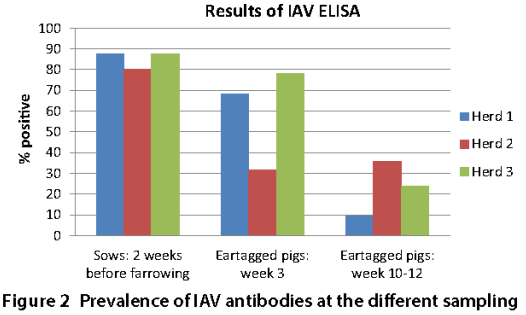

Figure 2 Prevalence of IAV antibodies at the different sampling times of both sows and piglets of the three herds. The columns present the proportion of IAV seropositive sows 2 weeks before farrowing and seropositive piglets at weeks 3 and 10–12.

IAV and IAV antibodies—sows

The majority (80–87.5%) of all included sows tested positive for antibodies against IAV 2 weeks before farrowing (Figure 2). In Herd 1, two sows were shedding IAV in the farrowing unit at week 1, and one of these sows was antibody negative 2 weeks before farrowing. The exact same pattern was observed in Herd 3, where two sows were also shedding IAV in the farrowing unit, and one tested negative for IAV antibodies 2 weeks before farrowing. In Herd 2, none of the tested sows were virus positive in the farrowing unit (Additional file 1).

Clinical signs

A statistically significant correlation was identified in Herd 1 between the IAV-positive litters/pens and an increased coughing index compared to the negative litters/pens (Table 5).

An additional statistically significant correlation was identified in Herd 2 between the presence of serous nasal discharge and the individual pig testing positive for IAV in the nasal swabs. This correlation was observed at week 1 and week 5 and equally when looking at the total number of infected pigs (Table 6).

As 69% of all the individual pigs of Herd 3 were positive for IAV at some point during the study (Table 4), it was not possible to obtain a significant correlation for any of

Table 5 Mean coughing index (CI) of virus-positive and -negative animals

Mean CI	Week 1	Week 3	Week 5	Total
Herd 1				
Virus positive	0.209	0.381	0.032	0.263
SD	0.21	0.28	0.04	0.26
Virus negative	0.026	0.223	0.05	0.089
SD	0.03	0.19	0.03	0.13
P-value	0.007	0.21	0.44	0.006
Herd 2				
Virus positive	0.042	0.348	0.109	0.168
SD	0.05	0.16	0.08	0.16
Virus negative	0.109	0.56	0.088	0.234
SD	0.12	0.53	0.05	0.36
P-value	0.40	0.41	0.48	0.48
Herd 3				
Virus positive	0.065	0.186	0.083	0.108
SD	0.04	0.09	0.03	0.08
Virus negative	0.090	0.239	0.106	0.166
SD	0.07	0.26	0.05	0.2
P-value	0.40	0.64	0.45	0.26

The results were considered significant at $P < 0.05$. "SD" is the standard deviation.

Table 6 Prevalence of nasal discharge of virus-positive and -negative animals

	Week 1	Week 3	Week 5	Total
Herd 1				
Virus positive	23% (3/13)	30.1% (4/13)	0% (0/3)	36.8% (7/19)
Virus negative	12.5% (8/64)	47.3% (26/55)	50.8% (30/59)	35.9% (64/178)
P-value	0.58	0.43	0.26	0.861
Herd 2				
Virus positive	100% (6/6)	70% (7/10)	83.3% (10/12)	82.1% (23/28)
Virus negative	44% (26/59)	32.7% (17/52)	34% (16/47)	37.3% (59/158)
P-value	0.03	0.062	0.006	< 0.0001
Herd 3				
Virus positive	77.8% (21/27)	91.3% (21/23)	78.6% (22/28)	82% (64/78)
Virus negative	62.7% (32/51)	81.8% (45/55)	86% (43/50)	76.9% (120/156)
P-value	0.26	0.47	0.6	0.46

The results were considered significant at $P < 0.05$.

Table 7 Accumulated results of the clinical data from all three herds (CI and nasal discharge)

Mean CI	Week 1	Week 3	Week 5	Total
Herds 1, 2 and 3				
Virus positive	0.099	0.31	0.092	0.176
SD	0.12	0.22	0.07	0.18
Virus negative	0.072	0.348	0.078	0.17
SD	0.09	0.39	0.05	0.27
P-value	0.43	0.70	0.52	0.87
Prevalence of nasal discharge				
Virus positive	65.2% (30/46)	69.6% (32/46)	74.4% (32/43)	69.6% (94/135)
Virus negative	38% (66/174)	55.3% (88/162)	57% (89/156)	49.4% (243/492)
P-value	0.002	0.093	0.059	< 0.0001

Results were considered significant at $P < 0.05$. "SD" is the standard deviation.

the clinical signs, even though several signs of respiratory disease were observed in the herd.

An overall analysis was performed to investigate whether the above mentioned associations were also apparent when accumulating the results of all three herds (Table 7). The coughing index and nasal discharge for all herds at each of the sampling times and as a total over all sampling times were calculated. A significant correlation between nasal discharge and the individual pig testing positive for IAV in the nasal swabs was observed both at week 1 and week 5 and when looking at the total number of infected pigs. However, no significant correlation was found with regard to the coughing index.

Discussion

The results of the present study revealed that IAV was clearly circulating in the farrowing unit, as well as in the start of the nursery period. The majority of the litters encountered IAV at some point during the study, and the true prevalence of IAV-infected individuals was probably higher since the pigs were not sampled every week. To our surprise, IAV was detected at a high prevalence even in piglets at 1 week of age, which to the best of our knowledge has not been described before. Overall, 98% of all the infected pigs tested positive within the first 5 weeks of life, even though more than 80% of the sows were seropositive for antibodies against IAV at farrowing. The high prevalence of seropositive piglets at week 3 (68–78%) in Herd 1 and Herd 3 indicated that the piglets did receive MDAs from the sows. Nevertheless, the results also revealed that these pigs still became infected with IAV at an early age. This can either be due to the level and specificity of the MDAs absorbed by the piglets [25], which was not tested in this study, or due to a lack of protection through MDA, which several studies have indicated [11, 26–31]. Interestingly, the piglets of Herd 2, which did not have a high rate of seropositive pigs at week 3 (31.5%), did not

show an overall higher amount of infected individuals. Moreover, Herd 2 had the highest number of seropositive pigs by the end of the study, suggesting that more pigs from this herd have elicited an active immune response to IAV and thereby may be less susceptible to reinfection. This is in accordance with the results from Loeffen et al. [30], who suggested that the presence of MDA in the piglets hindered an active immune response and that these pigs elicited a weaker immune response in response to a secondary IAV infection even with the same subtype.

The differences in the age of infection observed between the batches could be explained by the majority of the batches being in different stables, presumably with different infection pressures, different mixing of age groups and other differences in flow and management factors. Additionally, the presence and prevalence of IAV-positive sows could be a possible factor when considering batch-to-batch variation. In this study, it was observed that three out of the four positive sows also had a positive litter at week 1, which could indicate that the sows affect the transmission dynamics.

Overall, few pigs were infected at the end of the nursery period in three herds, indicating a good chance of having IAV-negative pigs at the time of transfer to the finisher section. However, at weeks 10–12, the prevalence of IAV antibody-positive animals was low, which could indicate that the majority of the pigs would not be protected against IAV reinfection in the finisher farm. Two previous studies have tested this hypothesis and tried to reinfect previously infected piglets with the same strain after the decline in MDA. In one of the studies, the piglets were found to be primed, and no reinfection was possible [29], whereas the other study showed a weakened immune response in the presence of MDA and showed that reinfection was possible in some of the pigs [30]. The decline in IAV antibodies observed at the last sampling time was in accordance with other studies that have found that MDA persists in piglets for approximately 10 weeks [11, 12, 32, 33].

Nonconsecutive shedding of IAV was found in Herd 1 and Herd 3, where piglets were shedding virus at week 1 and again at week 5, with 4 weeks in between. Other studies investigating the IAV dynamics [12, 19] also found pigs that tested positive for the same IAV subtype at nonconsecutive sampling times, which suggested that reinfection with the same virus was possible. An explanation for why we did not see more cases of reinfection could be the detection limit of the PCR assay, which would not detect pigs with a low viral load. The number of positive pigs over a minimum of two consecutive sampling times indicated that individual pigs had viral excretion for more than 2 weeks, which would suggest the presence of "prolonged IAV shedders". However, as the pigs were not sampled daily, we cannot rule out that these pigs either became reinfected with IAV between the two sampling times or that an environmental contamination of the sample could have occurred. More studies with daily samplings should be performed to prove the concept of prolonged IAV shedders. If we consider that the pigs were in fact true "prolonged IAV shedders", it is important to take this into consideration in the control measures for IAV, as they will increase the transmission rate. Previous studies have found prolonged shedding time to be correlated to the presence of MDA at the time of infection [26, 27, 30]. This phenomenon should be investigated in more detail because it may be an unwarranted effect of sow vaccination or immunity due to prior infections. A contributing factor to Herd 3 having a much higher prevalence of IAV may be related to the organization of the farrowing unit. Herd 3 only had two large farrowing stables, and so a division into age groups was impossible. Herd 1 and Herd 2, on the other hand, had a higher number of farrowing stables, making it possible to keep the youngest and the oldest pigs more separated, even though a clear sectioning was not possible. This underlines the importance of separation of age groups and strict all-in/all-out strategies when fighting viral pathogens such as IAV [34].

The subtypes found in the three herds represent the subtypes that are currently circulating in Denmark. The most prevalent subtype H1avN2sw was found in Herds 1 and 3, and Herd 1 also had one pig in the nursery unit that tested positive for a different subtype H1avN1av. As none of the pigs in the farrowing unit were infected with this subtype, combined with the low seroprevalence at the end of the nursery period, this different subtype poses a risk of a secondary IAV infection.

Although a descriptive study is not designed to evaluate any associations, the observations regarding influenza and clinical signs were analyzed, and the clinical data showed that there was a significant correlation between being positive for IAV in nasal swabs and clinical signs of respiratory disease. In Herd 1, an increased coughing index was observed. However, this correlation was not observed when accumulating the results of all three herds. In Herd 2, a significant correlation was observed between serous nasal discharge and the presence of IAV, and this correlation was also significant when accumulating the results of all three herds. This indicated that IAV had an impact on health in these enzootically infected herds.

IAV was detected in two out of 16 sows in both Herds 1 and 3, which showed sows as a potential source of exposure of the piglets in the herds and as a possible source of new IAV introductions into the farrowing unit as previously proposed [11, 35]. All piglets from three of the four IAV shedding sows tested positive for IAV at week 1, which could indicate that the virus was transmitted between piglets and sows. However, another explanation could be that both sows and piglets were infected by aerosols and fomites, since the infection pressure in the farrowing unit was quite high. Cases in which both sows and piglets are found positive at the same time need to be further investigated to evaluate the risk of sows transmitting influenza to piglets during the farrowing stage.

In each herd, one of the IAV-positive sows tested negative for IAV antibodies 2 weeks before farrowing, and 1 week after farrowing, these sows were shedding IAV, which indicated that the sows were most likely infected at entry into the farrowing unit, where an abundant circulation of IAV was present. These results emphasize the importance of having a clear introduction strategy of incoming gilts because gilts may either be seronegative if they originate from a non-IAV-infected herd or have antibodies against a different variant of IAV. Exposure to IAV by vaccination before insemination and before farrowing should be considered to reduce the risk of the sows being infected during pregnancy or when entering the farrowing unit. Indeed, gilts have previously been shown to be a contributing factor to IAV persistence at the herd level [18, 36]. Quarantine measures and testing of incoming gilts should be performed to avoid the introduction of new IAV strains into the herd, causing an epizootic outbreak.

All the herds included in the study were enzootically infected with IAV, and signs of clinical impacts were evident. A high infection pressure of IAV was discovered in both the farrowing unit and the start of the nursery period. Interestingly, these results indicated that most of the IAV infections occurred at an age when the piglets were considered clinically protected through MDA. Overall, 98% of all the infected piglets became infected before reaching 6 weeks of age. This indicated that MDAs might not provide optimal protection against IAV, and other control measures, such as improved external and internal biosecurity, should be considered when selecting a strategy for controlling IAV. Finally, sows should be considered highly important players in ongoing IAV transmission and as a possible source of new IAV introductions.

Additional file

Additional file 1. Overview of the antibody and virus shedding status of the sows and ear-tagged pigs at the different sampling times. The table shows the four different batches of sows and their respective piglets at the different sampling times. The numbers indicate the ear tag number of the piglets, while the sows are numbered from 1–16. Italic letters indicate that the pig was not blood sampled, bold letters indicate an IAV antibody seropositive ear-tagged pig or sow, and normal letters indicate an IAV antibody seronegative ear-tagged pig or sow. "+IAV" indicates the nasal swab of the individual pigs or sows that tested positive in the quantitative real time RT-PCR targeting the matrix gene of IAV. If a box is empty, it indicates that the ear-tagged pig is either dead or not sampled.

Abbreviations

Av: avian; HA: hemagglutinin; Hu: human; NA: neuraminidase; IAV: influenza A virus; MDA/MDAs: maternally derived antibody/antibodies; PCR: polymerase chain reaction; Pdm: pandemic; PPRSv: porcine respiratory and reproductive syndrome virus; PRDC: porcine respiratory disease complex; Sw: swine; swIAV: swine influenza A virus.

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Authors' contributions

PRH conceived the study, performed the analysis of the samples, interpreted the results and drafted the manuscript. IL helped with the coordination of the sampling and interpretation of the results and corrected the manuscript. CSK helped with the coordination of the sampling and interpretation of the results and corrected the manuscript. JSK helped with the interpretation of the results and corrected the manuscript. SW assisted in the overall planning of the study and corrected the manuscript. LEL planned the overall study, helped with the interpretation of the study and corrected and approved the final draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Additional file 1.

Herd 1	*Antibodies and IAV shedding*															
	Batch 1				Batch 2				Batch 3				Batch 4			
	SOW 1	**SOW 2**	**SOW 3**	**SOW 4**	**SOW 5**	**SOW 6**	**SOW 7**	SOW 8 + IAV	**SOW 9**	**SOW 10**	**SOW 11**	SOW 12	**SOW 13**	**SOW 14**	**SOW 15 + IAV**	**SOW 16**
Week 1	*11*	*20*	*6*	*2 + IAV*	*22 + IAV*	*29*	*36*	*41*	*43*	*51*	*55*	*66*	*75*	*68*	*58 + IAV*	*80 + IAV*
	14	*8*	*12*	*16 + IAV*	*25 + IAV*	*30*	*32*	*39*	*42*	*49*	*56*	*63*	*72*	*71*	*59 + IAV*	*81*
	19	*5*	*17*	*18*	*23*	*31*	*35*	*40*	*45*	*50*	*52*	*64*	*76*	*67*	*57 + IAV*	*77*
	15	*4*	*13*	*1*	*24 + IAV*	*27*	*34*	*38*	*46*	*48*	*53*		*74*	*69*	*61 + IAV*	*78 + IAV*
	3	*10*	*9*	*7*	*21 + IAV*	*28*		*37*	*44*	*47*	*54*		*73*	*70*	*60 + IAV*	*79*
Week 3	**11**	20	**6**	2	**22 + IAV**	**29**	**36**	41	43	**51**			**75**	**68**	*58*	**80**
	14	8 + IAV	**12**	16	**25**	30	**32**	39	**42 + IAV**	**49**		63 + IAV	**72**	*71*	**59**	*81*
	19	5	**17 + IAV**		**23**	31	**35**	40	**45**	**50 + IAV**	*52*	64 + IAV	*76*	*67*	*57 + IAV*	**77 + IAV**
	15	4	13		**24**	27	*34*		**46**	**48**			**74**	**69**	**61**	78
	3	10 + IAV	**9**		*21*				**44 + IAV**	**47**	*54*		*73*	*70*	**60**	79
Week 5	*11*	*20*	*6*	*2 + IAV*		*29*	*36*	*41*	*43*				*75*	*68*		*80*
	14	*8*		*16*	*25*	*30*	*32*	*39*	*42*	*49*		*63*	*72*	*71*	*59*	*81*
	19 + IAV	*5*	*17*		*23*	*31*	*35*		*45*	*50*	*52*	*64*	*76*	*67*	*57*	*77*
	15	*4*	*13*		*24*		*34*		*46*	*48*			*74*	*69*	*61*	*78*
	3	*10*	*9*		*21*	*28 + IAV*			*44*	*47*	*54*		*73*	*70*	*60*	*79*
Week 10-12	11	**20**	6	2	*22*	**29**	36	41	43				75	68		80
	14	8		16	*25*	30	32	39	42	49		63	72	71	59	81
	19	**5**	17		23	*31*	35		45	50	52	64	76	67	57	
	15	4	13		24	27	34		46	*48*			74	69	61	78
	3	10	9		21	28		37	44	47			73	70		79

Herd 2	*Antibodies and IAV shedding*															
	Batch 1				Batch 2				Batch 3				Batch 4			
	SOW 1	**SOW 2**	**SOW 3**	**SOW 4**	**SOW 5**	**SOW 6**	**SOW 7**	SOW 8	**SOW 9**	**SOW 10**	SOW 11	**SOW 12**	**SOW 13**	**SOW 14**	**SOW 15**	**SOW 16**
Week 1	*105*	*111*	*127*	*106*	*145*	*121*	*118*	*134*	*151*	*143*	*140 + IAV*		*144*	*164 + IAV*	*175*	*170*
	102	*113*	*129*	*107*	*137*	*123*	*117*	*131 + IAV*	*152*	*150*			*158*	*162*	*166*	*171*
	104	*115*	*128*	*109*	*138*	*120*	*119*	*136 + IAV*	*153*	*142*			*157*	*163*	*167*	*174*
	101	*112*	*126*	*108*	*139*	*122*	*116*	*133 + IAV*	*154*	*141*			*159*	*161*	*176*	*173*
	103	*114*	*130*		*135*			*132 + IAV*	*155*				*156*			*172*
Week 3	*105 + IAV*	*111*	**127**	106	145	**121**	118	134	151	143	140		144	**164**	175	**170**
	102 + IAV	**113**	*129*	107	*137*	**123**	117	**131**	152	150 + IAV			158	162 + IAV	**166**	171
	104 + IAV	*115 + IAV*	**128**	109		120	119	136	153	142 + IAV			157	**163**	**167**	174
	101 + IAV	112	126	108	139	**122**		133	**154**	141			159	161	*176*	**173**
	103 + IAV	**114**	*130*		135 + IAV			132	155				156			**172**
Week 5	*105*	*111*	*127 + IAV*		*145 + IAV*	*121*	*118*	*134*		*143*	*140*		*144*	*164*	*175*	
	102	*113*	*129*	*107*	*137*	*123*	*117*	*131*	*152*	*150*			*158*	*162*		*171*
	104	*115*	*128 + IAV*	*109 + IAV*	*138*	*120*	*119*	*136*	*153*	*142*			*157 + IAV*	*163 + IAV*	*167*	*174*
	101	*112*	*126 + IAV*	*108*	*139*	*122*		*133*	*154 + IAV*	*141*			*159*	*161*	*176*	*173 + IAV*
	103	*114*	*130 + IAV*		*135 + IAV*			*132*	*155*							*172 + IAV*
Week 10-12	105	111	127	**106**	**145**	121	118		151	143	140			**164**		
	102		129 + IAV	**107**	137	**123**			152					162		
	104	115	128	**109**	**138**	**120**	**119**	**136**	153	142				**163**		174
	101	112		**108**	139	**122**		133	**154**	**141**			159		176	
	103		130					132	155				156			

Herd 3	*Antibodies and IAV shedding*															
	Batch 1				Batch 2				Batch 3				Batch 4			
	SOW 1	**SOW 2**	**SOW 3**	**SOW 4**	SOW 5 + IAV	**SOW 6**	**SOW 7**	**SOW 8**	**SOW 9**	**SOW 10**	**SOW 11**	**SOW 12 + IAV**	SOW 13	**SOW 14**	**SOW 15**	**SOW 16**
Week 1	*401*		*411 + IAV*	*416*	*421 + IAV*	*426*	*431 + IAV*	*436*	*441*	*446 + IAV*	*451*	*456 + IAV*	*461*	*466*	*471*	*476*
	402 + IAV	*407*	*412 + IAV*	*417*	*422 + IAV*	*427*	*432 + IAV*	*437*	*442*	*447 + IAV*	*452*	*457 + IAV*	*462*	*467*	*472*	*477*
	403 + IAV	*408*	*413 + IAV*	*418*		*428*	*433 + IAV*	*438 + IAV*	*443*	*448 + IAV*	*453 + IAV*	*458 + IAV*	*463*	*468*	*473*	*478*
	404	*409*	*414 + IAV*	*419*	*424 + IAV*	*429*	*434*	*439*	*444*	*449 + IAV*	*454*	*459 + IAV*	*464*	*469*	*474*	*479*
	405	*410*	*415 + IAV*	*420*	*425 + IAV*	*430*	*435 + IAV*	*440*	*445*	*450 + IAV*	*455*	*460 + IAV*	*465*	*470*	*475*	*480*
Week 3	**401 + IAV**		**411**	**416**	421	**426**	431	436 + IAV	441	**446**	**451 + IAV**	**456**	461	**466**	**471 + IAV**	476
	402 + IAV	**407**	412	**417**	422	**427**	**432**	**437 + IAV**	442	**447**	**452 + IAV**	**457**	**462**	**467 + IAV**	**472 + IAV**	477
	403 + IAV	**408**	**413**	*418*		**428**	**433**	**438 + IAV**	443	**448**	**453 + IAV**	**458**	463	**468 + IAV**	**473 + IAV**	478
	404 + IAV	**409**	**414**	**419**	424	**429**	434	439 + IAV	444	**449**	**454 + IAV**	**459**	**464**	**469**	**474 + IAV**	479
	405 + IAV	**410 + IAV**	**415**	**420**	**425**	**430**	**435**	440 + IAV	445	**450**	**455 + IAV**	**460**	465	**470 + IAV**	475	480
Week 5	*401*		*411*	*416*	*421 + IAV*	*426*	*431*	*436 + IAV*	*441 + IAV*	*446 + IAV*	*451*	*456 + IAV*	*461*	*466*	*471 + IAV*	*476*
	402	*407*	*412*	*417*	*422 + IAV*	*427 + IAV*	*432*	*437*	*442 + IAV*	*447 + IAV*	*452 + IAV*	*457 + IAV*	*462*	*467*	*472 + IAV*	*477*
	403	*408*	*413*	*418*		*428*	*433*	*438*	*443 + IAV*	*448 + IAV*	*453 + IAV*	*458 + IAV*	*463*	*468*	*473 + IAV*	*478*
	404	*409*	*414*	*419*	*424*	*429*	*434 + IAV*	*439*	*444 + IAV*	*449 + IAV*	*454 + IAV*	*459 + IAV*	*464*	*469*	*474 + IAV*	*479*
	405	*410*	*415*	*420*	*425*	*430*	*435*		*445 + IAV*	*450 + IAV*	*455*	*460 + IAV*	*465*	*470*	*475*	*480 + IAV*
Week 10-12	**401**		**411**	416	**421**	426	431	**436**	441	446	451	456	**461**	466	471	476
	402	**407**	412	417	**422**	427	432	437	442	447	452	457	462	467	472	477
		408	**413**	418		428	433	438	443	448	453	458	463	468	473	478
	404	**409**	**414**	**419**	424	429	434	*439*	444	449	454	459	**464**	469	474	479
	405	**410**	415	**420**	**425**	430	435		445	450	455	460	465	470	475	**480**

Manuscript 2

Limited impact of influenza A virus vaccination of piglets in an enzootic infected sow herd

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Abstract

Recent studies have questioned the effect of maternal derived antibodies (MDAs) to protect piglets against infection with influenza A virus (IAV). Lack of protection by MDAs has encouraged the use of alternative vaccination strategies targeting young piglets in an attempt to stimulate an early antibody response. There is a lack of studies documenting the efficacy of piglet vaccination. In the present study, we monitored a group of vaccinated and non-vaccinated piglets in a Danish sow herd that initiated piglet vaccination with ¼ dose of an inactivated swine influenza vaccine at the time of castration.

A total of 160 piglets from 11 sows were included and either vaccinated with 0.5 ml inactivated swine influenza vaccine or sham-vaccinated. From week 0 until week 6, all included piglets were clinically examined and nasal swapped once per week and weighed at weeks 0, 3 and 6. Blood samples were collected from sows at week 0 and from piglets at week 3.

Vaccination of piglets had limited effect on clinical signs, body weight, antibody development and viral shedding, within the first 6 weeks of life. At least 50 % of all pigs of each treatment group tested positive for IAV at week 2, and very early onset of IAV shedding was observed. In total, 18 pigs were IAV positive in nasal swabs for more than one consecutive sampling time indicating prolonged shedding and 14 pigs were IAV positive with negative samplings in between indicating re-infection with the same IAV strain.

Keywords influenza A virus, swine influenza, piglet vaccination, influenza vaccination, recurrent shedding, reinfection

Introduction

Influenza A virus (IAV) infection in pigs can lead to clinical signs of respiratory disease and compromised animal welfare, increased use of antibiotics and negative impact on the productivity (Fablet et al., 2012; Opriessnig et al., 2011; Vincent et al., 2014). Furthermore IAV is a zoonotic disease, which leaves swine herds as a reservoir for possible future human IAV pandemics (Garten, 2009; Torremorell et al., 2012). These factors emphasize the need for an optimal control strategy to minimize the number of IAV positive pigs and herds. Recent studies documented changes in the IAV dynamics, and it is now clear that epizootic infections, in most cases, will result in the herd being enzootically infected (Cador et al., 2017; Cappuccio et al., 2017; Diaz et al., 2017b; Ferreira et al., 2017a; Loeffen et al., 2009; Pitzer et al., 2016; Rose et al., 2013; Simon-Grifé et al., 2012). Several studies investigated the effects and benefits of maternally derived antibodies (MDAs) in protection

against IAV infection in the piglets, and these studies showed that MDAs do not provide full protection against IAV infection and clinical signs (Allerson et al., 2014; Cador et al., 2016c, 2016b; Corzo et al., 2014; Diaz et al., 2017a; Markowska-Daniel et al., 2011; Rose et al., 2013; Simon-Grifé et al., 2012). Moreover, presence of MDAs at the time of primary infection may impair not only development of protective antibodies but also the memory response, making the piglet susceptible for a reinfection even with the same strain (Cador et al., 2016a; Deblanc et al., 2018; Loeffen et al., 2003). A previous study revealed that IAV infection can occur from three days of age despite the presence of MDA and IAV is highly prevalent in both the farrowing unit and in nursery pigs early after weaning (Corzo et al., 2014; Ferreira et al., 2017b; Rose et al., 2013; Ryt-Hansen et al., 2019; Simon-Grifé et al., 2012). Currently, there is no vaccine licensed in Europe for use in pigs below 8 weeks of age. Thus, a different approach to control IAV in young piglets e.g. by vaccination is needed. The aim of the present study was therefore to monitor clinical signs, weight, antibody development and viral shedding in piglets in a Danish herd that vaccinated piglets with a ¼ dose (0.5 ml) of an inactivated swine influenza vaccine (Respiporc FLU3) per piglet at the time of castration.

Materials and methods

The herd

This case took place in a Danish herd with 900 sows and 3500 pen places for nursery pigs. The herd was according to the Danish Specific Pathogen Free program (Svineproduktion, n.d.) declared free from Porcine Reproductive & Respiratory Syndrome Porcine virus (PRRSv). No strict all in/all out strategy was performed in any of the units, and stables were only washed and disinfected with hydrated lime between the different sows in the farrowing unit. No quarantine stable was present for incoming gilts. In the farrowing unit, a high number of nursing sows were used and the piglets were mingled more than once. No vaccination against IAV had been used previously in the herd. All piglets were vaccinated against *Mycoplasma hyopneumoniae* at day 4. Recurrent problems with respiratory disease were seen and the herd had previously tested positive for influenza A virus (subtype H1avN2).

Study design

A total of 160 piglets from 11 sows that farrowed on the same day were selected for the study. To assure colostrum intake, no litter equalization was allowed before castration at day 4, where all piglets were ear tagged with consecutive numbers. All movements were recorded. Pigs with an odd ear tag number were injected intramuscularly in the neck with 0.5ml Respiporc FLU3 (VAC),

corresponding to a quarter of a dose and piglets with an even ear tag number were injected with 0.5 ml of an isotonic Sodium Chloride solution, 9 mg/ml (control).

Data sampling

Clinical examination of all ear tagged pigs was performed weekly and included registration of nasal discharge, conjunctivitis, lacrimation and fecal soiling. In addition, the pigs were evaluated for body score and "other diseases" including clinical signs of lameness, wounds, eczema, umbilical hernia and CNS symptoms. Rectal temperature was measured at the time of vaccination and one-day post vaccination on all ear tagged pigs.

