Dansk Veterinær Konsortium

KØBENHAVNS UNIVERSITET | STATENS SERUM INSTITUT

Surveillance of swine influenza A virus in Denmark

Annual report 2023

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Kort sammenfatning af nøgletal

Der blev i 2023 modtaget 478 indsendelser (1672 prøver) til undersøgelse for svineinfluenza virus (swIAV). I alt indsendte 378 besætninger med forskellige CHR-numre prøver til overvågningen, og 239 besætninger testede positive mindst én gang i løbet af året, svarende til en andel af swIAV-positive besætninger på 63 %. Andelen af swIAV-positive besætninger, der testede positive for H1pdm (1A.3.3.2) i mindst én prøve i løbet af året, var 44 % (106/239 besætninger). HA- og NA-kombinationen (defineret som subtypen) blev bestemt for 236 indsendelser i 2023, hvilket svarer til 80 % (236/293) af de swIAV-positive indsendelser. Detaljeret genetisk karakterisering af hovedparten af de positive prøver viste en stor diversitet af swIAV i danske svin med en blanding af gener fra både grise og mennesker.

I 2023 blev der foretaget en aktiv screening af 25 besætninger med udegående grise (økologiske og friland). Syv af de 25 besætninger testede positive for influenza A virus svarende til 28 % af besætningerne. Fire af disse besætninger var økologiske besætninger, mens tre var frilandsbesætninger. Fem af de syv IAV-positive besætninger viste kliniske tegn på luftvejssygdom. Adskillige forskellige HA- og NA-kombinationer blev påvist med høj grad af lighed med vira fundet i det passive swIAV-overvågningsprogram, men fugleinfluenzavirus blev ikke påvist i besætningerne.

Opsummering

Der blev i 2023 modtaget 478 indsendelser, repræsenterende 378 forskellige besætninger (CHR numre) til undersøgelse for svineinfluenza virus (swIAV), hvilket er en lille nedgang i antallet af indsendelser i forhold til 2022, men markant lavere end i 2019-2021. Andelen af swIAV positive indsendelser viste et mindre fald i forhold til 2022, men var stadig over 60 %, hvilket er markant højere end alle tidligere år i overvågningen (2012-2021). Andelen af swIAV positive indsendelser, som indeholdt HA genet fra H1N1pdm09 oprindelse (H1pdm) steg til 40 %, hvilket formentlig var drevet af stigningen i H1pdm09N1av og en mindre stigning i H1pdm09N2sw.

Der er i 2023 for første gang gennemført en aktiv overvågning for swIAV og aviær influenza (AIV) i danske frilands- og økologiske besætninger. Resultatet viste en lavere andel (28%) af swIAV positive besætninger sammenlignet med andelen af positive fundet i den passive overvågning, hvor størstedelen af besætningerne har konventionel produktion. Der var ingen forskel på hvilke genotyper, der blev påvist i disse besætninger sammenlignet med besætningerne inkluderet i den passive overvågning og vigtigst af alt blev der ikke påvist virus der stammer fra fugle.

Pga. en målrettet indsats, er antallet af indsendelser, som er blevet karakteriseret ved fuld genom sekventering, steget i forhold til tidligere år, og resulterede i 192 genotypede swIAV, hvilket udgør et essentielt dataset til undersøgelse af genkonstellationer, genetiske markøer og evolution. Det var tydeligt at den interne gen kassette ”PPPPPA” (P = pandemisk H1N1pdm09 and A = Eurasian avian-like svine H1N1 oprindelse) var stigende i prævalens for alle forskellige HA og NA kombinationer. Selv en human sæsoninfluenza H3N2 virus, som blev detektereret i 2023, havde denne interne kassette, hvilket resulterede i et nyt såkaldt ”triple-reassortant” virus. Denne generelle stigning i tilstedeværelsen af PPPPPA kassetten i danske swIAV indikerer, at netop denne genkonstellation er favorabel for virus. De interne gensegmenter koder for proteiner der er vigtige for både replikation og det innate værtsrespons mod influenza infektion, hvilket kan forklare naturlig selektion af disse gensegmenter. I 2023 er etableringen af nye in-vitro assays for at kunne udføre en biologisk karakterisering af udvalgte danske swIAVs påbegyndt. De foreløbige resultater afslørede vigtige forskelle mellem de forskellige swIAV genotyper, som kan være med til at forklare fremgangen af specifikke swIAV genotyper, og tilmed give et indblik i det zoonotiske potentiale af disse virus. Et eksempel herpå var at et svine H1pdmN1pdm-2 virus, som ligner det virus fra

den første danske zoonotiske case i 2021, var i stand til at inficere humane lungeceller bedre end human sæson influenzavirus i et in-vitro assay, der evaluerer virus replikationen i forskellige cellelinjer. Et af de andre in-vitro assays, som tester swIAVs evne til at regulere værtcellers innate antivirale respons på infektion, viste, at H1pdmN1av-1 genotypen var i stand til at reducere produktionen af to vigtige antivirale proteiner i humane lungeceller og samtidig bibeholde en effektiv replikation.