Weighing of all pigs was performed at weeks 0, 3 and 6 on a piglet scale approved for pigs weighing between 200 g and 30 kilos (Bjerringbro Vægte Aps, Denmark).

Blood samples were drawn from *vena cava cranialis* of all ear tagged pigs at week 3 and from *vena jugularis* of the sows at week 0. All blood samples were collected in a vacutainer serum tube (Becton Dickinson, Denmark). The blood samples were kept at 5°C for a maximum of two days, until they were centrifuged at 3000rpm for 10 minutes and the resulting serum was immediately analyzed.

Nasal swabs were collected from all piglets at the time of vaccination (week 0) and weekly thereafter until week 6. The swab, a small rayon swab (Medical Wire, UK), was introduced in both nostrils where it was turned 360 degrees. Afterwards, the swab was transferred into the Sigma Virocult media (Medical Wire, UK), and kept at 2-8°C for a maximum of two days.

Laboratory analyses

Serology

The blood samples from sows and pigs were tested in a blocking ELISA that detects antibodies targeting the NP gene, which is conserved among the different Influenza A subtypes (IDEXX; Influenza A Ab Test; IDEXX Laboratories, Inc.)

Pooling of samples and RNA extraction:

The Sigma Virocult media (MWE, England) containing the swab was whirled using a Vortexer and poured into a 1.5 ml tube (Eppendorf), wherefrom 100µL were withdrawn for the pool. Five nasal swabs representing five pigs were pooled in same tube. Vaccinated and control animals were pooled in separate tubes. The pool was mixed and centrifuged and subsequently 200µL was withdrawn and transferred to a tube containing 400µL RLT-buffer (QIAGEN, Copenhagen, Denmark) containing 2-

Mercaptoethanol (Merck, Darmstadt, Germany). The RNA was extracted from the sample using the RNeasy mini kit (QIAGEN,) automated on the QIAcube (Qiagen, Hilden, Germany) using the large sample protocol version 2.

Real time RT-PCRs

A previously published quantitative real time RT-PCR assay targeting the matrix gene of all IAVs (Nagy et al., 2010) was utilized to determine if a pool was positive for influenza A virus. Briefly, the OneStep RT-PCR kit (QIAGEN) was used with the published primers, and all PCR reactions were run on the Rotor-Gene Q (QIAGEN) using the following program: 50 °C, 30 min; 95 °C, 15 min; cycling 45x (95 °C, 10 secs, 60 °C 20 secs, 64 °C 1 sec, 68 °C 1 sec, 72 °C 30 secs). All PCR reactions were run in duplicates, and the sample was only considered positive if both duplicates gave a Ct value. A pool was considered positive if it had a Ct value <36. If a pool tested positive, the RNA was extracted from the individual samples of the pool, using the same method as described above. The RNA was then subjected to the same quantitative real time RT-PCR assay as described above to determine which of the individual pigs that was positive for IAV. All positive individual samples with a Ct value <31 were then tested in a multiplex real time RT-PCR assay for the determination of the Influenza A subtype as previously described (Ryt-Hansen et al., 2019).

Viral isolation and NGS

One random nasal swab, with a low Ct value, was selected for viral isolation in MDCK cells. The MDCK cells were incubated at 37°C in a 5% CO2 atmosphere in a viral growth media containing Minimum Essential Medium Eagle (MEM) (Gibco, Carlsbad, CA, USA), 5% inactivated fetal calf serum (FCS), Non-essential amino acids (NEAA), 2 mM L-glutamine and Penicillin-streptomycin. The nasal swab was subjected to sterile filtration using a 0.45µM Millex-HP Millipore filter (Merck, Germany), and 200µL was then used for inoculation of cells along with media containing MEM, NEAA, 2 mM L-glutamine, Penicillin-streptomycin and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated Trypsin. After 24 hours, the supernatant was harvested. The RNA was extracted from the supernatant as described earlier, and the RNA used as a template for a modified version of the one-tube PCR protocol as previous described (Kai Lee, 2016). In short, the primers MBTuni-12 and MBT-uni13 were used together with the SuperScript III RT/Platinum Taq High Fidelity kit (Invitrogen, Denmark), and run on the T3 termocycler (Biometra, Denmark) with the following conditions: 42 °C, 60 min, 94 °C, 2 min, 5x (94 °C, 30 sec – 45 °C, 30 sec - 68 °C, 180 sec), 31x (94 °C, 30 sec – 57 °C, 30 sec – 68 °C, 180 sec) and 68°C, 7 min. The PCR products were visualized with UV-light on a 0.8% agarose E-gel (Thermo Fisher Scientific) and then purified with

the High Pure PCR Product Purification Kit (Roche, Denmark). Thereafter, the sample was sent for whole genome sequencing on the Illumina MiSeq platform at Statens Serum Institut (Copenhagen, Denmark).

Conventional PCR and Sanger's sequencing

Samples from pigs either testing positive for IAV for more than two consecutive sampling times, and samples from pigs testing positive for IAV at non-consecutive sampling times with minimum two negative sampling times in between, were chosen for Sanger's sequencing of the hemagglutinin (HA) and neuraminidase (NA) genes. The primers listed in Table 1 were used for the PCR along with the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit (Thermofisher Scientific, Copenhagen, Denmark). The PCR was run on the T3 thermocycler (Biometra, Fredensborg, Denmark) with the following program for the HA gene: 55°C for 30 min, 94 °C for 2 min, 4x (94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 180 sec), 41x (94 °C for 30 sec and 68 °C for 210 sec) and 68°C for 10 min and the following program for the NA gene: 54 °C for 30 min, 94 °C for 2 min, 4x (94 °C for 30 sec, 58 °C for 30 sec and 68 °C for 180 sec), 41x (94 °C for 30 sec and 68 °C for 210 sec) and 68°C for 10 min. The PCR product was thereafter visualized on a gel and purified, as described above for the NGS sequencing. The purified PCR products were sent for Sanger's sequencing with the PCR primers at LGC Genomics (Berlin, Germany).

Generation of consensus sequences

The whole genome sequencing data was imported into the program CLC genomics Workbench version 11.0.1. The reads were paired and trimmed, and then mapped against a set of 22 reference segments, covering all IAV segments known to circulate in Denmark. The mapping result with the highest number of reads and the correct consensus length was used to extract a consensus sequence for each of the eight genome segments. The HA consensus sequence was translated into amino acids. The Sanger's sequencing data was imported into CLC main workbench version 8, and were assembled against reference sequences of the HA and NA genes. The reference sequences used were sequences with the highest scoring sequence identity to the forward and reverse reads using the function "BLAST against NCBI". The sequences where then manually proofread and the consensus sequence of each sample was extracted and translated to amino acids.

Analysis of the consensus sequences

To confirm the subtype(s) of the IAV strain circulating in the herd, all HA and NA genes were aligned with contemporary HA and NA sequences obtained in the Danish annual swine IAV

surveillance and a neighbor-joining tree was constructed. Thereafter, all HA and NA nucleotide and amino acid consensus sequences were aligned using the MUSCLE algorithm (C. Edgar, 2013) and compared using the "pairwise comparison" tool in CLC main workbench version 8. A similar, alignment and comparison was also performed for both the HA and NA consensus sequences of the herd strain against the respective vaccine consensus HA and NA sequence. The HA of A/swine/Haselünne_IDT_2617_2003 (H1N1) with accession number: GQ161124 (nucleotides) and ACR39185 (amino acids) was used for the HA alignment and the NA of A/swine/Bakum/IDT1769/2003 (H3N2) with accession number GQ161100 (nucleotides) and ACR39300 (amino acids) was used for the NA alignment. To investigate if amino acid differences between the herd strains and the vaccine strain were in any of the known antigenic sites of the HA protein (Caton et al., 1982; Manicassamy et al., 2010; Rudneva et al., 2012; Yang et al., 2012), the location of the sites were annotated to each amino acid sequence in the alignment. Finally, to reveal any sequence differences between the HA sequence obtained from the same pig at different sampling times (prolonged or recurrent shedders) the HA consensus sequences of the same pig were individually aligned and compared as described above, and the same antigenic sites were annotated to the amino acid sequences. The consensus sequences of each internal gene (M, NS, NP, PA, PB1 and PB2) was investigated for the closest sequence identity in GenBank using the function "BLAST against NCBI" to determine if they were of avian of pandemic (A(H1N1)pdm09) origin. In addition, to confirm the BLAST results, all the internal genes were separately aligned with contemporary internal gene sequences obtained in the Danish swine IAV surveillance program and a neighbor-joining tree was constructed.

Statistical analyses

Sample size calculations were based on an average weight at weaning of 6.0 kg with a standard devia tion of 1.0 kg. With a significance of 5 %, a power of 80 %, the required samples size of a two-sided test, was 63 piglets per group to prove a difference in weight at weaning of 0.5 kg statistically signifi cant (Houe et al., 2004).

For comparison of clinical signs (lacrimation, nasal discharge or conjunctivitis) between VAC and control pigs a Pearson's Chi-squared Test was performed.

For an overall statistical comparison of means from normally distributed data (e.g. mean weight between VAC and control pigs), a Student's t-Test was performed. In addition, the pigs were divided into two groups based on the average Ct value of all positive nasal swabs during the entire study period. Pigs with a Ct value higher than the average Ct value were defined as "low infection level", and pigs with a lower or equal Ct value than the average were defined as "high infection level".

Since the two treatment groups were equally distributed according to infection level the analysis of "low infection level"/"high infection level" were done without including vaccination status. The relationship between infection level (low/high) and the mean weight were analyzed with a Student's t-Test and clinical signs with a Pearson's Chi-squared Test. Furthermore, the impact of transfer of pigs between sows on clinical signs, infection level and weight were analyzed with Pearson's Chi-squared Test and a Student's t-Test, respectively. The antibody status of the sows (positive/negative) at week 0 was tested for correlation to the number of virus positive and negative piglets at the different sampling times, using a Pearson's Chi-squared Test.
Each time point (0-6) was analyzed both separately and in total. Statistical analyses were computed in R version 3.5.1 (R Core Team 2018).

Results

A total of 160 piglets from 11 sows were included in the study at week 0, of which 80 received vaccination with Repsiporc FLU3 and 80 were sham vaccinated with Sodium Chloride solution, 9 mg/ml (control). Between week 0 and week 2, 52 piglets died due to diarrhea. By the end of the study, 102 piglets were still alive (Table 2).

Clinical signs

One day after vaccination, none of the pigs experienced pyrexia. The mean rectal temperature was 38.8°C (sd=0.6) in the vaccinated group and 38.8 (sd=0.6) in the control group.

Results of the weekly clinical examinations of the pigs in both treatments groups independently of their IAV status are shown in Table 2. When considering the weekly clinical signs of the pigs in both treatments groups, both positive and negative correlations were discovered, and no significant difference was consistent over more than one sampling time (Table 2).

The total number of pigs showing any of the clinical signs over the whole study period (sum of all weeks) was compared to the treatment group, stable unit, IAV presence and level, body condition score, transfer between sows and antibody status of the sows. No significant difference was observed in any of the clinical signs between the treatment groups. However, a strong significant correlation ($p<0.001$) was found between the presence of nasal discharge and the presence of IAV in the nasal swabs, independently of the treatment group. The presence of at least one of the three clinical signs of respiratory disease; nasal discharge, conjunctivitis or lacrimation also had a significant correlation to the presence of IAV independently of the treatment group ($p = <0.05$). A slightly weaker and non-significant correlation ($p = 0.078$) between conjunctivitis and the presence of IAV was also found,

independently of the treatment group. No correlation between lacrimation and the presence of IAV was found, and no correlation was found between the infection level (high or low) and the clinical signs. Interestingly, the transfer of pigs between sows had both a negative impact of the body condition score, fecal soiling and increased the number of pigs showing one of the three clinical signs correlated to respiratory disease (Supplementary table 1).

The summed number of each of the clinical signs over the whole study period of the virus positive pigs exclusively are listed in Table 3. No significant difference was found when comparing the degree of each of the clinical signs between the virus positive vaccinated pigs and the virus positive control pigs.

Body weight

The mean body weight of the vaccinated group were1.34kg (sd = 0.4) at week 0, 5.19kg (sd = 1.4) at week 3 and 8.25kg (sd = 1.9) at week 6, whereas the mean weight of the control group were 1.32kg (sd = 0.4) at week 0, 5.10kg (sd = 1.5) at week 3 and 7.83kg (sd = 2.4) at week 6 (Figure 1). No significant differences in average body weights were observed between the vaccinated pigs and control pigs at any of the three sampling times. However, a significant lower bodyweight was revealed in the IAV positive pigs at week 6 compared to IAV negative pigs, which weighed 1.7 kg more. Furthermore, the infection level (defined as higher or lower than the average Ct value (29.2)) also had a significant impact on the body weight at week 6, as the pigs with a "high infection level" weighed 1.47 kg less than the pigs with "low infection level" (Supplementary table 2).

Serology

The blood samples obtained from the sows at week 0 revealed that the majority of the sows (9/11) were positive for antibodies against IAV.

There was no significant difference in the percentage of seropositive piglets at week 3 between the two treatment groups in that 80 % of the controls and 73 % of the vaccinated pigs were antibody positive and the average OD-ratio did not differ significantly between the two treatment groups (control: 0.48 and vaccinated: 0.49) (data not shown). Approx. 80 % of the piglets from both treatment groups were seropositive at week 3, revealing no significant differences ($p > 0.05$) in the number of seropositive piglets between the two groups.

Prevalence of IAV

The percentage of piglets testing positive for IAV in nasal swabs over the study period are shown in Figure 2. Already at the time of castration (day three to four), eight piglets tested positive for IAV in nasal swabs. The prevalence of virus positive piglets then increased markedly and at week 2 approx. 50 % of all pigs of both groups tested positive for IAV in the nasal swabs. At week 3, a decrease in the prevalence was observed and then the prevalence increased again after weaning (week 4), where most of the pigs were mixed in the nursery stables. The only significant difference in the number of IAV positive pigs between the two groups was observed at the first sampling in the nursery unit (W4N4) where a significant higher number of IAV positive piglets were present in the control group compared to the vaccinated group. The results revealed that all but one pig, which survived throughout the study period tested positive for IAV at some point during the study, resulting in no overall difference in the total number of infected pigs between the two treatment groups (Supplementary table 3).

The percentage of virus positive pigs originating from either an antibody positive or negative sow is shown in Figure 3. Although the number of seronegative sows was very low (2/11 sows), there was a significant higher number of pigs from antibody negative sows that became infected during the first three sampling times at weeks 0, 1 and 2 compared to piglets from the seropositive sows. In contrast, the prevalence of positive piglets was the same at weeks 3-5 irrespectively of the antibody status of the sow, but, interestingly, significantly more pigs from the antibody positive sows were infected at week 6. However, no significant difference was observed in the total percentage of IAV positive piglets between the two treatment groups.

Duration of shedding time and virus subtype:

In total, seven pigs from the vaccinated group and eleven pigs from the control group tested positive for IAV in the nasal swabs over three consecutive sampling times and one piglet from the control group tested positive for IAV over four consecutive sampling points. We defined these pigs as “prolonged shedders”. Furthermore, 14 pigs tested positive for IAV at nonconsecutive sampling points separated by two or three sampling times. This number included eleven nonconsecutive shedders from the vaccinated group and three from the control group (Supplementary table 3). We defined these pigs as “recurrent shedders”.

All samples of the study with a Ct value <31 (n=45) were subtyped by a multiplex RT-real time PCR and revealed the herd subtype to be of H1avN2sw origin (data not shown).

Level of virus in nasal swabs:

Figure 4 list the viral load (estimated as average Ct values) in the nasal swabs of the positive pigs from both treatment groups during the study period. The only significant difference in viral load was in the pigs in the nursery unit at week 4. Unexpectedly, the vaccinated pigs had a higher level of viral shedding compared to unvaccinated controls.

The range in average Ct values of the two treatment groups was between 26.3 and 33.9 (Table 2). The average Ct value based on all the results of all piglets at all sampling times was 29.2, and this value was, as previously mentioned, used as a cut off for defining the pigs as having a "high infection level" or a "low infection level". When using this definition of infection level it was evident that there was no significant difference between the numbers of pigs with high and low infection level according to vaccination status (Table 2).

Genetic characterization of the herd IAV strain

Full genome sequences of all eight segments of the herd strain were obtained. The results revealed that the sample was of the H1avN2sw subtype, which agreed with the results of the multiplex RT-real time PCR. Moreover, the results of the BLAST revealed that the M, NP, PA, PB1 and PB2 genes were of the pandemic A(H1N1)pdm09 origin, whereas the NS gene was of avian origin. The full genome sequences from this study are available in the NCBI Genbank with the following accession numbers: MN249749-MN249756. The Sangers sequencing results derived from a total of 21 samples confirmed the HA and NA subtype and thereby documented that only one strain was circulating in the herd throughout the study period. The HA sequence identity of the viruses from the herd ranged between 98.7-100% at nucleotide level and 97.9-100% at amino acid level, and the NA sequence identity ranged between 99.2-100% at nucleotide level and 99-100% on the amino acid level.

Sequence identity of the herd IAV strain to the vaccine strain:

Pairwise comparison of the HA sequences of the herd strain and the HA sequence of the vaccine strain (Haselünne_IDT_2617_2003 (H1N1)) revealed a 89.5-90.2% identity on nucleotide level and 90.2-91.4% on amino acid level corresponding to 48-55 amino acid differences. Eight of these amino acid differences were found in antigenic sites (Sa, Sb, Cb, Ca1 and Ca2) (Caton et al., 1982; Manicassamy et al., 2010; Rudneva et al., 2012; Yang et al., 2012) and included the following differences compared to the vaccine strain; V90A, D91N, N142H, N159K, K170G, G172K, N173G and G239E. The position of amino acid changes were numbered according to the first Methionine (H1 numbering). The pairwise comparison of the NA sequences to NA sequence of the vaccine strain

(Bakum/IDT1769/2003 (H3N2)) revealed 88% sequence identity on the nucleotide level and 89 % on the amino acid level.

Sequence identity among IAV strains isolated from the prolonged- and the recurrent shedders

As mentioned earlier, 18 pigs tested positive for IAV over a minimum of three sampling times, which we defined as "prolonged shedders", and 14 pigs in total tested positive for IAV twice separated with two to three sampling times, which we defined as "recurrent shedders". Consensus sequences of the HA gene from at least two different sampling times were successfully obtained from four recurrent shedders (N78, V81, V85 and V89) and from five prolonged shedders (N36, N38, V45, V57 and N136). For the prolonged shedders no or few (≤5) nucleotide changes were observed between sequences obtained from the same pig at different sampling times, and only in two of the pigs, the nucleotide difference resulted in amino acid changes (I387S, V389I for pig N38 and K40N for pig N136). Pig V45 was categorized as a "prolonged shedders" but - despite it only tested negative in week 5 – the piglet also behaved as a "recurrent shedder" as it first tested positive for IAV at week 2, 3 and 4, and then tested negative at week 5 and positive again at week 6 with a relatively low Ct value. Sequencing of samples from pig V45 revealed that at weeks 2 and 3 the HA gene was 100% identical, whereas at week 6, 12 nucleotide mutations had occurred, resulting in seven amino acid changes. The HA gene was sequenced from five pigs including V45 with recurrent infections. All recurrent shedders showed between one and 17 nucleotide substitutions between samplings, and in all pigs at least one of the nucleotide changes resulted in amino acid changes between samplings. Of the five pigs with recurrent infection, identical amino acid changes were shared at position 159, 235, 331, 387 and 389 in that at least two of the pigs showed one of the stated mutations. Interestingly, three of these five mutations were located in HA1 which encodes the globular head of the HA protein (Steinhauer and Skehel, 2002), which is the main target for neutralizing antibodies. In addition, one of these positions (159) was located in the antigenic site Ca2, and the same mutation K159R occurred in two different pigs (N78 and V81). The HA and NA sequences of this study are available in the NCBI Genbank with the following accession numbers: MN263260-MN263291.

Discussion

No adverse effects of vaccination were encountered during the study indicating that vaccination of very young piglets is safe despite of it being "off label" use of the vaccine. The impact of vaccination of piglets was, however, vague. Significant differences in clinical signs between the vaccinated and the control group was observed, when analyzing each sampling time separately, however, the

difference was not always to the benefit of the vaccinated pigs and they were not consistent over more sampling times. In addition, when investigating the clinical signs of only the virus positive pigs of the two treatment groups no evidence of clinical protection was identified in the vaccinated group. Furthermore, the body weights at weeks 0, 3 and 6 were not significantly different between the two treatment groups, which suggested that vaccination of very young piglets did not results in a higher average daily weight gain. This was further emphasized by the fact that no difference in the prevalence of pigs with a poor body condition score between the vaccinated and the control pigs was observed.

Additional interesting observations were made in relation to this study. For example, the transfer of the piglets between different sows or pens were shown to have a negative impact on the body weight, the body score, fecal soiling and it resulted in more pigs with one or more clinical signs of respiratory disease independent of IAV vaccination. This underlines that transfer of pigs between pens presents a major risk factor for comprised health and might not have the desired effect in weight gain either.

The overall prevalence of IAV in both the vaccinated and the control group was very high and there were no overall differences between the two treatment groups. The only significant difference in the number of pigs testing positive for IAV was observed at week 4 among the weaned pigs in the nursery unit. At this time point, a significant higher number of IAV positive pigs were identified in the control group compared to the vaccinated group. This suggested that vaccination of the piglets during week 1 decreased the number of susceptible pigs after weaning. However, the number of pigs present in the nursery at week 4 was limited, and the difference in susceptibility was not sustained in the following weeks and, as mentioned above, the overall prevalence did no vary between the two groups. In addition, the IAV positive vaccinated pigs present in the nursery at week 4 had a significantly lower average Ct value, indicating that the vaccinated pigs shed more virus. Therefore, the impact of difference observed at week 4 is inconclusive. It could be argued that the study design itself made it difficult to observe any benefit of vaccination, as vaccinated and control pigs were present in the same pen and in the same stable. However, the advantages of the chosen study design were the presence of natural transmission dynamics of IAV in the pens and a no bias in relations to the sows.

The clinical observations and examination of IAV in nasal swabs confirmed that the presence of IAV was correlated to clinical signs of respiratory disease. Especially nasal discharge was strongly correlated with detection of IAV in the nasal swab, which confirms the result of a previous study

investigating the impacts of IAV in the farrowing and nursery units (Ryt-Hansen et al., 2019). In addition, the presence of IAV at week 6 was also correlated with a lower weight. Together, these results emphasize that IAV indeed have a clinical and economic impact in swine herds as described in other studies (Brown et al., 1993; Er et al., 2014; Ferrari et al., 2009; Loeffen et al., 2009; Ryt-Hansen et al., 2019; Van Reeth et al., 1996).

The results from the antibody ELISA test of the piglets at week 3 indicated that vaccination of piglets during week 1 did not increase the prevalence of seropositive piglets in week 3 compared to unvaccinated pen-mates in that approx. 20% of the piglets from both groups were seronegative in week 3. This could be due to the vaccination procedure used in this herd. According to the Summary of Product Characteristics (SPC) for Respiporc FLU3 the dose per pig is 2 ml administered twice. The choice to use only 0.5 ml once was made by the veterinary practitioner to reduce the price and risk of side effect when vaccinating 4 day old piglets. Another explanation for the lack of seroconversion could be that the piglet's immune system was not able to respond to this vaccination at such an early age. Nevertheless, previous studies on piglet vaccination against PCV2 at day 5 and *Mycoplasma hyopneumoniae* at one week of age (O'Neill et al., 2011; Reynolds et al., 2009; Wilson et al., 2013) suggested that the immune system is indeed able to respond efficiently to vaccination. Another more likely explanation is that the presence of maternal derived antibodies interfered with the vaccination (Loeffen et al., 2003; Renshaw, 1975), and hindered an active immune response in the piglet. However, lack of seroconversion was also seen in piglets from seronegative sows and the number of infected and vaccinated pigs from seropositive- and seronegative sows did not differ. Since no samples were taken from the colostrum or from newborn pigs, the possibility that the piglets did receive MDA despite the seronegative status of the sow cannot be confirmed.

The most likely explanation for the lack of effect of vaccination in the present herd was the fact that the piglets were infected very early in life – before a response to vaccination could be anticipated. Piglets at only four days of age became infected and the peak of infection was observed already at week 2. These results confirm the results of a previous study (Ryt-Hansen et al., 2019) and emphasize that in some herds it is extremely challenging to stimulate an antibody response before the pigs are naturally infected. Moreover, the low dosing regimen used, the inhibitory effect of MDA and strain diversity may also have an impact on the efficacy of the vaccine. Indeed, the level of homology between the vaccine strain and the circulating herd strain should be taken into consideration when evaluating the effect of vaccination. The HA of the circulating strain of the herd and the HA of the vaccine strain were of the same avian lineage, but shared only 90.2-91.4 % amino acid identity. A clear correlation between amino acid homology and level of cross protection has not

been finally established for swIAV, however, some of the amino acid differences between the vaccine strain and the field strain were located in specific antigenic sites, indicating that these differences might have an impact of antibody binding to the HA gene. However, further studies are needed to evaluate the impact of this finding. Moreover, the NA genes of the circulating strain and the vaccine strains were also found to vary. The internal genes of the circulating strain were, with the exception of the NS gene, of pandemic A(H1N1)pdm09 origin. However, the vaccine (Respiporc FLU3) does not include any component of A(H1N1)pdm09 origin. A previous study has shown that some of the protective antibodies induced by whole virus inactivated vaccine are also directed against the internal genes such as the M or NP genes (Vincent et al., 2017). Therefore, it could be speculated that the mismatch between the internal genes of the vaccine- and field strain impaired the efficacy of the vaccine; however, more studies are needed to document this rather controversial hypothesis. A modified live viral vaccine (MLV) for intra nasal use has recently been released for use in the US and has been shown to be effective when used in piglets as early as 1 day of age in the presence of MDA (Genzow et al., 2018; Vincent et al., 2012, 2007). The difference between the two vaccines is that inactivated vaccines mainly stimulates production of IgG, whereas the MLV vaccine also induces a local IgA response (Busquets et al., 2010; Gould et al., 2017; Loeffen et al., 2003; Renegar et al., 2004; Seibert et al., 2013).

Previous studies have suggested the possibility of pigs becoming re-infected with the same strain (Chamba Pardo et al., 2019; Diaz et al., 2017a; Ryt-Hansen et al., 2019; Simon-Grifé et al., 2012) and furthermore, piglets infected with IAV in the presence of MDA have been shown to have prolonged IAV shedding time (Loeffen et al., 2003; Rose et al., 2013). The present study design included very frequent sampling, which allowed us to study this in more details. Indeed, prolonged (consecutive) shedding of IAV for 2-3 weeks were documented along with recurrent (non-consecutive) shedding indicating reinfection with the same subtype. To sustain that the pigs indeed were infected with the same strain, viral sequences were obtained from pigs considered to be prolonged shedders and from pigs considered to be re-infected. The sequence data clearly showed that only one virus strain of the H1avN2sw subtype was circulating in the herd, which is the most prevalent subtype found in Denmark (Watson et al., 2015). Furthermore, the sequences obtained from the prolonged shedders revealed very close sequence homology, which indicated that these pigs were shedding the same virus for at least 14 days. Few to no nucleotide changes were observed in these animals. Similarly, the sequences obtained from the recurrent shedders, indicated that pigs were indeed capable of being re-infected with the same strain. However, in contrast to the prolonged shedders, the HA sequence data revealed significant differences between the strain collected at the

"first" infection and the "second" infection, and some of these changes were consistent between different re-infected pigs. This indicated a strong selective pressure because the amino acid changes clustered in known antigenic sites of the HA gene. This finding is very important in large swine herds chronically infected with IAV, because it will drive the evolution of viral variants towards variants that can escape the immunity against the field strain. Indeed, we have preliminary data that indicates that IAV in chronically infected herds behave similar to human seasonal viruses and undergo a stair-wise evolution and accumulate mutation in antigenic important sites (Manuscript 4). These findings emphasizes that viral drift of IAV is a reality also in swine and should be considered in cases of failure in controlling the infection – especially in larger and un-sectioned herds. Apart from genetic drift, the presence of MDA at the time of infection may also prone for re-infection. As MDAs have been shown to weaken an active immune response to initial IAV infection, there is a risk that when the MDAs wane, the pig can become susceptible for re-infection with the same strain. Several studies have indeed shown that the presence of MDA at the time of IAV infection weakens a subsequent active immune response (Deblanc et al., 2018; Loeffen et al., 2003; Markowska-Daniel et al., 2011; Niewiesk, 2014; Rose et al., 2013). However, actual reinfection has only been shown in one study (Loeffen et al., 2003). The accumulating evidence of prolonged shedding and re-infection with the same subtype and even strain should be taken into consideration when investigating IAV dynamics and transmission in the field.

Conclusion

The results of this study in a single herd revealed that there was no beneficial effect of piglet vaccination with a quarter of a dose of Respiporc FLU3 at the time of castration. The main reason for the lack of protection was probably the early infection time combined with the reduced dosing regimen used, which made it challenging to obtain a vaccine response before infection. The results of the study confirmed that IAV infection results in clinical signs of respiratory disease and that a negative effect on body weight should be expected in herds with circulating IAV in the farrowing and nursery unit. In addition, more evidence of prolonged shedding of IAV and re-infections with the same strain were provided and the results emphasized that the dynamics of IAV in the herds are even more complex than previously perceived. IAV is indeed a fast evolving pathogen and viral drift might be the reason for re-infections and thereby present an additional challenge for the control of the disease in the field.

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Table 1. Primers for conventional PCR of the HA and NA genes

HA-gene	Sequence:
Forward Primer (pQE-HA-S-F)	5'- CGG ATA ACA ATT TCA CAC AGA GCA AAA GCA GGG GAW AAT W -3'
Reverse Primer (pQE-HA-R)	5'- GTT CTG AGG TCA TTA CTG GAG TAG AAA CAA GGG TGT TTT -3'
NA-gene	
Forward Primer (pQE-NA-F)	5'- CGG ATA ACA ATT TCA CAC AGA GCA AAA GCA GGA GT -3'
Reverse Primer (pQE-NA-R)	5'- GTT CTG AGG TCA TTA CTG GAG TAG AAA CAA GGA GTT TTT T -3'

W = A or T according to the IUPAC nucleotide code.

Table 2. Overview of total number of pigs, % IAV positive pigs, mean Ct values, infection level and clinical signs between the two groups (VAC and control) over each of the 6 sampling times and accumulated as a total

Age (week)	**0 (vaccination)**	**1**	**2**	**3**	**4F**	**4N**	**5**	**6**	**Total**
Stable unit	Farrow	Farrow	Farrow	Farrow	Farrow	Nursery	Nursery	Nursery	
Number of piglets									
Total	160	123	108	104	53	49	101	102	800
VAC	80	61	56	55	28	26	53	54	413
Control	80	62	52	49	25	23	48	48	387
Influenza virus positive pigs, *n* (%)									246
VAC	3 (3.8)	10 (16.4)	30 (53.6)	11 (20.0)	17 (63.0)	**7 (26.9)**	23 (43.4)	20 (37.0)	121
Control	5 (6.3)	9 (14.5)	26 (50.0)	10 (20.4)	14 (56.0)	**17 (73.9)**	28 (58.3)	16 (33.3)	125
Ct among virus positive pigs, mean									
VAC	33.90	30.26	28.15	28.56	28.15	**26.30**	30.37	30.57	29.2
Control	30.11	30.86	26.39	26.84	28.47	**30.90**	30.05	30.32	29.1
Influenza virus, pigs with high shedding (Ct < 29.19), *n* (%)									
VAC	0 (0)	2 (3.2)	14 (25.0)	3 (5.4)	5 (23.8)	4 (15.4)	7 (13.2)	5 (9.3)	40 (9.7)
Control	2 (2.5)	1 (1.6)	15 (28.8)	7 (14.3)	6 (33.3)	3 (13.0)	8 (16.7)	5 (10.4)	47 (12.1)
Clinical signs, *n* (%)									
Lacrimation									25 (3.1)
VAC	4 (5.0)	1 (1.6)	0 (0.0)	0 (0.0)	2 (7.1)	0 (0.0)	1 (1.9)	0 (0.0)	8 (1.9)
Control	10 (12.5)	2 (3.2)	1 (1.9)	2 (4.1)	1 (4.0)	0 (0.0)	1 (2.0)	0 (0.0)	17 (4.4)
Nasal discharge									380 (47.6)
VAC	8 (10.0)	33 (54.1)	21 (37.5)	39 (70.9)	23 (82.1)	**3 (11.5)**	28 (52.8)	**49 (90.7)**	204 (49.4)
Control	11 (13.8)	22 (35.5)	12 (23.1)	33 (67.3)	22 (88.0)	**10 (43.5)**	34 (69.4)	**32 (69.6)**	176 (45.6)
Conjunctivitis									289 (36.2)
VAC	14 (17.5)	22 (36.1)	30 (53.6)	**34 (61.8)**	17 (60.7)	12 (46.2)	9 (17.0)	17 (31.5)	155 (37.5)
Control	24 (30.0)	25 (40.3)	30 (57.7)	**13 (26.5)**	10 (40.0)	8 (34.8)	12 (24.5)	12 (26.1)	134 (34.7)
Pigs with either lacrimation, nasal discharge or conjunctivitis									540 (67.6)
VAC	24 (30.0)	48 (78.7)	39 (69.6)	46 (83.6)	23 (82.1)	14 (53.8)	**31 (58.5)**	50 (92.6)	275 (66.6)
Control	37 (46.3)	42 (67.7)	38 (73.1)	35 (71.4)	22 (88.0)	16 (69.6)	**39 (79.6)**	36 (78.3)	265 (68.6)
Poor Body Score									294 (36.8)
VAC	41 (51.2)	23 (37.7)	14 (25.0)	15 (27.3)	6 (22.2)	9 (34.6)	**13 (24.5)**	17 (31.5)	138 (20.3)
Control	43 (53.8)	26 (41.9)	12 (23.1)	13 (26.5)	8 (32.0)	11 (47.8)	**23 (46.9)**	20 (42.6)	156 (22.2)
Fecal soiling									170 (21.3)
VAC	24 (30.0)	17 (27.9)	10 (17.9)	5 (12.7)	1 (3.6)	2 (7.7)	17 (32.1)	6 (11.1)	84 (20.3)
Control	24 (30.0)	17 (27.4)	9 (17.3)	7 (10.2)	2 (8.0)	2 (8.7)	18 (36.7)	9 (19.6)	86 (22.3)

The results are listed as pig age group (in weeks) along with the stable unit in which the pigs were present when sampled. The pigs are further divided into treatment group (VAC vs Control) when tested against the different parameters. Statistically significantly different results ($p<0.05$) between the VAC/Control groups ($p<0.05$) are highlighted in bold.

Table 3. Prevalence of clinical signs in IAV positive pigs of the two treatments groups

	Lacrimation	**Nasal discharge**	**Conjunctivitis**	**Respiratory signs ≥ 1**	**Poor Body Score**	**Faecal soiling**
Observations in total, *n*	799	799	799	799	799	799
Cases, *n* (% of total)	24 (3.1 %)	376 (47.6 %)	286 (36.2 %)	537 (67.6 %)	294 (37.0 %)	170 (21.3 %)
Virus positive, *n*	4	142	100	182	79	55
VAC, *n*	1 (25 %)	72 (50.7 %)	57 (57 %)	89 (49.9 %)	31 (39.2 %)	22 (40 %)
Control, *n*	3 (75%)	70 (49.3 %)	43 (43 %)	93 (51 %)	48 (60.8 %)	33 (60 %)

91

Figure 1. Boxplot showing the weight of the pigs of the two groups (control vs vaccine) at the three weighing times (week 0, 3 and 6)

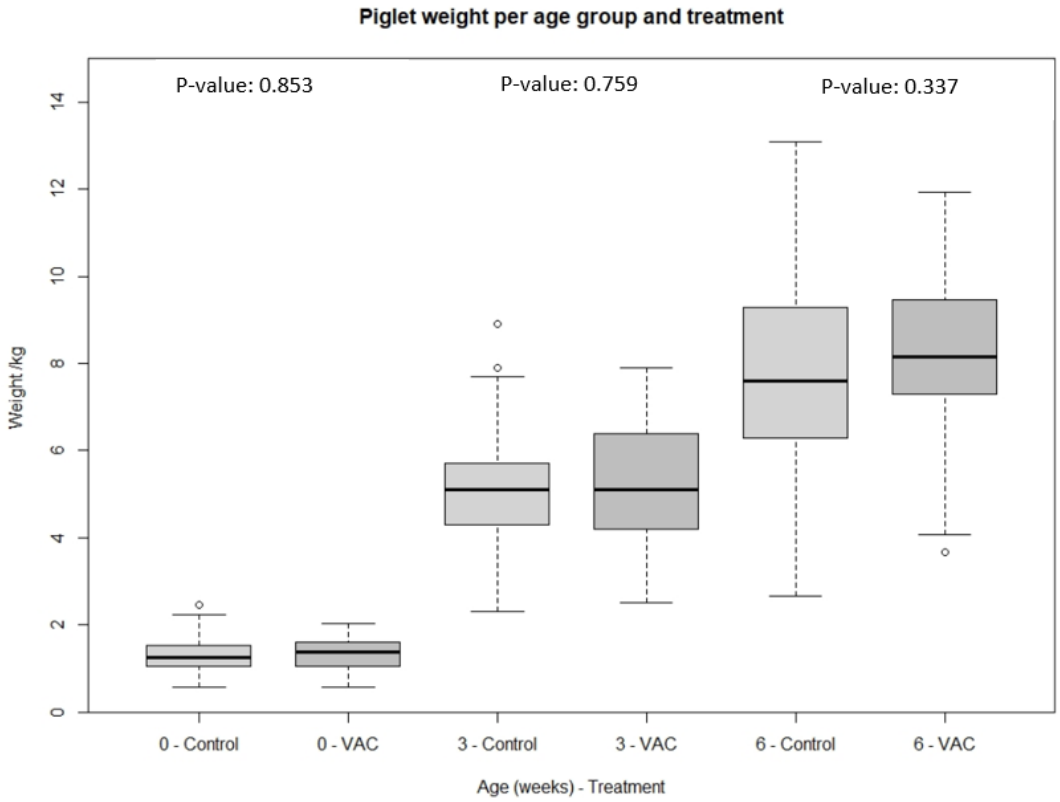
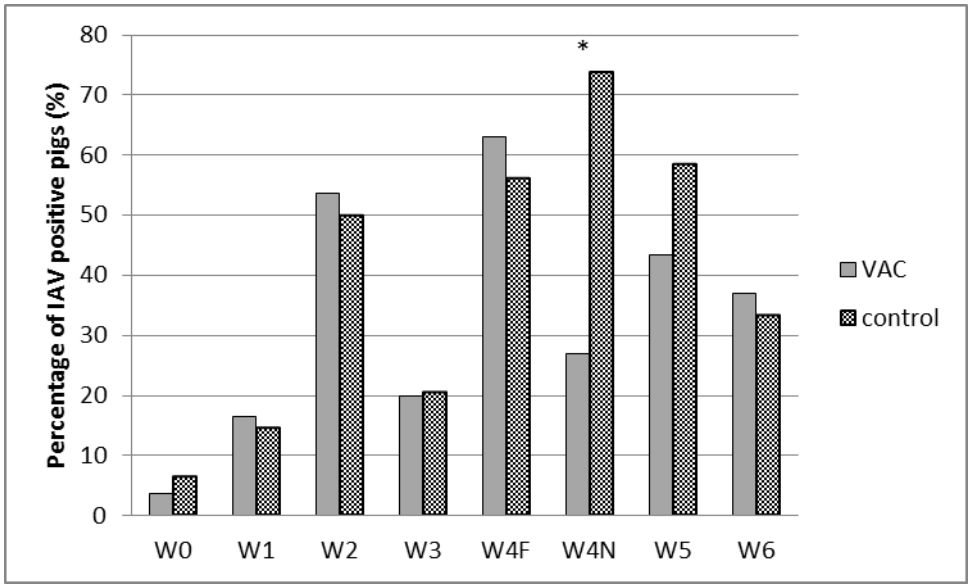
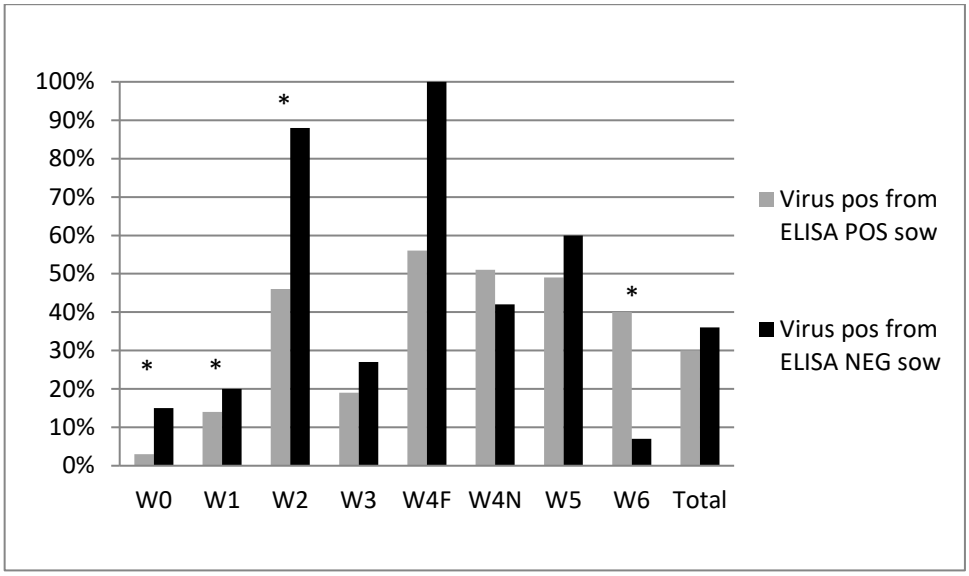


Figure 2. Prevalence of IAV in nasal swabs of the control and vaccinated (VAC) groups over the different sampling times (W0-W6)



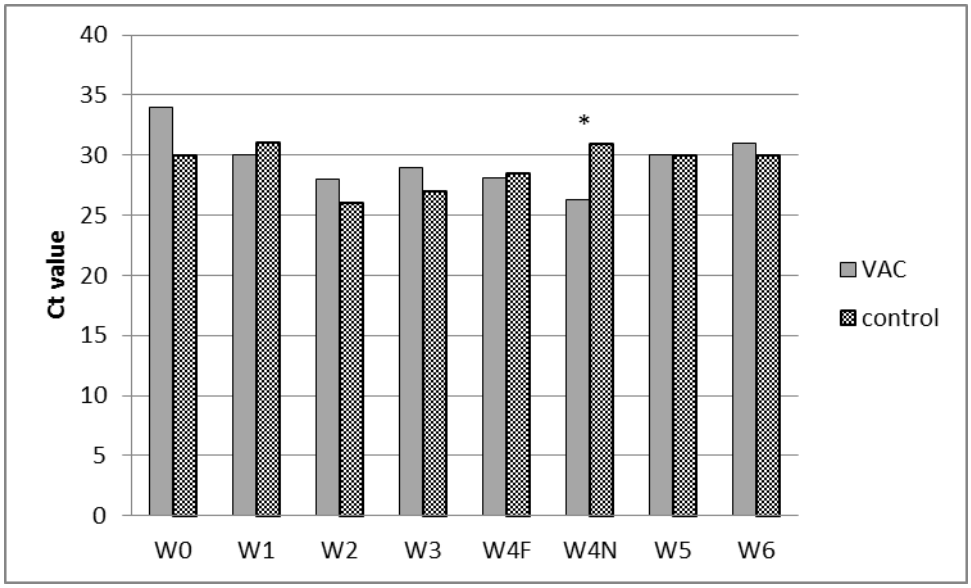
W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit. * Indicates a significant difference ($p<0.05$) between the two groups.

Figure 3. Prevalence of IAV positive pigs from seropositive or seronegative sows over the different sampling times (W0-W6)



W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit. * Indicates a significant difference ($p<0.05$) between the two groups.

Figure 4. Average Ct values of the IAV positive pigs of the control and vaccinated (VAC) groups over the different sampling times (W0-W6)



W4F: samples taken in week 4 in the farrowing unit. W4N: samples taken in week 4 in the nursery unit. * Indicates a significant difference ($p<0.05$) between the two groups.

Supplementary table 1. Differences in the overall prevalence of clinical signs between subgroups

Clinical sign	Lacrimation	Nasal discharge	Conjunctivitis	Respiratory signs ≥ 1	Poor Body Score	Faecal soiling
Total number of observations, *n*	799	799	799	799	799	799
Cases, *n* (%)	25 (3.1)	380 (47.6)	289 (36.2)	540 (67.6)	294 (37.0)	170 (21.3)
Treatment group						
VAC	8 (1.9)	204 (49.4)	134 (34.7)	265 (68.7)	201 (36.7)	86 (22.2)
Control	17 (4.4)	176 (45.6)	155 (37.5)	175 (66.6)	91 (37.9)	84 (20.3)
Stable unit						
Farrowing unit	**23 (4.2)**	**224 (40.9)**	**219 (40.0)**	**354 (64.6)**	156 (40.3)	116 (21.2)
Nursery unit	**2 (0.1)**	**148 (61.7)**	**69 (28.8)**	**178 (74.2)**	138 (33.5)	54 (22.5)
Virus infection level						
Negative	20 (3.6)	**236 (43.0)**	186 (33.9)	**355 (64.7)**	215 (39.1)	115 (20.9)
Positive	4 (1.6)	**142 (57.7)**	100 (40.7)	**182 (74.0)**	79 (32.2)	55 (22.4)
Virus infection level						
Low (Ct > 29.19)	23 (96.8)	336 (47.5)	247 (34.9)	477 (67.4)	268 (37.8)	147 (20.8)
High (Ct ≤ 29.19)	1 (3.2)	42 (48.3)	39 (44.8)	60 (69.0)	26 (30.2)	23 (26.4)
Other diseases						
No	17 (2.8)	**317 (52.6)**	208 (59.0)	414 (68.7)	**192 (31.9)**	121 (20.1)
Yes	8 (4.1)	**63 (32.3)**	80 (41.0)	125 (64.1)	**102 (52.3)**	49 (25.1)
Body Score						
Normal	11 (2.2)	**273 (54.2)**	**143 (28.4)**	330 (65.5)	-	61 (12.1)
Poor	13 (4.4)	**106 (36.1)**	**146 (49.7)**	209 (71.1)	-	108 (36.7)
Transfers to different sow						
Never	20 (3.1)	317 (49.0)	195 (30.1)	**421 (65.1)**	**208 (32.1)**	**121 (18.7)**
Once	3 (3.8)	27 (34.6)	50 (64.1)	**61 (78.2)**	**42 (54.5)**	**27 (34.6)**
Twice	2 (2.7)	36 (48.6)	44 (59.5)	**58 (78.4)**	**44 (59.5)**	**22 (29.7)**
Sow SIV ELISA at farrowing						
Negative	9 (4.3)	**82 (39.0)**	68 (32.4)	**130 (61.9)**	**52 (24.8)**	**28 (13.3)**
Positive	16 (2.7)	**298 (50.6)**	221 (37.5)	**410 (69.6)**	**242 (41.1)**	**142 (24.1)**
Sow parity						
First parity	8 (4.9)	**53 (32.3)**	**104 (63.4)**	**126 (76.8)**	**106 (65.0)**	**74 (45.1)**
Second and third parity	17 (2.7)	**327 (51.5)**	**185 (29.1)**	**414 (65.2)**	**188 (29.6)**	**96 (15.1)**

Statistically significantly different results between subgroups ($p<0.05$) are highlighted in bold.

Supplementary table 2. Correlation between body weight and different subgroups

Age (week)	**0 (birth)**		**3**		**6**	
Stable unit	**Farrowing unit**		**Farrowing unit**		**Nursery unit**	
	n (piglets)	**Weight/kg mean (sd)**	**n (piglets)**	**Weight/kg mean (sd)**	**n (piglets)**	**Weight/kg mean (sd)**
Body weight, mean (sd)	160	1.33 (0.4)	104	5.15 (1.5)	102	8.05 (2.2)
Treatment group						
VAC	80	1.34 (0.4)	55	5.19 (1.4)	54	8.25 (1.9)
Control	80	1.32 (0.4)	49	5.10 (1.5)	48	7.83 (2.4)
Virus infection level[1]						
Negative	152	**1.31 (0.4)**	83	5.09 (1.5)	66	**8.62 (2.1)**
Positive	8	**1.61 (0.4)**	21	5.37 (1.1)	36	**6.93 (1.8)**
Virus infection level[1]						
Low (Ct > 29.19)	158	**1.31 (0.4)**	94	5.13 (1.5)	92	**8.16 (2.1)**
High (Ct ≤ 29.19)	2	**1.87 (0.8)**	10	5.31 (0.7)	10	**6.69 (2.1)**
Other diseases[1]						
No	83	1.36 (0.4)	83	**5.33 (1.5)**	94	8.09 (2.1)
Yes	77	1.29 (0.4)	21	**4.43 (1.1)**	6	7.60 (3.6)
Body Score[1]						
Normal	76	**1.58 (0.3)**	76	**5.77 (1.1)**	64	**9.12 (1.7)**
Poor	84	**1.09 (0.3)**	28	**3.45 (0.7)**	37	**6.24 (1.6)**
Transfers to different sow						
Never	131	1.32 (0.4)	86	**5.35 (1.5)**	83	**8.26 (2.2)[2]**
Once	18	1.30 (0.3)	7	4.66 (0.5)	9	7.25 (2.0)
Twice	11	1.39 (0.2)	11	**3.90 (0.8)**	10	**7.03 (1.9)[2]**
Sow SIV ELISA at farrowing						
Negative	27	1.29 (0.4)	15	5.82 (1.5)	15	**9.35 (2.3)[3]**
Positive	133	1.33 (0.4)	89	5.03 (1.4)	87	**7.82 (2.1)**
Sow parity						
First parity	58	**1.13 (0.3)**	15	**4.31 (0.7)**	16	7.20 (2.0) [2]
Second and third parity	102	**1.43 (0.4)**	89	**5.29 (1.5)**	86	8.21 (2.2) [2]

Statistically significantly different results between subgroups ($p<0.05$) are highlighted in bold.[1] Status at the day of examination. [2]: $p < 0.1$. [3] difference caused by one litter from one ELISA negative sow.

Supplementary table 3. Results of the RT real-time PCR from the two treatments groups at the different sampling times (week 0-week 6)

Vaccination	W0	W1	W2	W3	W4	W5	W6
V1	neg	†	†	†	†	†	†
V3	neg	neg	neg	17.98	neg	neg	neg
V5	neg	†	†	†	†	†	†
V7	neg	neg	†	†	†	†	†
V9	neg	neg	†	†	†	†	†
V11	neg	34.4	†	†	†	†	†
V13	neg	†	†	†	†	†	†
V15	neg	19.07	31.19	neg	neg	29.44	neg
V17	neg	†	†	†	†	†	†
V19	neg	†	†	†	†	†	†
V21	neg	†	†	†	†	†	†
V23	neg	†	†	†	†	†	†
V25	neg	†	†	†	†	†	†
V27	neg	†	†	†	†	†	†
V29	neg	†	†	†	†	†	†
V31	neg	neg	32.21	20.14	neg	34.17	neg
V33	neg	neg	19.74	32.53	neg	34.7	neg
V35	neg	neg	neg	22.87	neg	neg	33.98
V37	neg	neg	32.65	30.44	neg	32.81	neg
V39	neg	neg	31.33	31.19	neg	neg	33.29
V41	neg	neg	neg	32.4	neg	34.13	33.39
V43	neg	neg	23.82	30.92	neg	neg	neg
V45	**neg**	**neg**	**16.89**	**30.95**	**30.93**	**neg**	**26.37**
V47	neg	†	†	†	†	†	†
V49	neg	neg	neg	neg	16.62	neg	33.27
V51	neg	†	†	†	†	†	†
V53	neg	†	†	†	†	†	†
V55	neg	neg	neg	neg	30.5	32.81	22.07
V57	**neg**	**neg**	**neg**	**neg**	**16.54**	**24.27**	**31.38**
V59	neg	NA	neg	neg	31.42	NA	32.61
V61	neg	neg	†	†	†	†	†
V65	neg	neg	neg	neg	28.25	34.51	32.54
V67	neg	neg	20.57	neg	26.22	27.4	33.69
V69	neg	33.43	22.39	neg	27.46	34.36	33.26
V71	neg	32.62	†	†	†	†	†
V73	neg	†	†	†	†	†	†
V75	neg	†	†	†	†	†	†
V77	neg	31.28	21.6	neg	30.34	neg	31.1
V79	neg	33.12	30.83	neg	29.82	neg	neg
V81	**neg**	**16.16**	**31.86**	**neg**	**neg**	**neg**	**29.1**
V83	neg	34.69	27.58	neg	neg	neg	neg
V85	**neg**	**neg**	**28.99**	**neg**	**neg**	**neg**	**24.88**
V87	neg	neg	31.2	neg	neg	neg	34.06
V89	**neg**	**33.87**	**30.31**	**neg**	**neg**	**neg**	**31.62**
V91	neg	neg	24.55	neg	†	†	†
V93	neg	†	†	†	†	†	†
V95	neg	†	†	†	†	†	†
V97	neg	neg	neg	neg	30.94	neg	neg
V99	neg	neg	neg	neg	29.77	neg	neg
V101	neg	neg	neg	neg	neg	neg	neg
V103	neg	neg	neg	neg	30.56	20.81	neg
V105	neg	neg	neg	neg	neg	33.45	neg
V107	neg	neg	†	†	†	†	†
V109	neg	NA	32.63	neg	31.23	33.34	neg
V111	33.25	neg	32.76	neg	neg	33.14	neg
V113	neg	neg	33.55	neg	neg	33.19	neg
V115	neg	neg	28.2	neg	neg	neg	neg
V117	neg	neg	†	†	†	†	†
V119	34.37	neg	33.46	neg	neg	32.75	neg
V121	34.09	neg	18.76	31.42	neg	neg	neg
V123	neg	neg	neg	neg	22.48	25.63	neg
V125	neg	neg	neg	neg	30.86	neg	neg
V127	neg	neg	neg	neg	26.27	neg	neg
V129	neg	neg	30.09	33.28	30.23	neg	neg
V131	neg	neg	neg	neg	17.41	27.75	neg
V133	neg	neg	neg	neg	25.61	23.37	neg
V135	neg	neg	neg	neg	30.17	neg	neg
V137	neg	neg	neg	neg	30.18	29.86	neg
V139	neg	neg	neg	neg	30.49	neg	neg
V141	neg	neg	neg	neg	32.14	neg	neg
V143	neg	neg	neg	neg	neg	22.32	18.67
V145	neg	neg	neg	neg	neg	neg	32.08
V147	neg	neg	neg	neg	neg	neg	32.94
V149	neg	neg	28.49	neg	neg	32.67	neg
V151	neg	neg	neg	neg	neg	31.64	neg
V153	neg	neg	30.69	neg	neg	neg	neg
V155	neg	neg	30.82	neg	neg	neg	neg
V157	neg	neg	30.69	neg	neg	neg	31.13
V159	neg	neg	27.32	neg	neg	neg	neg
V161	neg	33.94	29.19	†	†	†	†

Control	W0	W1	W2	W3	W4	W5	W6
N2	**neg**	**neg**	**neg**	**neg**	**28.32**	**31.93**	**neg**
N4	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N6	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N8	**neg**	**30.98**	**31.6**	**neg**	**20.53**	**27.9**	**neg**
N10	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N12	**neg**	**32.74**	**†**	**†**	**†**	**†**	**†**
N14	**31.76**	**31**	**24.51**	**neg**	**30.86**	**30.85**	**neg**
N16	**25.75**	**†**	**†**	**†**	**†**	**†**	**†**
N18	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N20	**32.94**	**neg**	**†**	**†**	**†**	**†**	**†**
N22	**neg**	**29.97**	**28.42**	**†**	**†**	**†**	**†**
N24	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N26	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N28	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N30	**neg**	**neg**	**30**	**23.44**	**neg**	**32.09**	**neg**
N32	**32.3**	**neg**	**29.95**	**19.74**	**31.24**	**neg**	**neg**
N34	**neg**	**neg**	**25.26**	**27.09**	**neg**	**neg**	**neg**
N36	**neg**	**neg**	**30.18**	**19.98**	**27.49**	**32.37**	**neg**
N38	**neg**	**neg**	**30.08**	**28.11**	**30.31**	**neg**	**neg**
N40	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N42	**neg**	**neg**	**27.98**	**32.32**	**neg**	**31.74**	**neg**
N44	**neg**	**neg**	**neg**	**31.71**	**neg**	**33.82**	**neg**
N46	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N48	**neg**	**neg**	**neg**	**neg**	**28.21**	**21.9**	**neg**
N50	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N52	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N54	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N56	**neg**	**neg**	**neg**	**neg**	**29.24**	**20.71**	**33.09**
N58	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N60	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N62	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N66	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N68	**neg**	**neg**	**neg**	**NA**	**30.11**	**33.43**	**19.74**
N70	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N72	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N74	**neg**	**neg**	**neg**	**neg**	**18.38**	**28.88**	**31.76**
N76	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N78	**neg**	**34.14**	**18.4**	**neg**	**neg**	**34.78**	**20.75**
N80	**neg**	**neg**	**30.1**	**neg**	**neg**	**neg**	**neg**
N82	**neg**	**neg**	**31**	**neg**	**neg**	**17.08**	**33.04**
N84	**neg**	**34.09**	**30.12**	**neg**	**neg**	**neg**	**neg**
N86	**neg**	**neg**	**22.56**	**neg**	**neg**	**neg**	**32.84**
N88	**neg**	**neg**	**17.94**	**neg**	**neg**	**33.52**	**34.52**
N90	**neg**	**neg**	**23.32**	**neg**	**33.1**	**neg**	**neg**
N92	**neg**	**neg**	**19.59**	**neg**	**33.45**	**neg**	**neg**
N94	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N96	**neg**	**neg**	**†**	**†**	**†**	**†**	**†**
N98	**neg**	**neg**	**34.92**	**neg**	**neg**	**33.24**	**33.73**
N100	**neg**	**neg**	**neg**	**neg**	**neg**	**neg**	**34.72**
N102	**neg**	**neg**	**neg**	**neg**	**29.75**	**31.3**	**28.48**
N104	**neg**	**neg**	**neg**	**neg**	**neg**	**33.09**	**31.4**
N106	**neg**	**neg**	**neg**	**neg**	**neg**	**neg**	**33.54**
N108	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N110	**neg**	**neg**	**19.06**	**25.28**	**neg**	**neg**	**neg**
N112	**neg**	**neg**	**21.77**	**†**	**†**	**†**	**†**
N114	**neg**	**neg**	**26.94**	**32.46**	**33.12**	**neg**	**neg**
N116	**neg**	**neg**	**23.93**	**neg**	**32.9**	**33.03**	**29.01**
N118	**neg**	**neg**	**32.33**	**neg**	**33.25**	**25.77**	**neg**
N120	**neg**	**neg**	**18.89**	**neg**	**33.1**	**neg**	**neg**
N122	**neg**	**neg**	**neg**	**neg**	**33.75**	**neg**	**NA**
N124	**neg**	**neg**	**neg**	**neg**	**31.62**	**33.53**	**neg**
N126	**neg**	**neg**	**neg**	**neg**	**25.81**	**29.43**	**23.99**
N128	**neg**	**neg**	**neg**	**neg**	**33.51**	**neg**	**neg**
N130	**neg**	**neg**	**neg**	**neg**	**32.96**	**26.22**	**neg**
N132	**neg**	**neg**	**neg**	**neg**	**30.53**	**32.58**	**neg**
N134	**neg**	**neg**	**neg**	**neg**	**33.84**	**NA**	**neg**
N136	**neg**	**neg**	**neg**	**28.28**	**29.8**	**31.52**	**neg**
N138	**neg**	**neg**	**neg**	**neg**	**neg**	**neg**	**32.38**
N140	**neg**	**neg**	**neg**	**neg**	**34.19**	**neg**	**neg**
N142	**neg**	**†**	**†**	**†**	**†**	**†**	**†**
N144	**neg**	**neg**	**neg**	**neg**	**27.55**	**neg**	**32.07**
N146	**neg**	**neg**	**neg**	**neg**	**28.75**	**30.53**	**neg**
N148	**27.81**	**neg**	**neg**	**neg**	**neg**	**neg**	**neg**
N150	**neg**	**neg**	**23.89**	**neg**	**31.89**	**31.83**	**neg**
N152	**neg**	**32.06**	**33.51**	**neg**	**†**	**†**	**†**
N154	**neg**	**neg**	**neg**	**neg**	**34.12**	**30.93**	**neg**
N156	**neg**	**33.42**	**†**	**†**	**†**	**†**	**†**
N158	**neg**	**neg**	**neg**	**neg**	**NA**	**neg**	**neg**
N160	**neg**	**neg**	**neg**	**neg**	**21.83**	**27.26**	**neg**
N162	**neg**	**19.38**	**†**	**†**	**†**	**†**	**†**

“W” gives the week number, where “0” is the time of vaccination. V1-V161 and N2-162 indicates the ear-tag number of the individual pig, and the letter “V” indicates that the pig was vaccinated and “N” indicates that the pig was sham vaccinated. “†” indicates that the pig is dead. “NA” indicates that the pig was not sampled. “neg” indicates that the pig was negative in the PCR. The numeric values indicates the Ct value of the nasal swab at the given sampling time, and the background color indicates the level of IAV found in the sample, where dark green indicates a high amount of IAV and light green indicates a low amount of virus. The ear number highlighted in bold indicates the pigs originating from an IAV seronegative sow.

Manuscript 3

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

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Abstract

Influenza A virus (IAV) is a highly contagious pathogen in pigs. IAV infection causes respiratory disease and is thereby a challenge for animal health, animal welfare and the production economy. In Europe, the most widespread strategy for controlling IAV is implementation of sow vaccination programs, to secure delivery of protective maternally derived antibodies (MDAs) to the newborn piglets.

In this study we report a unique case, where a persistently IAV infected herd experienced an acute outbreak with a new IAV subtype and subsequently decided to implement a mass sow vaccination program. Clinical registrations, nasal swabs and blood samples were collected from four different batches of pigs before and after vaccination. Virus isolation, sequencing of the virus strain and hemagglutinin inhibition (HI) tests were performed on samples collected before and during the outbreak and after implementation of mass sow vaccination.

After implementation of the sow mass vaccination, the time of infection was delayed and the viral load significantly decreased. An increased number of pigs, however, tested positive at two consecutive sampling times indicating prolonged shedding. In addition, a significantly smaller proportion of the 10-12 weeks old pigs were seropositive by the end of the study, indicating an impaired induction of antibodies against IAV in the presence of MDAs. Sequencing of the herd strains revealed major differences in the hemagglutinin gene of the strain isolated before- and during the acute outbreak despite that, the two strains belonged to the same HA lineage. The HI tests confirmed a limited degree of cross-reaction between the two strains. Furthermore, the sequencing results of the hemagglutinin gene obtained before and after implementation of mass sow vaccination revealed an increased substitution rate and an increase in positively selected sites in the globular head of the hemagglutinin after vaccination.

Introduction

Influenza A virus (IAV) in swine (swIAV) is an enzootic virus of swine herds globally. SwIAV infects the cells of the respiratory tract, inducing clinical signs of respiratory disease and fever [1–3]. Additionally, IAV impairs the immune system, making the infected pig more susceptible to other pathogens [4–6]. During the last 10-20 years, the pig industry has undergone profound structural changes resulting in a significant increase in herd size and a continuous movement of pigs between production units. These changes has altered the dynamics of swIAV from an epizootic disease that resolved in a few weeks to a more enzootic situation with persistent circulation of the virus in same

herds for years due to the continuous exposure of naïve piglets [7–15]. These changes have emphasized that there is a need for effective control measures at the herd level, and have resulted in a marked increase in the sales of IAV vaccines. One of the most used vaccines on the European market is Respiporc FLU3, which is an inactivated, adjuvanted, whole virus trivalent vaccine including the subtypes; H3N2sw, H1avN1 and H1huN2 [16]. This vaccine also provides protection against H1avN2sw, which is the most prevalent subtype found in Denmark [17].

As pigs have an impermeable epitheliochorial placenta, newborn piglets depend on immunoglobulins from the sow colostrum for protection against infections during the first weeks of life [18]. Sow vaccination is therefore a widely used strategy for the prevention of many porcine pathogens. However, recent reports indicate that the impact of MDAs may be more complex than previously perceived [8,19–24]. Described examples of “unwanted" effects of MDAs include impaired/delayed development of immunity, prolonged shedding periods and an increased risk of enzootic IAV infection at the herd level [8,19,20,24–26].

The high mutation rate of RNA viruses enable them to rapidly evolve variants with a better fitness and/or modified antigenicity, capable of evading the immune system [27,28]. The surface protein hemagglutinin (HA) of IAV is more variable than the other viral proteins, consistent with the fact that it is the major target for neutralizing antibodies [29–32]. Especially mutations in the globular head of the hemagglutinin protein, which includes the receptor binding site for host cell entry and five specific antigenic sites/epitopes (Sa, Sb, Ca1, Ca2 and Cb) have been shown to modify the binding of neutralizing antibodies [33–37].

The continuous circulation of a huge variety of antigenically distinct variants of swIAV provides a significant challenge for the control of swIAV, because herd immunity may be compromised by introduction of new strains and/or by emergence of antigenically different variants within the herd. There is, however, a lack of controlled field studies on the swIAV dynamics in these herds.

A Danish sow herd that had been persistently infected with a swIAV of the H1avN1 subtype for years suddenly experienced an acute outbreak involving an H1avN2sw strain. By a co-incidence, this herd was included in another project and therefore we were able to perform a prospective study including observations and samplings both prior to, during, and after the outbreak. The aim of the study was to examine the clinical impact, the viral dynamics, as well as the genetic and antigenic variability of circulating strains prior to, during and after the acute outbreak. Following the acute outbreak, the herd decided to start a mass sow vaccination program, and therefore the study was

extended to include exploration of the effects of a mass sow vaccination initiated during an acute outbreak.

Materials and methods

Ethical statement

The study was carried out in strict accordance with the guidelines of the Good Experimental Practices (GEP) standard adopted by the European Union, and all experimental procedures were performed in accordance with the recommendations provided by the National Veterinary Institute of Denmark. All samples were collected by a trained veterinarian and with the farmers consent.

Herd

The herd was located in the northwestern part of Jutland, Denmark and had 600 sows with a main production of 30 kilos pigs and a small production of finisher pigs. The herd had a known health status according to the Danish Specific Pathogen Free program [38] termed "Blue SPF + AP2 + PRRS Type 1", indicating that the herd was serologically positive for *Actinobacillus pleuropneumoniae* type 2 and PRRSv type 1. However, both of these pathogens were under control. In addition, the health status specified that herd was declared free from *Mycoplasma hyopneumoniae*, *Brachyspira hyodysenteriae*, *Pasteurella multocida*, *Haematopinus suis* and *Sarcoptes scabiei* var. *suis*. The herd bought all new gilts from an external source, which had an identical health status. The replacement rate of the sows was approx. 50 %/year. All piglets were weaned batch-wise at four weeks of age and placed in empty nursery stables where they were allocated to pens according to size. The herd had four farrowing stables and ten nursery stables. All stables were washed with high pressure and disinfected using hydrated lime between batches. No strict "all in all out" flow of pigs was maintained in any of the stables.

Study design and sampling

As it was the plan to include the herd in another IAV study [39], it was screened for the presence of IAV in December 2016. At the screening, 30 nasal swabs were collected which included five nasal swabs obtained from one-week-old piglets (farrowing unit), five nasal swabs of three-week-old piglets (farrowing unit), 10 nasal swabs from five-week-old weaners (nursery) and 10 nasal swabs from 9-week-old weaners (nursery).

In February 2017, the herd veterinarian reported an increase in respiratory problems in the farrowing unit, and increased secondary bacterial infections in nursery pigs. The 1st round of sampling was

carried out from March to June 2017. The sampling round included four batches of sows with farrowing dates one-week apart. At farrowing, five piglets of each sow were ear-tagged. Nasal swabs were collected from the ear-tagged pigs at week 1, 3, 5 and 10-12 and blood samples were collected at week 3 and week 10-12. The exact same study design was conducted for the 2nd round of sampling, which was carried out from May to August 2017 after implementation of mass sow vaccination with Respiporc Flu®3 (IDT Biologika GmbH, Dessau-Rosslau, Germany) (Fig 1AB). All sows with farrowing dates from May and onwards were vaccinated for the first time in the third week of March, and for the second time three weeks later. To avoid a mix of unvaccinated and vaccinated sows in the farrowing unit for the 2nd sampling, the first batch of sows included farrowed the last week of May, five weeks after their 2nd vaccination. The following sow batches were thereby six, seven and eight weeks post 2nd vaccination. A timeline showing the different sampling rounds in relation to IAV occurrence and vaccination is presented in Fig 1A.

Blood samples and nasal swabs were collected from the sows and the piglets at different time points according to Fig 1B. The blood samples were collected, using a vacutainer serum tube (Becton Dickinson, Denmark), from sows and piglets in *vena jugularis* and *vena cava cranialis* respectively. The blood samples were kept at 5°C for a maximum of 2 days. Subsequently, the samples were centrifuged at 3000rpm for 10 minutes, and the serum stored at -20 ^{0}C until test.

The nasal swabs were collected with a small or large rayon swab (Medical Wire, UK) according to the size of the animal. The swab was inserted and turned 360 degrees in both nostrils of each pig, and then immersed into the Sigma Virocult media (MWE, England). The samples were kept at 5° C for a maximum of 2 days until pooling and RNA extraction. Extracted RNA was kept at -80 ^{0}C until use.

Clinical registrations

The clinical registrations were performed as previously described [39]. Briefly, a coughing index for the pen including minimum one ear tagged pig was calculated and individual clinical signs including dyspnea, lacrimation, nasal discharge, conjunctivitis, fecal soiling, body condition, limping and hernia were recorded for ear-tagged pigs.

Pooling of nasal swabs, RNA extraction and quantitative real time RT-PCR

The pooling, RNA extraction and quantitative real time RT-PCR was performed as previously described [39]. Briefly, the nasal swabs were pooled litter-wise for the piglets and batch-wise for the sows. RNA was extracted from the pools using the RNeasy mini kit automated on the QIAcube (QIAGEN). Then, a previously published quantitative real time RT-PCR assay targeting the matrix

gene influenza A [40] was used to determine if a pool was positive for IAV. Samples with a Ct value <36 was considered positive. If a pool was positive, the RNA was extracted from the individual samples and then tested by quantitative real time RT-PCR as described above. The positive individual samples with a Ct value <31 were tested in a previously described multiplex quantitative real time RT-PCR [39], to determine the IAV subtype.

Viral isolation and sequencing

At least one positive individual sample of each batch with a Ct value < 31 were chosen for PCR amplification of the HA and NA genes followed by Sanger sequencing as previously described (Manuscript 2). In addition, the sample with the lowest ct value from the initial screening and five samples with the lowest ct value from the 1st and 2nd sampling round were chosen for isolation in Madin-Darby Canine Kidney (MDCK) cells and sequenced on the Illumina Miseq platform as previously described (Manuscript 2).

Analyses of the viral sequences

The generation of consensus nucleotide and amino acid sequences of the Sanger's sequencing data and the Illumina sequence data was performed as previously described (Manuscript 2) using the program CLC Main Workbench version 8 for the Sanger sequencing reads and CLC Genomics Workbench version 11.0.1 for the Illumina reads. All sequences are available in NCBI Genbank with the following accession numbers: MN410726-MN410785 (Sanger sequences) and MN410796-MN410883 (Illumina sequences).

The consensus nucleotide- and amino acid-sequence of each gene (HA, NA, M, NP, NS, PB1, PB2 and PA) derived from the Sanger and Illumina sequencing were aligned using the MUSCLE algorithm [41] in CLC Main Workbench version 8. The subtype(s) of the IAV strain circulating in the herd were checked by constructing a phylogenetic tree (using neighbor joining) that included both contemporary HA and NA sequences, obtained in the Danish annual swine IAV surveillance, and also the HA and NA sequences from the present study, aligned using MUSCLE. Thereafter, the location of known antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) of the H1 gene [33,35,36,42,43] were identified in the HA amino acid alignment of the present study, and examined manually for differences/mutations. For the remaining genes a BLAST analysis was performed against NCBI Genbank [44] to determine the closest sequence match and thereby the origin (avian or pandemic). Finally, the sequences were subjected to a pairwise comparison, to reveal the overall sequence identity.

Viral evolution of the HA gene

We used programs from the software package BEAST2 version 2.5.2 [45] to estimate the substitution rate for the HA gene both before vaccination (during the 1st sampling round) and after mass sow vaccination (during the 2nd sampling round). Specifically, the substitution model was specified to be HKY with gamma distributed rates over sites, with a strict clock model, and using tip dates (sampling dates). The following priors were specified: The tree model was set to "Birth Death Skyline Serial", which is used when lineages are sampled sequentially through time. The reproduction number was set to be between 0 and 10 with a log normal distribution. The "BecomeUninfectiousRate" was estimated to be approximately 52 per year (corresponding to an average time being infectious of 1 week) with a log normal distribution and $CI_{95\%}$ = [44.4-224]. The clock rate was set as a log normal distribution with a mean value of 0.001 and, which is estimated to be substitution rate of RNA viruses, with a $CI_{95\%}$ = [3.95 x 10^{-5}-0.005]. The gamma shape prior and the kappa prior were left at the default values. A gamma distribution of the "origin prior" was chosen with an alpha value of 0.5 and a beta value of 2. Lastly, the sampling proportion prior was set to a log normal distribution with a mean value of 0.001 and $CI_{95\%}$ = [3.95 x 10^{-5}-0.005]. The chain length was set to 10.000.000 with a log every 1000, and the MCMC was run twice. The program BEAUti2 [45] was used to set up the analysis, and Tracer version 1.7.1 [46] was used to inspect the results and check for convergence of the MCMC runs.

The program CODEML in the program package PAML [47] was used to investigate whether there were positively selected sites in the two datasets. Specifically, we did this by comparing the fits of CODEML's Model 1a (M1a) and Model 2a (M2a) (NSsites = 1 and 2). In these models, selection is quantified using the dN/dS ratio (the ratio between the rate of non-silent substitutions per non-silent site and the rate of synonymous substitutions per synonymous site). A dN/dS ratio larger than 1 indicates the presence of positive selection (there are more amino-acid changing substitutions than expected for random reasons). M1a is a two-parameter model, which assumes two classes of codons, one class with negatively selected sites (dN/dS < 1) and one with neutral sites (dN/dS = 1), whereas M2a is a three-parameter model, which includes an additional class of positively selected sites (dN/dS > 1) [48]. If M2a fits the data significantly better than M1a (given the extra parameters in the model), then this is statistical evidence for the presence of positive selection in some codons. The Bayes Empirical Bayes (BEB) procedure [49] implemented in CODEML, was used to identify which sites that were positively selected. Model fits were compared using the Akaike Information Criterion (AIC), and Akaike weights, and also using likelihood ratio tests [50,51]. Moreover, an additional

CODEML analysis, was used to determine the average global dN/dS (ω) value for the HA genes (NSsites = 0)[48,52].

We also used the program MrBayes [53] to estimate both clock rates and the presence of positively selected sites simultaneously. Specifically the codon model with gamma distributed rates was specified as: lset nucmodel=codon omegavar=ny98 rates=gamma, and report possel=yes site omega=yes. Node Dating was specified using the function "calibrate" to add a fixed sampling time to each sequence. The following priors were set for each data set: prset brlenspr=clock:uniform clockratepr=normal treeagepr=truncatednormal nodeagepr=calibrated. The data analysis was performed using two parallel runs for 3.000.000 generations with a sample frequency of 600. The phylogenetic tree was inferred in a Bayesian framework and with MCMC sampling of posterior probabilities. Tracer version 1.7.1 [46] was used to inspect results and check for convergence of the two MCMC runs. Tree visualization was performed using FigTree version 1.4.4 [54]

Influenza ELISA

All blood samples were tested for antibodies against all Influenza A types using a commercially available blocking ELISA (IDEXX; Influenza A Ab Test; IDEXX Laboratories, Inc.). This test targets a conserved epitope in the nucleoprotein (NP) of influenza A virus. The OD values of the samples were divided by the OD value of the negative control to determine the S/N ratios. Samples were regarded as positive if they had an S/N ratio <0.6 and negative if it had an S/N ration ≥0.6.

Hemagglutinin inhibition (HI)-test

The HI-tests were performed to determine the specific antibody titers of the sows of the 1st and 2nd sampling rounds, against the three different viral strains of the study: the enzootic IAV strain found at the screening test (P5-U4) and two different variants of the epizootic IAV strain – one isolated before (HB4) and one isolated after (VB4) the implementation of mass sow vaccination. To test if the vaccinated sows had indeed been vaccinated, an additional HI-test was performed including an H3N2 isolate, similar to the H3N2sw included in the vaccine. Immune sera raised against Respiporc FLU 3 and against the H1N1 component of the vaccine were used as controls. First, a hemagglutination (HA) test was performed to determine the HA titer of each viral isolate, and four HA-units (HAU) of the viral isolates were used as antigen for the HI-test. The sera were inactivated at 56ºC for 30 minutes and then treated with receptor-destroying enzyme (RDE). Then, the sera were mixed with 50 % erythrocytes to remove specific inhibitors of haemagglutination and agglutination factors. Two-fold serum dilutions were tested against the four isolates, starting at a dilution of 1:20

followed by the addition of 0.6% guinea pig red blood cells. The titers were expressed as the reciprocal of the highest dilution of serum inhibiting the four HAU, and subjected to log2 transformation for statistical analysis. The average mean log2 values were subsequently converted back to average HI-titers. An HI-titer <20 were considered negative.

Statistics

For each sampling round, a statistical analysis was performed comparing the prevalence of IAV-positive and IAV-negative individuals at each sampling time (weeks 1, 3, 5 and 10–12) with the presence of one of the clinical signs registered at the individual level using a Pearson's Chi squared Test. The same test was performed for comparing a difference in prevalence of seropositive and seronegative pigs at week 3 and week 10-12 between the two sampling rounds.

For an overall statistical comparison of means from the normally distributed data (CI, HI-titer, substitution rate and omega values etc.) a Student's t-Test was performed comparing both IAV positive and negative pigs and comparing the results of the 1st and 2nd sampling round [55].

All statistical analysis and graphs were completed using GraphPad Software [55] and Microsoft Excel. P-values below 0.05 were considered statistically significant.

Results

In total, 30 screening samples were collected from two different age groups in the farrowing and nursery unit, respectively. At the 1st and 2nd sampling round 16 sows and 80 ear-tagged pigs were included, respectively. The number of ear-tagged pigs varied slightly between samplings due to mortality or ability to locate the ear-tagged pigs (Table 1).

IAV at the screening test

At the screening test in December 2016, the three- and five-weeks old pigs tested positive for IAV in nasal swabs, while the one-week old piglets and nine-week old weaners were negative.

IAV and IAV antibodies – 1st sampling round (before mass sow vaccination)

Test of serum for antibodies against IAV by ELISA revealed that all of the sows of the four batches were seropositive two weeks before farrowing (Fig 2A). In total, 81 % of the three-week old piglets were seropositive, whereas the number of seropositive piglets decreased to 31 % at week 10-12 (Fig 2A). At week 1, 73.7 % of all the piglets tested positive for IAV in nasal swabs (Fig 2A). One of

these piglets also tested positive at week 3, but the remaining pigs were negative for IAV at weeks 3, 5 or 10-12 (Table 1).

IAV and IAV antibodies - 2nd sampling (after mass sow vaccination):

Test of serum for antibodies against IAV by ELISA revealed that all of the sows in the four batches were seropositive two weeks before farrowing and that 82.3 % of the three-week old piglets were seropositive. Conversely, a significantly lower ($p = 0.006$) number of 10-12 week-old-pigs were seropositive in the 2nd sampling round compared the 1st sampling round (31%). After vaccination, the number of pigs that tested positive for IAV in nasal swabs at week 1 was significantly reduced ($p < 0.001$) as only 9.3% were positive (Fig 2B). However, compared to the 1st sampling round, a significant increased ($p < 0.001$) number of IAV positive pigs at the subsequent three samplings were observed (week 3, 5 and 10-12), since 45.1 %, 54 % and 15.3 % of the pigs tested positive for IAV in nasal swabs at week 3, 5 and 10-12, respectively (Fig 2B and Table 1).

Differences in viral shedding between the 1st and 2nd sampling

The comparisons of the total number of individual pigs that were infected at least once during the study period, the number of pigs that tested positive at two consecutive sampling times, which we defined as "prolonged shedders" and the average Ct value at the different sampling times are shown in Table 2. No statistical significant difference was observed in the total percentage of pigs being infected at least once during the study period between the 1st and 2nd sampling rounds. However, a statistical significant increased number of "prolonged shedders" ($p < 0.001$) was observed at the 2nd sampling round after the implementation of mass sow vaccination. Moreover, a marked significant difference ($p < 0.0001$) was identified in the average Ct values between the two sampling rounds, indicating a significant decrease in viral shedding after implementation of the mass sow vaccination program.

Clinical signs

The average coughing index (CI) for the IAV positive and negative litters/pens at the different sampling times and the presence of nasal discharge at the different sampling times compared to the number of pigs testing positive or negative for IAV are shown for the 1st and 2nd sampling rounds in Table 3 and Table 4, respectively. No correlation was observed between the CI and the number of pigs with nasal discharge and the presence of IAV at the 1st sampling. Conversely, a significant correlation was seen between the presence of IAV and an increased coughing index at week 1, week 5 and over all samplings in the 2nd sampling round. Moreover, a significant correlation between the

presence of IAV and nasal discharge was seen at week 1 and over all samplings in the 2nd sampling round. No significant correlations between IAV and the presence of dyspnea, lacrimation, conjunctivitis, fecal soiling, body condition, limping and hernia were revealed in any of the sampling rounds (data not shown).

Subtyping and strain-characterization – screening test (the enzootic IAV)

The results of the multiplex real time rt-PCR tests revealed that all the samples obtained at the time of the screening test were the H1avN1 subtype. This was consistent with the phylogenetic analyses of the HA and NA consensus sequences. The BLAST results revealed that all the internal genes were of avian-like H1Nx origin. The same subtype had been identified in the herd earlier trough diagnostic samples obtained by the herd veterinarian (personal communication).

Subtyping and strain-characterization – 1st sampling round (before mass sow vaccination)

The multiplex PCR tests revealed that all the samples obtained during the 1st sampling round, prior to mass-vaccination, belonged to the H1avN2sw subtype. This was in accordance with the HA and NA consensus sequences derived from the Illumina and Sanger's sequencing. The BLAST analysis of the internal genes revealed that the M, NP, PB1, PB2 and PA gene originated from the H1N1pdm09 subtype, whereas the NS gene were of avian-like H1Nx origin. A pairwise comparison of all HA sequences (n=18) obtained from pigs sampled before vaccination revealed a close identity with 0-6 nucleotide differences corresponding to a sequence identity of 99.62-100 %. The comparison also included two HA consensus sequences derived from the same pigs at two different sampling times (weeks 1 and 3), indicating that the pigs tested positive for the same strain for minimum two weeks. The full length of the HA gene were not obtained from all of the HA consensus sequences and therefore 21 nucleotides corresponding to seven amino acids were removed from the 5'end the all of the HA consensus sequences before further analysis.

Subtyping and strain-characterization – 2nd sampling round (after mass sow vaccination)

The multiplex PCR revealed that all the positive samples obtained from the 2nd sampling round were of the H1avN2sw subtype. This result was consistent with the HA and NA consensus sequences derived from the Illumina and Sanger's sequencing. Equal to the 1st sampling, the BLAST analysis of the internal genes revealed that the M, NP, PB1, PB2 and PA gene were of H1NXpdm09 origin, whereas the NS gene were of avian-like H1Nx origin. A pairwise comparison of all HA sequences (n=19) from pigs sampled after vaccination revealed a close identity with 0-15 nucleotide differences corresponding to a sequence identity of 99-100 %. The comparison also included seven HA

consensus sequences derived from the same three pigs at two-three different sampling times, revealing that these pigs were positive for the same strain for two-seven weeks. To ensure an equal length of the HA sequences 21 nucleotides corresponding to seven amino acids were trimmed from the 5'end before further analysis.

Comparison of HA consensus sequences obtained at the screening test and during the 1st sampling round

The avian-like HA genes from isolates collected at the screening test and at the 1st sampling round revealed a sequence identity of 87% at the nucleotide level and 89 % at the amino acid level, which equaled 59-61 amino acid differences. The majority of the mutations were present between amino acid Nos. 91-307 (numbering from 1st methionine), which includes the HA1 part of the HA gene, that encodes the globular head and contains the receptor binding site for host-cell-entry. Thirteen of the 54 changes were present in specific antigenic sites (Sa, Sb, Cb, Ca1 and Ca2), including S91N, P141S, D142N, P154S, N159S/R, R172G, R179S, K180R, T181S, K186Q, G187E, S203R and N212D (numbering from 1st methionine).

Comparison of the HA consensus sequences obtained at the 1st and 2nd sampling rounds (before and after mass sow vaccination)

Only minor differences were observed between the consensus sequences obtained at the 1st and 2nd sampling rounds (before and after the implementation of mass sow vaccination). On the nucleotide level, the HA genes were between 99-100 % identical on nucleotide level and 98-100 % identical on the amino acid level, corresponding to 0-8 amino acid changes. The amino acid HA sequences of both the 1st and 2nd sampling round contained a deletion at position 144 and therefore amino acid numbers above 144 would have 1 added to them to correspond to the current H1 numbering, from the first Methionine. Some amino acid differences between the sequences obtained from the 1st and 2nd sampling rounds, were in specific antigenic sites and included S159R (Ca2), which were present in 8/19 sequences from the 2nd sampling, T207A/S/N (Sb) present in 6/19 sequences form the 2nd sampling round and Q210H (Sb) present in 1/19 sequences from the 2nd sampling round.

Comparisons of the NA gene and the internal genes obtained at the 1st and 2nd sampling rounds (before and after mass sow vaccination)

The NA gene sequences of the H1avN2sw strain before and after vaccination were between 99-100 % identical corresponding to up to 14 nucleotide differences. Amino acid differences between isolates from the 1st and 2nd sampling rounds were identified in ten positions, however, the majority

of differences were only found in one or two sequences. The similarity of the remaining genes between the 1st and 2nd sampling rounds varied among genes but was generally high with a maximum of ten nucleotide differences. Only one amino acid difference in the PB1 gene (V724I) were consistent in all the sequences of the 2nd sampling round compared to the sequences of the isolates from the 1st sampling round. The number of nucleotide differences and the location of the amino acid differences in the NA- and internal genes are listed in S1 table.

Viral evolution of the HA gene - 1st sampling round (before mass sow vaccination)

Based on analysis using BEAST, the substitution rate for the HA gene before vaccination was estimated to be 0.00316 substitutions per site per year (SEM = 0.000032) corresponding to an average of 5.04 nucleotide substitutions per year for the entire gene (1596 nucleotides long in this dataset).

CODEML analysis of the 18 HA sequences derived from pigs of the 1st sampling round (before mass sow vaccination) suggested that positive selection was mostly absent. Thus, likelihood ratio testing indicated that M2a did not fit the data significantly better than M1a ($p > 0.05$). In addition, the Akaike weights for M1a and M2a were 0.63 and 0.37 respectively, suggesting somewhat higher support for the model without selection. Under model M2a (which was only weakly supported) there was some evidence for positive selection site at position 203 (probability of being positively selected, Pr+, estimated to be 0.81). The global dN/dS (ω) value was estimated to be 0.31, i.e., on average the gene is under medium strong negative selection (the tendency is to conserve the sequence).

The CODEML analysis makes no assumption about the substitution rate being clock-like (rates are instead free to vary on each branch of the phylogeny). However, since there is evidence that influenza sequences typically do evolve according to a molecular clock, it can be advantageous to use a model that explicitly makes that assumption (and which will then use much fewer parameters). We therefore also analyzed the data using MrBayes, and a model including both a strict clock rate, and a codon-based substitution model for estimating dN/dS rates (S1 Fig). In agreement with the CODEML analysis, there is weak evidence for positive selection on position 203 (Pr+ = 0.54), with an estimated dN/dS = 1.5. The average dN/dS rates for the negatively and positively selected sites were estimated to be 0.29 (negatively selected) and 1.81 (positively selected). Table 5 list estimated dN/dS and Pr+, and also whether the codon is a known antigenic site, for the codons with most support for being positively selected.

Viral evolution of the HA gene - 2nd sampling (after mass sow vaccination)

Using BEAST, the substitution rate for the HA sequences after vaccination was estimated to be 0.00357 substitutions per site per year (SEM = 0.0000176), corresponding to 5.7 nucleotide substitutions per year for the entire HA gene. This is 12 % higher than the rate estimated before mass sow vaccination (significantly different with $p < 0.001$).

CODEML-based analysis of the 19 HA sequences derived from pigs after vaccination strongly supported the presence of positive selection. Specifically, the Akaike weight for M1a and M2a was 0.00035 and 0.9996 respectively, and M2a (which includes a class of positively selected sites) thus has much higher support than M1a. Under model M2a, positions 159 and 207 showed very strong evidence (>99%) of being positively selected. An additional 9 sites were identified as being positively selected with a lower probability (<95%) (Table 5). In agreement with these results, the average dN/dS (global ω) value for the entire HA gene was estimated to be 0.35, and thereby slightly higher than that of the HA sequences before vaccination (0.31).

Analysis using MrBayes (where the model includes both a constant, clock-like substitution rate and a codon-based substitution model for estimating dN/dS) supported the conclusions from the CODEML-based analysis (Table 5 and S2 Fig). Thus, several sites now have support for being positively selected, with substantially higher estimated dN/dS rates. Specifically, the estimated dN/dS rates were 0.05 for negatively selected sites and 4.23 for positively selected sites. The dN/dS value for positive selection was significantly increased (p-value <0.0001) compared to the dN/dS value identified among the sequences before vaccination. The positively selected sites are presented and compared to the CODEML analysis in Table 5. Six of the sites were located in previously known antigenic sites or in B-cell or T-cell epitopes, and included the two codons (159 and 207) which were identified through both analyses, as having the highest probability of being positively selected.

Hemagglutinin inhibition test (HI-test)

The results of the HI-test of the sow sera from the unvaccinated sows during the 1st sampling and HI-titers of the vaccinated sows of the 2nd sampling are shown in Fig 3. The sera was tested against three different virus isolates: P5-U4, which were isolated from one of the screening samples, HB4 which was isolated from one of the nasal swabs from the 1st sampling, and VB4 isolated from the 2nd sampling round. The VB4 isolate had three amino acid changes (S159R, T207S and Q210H) compared to the HB4 isolate. For the HB4 isolate, serum dilutions were only made until 1:640 due to a lack of viral isolate.

The results revealed that all of the sows from the 1st sampling round had antibodies that reacted with the enzootic H1avN1sw strain (P5-U4) (mean log2 = 8.05; mean titer: 266). However, only six of the fifteen sows had antibodies towards the epizootic H1avN2sw strain (HB4) (mean log2 = 7.98/mean titer: 254) isolated before start of mass vaccination and, similarly, only four of the fifteen sows had antibodies towards the epizootic H1avN2sw strain (VB4) (mean log2 = 8.57/mean titer: 381) isolated after mass vaccination. All the vaccinated sows reacted with the enzootic H1avN1 (P5-U4) strain, and with a significant (p-value 0.025) higher titer (mean log2 = 9.26/mean titer: 612) than the sows of the 1st sampling round. Furthermore, an increase in the number of vaccinated sows (11/15) with antibodies reacting against the epizootic strain isolated prior to vaccination (HB2) was observed along with a higher, but not statistically significant, average titer of 364 (mean log2 = 8.5) (p = 0.209). Interestingly, only 9 out of 15 of the vaccinated sows reacted against the strain isolated after start of vaccination (VB4) albeit those that reacted had a high mean titer of 275 (mean log2 = 8.1). The sow sera of the vaccinated sows were also tested against an H3N2sw strain with high level of genetic similarity to the vaccine strain. The results of this test revealed that all vaccinated sows reacted against the H3N2 and with very high titers >1280 (data not shown), indicating that the sows had indeed been vaccinated.

Discussion

In this study, the effect of mass sow vaccination in relation to an outbreak with a new swIAV strain in a previously persistently infected herd was investigated. A unique dataset was collected before, during and after the outbreak. Samples collected during the acute outbreak revealed that the infections with IAV solely occurred in the farrowing unit, with the vast majority of piglets being infected at week 1. This infection pattern clearly changed during the 2nd sampling round, which was conducted after the implementation of mass sow vaccination. A clear delay in onset of infection was observed, as very few piglets were infected at week 1. However, at week 3, almost 50 % of the piglets were positive for IAV in nasal swabs, resulting in a high number of IAV positive piglets at weaning. At weaning (week 4), the piglets were mixed into the nursery, providing new naïve individuals for infection, which was most likely the reason for the peak of infection observed at week 5. The presence of IAV circulation in the nursery unit resulted in the presence of IAV positive pigs at the end of the nursery period, which increased the risk of IAV being transferred into the finisher unit. The delayed infection time and the significantly lower viral load observed in the pigs after mass sow vaccination, suggested that the vaccine provided some level of protection through the transfer of MDAs to the piglets. However, as previously described, the presence of MDA at the time of infection can increase the individual shedding time [8,20,58]. Indeed, a marked increase in numbers

of prolonged shedders, after the use of mass sow vaccination, was observed in this study. This increase in prolonged shedders and the delay in infection time resulted in a higher number of IAV positive pigs being moved around the production system, consequently spreading the IAV to all age groups present in the herd. This observation supports the modeling performed by Cador et al. [19] who concluded that the presence of MDA would extend the IAV persistence within the herd. Another consequence of the presence of MDA at the time of IAV infection can be a suppressed active immune response, which has been described by several studies both in regards to neutralizing antibodies, IgG, IgA and the T-cell responses [20,24–26]. In this study, it was observed that significantly fewer pigs were seropositive at week 10-12 from the 2nd sampling (after mass sow vaccination) compared to the 1st sampling (before mass sow vaccination), suggesting that the presence of MDAs, also in this study, impaired the development of antibodies. The study did not provide data to elucidate if these seronegative pigs are protected against subsequent infection with the same IAV strain despite the lack of measurable antibodies or if they have developed a sufficient memory response. Re-infection with the same subtype after the MDA has declined has only been shown in one study [20] and needs to be investigated further.

The vaccine used in this study is approved for use in sows and pigs older than 56 days. The specific product characteristics (SPC) recommend a basic immunization of two doses applied with three weeks interval to obtain between four-six month immunity according to the age of the pig at the time of vaccination. However, when gestating sows are boosted two weeks before farrowing, the SPC of the vaccine claims to protect piglets against clinical signs of disease the first 33 days of life through transfer of MDAs [59]. In this study, mass sow vaccination was performed, meaning that while the sows included in the 2nd sampling all had received two vaccinations, they did not receive a booster two weeks before farrowing. However, this vaccination strategy is widely used in Denmark, and a protection of the piglets is expected. Clinical registrations were obtained, to reveal a possible clinical protection of the piglets, as a result of mass sow vaccination. Since different age groups became infected during the 1st (week 1) and 2nd (weeks 3-12) sampling rounds it is difficult to compare the results. However, a higher coughing index and the presence of nasal discharge was correlated with the presence of IAV in nasal swabs during the 2nd sampling round, indicating that vaccination of sows did not provide protection against upper respiratory tract infections. Since Denmark is almost free of swIAV of the H3Nx subtypes, antibodies against this subtype can be used as a marker of vaccination with quite high sensitivity, and therefore we could confirm that the sows of the 2nd sampling round had indeed been vaccinated.

The results of this study indicated that mass sow vaccination during an acute outbreak might not be an optimal control strategy if the goal is to protect piglets against infection. However, it should be emphasized that the study was only performed in one herd. Furthermore, it is also important not to undermine the effect the vaccine might have had on protection of the sows, both during the gestation and when entering the farrowing unit, were circulation of IAV thrives [39]. In general, vaccination of naïve herds without IAV circulation is warranted, as an IAV introduction probably will lead to a major outbreak until the herd immunity has been built up. On the other hand, before initiating mass sow vaccination it is important to consider the impacts of the MDAs on the transmission dynamics.

Another important aspect with regard to protection achieved through antibodies was demonstrated in this study, as the herd, which was persistently infected with an H1avN1 strain, had a massive outbreak by an H1avN2sw strain. These two IAV strains have the same avian-like H1 gene, and thereby some level of cross protection was expected. However, it was clear from the 1st sampling round that most of the piglets became infected in week 1, despite most of them likely having received MDAs from their seropositive mothers. Characterisation of the genetic differences of the enzootic strain found at the screening test and the epizootic strain found during the 1st sampling revealed major differences. First of all, the HA genes, while both being of avian-like H1 origin, were only approx. 88 % identical, with several amino acid differences. A large proportion of these differences were located in parts of the HA gene encoding the globular head, and in locations corresponding to specific antigenic sites. Furthermore, the HI-results revealed that there was a weak cross-protection between the two strains, as 100 % of the sows of the 1st sampling round showed a reaction against the enzootic strain H1avN1 as opposed to only 40 % showing a reaction against the epizootic strain H1avN2sw. In addition, differences in the other genes might also have an impact on the level of cross-protection between the two strains. The results indicated that some swIAV strains, belonging to the avian-like H1 clade, have undergone antigenic drift to a degree that has abolished serological cross-reaction. This also raises the question if the avian-like H1 strain included in commercially available vaccines, provides cross-protection to all the different variants of the H1 avian-like subtypes. Indeed, a German study has investigated the genetic and antigenic diversity of the avian-like H1Nx viruses in several European countries, and documented an extensive diversity within this subtype, revealing an antigenic difference of up to ten antigenic units (AU) (personal communication Professor Tim Harder, FLI, Germany). In humans, the seasonal IAV vaccines are updated when the new strain differs four AU from the vaccine strain. Thus, this clearly suggests that a more regular update of strains in IAV vaccines intended for use in swine could be beneficial, but

unfortunately, the European Medical Authority (EMA) does not allow for updates of veterinary IAV vaccines without going through a new licencing.

Differences in evolutionary dynamics were also observed between the viral sequences obtained from the 1st sampling round (before vaccination) and the 2nd sampling round (after mass sow vaccination). The molecular clock-based analysis, which takes sampling dates into account, revealed a significant increase in the nucleotide substitution rate in sequences obtained from pigs originating from vaccinated sows, compared to the ones originating from non-vaccinated sows. Furthermore, analysis using CODEML and MrBayes showed a substantial increase in positive selection (both the number of sites, and the strength of selection) after vaccination. Interestingly, several of the positively selected sites found in the sequences were located in the globular head of HA and in known antigenic sites. An isolate containing mutations in three sites (positions 159, 203, and 207) with strong support for being positively selected, was included in the HI-test. The results of the HI test showed that the number of sows from both the 1st and 2nd sampling that developed antibodies that recognized this isolate was decreased compared to the number of sows that reacted with the isolate without these three mutations. Though not significant, this suggested that the mutations had an impact on antibody binding. In conclusion, the evolutionary analysis clearly showed that an increase in the general substitution rate of the HA gene and positive selection in codons encoding antigenic sites occurred between the 1st and 2nd sampling round and that this had a negative impact on antibody binding. It is known that the global population immunity against human seasonal IAV strains leads to selection of escape mutants [33,36,60,61], but to our knowledge, this has not been described for IAV infection in swine under field conditions. One explanation for the expanded diversity and the positive selection of escape mutants could be that the increase in the infection time of the individual pig we observed after mass sow vaccination increased the likelihood of mutations through viral drift. In the present study, it is not possible to conclude if this change in viral diversity were due to the use of the vaccine or if it was driven by the immunity raised against the circulating field strain. Further studies are needed to explore the impact of herd immunity, vaccination and the generation of escape mutants in swine.

Conclusion

The results of this study provided unique data on a case, where a previously persistently infected herd experienced an outbreak with a new subtype of IAV and it was thereafter decided to start mass sow vaccination. The genetic analysis revealed that differences within the same IAV subtype can lead to a lack of cross-protection toward a similar strain and consequently to an acute outbreak. The presence of MDAs in piglets during infection resulted in a lower viral load, but also in an increased

shedding time and an impaired active immune response to infection. The increased shedding time along with the presence of maternal derived antibodies could be factors driving the positive selection of the HA gene, which in time might lead to the generation of escape mutants.

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Table 1. Number of pigs testing positive for IAV in nasal swabs in the different batches at week 1, 3, 5 and 10-12 during the 1st and 2nd sampling

	Batch 1		Batch 2		Batch 3		Batch 4		Total	
Sampling:	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Week 1	16/20	4/19	12/19	0/20	9/18	3/19	19/19	0/17	56/76	7/75
Week 3	0/18	4/18	0/19	15/20	0/17	6/18	1/19	7/15	1/73	32/71
Week 5	0/18	8/18	0/19	4/20	0/18	14/19	0/19	14/17	0/74	40/74
Week 10-12	0/17	1/18	0/18	3/19	0/14	2/19	0/18	5/16	0/67	11/72

The values are given as the number of pigs testing positive of IAV in nasal swabs out of the total number of pigs sampled at the given sampling time.

Table 2. Prolonged shedders, total number of infected individuals and Ct values of the 1st and 2nd sampling

	1st sampling	2nd sampling	P-value:
No. of prolonged shedders	1.8 % (1/56)	28.3 % (17/60)	<0.001
No. of infected individuals	73.7 % (56/76)	80 % (60/75)	0.467
Average ct value:			
Week 1	25.4	31.3	0.0014
Week 3	20.92	30.7	-*
Week 5	-	31.9	-
Week 10-12	-	32	-
Total:	25.11	31.3	<0.0001

The percentage of prolonged shedders is calculated based on the total number IAV positive pigs during the study. The total percentage of infected pigs during the study is calculated compared to the number of pigs at the beginning of the study. *In the 1st sampling round only one pig was positive at week 3 and therefore no p-value could be estimated for the difference in average Ct value.

Table 3. Differences in mean coughing index between the 1st and 2nd sampling round

	Week 1		Week 3		Week 5		Week 10-12		Total	
Sampling round:	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Virus positive:	0.14	0.13	1.14*	0.37	-	0.23	-	0.02	0.2	0.23
SD:	0.1	0.11	0	0.19		0.2		0.01		0.21
Virus negative:	0.12	0.07	0.6	0.47	0,17	0.08	0.05	0.03	0.27	0.09
SD	0	0.06	0.38	0.4		0.1		0.03		0.18
p-value	0.77	0.06	0.07	0.50	-	0,05	-	0.51	0.49	0.0008

*only one registration as only one litter was positive

Table 4. Differences in the number of IAV positive and negative animals with nasal discharge between the 1st and 2nd sampling

	Week 1		Week 3		Week 5		Total*	
Sampling round:	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Virus positive:	60.7 % (34/56)	85.7 % (6/7)	100 % (1/1)	71.9 % (23/32)	0 % (0/0)	85 % (34/40)	61.4 % (35/57)	79.7 % (63/79)
Virus negative:	80 % (16/20)	39.7 % (27/68)	66 % (48/72)	74.4 % (29/39)	66 % (49/74)	67.5 % (23/34)	68 % (113/166)	56 %(79/141)
p-value	0.2	0.05	0.71	0.97	-	0.14	0.45	0.001

The parentages gives the number of pigs with nasal discharge out of the total number of positive or negative pigs. *Week 10-12 were not included as nasal discharge was difficult to evaluate when using a nasal wire to restrain the pigs.

Table 5. Identification of positively selected sites of the HA gene by MrBayes and CODEML

1st sampling round (before vaccination)				**2nd sampling round (after vaccination)**			
Codon:	ω value: Mb/CO	Pr+: Mb/Co	Antigenic site:	Codon:	ω value Mb/CO	Pr+ Mb/CO	Antigenic site:
22	1.1672	0.3905	-	13	2.1589	0.4676	-
159	1.1689	0.3913	+	**16**	3.2259/7.241	0.7100/0.821	-
203	1.4519/4.709	0.5427/0.806	+	**17**	2.5500/4.835	0.5521/0.53	-
241	1.1772	0.3952	-	**19**	2.5533/4.99	0.5527/0.548	-
385	1.1782	0.3957	-	101	2.2352	0.4841	-*
424	1.1743	0.3938	-	**159**	4.1941/8.612	0.9715/0.999	+
				203	3.0122/5.604	0.6562/0.631	+
				207	4.1222/8.57	0.9431/0.992	+
				210	2.5254/5.47	0.5469/0.605	+
				363	2.2431	0.4858	-
				365	2.6210/5.643	0.5674/0.625	-
				377	2.5860/4.778	0.5600/0.523	-
				420	2.5194/5.023	0.5456/0.552	-^
				471	2.4940/4.98	0.5401/0.547	-^

The number of the codon values are based on numbering from the first methionine in the HA gene and due to the deletion at position 144, values above have been added one. “ω value” gives the dN/dS ratio for the positive selected sites. “Pr+” gives the probability of the codon being positive selected. “Mb” gives the results of the MrBases analysis. “CO” gives the results of the CODEML analysis. Antigenic sites were defined as the previously published Sa, Sb, Ca1, Ca2 and Cb sites of H1 [33,34,36,43,44].The codon highlighted in bold are the codon positions, which were defined as positive selected sites in the both analysis. * located in a B-cell epitope identified in H1N1pdm09 [57]. ^ located in a T-cell epitope identified in human H1[58].

Fig 1. Overview on the timeline of the study in relation to IAV occurrence and vaccination (1A) and the study design (1B)

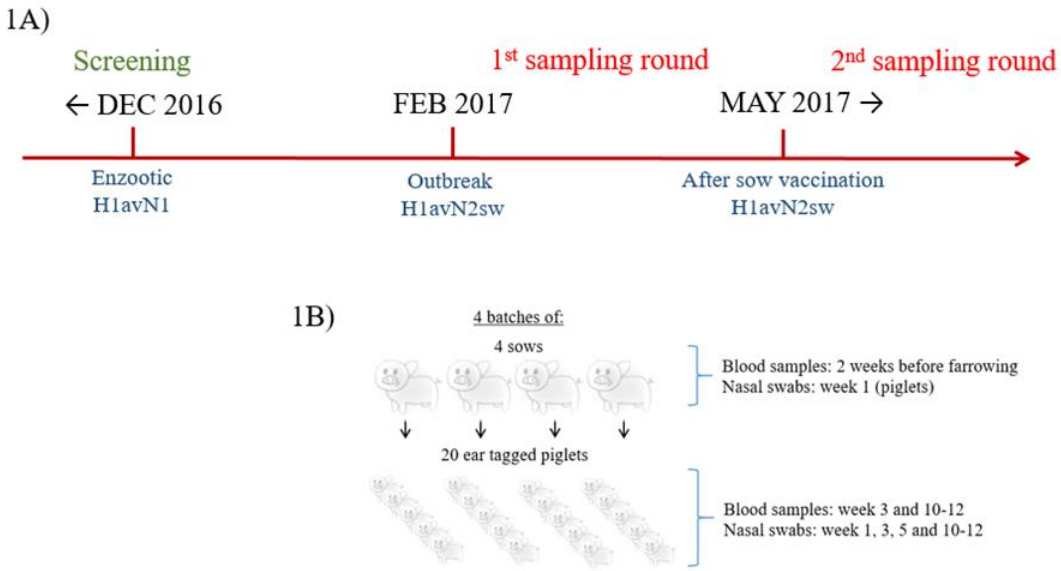
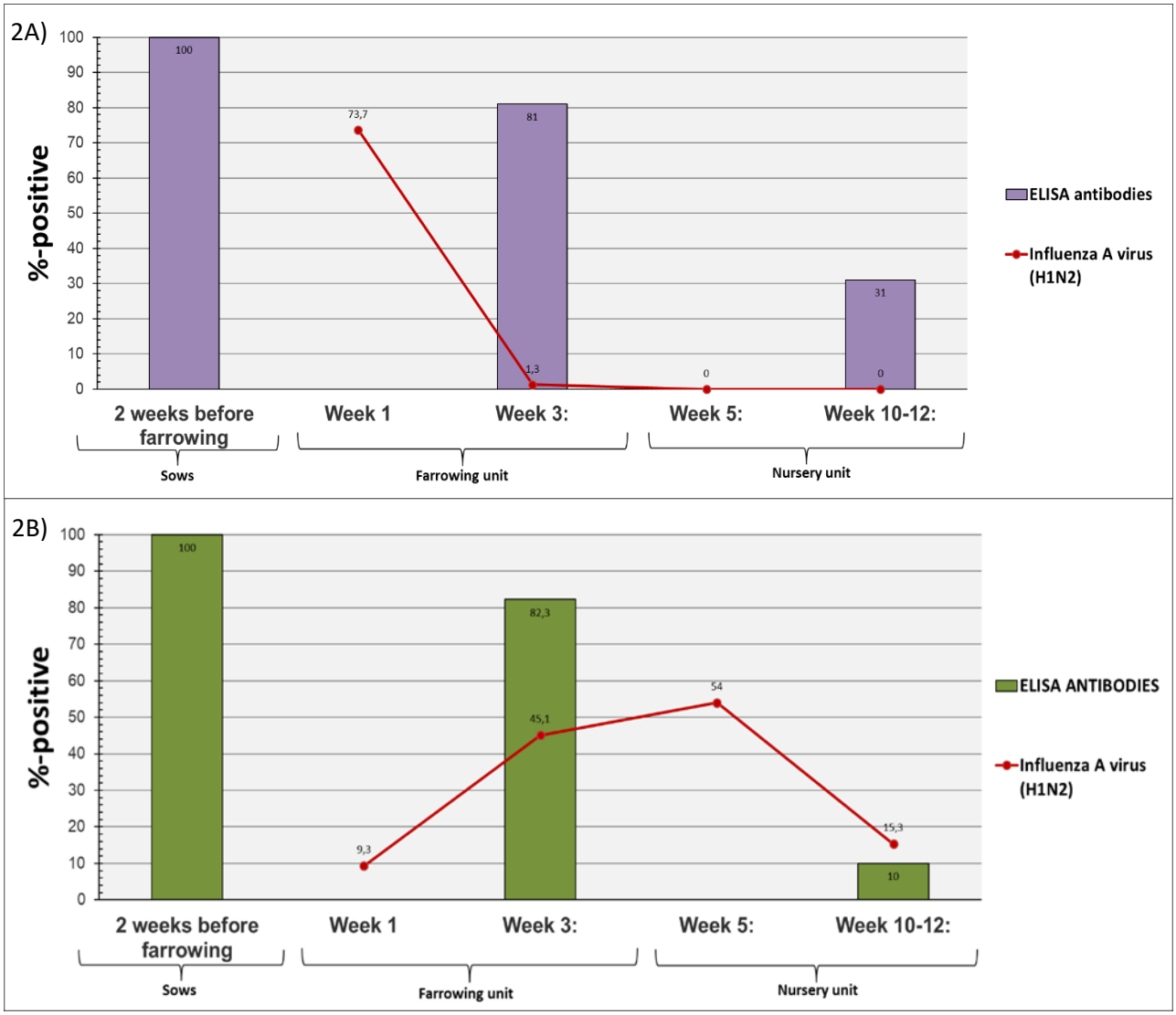
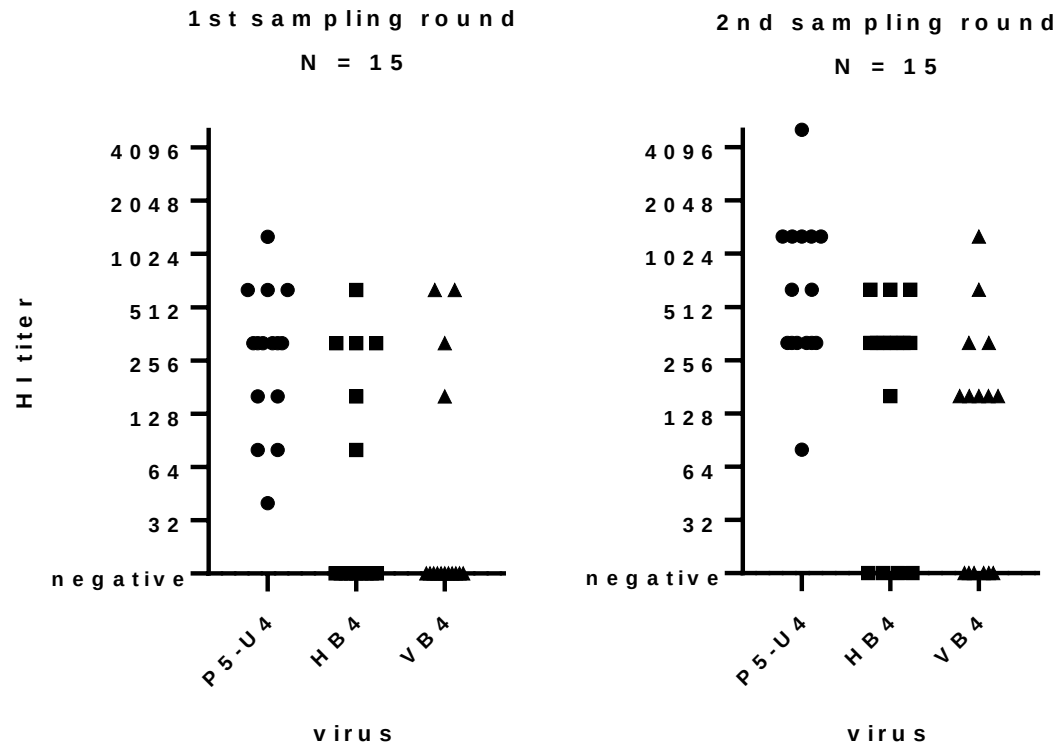


Fig 2. The percentage of seropositive sows and pigs and the summed percentage of the number of pigs testing positive for IAV in nasal swabs at the 1st (2A) and 2nd (2B) sampling round



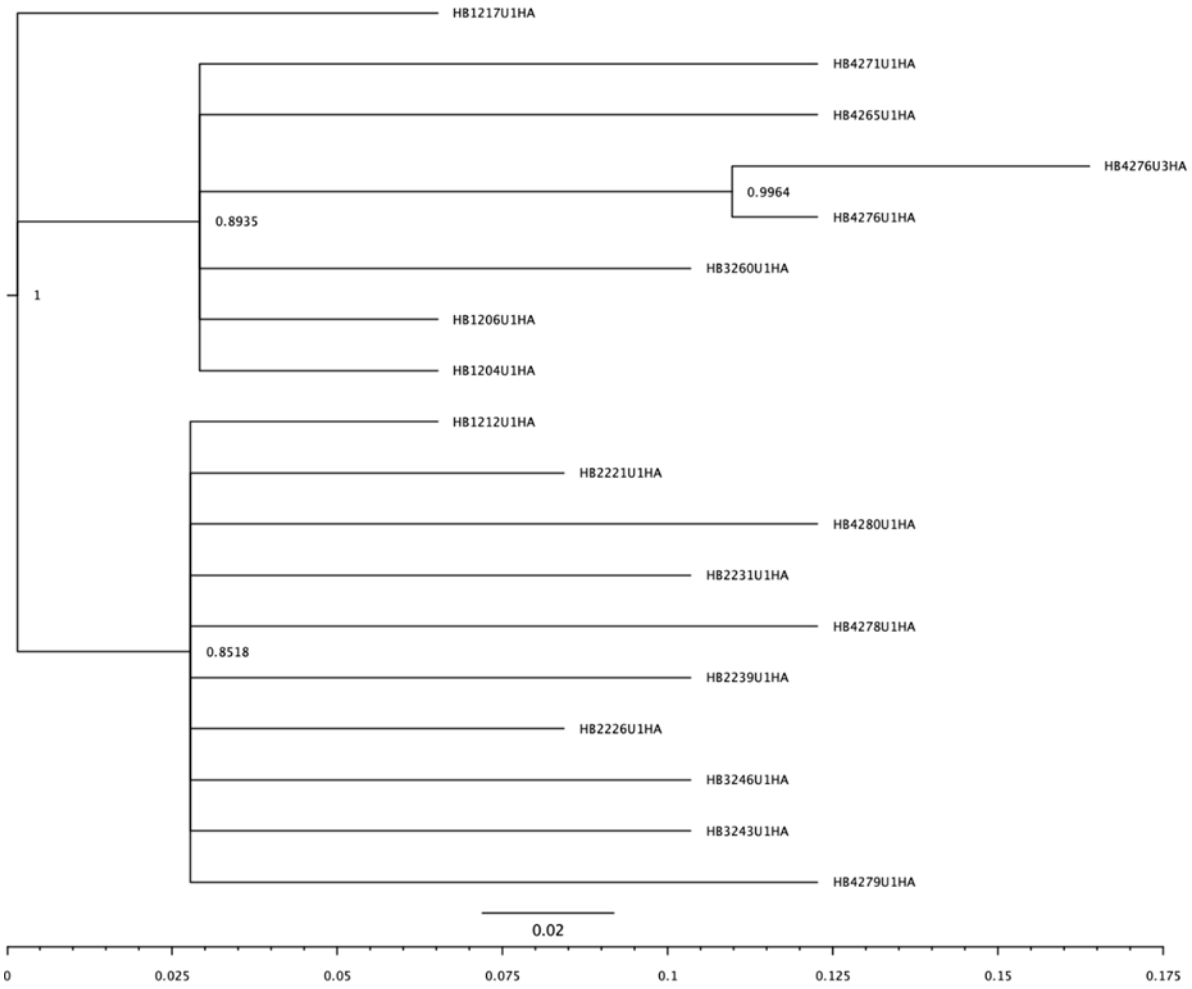
The columns shows the percentage of seropositive sow and pigs. The blood samples were taken 2 weeks before farrowing from the sows and at week 3 and week 10-12 from the pigs. The red line show the summed percentage of pigs at each sampling time (week 1, 3, 5 and 10-12) testing positive for IAV in nasal swabs. "2A" presents the results of the 1st sampling round (before mass sow vaccination), and "2B" presents the results of the 2nd sampling (after mass sow vaccination).

Fig 3. Results of hemagglutination inhibition (HI) test of sow sera collected during the 1st (left) and 2nd (right) sampling round



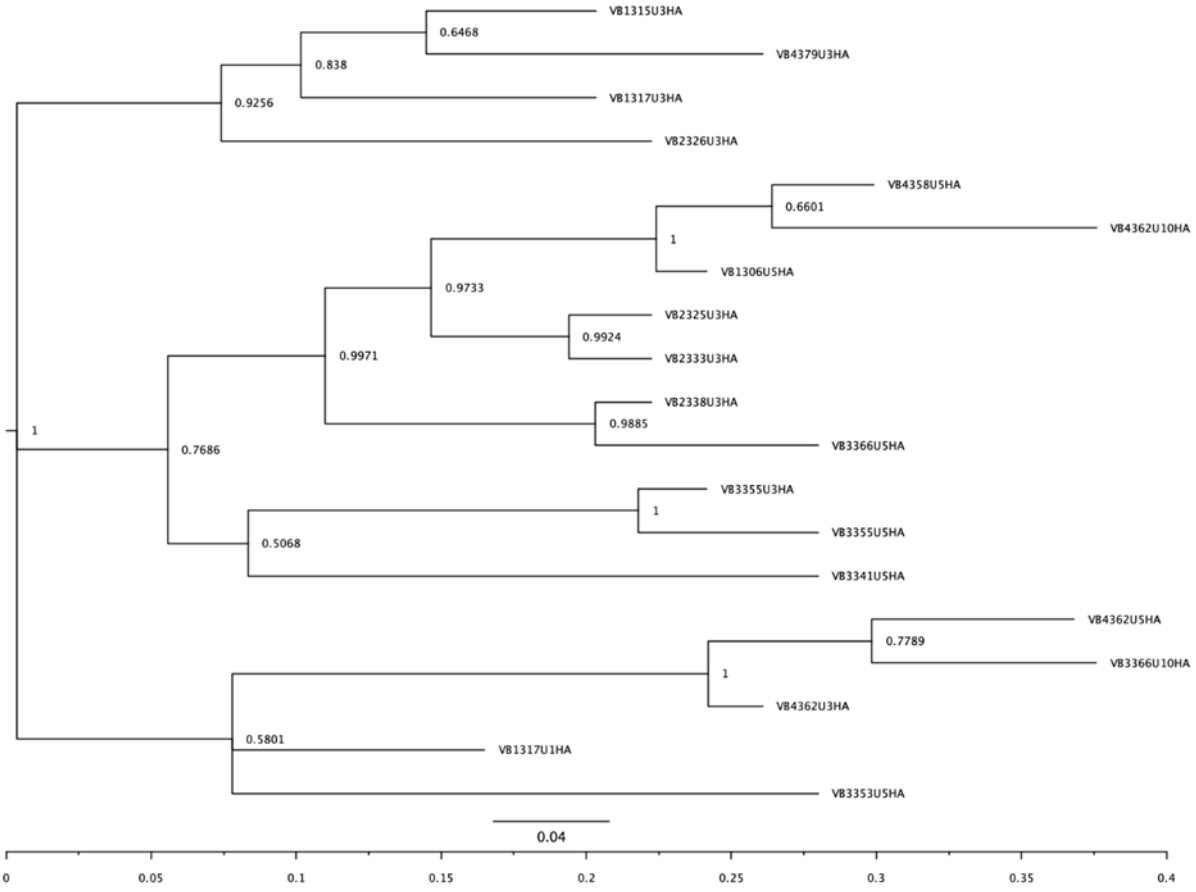
Each sow sera was tested against three different virus: P5-U4 isolated from a pig sampled during the screening; HB4 sampled from a pig during the 1st sampling round and before vaccination and VB4 collected during the 2nd sampling round after start of mass sow vaccination. Negative samples were samples with a HI titer below 20.

S1 Fig. Bayesian strict molecular clock tree of the HA sequences of the 1st sampling



The x-axis represents time in years. Node labels represent posterior probabilities. The sequences are named as follows: HB indicates that the sequences were obtained in the first sampling round, and the following cipher gives the batch-number. The next three ciphers gives the ear tag number of the pig and “U1”, “U3”, “U5” and “U10” indicates the sampling time according to week 1, 3, 5 and 10-12. “HA” indicates that the sequences encodes the hemagglutinin gene.

S2 Fig. Bayesian strict molecular clock tree of the HA sequences of the 2nd sampling



The x-axis represents time in years. Node labels represent posterior probabilities. The sequences are named as follows: VB indicates that the sequences were obtained in the second sampling round, and the following cipher gives the batch-number. The next three ciphers gives the ear tag number of the pig and “U1”, “U3”, “U5” and “U10” indicates the sampling time according to week 1, 3, 5 and 10-12. “HA” indicates that the sequences encodes the hemagglutinin gene.

S1 table. Nucleotide and amino acid differences among NA and the internal genes of the sequences derived from the pigs of the 1st and 2nd sampling

Gene:	Pairwise comparison (nt-difference):	Amino acid differences	Position	No. of seq:
NA	0-14	I→T	56	5/24 2nd
		T→A	71	1/24 2nd
		K→R	75	2/24 2nd
		A→S	88	1/24 2nd
		K→E	93	3/24 2nd
		P→S	340	1/24 2nd
		G→C	381	1/24 2nd
		K→R	403	2/24 2nd
		G→V	414	3/14 1st
		G→S	454	1/24 2nd
NS	0-4	A→S	23	1/5 2nd
		D→N	24	1/5 1st
		R→H	59	1/5 1st
NP	0-8	S→G	351	1/5 1st
		M→L	380	1/5 2nd
M	0-4	-	-	-
PB1	0-10	I→V	368	1/5 2nd
		R→Q	584	1/5 2nd
		V→I	724	5/5 2nd
PB2	0-7	D→E	60	1/5 2nd
		R→S	369	2/5 2nd
PA	0-7	M→I	12	1/5 1st
		I→V	30	1/5 2nd
		I→L	118	1/5 1st and 5/5 2nd
		I→V	330	2/5 2nd
		K→N	360	1/5 2nd
		V→I	432	3/5 2nd
		S→F	709	1/5 1st

The first columns describes the different genes. The second column describes the results of the pairwise comparison performed on the nucleotide consensus sequences. The third column describes the differences in amino acids according to the IUPAC codes. The forth column gives the position according to numbering from the first Methionine. The fifth column gives the number of sequences which had the given mutation compared to total number of sequences obtained from the samplings; 1st = 1^{st} sampling and 2nd = 2^{nd} sampling.

Manuscript 4

Substantial antigenic drift in the hemagglutinin protein of swine influenza A virus

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In preparation

Abstract

Like other RNA viruses, human influenza A virus (IAV) has a high mutation rate. This leads to the gradual accumulation of nucleotide and amino acid substitutions, which occasionally results in the emergence of immune-escape variants that are able to re-infect previously exposed individuals ("antigenic drift"). Historically, outbreaks of influenza in swine herds have had an acute pattern and often resolved within a few weeks and therefore the degree of viral antigenic drift in swine were considered to be much lower than of humans. Concurrently with the increase in herd size, the epidemiology of swIAV has nevertheless changed and swIAV is now enzootic in many herds, which may influence the evolution of the virus. The aim of this repeated cross-sectional study was to assess the persistence and viral evolution of swIAV by analysing monthly nasal swabs obtained from an enzootically infected herd over a one-year period.

At each sampling, 64 nasal swabs were collected from sows, piglets and nursery pigs, and from each sampling, the swIAV sequences were obtained. For each sampled litter/pen a coughing index was calculated.

We found that swIAV persisted in the herd throughout the one-year period, since the virus was detected in at least one sample from each month. The highest prevalence of swIAV was observed in three-week-old-litters while the highest viral load was identified in one-week-old litters. We found that 33 % of the included sows tested positive for swIAV in nasal swabs, suggesting that they played an important role in the transmission. Phylogenetic analysis found the evolution of the HA gene to be largely clock-like (mutations accumulate at a roughly constant rate), suggesting that most observed mutations were neutral. The phylogenetic tree revealed a pectinate topology typical of human influenza A virus, indicating repeated genetic bottlenecks where a single immune-escape mutant was selected and became the ancestor of the next wave of infection. The importance of immune-escape was further emphasized by evidence of positive selection in known antigenic sites including both B-, and T-cell epitopes. We conclude that sustained antigenic drift similar to that seen in humans, should be considered also for swine adapted viruses.

Introduction

Novel influenza A viruses (IAV) can develop through two different mechanisms; genome reassortment (antigenic shift) and gradual accumulation of mutations (antigenic drift). Genome reassortment occurs as a consequence of the segmented genome of IAV, when RNA segments originating from different subtypes/strains are mixed during assembly of progeny virions, leading to

the formation of new subtypes/strains that may have novel antigenic properties [1,2]. Antigenic drift is a much slower process where the error prone RNA polymerase causes misincorporation of nucleotides during genome replication [3,4]. Mutations in coding regions of the viral genome are either synonymous or non-synonymous. Non-synonymous mutations occurring in immunogenic epitopes can undergo positive selection driven by host immunity, and may lead to the virus escaping e.g. neutralizing antibodies. The major epitopes of IAV, also termed antigenic sites, are located on the globular head of the hemagglutinin (HA) molecule, which is encoded by the HA1 domain. Several antigenic sites has been identified for both the H1 and H3 subtypes [5–10]. For humans, positive selection in these sites has been documented [11–14], and as little as one mutation in an antigenic site has been shown to affect the vaccine effectiveness [15,16]. Human influenza vaccines are therefore, evaluated twice a year to prevent mismatches between vaccine strain and circulating strains [17].

The rate of antigenic drift of swine IAV (swIAV) has generally been believed to be much lower than that of human IAV, mainly due to the short lifespan of pigs and the acute nature of the infection historically seen in pig herds, which limits the impact of pre-existing immunity [3,18–22]. Consequently, the swine influenza vaccines are updated less frequently [23]. Previous studies on the antigenic drift of swine hemagglutinin of the H1 or H3 subtypes, has mainly focussed on the global or national evolution [19,24–29]. For the H1 subtype in swine the nucleotide substitution rates have been estimated to range between 1.9-4.4 x 10^{-3} per site per year [11,30–33], whereas the nucleotide substitution rate of the swine H3 subtype has been documented to be as high as 6 x 10^{-3} per site per year [34]. While the highest rates are comparable to that of human H1 subtypes, the selection pressure expressed as the ratio of nonsynonymous to synonymous mutations has been found to be lower in swine compared to humans [11,22,35,36].

Over the past ten years, the understanding of swIAV circulation in swine herds has changed and it is now recognized that an infection with swIAV is likely to result in an enzootic infected herd [37–44]. This is probably a consequence of increasing herd sizes, which provides a continuous flow of naïve piglets [45,46]. The possible impact that herd level persistence of IAV might have on antigenic drift over time in a specific population of pigs, has, to the best of our knowledge, never been investigated. However, we believe it is highly important to get an increased understanding of antigenic drift occurring within single herds, as it can help explain the high genetic diversity within swIAV lineages documented in large-scale investigations and surveillance programs [25–27,29,47,48]. Importantly, if positive selection comparable to that observed in human IAV occurs in swine IAV, the possible effects on herd immunity and vaccination, should be taken into consideration when designing

vaccines and evaluating swIAV control programs. We here report the results of a repeated cross-sectional study where we investigated the dynamics and viral evolution within a single sow herd over a one-year period.

Materials and methods

Herd description

The herd consisted of 480 sows and 2000 nursery pigs. The farrowing stables were divided into two units and had no sectioning between different age groups, with weekly farrowings. The farrowing unit was cleaned once a year, without the use of disinfectants. At four weeks of age, piglets were weaned into a heated nursery. The nursery stables contained seven separate rooms with separate airflow, and all rooms were cleaned and disinfected between batches. The nursery pigs were housed in the nursery, until they were sold at approx. 30 kilos. Gilts were recruited internally and were subjected to eight weeks of quarantine from 12 weeks-of-age. Thus, no pigs were introduced into the herd during the study period. According to the Danish Specific Pathogen Free program [49], the herd was free from infection with *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* serotype, 2, 6, and 12, PRRSv type 1 and 2, *Brachyspira hyodysenteriae*, *Pasteurella multocida*, *Sarcoptes Scabiei* var. *Suis* and *Haematopinus suis*. The herd used a low number of nursing sows and minimized cross-fostering of piglets. No vaccination against IAV was performed. The herd experienced recurrent respiratory symptoms in both the farrowing and nursery unit, and tested positive for IAV in July 2017, where the subtype "H1avN2sw" was diagnosed by full genome sequencing.

Study design

Nasal swabs were collected monthly from November 2017 to October 2018. The nasal swabs were obtained from 20 piglets from four one-week-old litters (five piglets per litter), 20 piglets from four three-week-old litters (five piglets per litter) and 20 pigs from four pens with five-week-old nursery pigs (five pigs per pen). In addition, nasal swabs were collected from each sow of the one-week-old litters, and the parity of the sows was recorded. In total 64 nasal swabs were collected each month corresponding to 768 nasal swabs obtained over the full year. The individual samples and sequences were given an ID ranging from F1-F12 according to which month they were sampled, F1 being the first month (November 2017) and F2 being the second month etc. Moreover, the sample ID also included the age of the pigs, W1, W3 and W5 indicating week 1, 3 and 5, respectively. In addition,

sequences obtained previously in July 2017, was included in the genetic analysis and named "W00_W1_01".

Sampling

Nasal swabs were collected from the sows and piglets using a large or small rayon dry swab (Medical Wire, UK), respectively. The swabs were inserted into each nostril and turned 360 degrees. Thereafter, the swab was inserted in a tube containing 1ml Sigma Virocult media (Medical Wire, UK) and kept at 2-8°C for maximum 24 hours before being processed.

Coughing index

For each litter/pen, included in the sampling, a coughing index was calculated, as described in a previous study [44]. Briefly, the coughing index was calculated by dividing the total number of coughs and sneezes with the number of pigs in the litter/pen multiplied by the time observed (three minutes).

Pooling and RNA extraction

All the nasal swabs from pigs were initially pooled prior to extraction. Subsequently, the two most positive pools of each sampling time were identified, and the individual nasal swabs of the pools were subjected to RNA extraction and real-time RT PCR, to identify samples for viral isolation and sequencing. All nasal swabs were mixed using a Vortexer, and 100µL was extracted for the pool. All individual samples (excluding the samples from sows) were pooled litter-wise, with five nasal swabs in each pool. The pools were mixed and centrifuged. Subsequently, 200µL suspension was transferred to a new tube containing 400µL RLT-buffer (QIAGEN, Copenhagen, Denmark) with 2-Mercaptoethanol (Merck, Darmstadt, Germany). Subsequently, the RNA was extracted using the RNeasy mini kit (QIAGEN) automated on the QIAcube (QIAGEN) according to large sample protocol version 2.

Real-time RT PCR

In order to determine if a pool was positive for IAV, a previously published real-time RT PCR assay targeting the matrix gene of IAV [50] was adopted. Briefly, the published primers and the OneStep RT-PCR kit (QIAGEN) was used for the PCR mix, which was subsequently run on the Rotor-Gene Q (QIAGEN) using the following program: 50°C, 30 min; 95°C, 15 min; cycling 45x (95°C for 10 secs, 60°C for 20 secs, 64°C for 1 sec, 68°C for 1 sec, 72°C for 30 secs). All samples were run in duplicates and the pool was only considered positive if both samples gave a positive result and had a

Ct value < 36. All positive samples with a Ct value < 31 were tested to determine the IAV subtype using the previously published multiplex real-time RT PCR assay [51] with the modifications described in a previous study [44] and run on the Rotor-Gene Q (QIAGEN). The individual samples of two most positive pools of each sampling were also tested. Positive individual samples were selected for viral isolation and sequencing.

Viral isolation and NGS

The monthly nasal swab with the lowest Ct value was selected for viral isolation. The nasal swabs were first subjected to sterile filtration using a 0.45µM Millex-HP Millipore filter (Merck, Germany) and then grown in Madin-Darby Canine Kidney (MDCK) cells under the conditions as described in a previous study (Manuscript 2). After incubation, the RNA was extracted from the supernatant of each cell isolate using the same method as described above, however performed manually. Subsequently, the RNA was subjected to PCR amplification of each IAV segment and prepared for sequencing on the Illumina MiSeq platform using the methods described in a previous study (Manuscript 2).

HA and NA amplification and Sanger sequencing

Additional individual nasal swabs, which had Ct values < 31, of each sampling were subjected to HA and NA PCR amplification and subsequent Sanger sequencing using the same methods as described in a previous study (Manuscript 2).

Consensus sequence generation

The determination of consensus nucleotide and amino acid sequences based on the Illumina and Sanger sequencing data was done as previously described (Manuscript 2) using the program CLC genomics Workbench version 11.0.1 and CLC main workbench version 8, respectively.

Characterization of the herd swIAV strain

The nucleotide and amino acid consensus sequences of each of the eight gene segments were aligned using the MUSCLE algorithm [52] in CLC main workbench version 8. The sequences of the alignments were compared using the pairwise comparison tool. In addition, the lineage of each gene segment was determined by aligning the respective sequences with contemporary swIAV sequences obtained in the Danish swine IAV surveillance program and subsequently neighbor-joining trees were constructed. Furthermore HA amino acid sequences were annotated for known antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) [5,6,8,53,54], B-cell epitopes [55,56] and T-cell epitopes [57–60], which

were then manually checked for variation. Potential asparagine-linked glycosylation sites of the HA protein was predicted by the program NetNGlyc 1.0 Server [61].

Molecular clock analysis and positively selected sites

Neighbor joining trees were constructed for each of the eight gene segments using the CLC main workbench version 8 software. The eight gene segments obtained from the diagnostic sample of the same herd sampled approx. 4 months earlier (July 2017), were included in all analyses and used as an outgroup. The resulting tree, including information on sampling dates, was subsequently checked for the presence of a temporal signal (i.e., whether nucleotide changes accumulate proportionally to elapsed time) using the program TempEST [62]. Thereafter, the software package BEAST2 version 2.5.2 [63] was used to determine the substitution rate of each of the eight gene segments. Specifically, the substitution model was specified to be HKY with gamma distributed rates over sites, with a strict clock model, and using tip dates (sampling dates). The following priors were specified: The tree model was set to "Birth Death Skyline Serial", which is used when lineages are sampled sequentially through time. The reproduction number was set to be between 0 and 10 with a log normal distribution. The "BecomeUninfectiousRate" was estimated to be approximately 52 per year (corresponding to an average time being infectious of 1 week) with a log normal distribution and $CI_{95\%}$ = [44.4-224]. The clock rate was set as a log normal distribution with a mean value of 0.001 and, which is estimated to be substitution rate of RNA viruses, with a $CI_{95\%}$ = [3.95×10^{-5}-0.005]. The gamma shape prior and the kappa prior were left at the default values. A gamma distribution of the "origin prior" was chosen with an alpha value of 0.5 and a beta value of 2. Lastly, the sampling proportion prior was set to a log normal distribution with a mean value of 0.001 and $CI_{95\%}$ = [3.95×10^{-5}-0.005]. The chain length was set to 10.000.000 with a log every 1000, and the MCMC was run twice. The program BEAUti [63] was used to set up the analysis with all priors. Summaries of results and checking of MCMC convergence was done using the program Tracer, version 1.7.1 [64].

The program CODEML of the PAML package [65] was used to identify positively selected sites in all 8 genome segments. Specifically, we did this by comparing the fits of CODEML's substitution models 1a (M1a) and 2a (M2a) (NSsites = 1 and 2). These substitution models include parameters for the ratio between the rate of non-synonymous substitutions per non-synonymous sites and the rate of synonymous substitutions per synonymous site (the dN/dS ratio, also indicated by ω). A dN/dS ratio above 1 indicates positive selection (there are more amino acid changing substitutions then expected). M1a includes two categories of codons – some under negative selection (dN/dS ratio < 1)

and some codons where mutations are neutral (dN/dS ratio = 1). The model M2a includes 3 categories of codons – the same two as M1a plus an additional category of codons under positive selection (dN/dS ratio > 1). If M2a fits a dataset significantly better than M1a, then there is evidence of positive selection in some codons (and the identity of these codons is also found during model fitting). The fit of each model was compared using the Akaike Information Criterion (AIC) and likelihood ratio tests [66,67]. In addition, the average dN/dS ratio (global ω ratio) of all HA sequences was also estimated using CODEML (NSsites = 0).

The program MrBayes [68] was used for reconstructing clock-based phylogenetic trees using codon-based substitution models, allowing simultaneous estimation of clock rates and detection of positively selected sites for the HA and NA gene segments. Specifically the codon model with gamma distributed rates was specified as: lset nucmodel=codon omegavar=ny98 rates=gamma, and report possel=yes site omega=yes. Node Dating was specified using the function "calibrate" to add a fixed sampling time to each sequence. The following priors were set for each data set: prset brlenspr=clock:uniform clockratepr=normal treeagepr=truncatednormal nodeagepr=calibrated. The data analysis was performed using two parallel runs for 3.000.000 generations with a sample frequency of 600. The phylogenetic tree was inferred in a Bayesian framework and with MCMC sampling of posterior probabilities. Each data analysis was performed in two parallel runs of 3.000.000 generations with a sample frequency of 600. The subsequent results were visualized using Tracer version 1.7.1 [64] and FigTree version 1.4.4 [69].

Statistics

Student's t-test was used to investigate if the average coughing index was significantly different between the IAV positive and IAV negative litters/pens. A chi-squared test was used to evaluate if 1st parity sows were more likely to be IAV positive compared to older sows, and the same test was also used to test if IAV positive sows were more likely to have an IAV positive litter compared to the IAV negative sows. All calculations were done using the GraphPad Software [70]. A likelihood ratio chi-squared test was used to test if M2a fit the data significantly better than M1a (indicating the presence of positively selected codons) [71]. Statistical significance was considered when the p-value was below 0.05.

Results

Presence of enzootic IAV

The results of real-time RT PCR targeting the matrix gene revealed that IAV was present at all monthly samplings. Some variations were however observed between months: for example very few litters/pens tested positive at F11 (September) and the Ct values of the sampled did not allow for sequencing (Table 1 and Figure 1). The results of the test of the pooled samples of each month, showed that 60 % of the one-week old litters, 69 % of the three-week old litters and 60 % of the pens with five-week old weaners tested positive over the entire study period. In total, 16 of 48 (33%) sows tested positive for IAV in the nasal swabs over the study period. The majority of IAV positive sows also had a positive litter (14/16). The prevalence of IAV positive litters from IAV positive sows (88%), was significantly higher than the prevalence of IAV positive litters from IAV negative sows (50 %) ($p = 0.03$). For first parity sows, seven of 15 (47 %) tested positive for IAV in nasal swabs as opposed to only nine of 33 (27 %) of the $\geq 2^{nd}$ parity sows. However, this difference was not significant (p value = 0.32).

Correlation between IAV and the coughing index

For each age group (weeks 1, 3 and 5) the average coughing index (CI) of the IAV positive and negative litters/pens, was calculated (Table 2). Overall, the mean CI was significantly higher in litters/pens that included at least one pig testing positive for IAV in nasal swabs (p value = 0.03). No significant differences were discovered within the individual age groups, but a tendency towards a higher coughing index in the IAV positive litters was most evident at week 1 (p value = 0.07).

Herd strain characterization

In total, ten full genome sequences were obtained from cultured isolates based on individual monthly samples. However, it was not possible to obtain viral isolates from F7 and F11. Full genome sequences from one sample obtained in July 2017 were additionally included. Moreover, 19 HA and NA sequences were obtained from RNA of individual nasal swabs of all samplings except from F11. Thus, in total 30 HA and NA sequences were generated, including 1-4 sequences from each sampling time (except F11).

The herd swIAV isolated throughout the study was of the H1N2 subtype, with a HA gene of Eurasian avian-like origin, and a NA gene of the swine adapted Hong Kong H3N2 origin. All gene segments of the internal gene cassette were of Eurasian avian-like origin. The HA gene segments had

a pairwise nucleotide sequence identity ranging between 98.6-100 % and, similarly, the NA gene segment had a pairwise nucleotide sequence identity ranging between 98.5-100 %. The M, NP, PA, PB1 and PB2 gene segments had a pairwise nucleotide sequence identity ranging between 99.2-100 % respectively, whereas the NS gene segments had a pairwise nucleotide sequence identity ranging between 97.5-100 %. All sequences will be available in NCBI Genbank.

Phylogenetic analysis and substitution rates

The TempEst analysis revealed that all eight gene-segments, but especially the HA gene (correlation coefficient 0.95), showed association between genetic divergence through time and sampling dates indicating that a phylogenetic molecular clock-analysis (using BEAST and MrBayes) was suitable for the sequences (Table 3). Using BEAST the nucleotide substitution rate for the HA segment was estimated to be 7.6×10^{-3} substitutions/site/year, corresponding to 13 nucleotide substitutions per year for the entire gene (which is 1698 nucleotides long in this dataset). Estimated substitution rates were also high in the NA and NS segments (6.9×10^{-3} and 5.7×10^{-3} substitutions/site/year respectively), while the remaining segments had substitution rates ranging from 1.1 to 2.9×10^{-3} substitutions/site/year.

Interestingly, the phylogenetic tree based on the HA sequences displays the same imbalanced (so-called "comb-like" or pectinate) topology as that which is typical for human influenza trees spanning multiple years. The main feature of this type of topology is the repeated bottlenecks where only a single lineage persists and forms the ancestor for subsequent lineages (Figure 2). This is most likely caused by repeated selection of a single immune-escape variant that becomes the founder of the next wave of infection. The phylogenetic tree based on NA sequences had a somewhat comb-like topology also (Figure 3), however, with fewer bottleneck events (although firm conclusions in this regard is made difficult by greater uncertainty about branching pattern, and the resulting high level of polytomies in the tree).

Positive selection

The program CODEML from the PAML package was used to test if positive selection was present in the eight gene segments. The results showed that the M2a model (indicating the presence of positive selection) fitted the HA sequences significantly better, whereas this was not the case for the remaining genes (Table 3). Estimates of dN/dS ratios for individual codons in the HA gene, under the M2a model, strongly indicated the presence of positive selection at position 553 (numbering from first methionine) in the HA2 part of the gene, which encodes the stalk region. Further analysis of the

HA amino acid alignment showed that a mutation from tryptophan to arginine at position 553 was present in four pigs at F4, F6 and F8. Interestingly, this specific position is located in a B-cell epitope identified in the A(H1N1)pdm09 virus and in a T-cell epitope identified among human seasonal H1N1 (Table 4). The average dN/dS ratio of the HA sequences was estimated to be 0.19, while the value for codon 553 was estimated at 1.6, supporting that this position was under positive selection. Analysis of dN/dS ratios for individual codons using MrBayes identified several additional sites having a dN/dS ratio below 1, but significantly higher ($p < 0.05$) than the average dN/dS ratio (0.19) (Table 4). The majority of these mutations were present in the HA1 part of the HA gene and included seven mutations in known antigenic sites and several of the positions were in other known B- or T-cell epitopes. Further investigation of HA sequences identified seven mutations (D142N, P154L, K172R, V233D, E239K, H300Y and I421F), that all showed a clear temporal pattern, where the given mutation became established at one time point and remained in all the following sequences until the end of the study.

Discussion

This study documented IAV persistence within a swine herd over a one-year period, supporting the increasing number of studies that have shown that IAV may persists within the herd [37–44]. IAV was abundantly present in the one-week old litters, which in addition was the age group that showed the highest average viral load. These findings supports the results of a pervious study performed by us, which identified IAV in nasal swabs of piglets from three days of age [44]. In general, a high percentage of litters were positive for IAV in the farrowing unit, probably as a consequence of only two farrowing stables being available, meaning that new-born piglets were housed side-by-side with piglets ready for weaning at 4 weeks of age. In turn, this provided an optimal environment for IAV transmission, as new naïve individuals were readily available for infection. The relatively high percentage of sows (33.3 %) that tested positive for IAV in nasal swabs in this study, suggested that the sows had an important role in the transmission dynamics. In addition, a significantly higher number of IAV positive sows also had an IAV positive litter, which suggested a transmission from sow-to-piglet or piglet-to-sow. These findings are in accordance with previous work [42–44,72], although more studies are needed to firmly determine the directionality of transmission between sows and piglets. The high number of sows found positive for IAV in this study and the fact that they were positive for approx. 1.5-2 weeks after being introduced into the farrowing unit, emphasize the importance of stimulating sow immunity, especially before entering an environment where IAV is circulating. If the sows are inadequately immunized when entering the farrowing unit, there is a high risk of IAV infection occurring a few days before birth, which potentially could lead to birth

complications and a lower milking yield, which in turn will result in compromised animal welfare and production economy. First parity sows were overrepresented among the IAV positive sows, even though this correlation was not statistically significant. In the herd investigated here, the internally recruited gilts were kept in a quarantine stable for eight weeks, and were thereby not exposed to the herd strain several weeks prior to re-introduction into the sow-herd. This could explain why the gilts seemingly were more prone to IAV, compared to the older sows continuously exposed to the herd strain. This further highlights that proper gilt immunization is important, through either natural exposure or vaccination.

The results of the study also highlighted the importance of herd management in the control of viral diseases. The same MCREBEL principles, which are widely used in controlling PRRSv infection [73], could also be a helpful tool in the prevention of IAV transmission within the herd. A clear sectioning and all in/all out management of weekly batches in the farrowing unit would most likely limit the IAV transmission significantly in the present herd.

The overall mean coughing index (CI) was significantly higher in IAV positive litters/pens. This supports our previous findings [44] which found a significant correlation between the CI and the pen/litter testing positive for IAV in nasal swabs. In turn, CI could be a helpful tool in identifying IAV positive litters/pens for diagnostics. Furthermore, the correlation to clinical symptoms of respiratory disease underline that IAV has an impact on the health and welfare of infected pigs.

Throughout the study, all eight genomic segments of the circulating H1avN2sw strain were highly similar (98.5-100 %) to the initial consensus sequence, supporting that only a single IAV variant was circulating in the herd during the study. This is consistent with the information that the herd operated as a closed herd with no import of pigs, as the gilts were internally recruited. In turn, this provided the optimal scenario to study the viral drift within a single IAV strain, as the risk of reassortment events was limited. A correlation between sampling time and genetic diversity was found for all eight segments of the IAV strain, with the strongest temporal signal being present in the HA gene. The phylogenetic tree based on HA amino acid sequences had the distinct comb-like topology also known from human HA sequences, with a main trunk that represents the pathway of advantageous (mostly immune escape) mutations, which have been selected over time. Conversely, the shorter site branches represents the isolates that died out, because they were not able to avoid the host immunity [16,74–76]. Likelihood-based analysis using CODEML, confirmed the presence of positive selection in the HA gene. Further inspection of the HA amino acid alignment showed how amino acid mutations at specific positions arose at a given time point and remained until the end of the study,

consistent with these amino acid changes being advantageous. All the mutations manually identified corresponded to positions in which the dN/dS ratio was estimated to be higher than the average (using Bayesian analysis). Several of these positions were located in previously known antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) or in other known B or T-cell epitopes, suggesting that positive selection occurred in immunogenic important sites. The single positively selected site (dN/dS ratio > 1) identified by the CODEML and Bayesian analysis, was located in the HA2 subunit of the HA gene, which encodes the stalk region. Interestingly, this position was included in a B-cell epitope proposed by a previous study investigating the A(H1N1)pdm09 subtype. In this study the reaction of the peptide encoding the epitope against a panel of swine sera raised against a panel of H1 and H3 subtypes were tested and showed a reaction, indicating this epitope to be highly immune reactive in pigs [56]. Moreover, the position was also included in a T-cell epitope defined in the human seasonal H1N1 subtype [57]. The results of the two studies indicate that the positive selection identified in position 553, could have an impact on the immunity, and thereby be important for immune escape variants of swIAV.

The nucleotide substitution rate calculated for the HA gene in this study exceeded the nucleotide substitution rate of 1.9 to 4.37 x 10^{-3} substitutions per site per year for swine H1Nx subtypes reported in previous studies [11,30–33]. The nucleotide substitution rate was also markedly higher than that reported for human H1N1 [11,77,78]. However, it is a well-known phenomenon, that when sequentially viral samples are collected over a short period of time, the clock rate is often estimated to be much higher compared to values estimated from samples collected over a longer period of time [79–81]. Therefore, it is difficult to compare the within-herd substitution rate calculated in this study, with the overall substitution rates calculated previously based on samples collected over several years and originating from many different herds or people. However, the nucleotide substitution rate of the NP gene corresponds to the results of an earlier study [82]. The average dN/dS (global ω ratio) of the HA sequences of this study was similar to that found in two studies investigating human H1N1 subtypes (ω ratio: 0.18-0.21) [83,84], but lower than that documented in two other studies (ω ratio: 0.24-0.38) [11,12]. The results of our study need to be confirmed by additional within-herd studies of the viral evolution, however, the data obtained in the present study suggested that the within herd evolution of swine IAV are comparable to that of human seasonal IAV. The rate of nucleotide substitution was high, and there was clear evidence for positive selection on the HA gene, especially in epitopes important for the adaptive and cellular immune response. Moreover, the topology of the phylogenetic tree indicated that immune escape variants were selected over time. Consequently, the results further confirmed that the antigenic drift of IAV in swine is comparable to seasonal human

IAV. The extensive antigenic drift with generation of escape variants could possibly lead to acute clinical swIAV outbreaks even in herds without an external IAV introduction. In addition, the intensive viral drift could also have a negative impact on vaccine efficacy, however, this need to be studied in more detail. Nevertheless, it is recommendable for all herds to get their IAV strain/s sequenced. Then, the herd veterinarian will have an additional tool in explaining disease developments and it will contribute to our general understanding of the viral evolution of IAV in swine. From a human health point of view, it is also important to understand the viral evolution of IAV in swine herds, as they represents a reservoir for generation of future human pandemics, as demonstrated by the 2009 human pandemic [85,86]. The risk of generating new human pandemics will probably increase as an increasing number of herds becomes persistently infected, keeping a constant evolving reservoir of IAV circulating in swine.

In conclusion, the present study confirmed other recent studies [39,43,45,72,87] that found that swIAV infections should be regarded as enzootic infections with long term within-herd persistence. Our results also revealed that this change in epidemiology potentially affected the viral evolution, measured as increased diversity and selection of escape mutants. Finally, persistent circulation of swIAV in swine herds increases the likelihood for generation of re-assortments between human and swine IAV strains.

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Table 1. Prevalence of swIAV positive litters/pens and sows at each sampling time

	week 1	week 3	week 5	sows
Nov	75% (3/4)	100% (4/4)	75% (3/4)	50% (2/4)
Dec	75% (3/4)	75% (3/4)	25% (1/4)	50% (2/4)
Jan	100% (4/4)	75% (3/4)	100% (4/4)	0% (0/4)
Feb	75% (3/4)	50% (2/4)	100% (4/4)	75% (3/4)
Mar	75 % (3/4)	75% (3/4)	50% (2/4)	100% (4/4)
Apr	50% (2/4)	75% (3/4)	75% (3/4)	25% (1/4)
May	100% (4/4)	50% (2/4)	75% (3/4)	25 % (1/4)
Jun	25% (1/4)	25% (1/4)	25% (1/4)	25% (1/4)
Jul	25% (1/4)	100% (4/4)	75% (3/4)	25% (1/4)
Aug	50% (2/4)	100% (4/4)	50% (2/4)	0% (0/4)
Sep	0 % (0/4)	50% (2/4)	50% (2/4)	0% (0/4)
Oct	75% (3/4)	50 % (2/4)	25% (1/4)	25% (1/4)
Total	60.5 % (29/48)	68.% (34/48)	60.5 % (29/48)	33.3 % (16/48)

The prevalence of swIAV at week 1, 3 and 5 were based on pooled samples, whereas samples from sows were tested individually.

Table 2 – Average Coughing Index (CI) in IAV positive and negative litters/pens

	week 1	week 3	week 5	Total
IAV positive	0.12 (SD = 0.14)	0.36 (SD = 0.27)	0.09 (SD = 0.08)	0.32 (SD = 0.23)
IAV negative	0.05 (SD = 0.08)	0.30 (SD = 027)	0.06 (SD = 0.04)	0.12 (SD = 0.17)
P-value	0.07	0.48	0.12	0.03

Table 3. The best fitting substitution model, temporal correlation coefficient and nucleotide substitution rate of each of the eight IAV gene segments

	M1a		**M2a**		**Significant difference**	**Correlation coefficient**	**Substitution rate**
	AIC	Akaike weight	AIC	Akaike weight	p-value		
HA	5494.30	0.1143	5490.20	0.8857	<0.05	0.95	7.6×10^{-3}
NA	4203.24	0.9478	4209.06	0.0521	>0.05	0.81	6.9×10^{-3}
M	2866.58	0.8810	2870.58	0.1189	>0.05	0.82	2.5×10^{-3}
NS	2531.02	0.8810	2535.02	0.1189	>0.05	0.68	5.7×10^{-3}
NP	4310.14	0.8810	4314.14	0.1189	>0.05	0.77	1.1×10^{-3}
PB1	6595.06	0.8810	6599.06	0.1189	>0.05	0.86	2.47×10^{-3}
PB2	6459.84	0.8810	6463.84	0.1189	>0.05	0.94	2.94×10^{-3}
PA	6170.36	0.8810	6174.36	0.1189	>0.05	0.60	2.13×10^{-3}

M1a = model 1, which describes neutral and negative selection. M2a = model 2, which describes neutral, negative and positive selection. AIC = Akaike information criterion. The level of statistical difference between the two models M1a and M2a is given in the column “significant difference”. The correlation coefficient of the TempEst analysis. The substitutions rate gives the results of the BEAST analysis expressed as nucleotide substitution rate per site per year.

Table 4. Identified positive selected sites and their amino acid changes, dN/dS ratios, probabilities and location in antigenic sites

Position (from 1st M)	Position (from DTLC)	Mutation	dN/dS ratio	PR+	Sequences showing the mutation	Antigenic site/epitope/glycol-sylation site
18	1	D→E	0.7982	0.2721	1/3 F8	T-cell
60	43	S→N	0.8255	0.2837	3/3 F8	B-cell
142	125	D→N	0.7816	0.2651	2/3 F6 1/1 F7 All F9-F12	Sa
154	137	P→L	0.8791	0.3065	1/4 F2 1/3 F3 2/4 F4 All F5-F12	Ca1
172	155	K→R	0.7672	0.2588	2/3 F6 1/1 F7 All F9-F12	Sa
173	156	N→D	0.7991	0.2725	All F1-F12	Ca1
187	170	G→R	0.8784	0.3062	1/4 F2 1/3 F3	Ca1
210	193	E→G	0.8746	0.3046	All F1-F12	Sb
227	210	F→Y	0.8215	0.2820	1/2 F1	B-cell
233	216	V→D	0.8407	0.2903	2/3 F6 All F7-F12	B-cell
239	222	E→K	0.7717	0.2607	2/3 F6 All F7-F12	Ca2
293	276	T→N	0.8282	0.2849	1/2 F1	Glyco
300	283	H→Y	0.8402	0.2898	1/4 F4 All F5-F12	-
305	288	S→N	0.8255	0.2838	1/4 F4 1/1 F5	-

					1/3 F6	
421	404	I→F	0.8102	0.2773	All F10-F12	T-cell
459	442	V→I	0.8036	0.2745	1/3 F3	T-cell
553	536	W→R	**3.156** 1.5882	**0.879** 0.6380	1/4 F4 1/3 F6 2/3 F8	B-cell and T-cell

Column 1 and 2 indicates the position of the positive selected sites identified by the Bayesian analysis (bold text) and in both the Bayesian analysis and CODEML (normal text). Column 3 indicates the mutation observed over time. Column 4 and 5 gives the dN/dS ratio and the probability (PR+) of the position being positive selected with the results of the Bayesian analysis in normal text and the CODEML results in bold text. Column 6 presents the sequences wherein the given mutation was identified, herein F1-F12 indicates the sequences where the given mutation was identified. Column 8 specifies if the mutation was located in an antigenic site (Sa, Sb, Ca1, Ca2 and Cb) [5,6,8,53,54], glycosylation site (Glyco) [61] or a B-cell [55,56] or T-cell epitope [57–60].

Figure 1. The average Ct value of the IAV positive litters/pens and sows at each sampling time

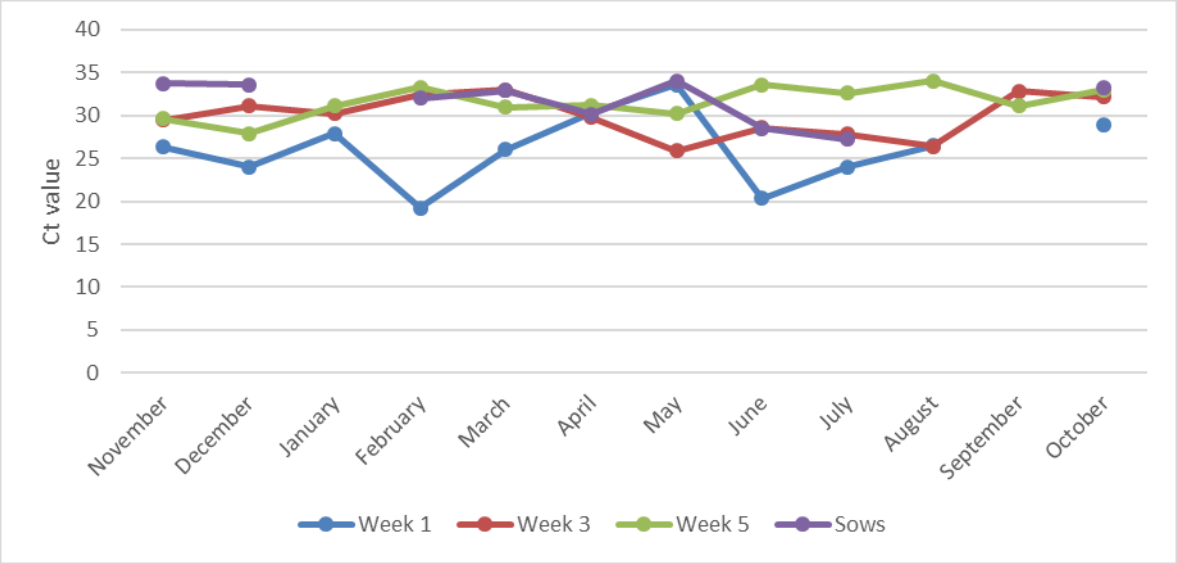
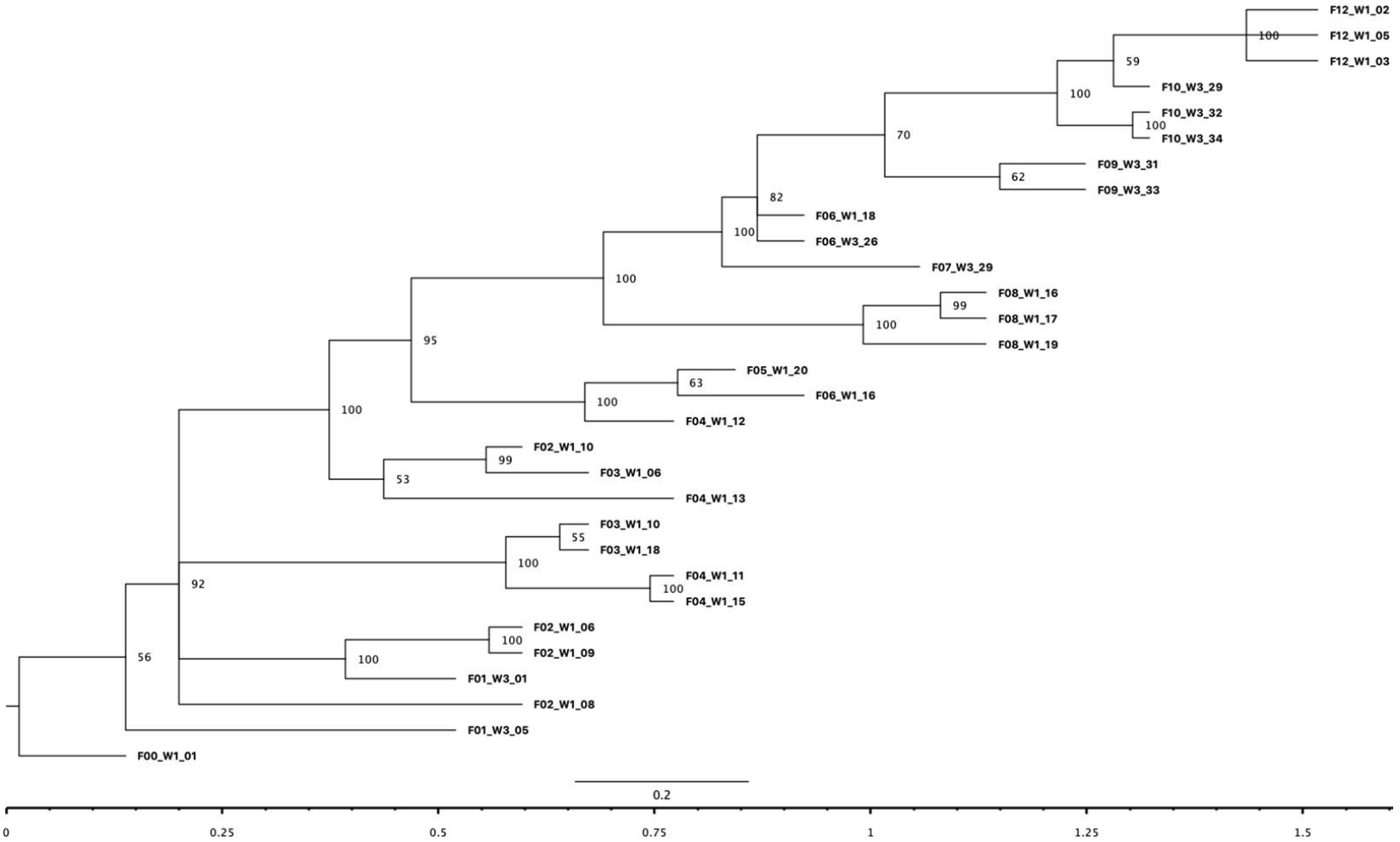
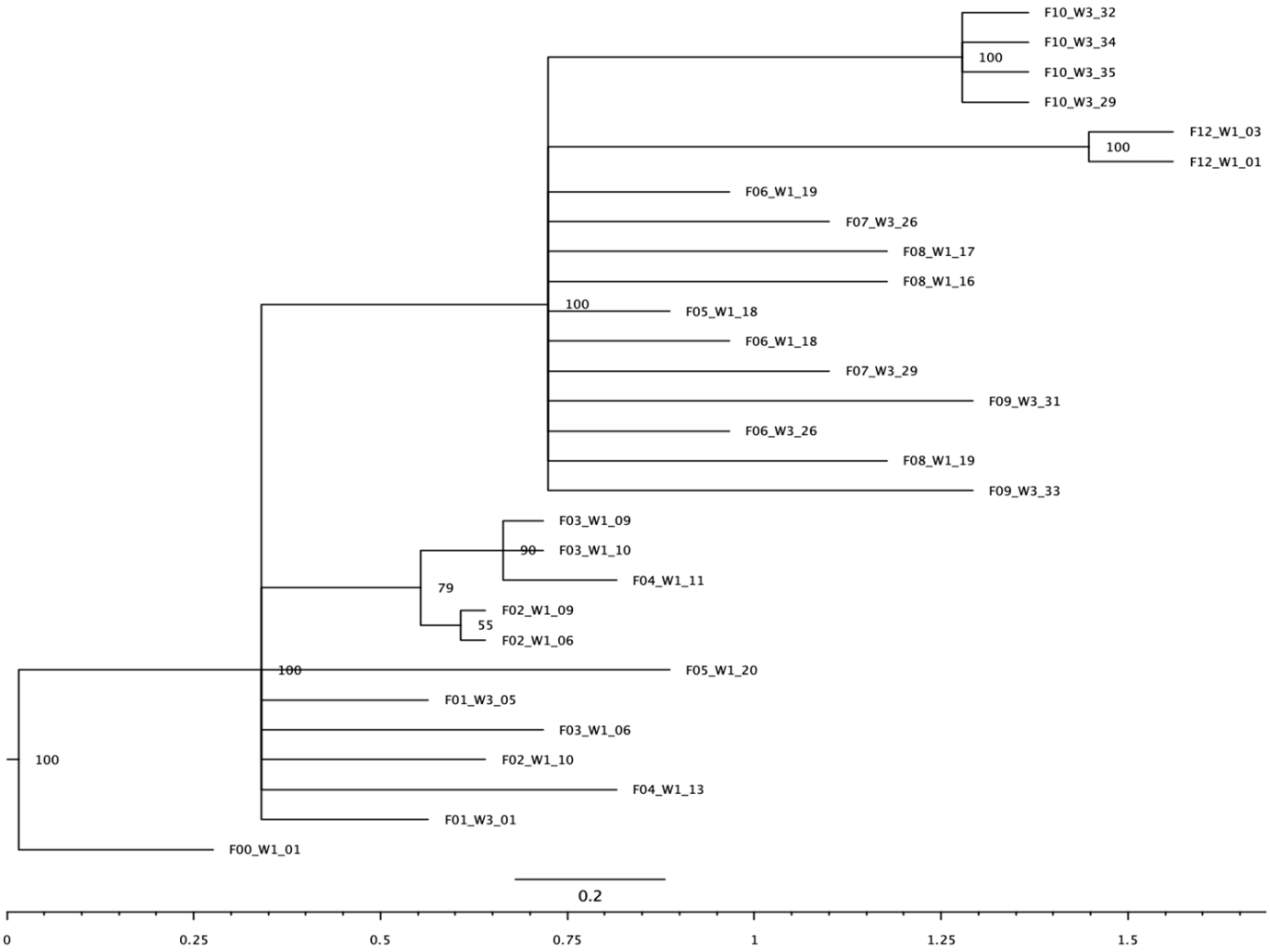


Figure 2. Bayesian strict molecular clock tree of the HA sequences



The x-axis represents time in years. Node labels represent posterior probabilities. F00_W1_01 sampled approx. four month before the actual study was used as outgroup.

Figure 3. Bayesian strict molecular clock tree of the NA sequences



The x-axis represents time in years. Node labels represent posterior probabilities. F00_W1_01 sampled approx. four month before the actual study was used as outgroup.

Part 4 – Discussion, conclusions and perspectives

Discussion and conclusions

Transmission dynamic of swIAV

The primary aim of the PhD project was to elucidate the transmission dynamics of swIAV in Danish swine herds. Based on the literature review and the findings obtained during the PhD project some important aspects of the transmission dynamics of swIAV were determined. Firstly, swIAV has been identified in pigs of all age groups, including suckling piglets, weaners, fatteners, sows and gilts [9–14]. Due to the general belief that the suckling piglets were somewhat protected against swIAV because of the presence of MDA, many previous studies have solely focused on infections of pigs after weaning. Fortunately, the longitudinal studies performed in the PhD project did include several samplings in the farrow unit and all four manuscripts revealed that swIAV was highly prevalent in piglets already at one week of age. Interestingly, clinical signs of respiratory disease were also documented in this age group. Manuscripts 1 and 2 disclosed that piglets at three to four days of age can be infected with swIAV, indicating that even newborn piglets can become infected and highlighting the importance of swIAV circulation and clinical impacts in the farrowing unit.

Even though only a few studies had focused on the role of the sows and gilts in the transmission dynamics [12–14], their results showed that both sows and gilts became infected with swIAV, and that especially gilts, were important for the transmission dynamics. There are several possible explanations for gilts playing a crucial role in the transmission dynamics in the herds. Firstly, incoming gilts are not tested for swIAV before entering the herd, and if a lack of or non-optimal quarantine measures are in force, the "new gilts" pose a risk for novel swIAV introduction. Secondly, gilts purchased from an external source may lack immunity towards the swIAV circulating in the receiving herd. Therefore, herds should ensure a proper quarantine for incoming gilts, and have a strategy for stimulating the immunity of the gilts before transferring them to stables with swIAV circulation. The gilts will otherwise become infected as soon as they enter the herd, and by that contribute to the persistence of swIAV at the herd level. In accordance with the above-mentioned studies [12–14], it was recorded in the present PhD project that both sows and gilts were indeed infected in the farrowing unit. Since piglets were also tested at the same time points, we were able to document that there was a clear association between the presence of swIAV in the sow/gilt and the presence of swIAV in their piglets. This strongly indicated a transmission between the sow/gilt and the piglets, which further emphasizes the importance of sows and gilts in the transmission dynamics in the farrowing unit. However, the directionality of transmission remains unknown and requires controlled experimental studies. In Manuscript 4, we found a higher

prevalence of swIAV in the gilts compared to sows, which could indicate that the gilts might be more prone to swIAV infections. This may in part be due to gilts having a weaker immunity towards the swIAV herd strain compared to sows, which have been exposed to the herd strain previously. However, the gilts described in Manuscript 4 were recruited internally, and therefore they should not be completely naïve to the herd swIAV. On the other hand, the gilts of this herd were moved to a quarantine facility already at 12 weeks of age, and by that, the immunity could have waned at the time they were re-introduced into the sow herd. In summary, both sows and gilts should be considered as an important part of the swIAV transmission dynamics. Gilts should be introduced into the herd with caution, and gilt immunization should be considered an important tool in reducing swIAV transmission within the herd. Moreover, sow and gilt immunization might also benefit the reproduction parameters as swIAV infection during gestation can cause stillbirths and abortions [185,187,190,192]. Additionally, since the swIAV positive litters were often linked to swIAV positive sows and gilts, immunization could also stimulate MDA uptake in the piglets, which could provide clinical protection [178,183,218,219].

Another important aspect concerning the swIAV transmission dynamics is the individual shedding time. Several studies have estimated the individual shedding time to be approx. one week [10,178,214]. However, increased shedding time in the presence of MDAs has also been documented [10,183]. The presence of so-called “prolonged shedders” was likewise described in Manuscript 1 and 3 where pigs tested positive for swIAV at a minimum of two consecutive samplings. In the first studies described in Manuscript 1, prolonged shedders were present in all herds, however at different prevalence. In the herd described in Manuscript 3, a significant increase in the number of prolonged shedders was observed after introducing mass sow vaccination, thereby supporting the correlation between the presence of MDAs at the time of infection and an extended shedding time. However, the study design utilized in the studies in Manuscripts 1 and 3 did not include very frequent sampling, and therefore the presence of prolonged shedders may have been over- or under estimated. Conversely, the study design chosen for the study described in Manuscript 2 included a more frequent (weekly) sampling, and the results were consistent with the previous findings of prolonged shedders. Moreover, in this study viral characterization was performed, revealing that only one swIAV strain was circulating in the herd, and thereby showing that the consecutive detection of swIAV in the same pigs was not due to infection with another swIAV strain. Moreover, the high identity between sequences obtained from the same pigs at different samplings, further supported the findings that the pigs indeed were infected with the same virus at consecutive samplings. Similar findings of consecutive shedding were presented in another study [13].

In summary, the results of the above-mentioned previous studies [10,183] and the studies included in this thesis, indicates that a shedding time of one week cannot always be expected for the individual pigs, and that the presence of MDA might be a driver for prolonged shedding. The presence of prolonged shedders is expected to contribute to an increased reproduction number (R_0), and hence an increased risk of transferring swIAV around the production system. It should, however, be noted that the amount of virus being shed, also is important for the transmission rate. Manuscript 3 described a clear increase in prolonged shedders after implementation of mass sow vaccination, since they constituted 28 % of the total number of infected pigs. At the same time, swIAV was transferred throughout the production units, potentially as a consequence of the number of prolonged shedders and the delayed time of infection resulting in swIAV positive pigs at weaning. Furthermore, the prolonged shedders were probably the main driver of the marked increase in genetic and antigenic drift observed after vaccination, thereby underlining the negative role of prolonged shedders in the herds.

An additional phenomenon concerning viral shedding observed in Manuscripts 1 and 2 was the presence of pigs testing positive for the same swIAV strain at two non-consecutive samplings, indicating re-infection (recurrent infection) with the same swIAV strain. This observation was supported by findings made in both an American [13] and a Spanish [11] study. The American study analyzed viral sequences obtained from the non-consecutive shedders and a high sequence identity was identified and the HA protein only differed in a few amino acids. Likewise, only one subtype was identified by multiplex RT PCR in the herds with recurrent shedders described in Manuscript 1, though this was not confirmed by sequencing. Conversely, in Manuscript 2, swIAV sequences obtained from recurrent shedders were characterized, and similar to the American study, only minor changes were observed within the HA gene between “first” and “second” infection. When investigating the specific amino acid changes occurring between the first and second infection, it was revealed that five of the amino acid changes observed at the second infection were shared among viruses obtained from different pigs defined as recurrent shedders. The majority of these amino acid changes were located in the globular head of the HA protein, as well as in one well-known antigenic site. These results suggest that antigenic drift could be responsible for creating escape mutants and thereby resulting in re-infections with the same swIAV strain. However, more studies are needed to verify the occurrence of re-infections with the same swIAV stain, preferably in a controlled experimental study setup. In addition, further studies should be carried out to investigate whether antigenic drift could be responsible for the re-infections. Several other factors might be relevant for evaluating recurrent shedding, including changes in MDA levels (discussed later) and development

of host immunity. It cannot be ruled out that some of the observed recurrent infections might be a result of environmental contamination of the sample. Several studies have documented that swIAV can be detected in air-samples within the herds [141,215,323]. As all samples obtained in our studies were based on nasal swabs, there was a risk of contamination when obtaining the sample. Moreover, the snout of the sampled pig, could also have been contaminated by other pigs via direct contact. However, the results obtained from the nasal swabs of the studies, clearly showed a high amount of negative samplings, and similar infections patterns were also observed among herds, indicating that environmental contamination of the sample was not significant. Moreover, the semi-quantitative measure of the viral load in nasal swabs (Ct values) obtained throughout the project, revealed higher viral load than the viral load in air and environmental samples found by others and by us (unpublished results). To mitigate the risk of contamination, deep throat samples or even tracheal flush samples could be considered, though this was not tried in our field studies because of practical and animal well-fare reasons.

Increasing evidence of the enzootic nature of swIAV within herds has been compiled during the last ten years [9–13,220,240,330]. The results obtained in Manuscripts 1, 2, 3, and especially in Manuscript 4, confirm the continuous circulation of the same swIAV strain within a single herd for months or even years. This underlines that introduction of swIAV, will most likely result in an enzootic circulation subsequent to the initial outbreak. Several of the above-mentioned factors play a role in the persistence of swIAV at the herd level. In addition, a general increase in herd size is favored by the industry, which in turn results in a continuous flow of naïve individuals into the herds, providing an optimal environment for swIAV circulation [240,331,332]. Therefore, as herds increase in size, the importance of herd-management and internal biosecurity becomes more and more important. The presence of prolonged and recurrent shedders could also be important for herd-level swIAV persistence and has rarely been taken into account when estimating reproduction numbers (R_0) and modelling swIAV infections [214,217,240,330]. As discussed in details later, MDAs might play an important role in the induction of prolonged and recurrent shedders, which should be taken into consideration when selecting control strategies. Several studies [178,183,214,218–220] including our studies have proven that swIAV infection can occur in pigs in the presence of MDA, and therefore young piglets should be considered highly important in the continuity of swIAV transmission. External factors can also influence the presence and transmission of swIAV in the herds. For example, the herd density in the area of a given herd is a risk factor for herd swIAV positivity [324,325], and therefore external biosecurity should also be prioritized. The introduction of

new pigs into the herd should also be considered as one of the key risk factors of novel swIAV introduction. Therefore, immunization and quarantine strategies should be implemented.

Seasonality of swIAV has been described in some studies, probably as a consequence of the impact that the outdoor temperature has on the indoor environment in the stables [15,23,311,318,320]. When the outdoor temperature decreases, it can result in decreased ventilation for maintenance of the indoor temperature, which in turn results in less frequent exchange of air, providing a better environment for swIAV transmission. Moreover, if herds are struggling in winter to keep the room temperature high, the pigs might be more susceptible to infections in general [333]. Nevertheless, it is important to realize that many studies, including ours, have failed to show an impact of the season [8,10–13,17,19], and therefore swIAV infections should always be considered as a cause of disease regardless of the season.

In conclusion, swIAV can potentially infect all age groups in the herds, as long as naïve individuals are present. The role of suckling piglets, sows and gilts in the transmission dynamics has been highlighted through the studies performed during the present PhD project, and in turn provides a stronger basis in deciding when and where to apply control efforts in the herds. The potential effect that MDAs have in generating prolonged shedders has been emphasized, and additionally cumulative evidence suggests that re-infection with the same swIAV strain is possible, and might be a consequence of antigenic drift generating escape mutants. These aspects all contribute to the understanding of the transmission dynamics, but also underscore the complexity of swIAV.

Clinical impact of swIAV

An additional aim of this PhD project was to investigate the clinical impact of swIAV. Previous studies have shown that the clinical signs observed in pigs are coherent with the classical flu symptoms of people [2,3,10,116,170,174]. Moreover, a number of studies have provided evidence of reduced body weight gain upon swIAV infection [7,182,183]. On the other hand, a number of experimental [3,171,181,214] - and field studies [9,184] have also observed pigs, which did not become ill following swIAV infection. Several factors might contribute to this. First, when performing experimental studies, the pigs are housed in a highly protected environment and are mainly obtained from herds with a high health status. It can therefore be difficult to reproduce clinical disease under such experimental conditions. Factors such as ventilation, air quality, pig density and lack of other pathogens, might also play a role in the clinical outcome of disease [320,323,334]. Moreover, a high level of MDAs present at the time of infection, can lead to complete clinical protection [178,183,218,219]. In our studies, clinical signs were evaluated in order to study

potential correlations to the presence of swIAV, both at individual level and at group level. The results of the studies clearly suggested a clinical impact of swIAV in the herds. In the herds described in Manuscript 1, 2 and 3, an association between the presence of nasal discharge and swIAV in the individual pigs was identified. Moreover, the results obtained from herds described in Manuscripts 1, 3 and 4, revealed that an increased coughing index was correlated with the presence of swIAV in the respective litter or pen. As the study described in Manuscript 2 included weighing of the pigs, we were able to find an interesting association between significantly reduced body weights in the six-week-old pigs that tested positive for swIAV. It is not clear, however, if the presence of swIAV at the day of sampling could already have caused a decreased body weight. It should be underlined that not all pigs included in our studies showed clinical signs of respiratory disease. This could both be explained by the fact that the pigs were only examined on one day, which thereby did not provide the full picture of disease development through the different stages of the infection and by the fact that some pigs might be subclinically infected [184,185].

In conclusion, swIAV has indeed a clinical impact in the herds, emphasizing that swIAV affects both animal welfare and health. There is evidence that swIAV can lead to a delayed growth rate, which can have significant economic consequences for the farmer. The fact that swIAV is part of the PRDC can contribute to increased disease outcomes with increased mortality and antibiotic treatments [6]. On the contrary, subclinical infections with swIAV should, to a certain extent, also be expected, due to the influence of both host factors such as immunity and current infection status and environmental factors. The results of the studies presented in the four manuscripts also emphasize that the impact of swIAV might vary between herds, thus underlying that herd factors such as management, structure and biosecurity level contribute to the disease manifestation.

Genetic diversity of swIAV

Another aim of the study was to examine the genetic variability of swIAV. As previously mentioned, IAV mainly evolve through two different mechanisms known as viral reassortment and genetic drift [88,332]. Several reassortment events between and within human, avian and swine IAV have occurred over time and have led to major pandemics and epidemics in humans and swine [1,142]. Different countries have been exposed to different IAV subtypes and lineages, and until 2009, there was a somewhat clear separation of swIAV lineages circulating in Europe and in North America [1]. However, after the introduction of A(H1N1)pdm09, the Eurasian avian-like H1N1 lineage has been introduced into North America and genes originating from the TRIG cassette have been introduced into Europe. Moreover, several new reassortment events have been observed since 2009

[1,18,41,288]. In Asia, swIAV containing the genes of American and European lineages has been circulating before the 2009 pandemic [285]. However, evidence still supports that the A(H1N1)pdm09 originated from Mexico [112]. It can be discussed, if other factors also contributed to the increased diversity of swIAV observed after 2009. First, the 2009 pandemic lead to increased focus on pigs as a reservoir for human pandemics, and resulted in the initiation of surveillance programs in several countries [1,16,19,20,311]. Moreover, the development of new sequencing techniques became available during the same years [86], and may also have played a role in the increase in novel subtypes and strains identified. The swIAV strain identified in the herd described in Manuscript 2 is an example of the different reassortment events that have occurred in Denmark. Firstly, the swIAV was an H1N2 subtype, which in itself is a reassortant of the Eurasian avian-like H1N1 subtype and the swine-adapted Hong Kong H3N2 subtype [181]. Secondly, the swIAV contains an internal gene cassette of pandemic origin with the exception of the NS gene having Eurasian avian-like origin. Since the introduction of A(H1N1)pdm09 in Denmark, an increase of Danish swine H1N2 isolates containing pandemic genes in the internal cassette has been observed [8]. A similar trend has been observed in other countries [17,18,41,274,286,317,335–337]. Therefore, it appears that the presence of the pandemic internal gene cassette is an advantage for swIAV strains. It can be argued whether this is due to an increased replication efficiency, as the polymerase genes are encoded by the internal gene cassette. In addition, the current vaccine used for the H1N2 in Denmark does not contain any components of the A(H1N1)pdm09 subtype [232], it can therefore be speculated whether swIAV containing pandemic internal genes might have an advantage in vaccinated pigs, due to a lack of antibodies raised against the pandemic internal gene cassette. However, antibodies targeting the surface proteins are the most important for clearing swIAV infections, whereas the antibodies targeting the remaining proteins play a minor role.

Manuscript 3 described a herd enzootically infected with a complete Eurasian avian-like H1N1, which subsequently experienced an outbreak with a Danish H1N2 subtype containing a pandemic internal gene cassette, with the exception of the Eurasian avian-like NS gene. No cross protection was observed between the two strains and the new swIAV strain completely eradicated the old swIAV strain in the herd. This case illustrated how difficult swIAV is to control as several different swIAV subtypes and strains are circulating. Moreover, the case illustrated the level of genetic drift that has occurred over time in the HA gene of the Eurasian avian-like H1Nx lineage. The HA genes of the enzootic strain and the outbreak strain were both of Eurasian avian-like H1Nx lineage, nevertheless a clear lack of cross protection was observed between the two strains. The sequencing results for the HA gene revealed major differences between the two HA genes, which likely

explained the lack of cross protection. The difference in the internal gene cassette and the NA gene may also have played a role in the lack of cross protection between the strains. The results of this study suggest that influenza A subtypes no longer can be regarded as serotypes.

Another interesting aspect of viral evolution is the level of genetic and antigenic drift occurring within the individual pigs. In Manuscript 3, it was clearly seen that after implementation of mass sow vaccination, the occurrence of prolonged shedders was increased. Concurrently, a clear increase in the viral diversity and the level of positive selection was observed. As genetic drift occurs naturally over time, it is expected that the longer swIAV is present in the individual, the more likely it is to change. In addition, the presence of antibodies will drive the selection of advantageous mutations. In Manuscript 2, indications of re-infections with the same subtype were observed in a number of pigs. The viral characterization of the swIAV originating from these recurrent shedders, revealed the manifestation of mutations in the globular head of the HA protein, as well as in an antigenic site. These results indicated that antigenic drift of swIAV could result in the generation of escape mutants [39,109,338,339], which in turn could facilitate re-infections with the same strain. However, additional studies are needed to confirm these findings, by including more frequent samplings and minimizing the risk of environmental contamination of the samples.

Manuscript 4 presented the results on the viral evolution of a single swIAV strain over a one-year period within a single herd. Surprisingly, substantial genetic and antigenic drift in the HA gene was observed in this study. The evolution of the herd swIAV strain resembled that of human seasonal IAV, as the phylogenetic analysis suggested the occurrence of repeated bottlenecks, resulting in the continuous selection of immune-escape variants. These results were in contrast to previous studies [97–100] and indicated that the evolution of swIAV is likely driven by pre-existing immunity similar to human seasonal IAVs [338]. Interestingly, the result presented in Manuscript 4 suggested that escape mutants were generated much faster than for human seasonal IAV [338]. The fast generation of escape mutants, supports and possibly explains the re-infections described in Manuscript 2, and aids in explaining how mass sow vaccination, stimulating MDA uptake in piglets, lead to the increase genetic and antigenic drift described in Manuscript 3. Moreover, the fast genetic and antigenic drift observed in this study could help explain the extensive diversity of swIAV lineages presented by previous studies [18,105,107,311,340–342]. However, further and more extensive studies are needed to confirm these rather controversial findings presented in Manuscript 4, and future studies should include antigenic mapping of the herd strain.

In conclusion, the results of the studies performed in the PhD project suggest that substantial antigenic and genetic drift occurs in swIAV both at individual level and at the herd-level. The antigenic drift of swIAV is most likely driven by the pre-existing immunity, and might be a consequence of the increased herd sizes, which ensure the enzootic circulation of swIAV, causing pigs to be repeatedly exposed to the herd strain. Consequently, generation of escape mutants, which potentially has substantial consequences for controlling swIAV should be expected. Moreover, it raises concern that swIAV variants constantly evolve in swine herds, with unknown zoonotic potential.

Vaccination against swIAV

The last aim of the PhD project was to evaluate the effect of different vaccine strategies adopted in Danish swine herds. The literature review presented a number of studies all describing the effect of maternally derived antibodies [178,183,214,218–220], which is what most vaccine strategies aim at stimulating through different sow vaccination programs. The only swIAV vaccines available for use in Europe are WIV vaccines, which mainly stimulate a systemic antibody response [87,231]. Antibodies stimulated by vaccination can subsequently be transferred to piglets through the colostrum [213]. Coherently with this process, the two major WIV vaccines on the European market claim a reduction of the spread of swIAV to lungs and clinical signs in vaccinated pigs [232,233], and a clinical protection of piglets originating from vaccinated sows [232].

Many of the studies investigating the effect of MDA were experimental studies [178,183,214,218,219]. Only in one of these studies the piglets originated from sows being naturally exposed to swIAV [183]. All other studies investigated piglets derived from sows that were vaccinated up to five times before farrowing [178,214]. Despite the use of excessive vaccination programs, all studies still showed piglets with low MDA levels, and in some studies, these piglets showed very similar results to the MDA negative piglets with regard to virus shedding and clinical signs [178,214,218]. This clearly suggested that the level of MDA determined the degree of protection of the piglets. In addition, these experimental studies used a highly homologous swIAV strain for challenge, which might also impact the level of protection [219,220,250]. Furthermore, all these experimental studies performed the initial swIAV infection in weaned piglets [178,183,214,218,219]. However, after weaning the MDA has potentially already decreased, and more importantly the vaccine only claims clinical protection of piglets through MDA until 33 days-of-age, which was before initial inoculation in the majority of the studies [178,183,214]. Therefore, none of the above-mentioned studies actually investigated the effect of the MDA in the period where

MDA is supposed to be present at the highest level, and where the veterinarian and the farmer often expect to see an effect of vaccination. The experimental studies therefore do not represent the actual situation in the field, since one would expect to find different level of MDA, as well as non-homologous circulating swIAV strains compared to the vaccine strain. In addition, the sows in the field are seldom exposed to multiple vaccinations right before farrowing. Nevertheless, the experimental studies [178,183,214,218,219] along with the few available field studies [220,221] still provide valuable information on the effects of MDA. Most of the studies showed clinical protection by MDA, but the effect was dependent on the level [178,183,218]. High levels of MDA at a young age generally provided complete clinical protection, whereas the clinical effects decreased along with decreasing levels of MDA and increasing age. As previously mentioned, some studies have shown that pigs with low levels of MDA respond in a similar manner to MDA negative pigs upon challenge with swIAV, which is an important aspect when considering the effect of vaccination in the field. Complete sterile immunity was not observed in any of the reviewed studies [178,183,214,218–221]. However, one field study showed a significant decrease in the number of swIAV infected piglets at weaning in batches originating from vaccinated sows [220]. Interestingly, a comparison between the HA genes of the herd strain and vaccine strain did not show any difference in antigenic sites. In addition, even though the sow vaccination was effective in reducing the number of piglets infected at weaning, the majority of pigs were infected with swIAV one week later. Moreover, a secondary peak of swIAV infections was observed in some of the batches originating from vaccinated sows, suggesting re-infections after MDA had waned. Another study [219] also showed significant reduction in the number of infected piglets receiving MDAs from sows vaccinated with the same swIAV strain, as used for subsequent challenge of the piglets. The results of these two studies indicate that the level of homology between the vaccine strain and the herd strain could be an important factor for vaccine efficacy. The impact of vaccine homology was also shown in a study by Vincent et al., which tested the level of protection against A(H1N1)pdm09 challenge, raised by four different WIV vaccines [250]. The importance of strain specific antibodies was further emphasized by the results of Manuscript 3, which clearly showed that genetic differences within the same swIAV lineage can have a pronounced effect on the level of cross-protection.

If considering homology between the vaccine strains and the herd strains to be important, it is quite conspicuous that the vaccine strains included in two of the most popular WIV vaccines in Europe are between 10 and 19 years old [232,233]. As highlighted in Manuscripts 3 and 4 as well as in several studies [18,105,107,181,311,340–342], swIAV show a vast genetic diversity, and several diverse strains are present within a single swIAV lineage. Therefore, a more frequent update of swIAV

vaccines should be considered. However, it is not simple to update swIAV vaccines. First of all the current European legislation does not allow for regular updates of the vaccine strains, without the vaccine obtaining a new license, which takes several years [206]. In addition, a major challenge is how to select new vaccine strains. As the diversity of the different lineages is large, how can the most broadly reacting strain be chosen? Should the common ancestor of the lineage be selected or would it be better to include several different strains within the same lineage, in order to represent different sub-clusters? The answers to these questions are not straightforward. However, it should be noted that swIAV WIV vaccines, in contrast to human IAV vaccines [343], contain adjuvants, which should ensure a broader immune response [206,236].

In the U.S., the importance of vaccine and herd strain homology has been taken into account, as an increasing numbers of autogenous vaccines are used [250]. In addition, the University of Minnesota Veterinary Diagnostic Laboratory offers HA-sequencing of herd swIAV strains to determine the best match to the commercial WIV vaccines [249]. A live-attenuated intra nasal swIAV vaccine is now also available on the U.S. market and has some advantages compared to the WIV vaccines available in Europe. Firstly, it can be used in newborn piglets, secondly it stimulates a mucosal antibody response and lastly no sign of VAERD has been related to vaccination upon heterologous challenge [253,255,340]. A disadvantage of the LAIV is that it can contribute in reassortment events, and create novel IAV with unknown potentials.

In Denmark, farmers and veterinarians are struggling to control swIAV infections in the farrowing unit, and consequently WIV vaccines are used “off label” in piglets. However, the effects of piglet vaccination described in Manuscript 2 were very limited. One of the main explanations for the lack of effect was most likely the early infection time, which in turn made it difficult to generate an immune response towards the vaccine before natural infection. Moreover, the vaccine scheme applied might also have had an impact on the effect, as the vaccine is normally used in a higher dose and as a “prime-booster” vaccination. If a herd mainly has swIAV infections in the nursery unit, it could be speculated that two vaccinations in the farrowing unit might have a subsequent effect on clinical signs in the nursery. However, additional studies are needed to investigate this.

It is the perception of several farmers and veterinarians that WIV vaccination has a poor impact on swIAV in the field. This is probably because they wrongly expect the vaccine to provide sterile immunity, and do not consider that the claim of the vaccine is mainly to reduce swIAV spread to the lungs and to reduce the severity of clinical signs in piglets younger than 33 days [232,233]. The effect of sow vaccination on disease in piglets should therefore be measured as a reduction in clinical

signs of respiratory disease, while in older pigs, as a reduction in cases of severe pneumonia. In addition, the possible unwarranted effects of MDA should also be taken into consideration when initiating vaccination. There is a risk of inducing prolonged and recurrent shedders. In addition, pigs infected in the presence of MDA, has been associated with a weakened immune response towards swIAV [10,178,183,214,218]. In manuscript 3, it was clearly documented that a significantly lower number of pigs seroconverted after primary infection after mass sow vaccination, and a significant increase in prolonged shedders was registered. Moreover, no clinical protection of piglets was observed after the use of mass sow vaccination. The possibility of inducing VAERD [226,227] should also be kept in mind thus highlighting the importance of proper diagnostics before initiating vaccination. However, as mentioned earlier, the effect that the WIV vaccines might have on the protection of the sows might be underestimated. The results of Manuscripts 1, 3 and 4 all suggested that sows and gilts contribute to the transmission of swIAV in the farrowing unit, and vaccination will aid in boosting their immunity towards swIAV. However, the SPC of the vaccine does not provide a clear instruction on how to vaccinate gilts and sows [232,233], and, as reviewed in the introduction of the thesis, there are several different ways to perform sow vaccination [206,241,249]. Furthermore, there is a lack of field studies describing the effect of different mass sow vaccination programs. Moreover, swIAV is a very difficult pathogen to control as it has a high reproduction number (R_0) [214] and as the herd size increases, a much higher number of naïve pigs are available for infection [331,332]. Therefore, the importance of separation between age groups, all in/all out managements of rooms and high internal biosecurity practices are vital in limiting the spread of swIAV within the herds [240,322] and might be just as important as vaccine strategies.

In conclusion, the efficacy of WIV vaccines will vary from herd to herd. Several factors will influence the effect of vaccination, for example the chosen vaccine scheme, the level of antibody responses, MDA uptake, the homology between the vaccine strain and the field strain and pre-existing immunity. Herd management factors, which affect the swIAV transmission dynamics, can potentially also influence the results of a given vaccination strategy. Therefore, control strategies should aim at both improving the management and biosecurity procedures in the herd as well as immunization. Generally, there is a need for recommendations for controlling swIAV, and efforts should be focused in adjusting expectations to WIV vaccines. Autogenous vaccines and intra nasal live-attenuated vaccines could potentially be a good alternative to WIV vaccines, but are currently not licensed in Europe.

Overall conclusions

The studies performed in the scope of this PhD project have generated results that significantly have increased our knowledge of swIAV transmission dynamics and document the enzootic nature of swIAV. For the first time, we proved that swIAV infections affect very young piglets, and that sows and gilts play a significant role in the dissemination of the virus in the farrowing unit. These results provide a solid basis for the implementation of more effective control measures in the future. Additionally, our studies provide further evidence of prolonged and recurrent swIAV infections with the same swIAV strain and reveal a tight link to the presence of preexisting antibodies at the time of infection. Clinical impacts of swIAV have been documented in all of the studies performed in the PhD project and include clinical signs of respiratory disease as well as reduced weight gain, highlighting the negative impact of swIAV on animal health and on the productivity.

The results of the PhD revealed that swIAV is prone to intensive genetic and antigenic drift and that positive selection occurs especially within the globular head domain of the HA protein. Finally, we found that initiation of mass sow vaccination can delay the onset of infection and reduce virus shedding, but can also lead to an increase in prolonged shedders, which in turn seems to drive virus evolution and eventually resulting in antigenic drift. The study evaluating the effect of the early piglet vaccination did not provide promising results for this control strategy, but did underline the clinical impact of swIAV infections and further supported the impact of prolonged and recurrent swIAV shedders.

Perspectives

The effects and potential "adverse effects" in the presence of MDAs at the time of infection need to be further investigated. It is crucial to understand the effects of MDAs, since most vaccine strategies are still based on stimulating MDA uptake in piglets. It is advisable to further investigate whether piglets infected in the presence of MDAs are prone to being re-infected with the same swIAV strain after MDA has declined and to investigate whether the infected piglets are primed after the initial infection, despite lack of measurable systemic antibodies. Future studies should include piglets infected naturally in the herd, which are subsequently transported to experimental facilities after weaning and re-challenged with the field swIAV strain after MDA decline. Such a study would provide a setup reflecting natural conditions, where MDA levels are different, and where pigs encounter the same subtype several times throughout the different parts of the production system. The immune response towards swIAV during both primary and secondary infections should be investigated more thoroughly, and should include assays for the detection of B-cells in the blood.

The results of such investigations could help to explore if some pigs are still primed for a secondary swIAV infection, even though no seroconversion is seen after primary infection in the presence of MDA. In addition, focus should also be on the innate immune response to swIAV, especially to investigate if the MDA levels at the time of infection have an impact on the innate immune responses.

Another possible study could include a screening of the presence of swIAV in gilts at the different levels of the production system. Herds, buying gilts from an external source, could preferentially be included in the investigation, thereby allowing for evaluation of different management systems for introducing external gilts in the herds. Including both herds, which are subject to different vaccine strategies, as well as herds where vaccination is not performed, could help evaluating if vaccine strategies aid in protecting the gilts during different stages in the production. Consequently, the results of such a study could contribute to a recommendation for quarantine use and vaccine strategy for gilts.

In addition, the occurrence of VAERD in the field needs to be examined in more detail. If VAERD is a consequence of heterologous vaccinations in the field, it emphasizes the importance of including sequencing, before selecting the vaccine to apply in the given herd. Moreover, the level of homology needed for not causing VAERD should be further examined and possible genetic markers should be identified. Another effect of vaccination that could be interesting to examine in more detail, is the level of antigenic drift that a vaccine can impose on swIAV. If an increased antigenic drift is confirmed, the effect of different vaccine types could be evaluated. It would be interesting to examine if a highly homologous vaccine poses a greater risk for the induction of escape mutants compared to less homologous vaccines. Moreover, the reverse-genetics system [344,345] recently established by our group, could be utilized for generating swIAV with an array of different amino acid changes in antigenic sites, which subsequently could be used for determining the impact of genetic changes both *in vitro* and *in vivo*. The same system could also be applied to investigate the possible advantage of swIAV having a pandemic internal gene cassette.

As mentioned in Manuscript 4, more studies are needed to elucidate the within-herd evolution of swIAV. Such studies would increase our general understanding on how swIAV evolves under natural conditions. If the substantial and fast antigenic drift documented in Manuscript 4 is confirmed, pigs could potentially be used as a model for predicting the evolution of human seasonal IAV.

Finally, there is a great need for studies evaluating optimal measures for controlling swIAV within the herds. First, it should be considered, if it is beneficial to completely eradicate swIAV in the herd,

or if strategies should instead be aimed at reducing the spread and clinical signs of the disease. Moreover, further field studies should be performed to evaluate the effect of different vaccine strategies, so that the current vaccine use could be improved and simplified. Vaccine composition should also be evaluated, so that the WIV vaccines include strains that cover the majority of the circulating swIAV strains. This is, however, a difficult task, and it is also a constant target for human IAV vaccine development.

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