De fylogenetiske analyser indikerede, at gruppen af H1av (1C.2.4) swIAV er den mest udbredte i danske grise, mens det for de pandemiske H1pdm09Nx (1A.3.3.2) virus stod klart at virus tilhørende to overordnede clusters cirkulerer: et indeholdende H1pdmN1pdm virus og et andet indeholdende H1pdmN1av virus. For N1 generne var der er klar gruppering i forhold til oprindelsen af det tilhørende HA gen, mens der for N2 gener ikke var nogen klar opdeling i grupper.

Den antigene karakterisering der er lavet på baggrund af hemagglutination-inhibition (HI) test viste, at der generelt var en manglende krydsreaktion mellem danske swIAV og fritte sera rejst mod de humane sæson H1 influenza vaccine stammer. En nedsat krydsreaktion mellem de danske swIAV 1C.2 (H1avNx) virus og svine sera rejst mod den trivalente svine influenza vaccine blev også observeret, og dette var specielt tydeligt for swIAV, der tilhørte 1C.2.4 grupperingen. Til gengæld var der en høj grad af krydsreaktion mellem alle de testede 1A.3.3.2 (H1pdm09Nx) virus og svine sera rejst mod den monovalente vaccine indeholdende H1N1pdm09. Virus fra den eneste påvisning af human sæson H3N2 virus i danske svine viste en manglende krydsreaktion til svine sera rejst mod den trivalente svine influenza vaccine, men en god reaktion til fritte sera rejst mod den seneste human H3N2 sæson influenza vaccine stamme.

Den store andel af fuld genom karakteriserede swIAVs gjorde det muligt at lave en detaljeret søgning for adskillige genetiske markøer, som tidligere er blevet identificeret i forbindelse med resistens, virulens eller zoonotisk potentiale. Adskillige af disse genetiske markøer blev fundet blandt de danske swIAV, hvor især mutationer beskrevet til at kunne forøge det zoonotiske potentiale blev fundet i PA og NP proteinerne. Derudover blev der for første gang fundet en mutation i PB2 proteinet i en H1avN2sw-1 genotype, som tidligere beskrevet som betydende for at ændre AIV's værtsspektrum fra fugle til pattedyr. Mutationen var dog en anden end den der normalt er blevet observeret i AIV. Det er også bekymrende at der i 2023 blev identificeret den første swIAV med 275Y mutationen, som er linket til resistens mod det antivirale middel oseltamivir (Tamiflu). Denne virus var af genotypen H1pdmN1av-2, som var den genotype, der gav anledning til den anden zoonotiske case i Danmark. De to virus med overnævnte mutationer er udvalgt til yderligere biologisk karakterisering, hvilket kun er muligt grundet den ekstra bevilling til overvågningsprogrammet for at opsætte de tidligere nævnte in-vitro assays. En anden måde at karakterisere swIAV *in-vivo* er ved at teste virus evne til at inficere fritter, som er en anerkendt model for human influenza infektion. To danske swIAVs; H1pdmN1av-1 og H1pdmN1av-2, er blevet testet for deres transmission i fritter, og resultaterne viste at begge virus effektivt kunne inficerer fritterne, og at luftbåren smitte var muligt for H1pdmN1av-1, hvilket understreger denne virus zoonotiske potentiale.

Alt i alt har den årlige swIAV overvågning, gennemført i 2023, givet et vigtigt indblik i spredningen, cirkulationen og evolutionen af swIAV i Danmark og var essentiel for kortlægningen af swIAV med specifikke molekylære markøer og karakteristika, der potentielt kan øge deres zoonotiske egenskaber.

Vigtigste fund:

- > 60 % swIAV positive indsendelser og en stigning i andelen af H1pdm09 (1A.3.3.2) positive indsendelser
- Stigning i fundet af den interne kassette "PPPPPA" i alle HA og NA kombinationer

- Påvisning i en besætning af en human sæsoninfluenza lignende H3N2 virus i danske svin, som var reassorteret og havde fået den interne kassette ”PPPPPA”. Der var ingen tegn på spredning til andre besætninger af dette virus.
- Blandt de danske H1av virus falder langt størstedelen under grupperingen “1C.2.4”, og disse virus viste generelt begrænset kryds-reaktion til H1av komponenten i den tilgængelige trivalente svine influenza vaccine.
- For første gang blev der påvist mutationer i PB2 og NA proteinerne, som er relateret til værtspektrum og neuraminidase inhibitor (Tamiflu) resistens.
- Virus af H1pdmN1pdm- og H1pdm09N1av genotyperne blev biologisk karakteriseret ved brug af *in-vitro* og *in-vivo* metoder, som påviste tilstedeværelsen af adskillige karakteristika, som tegn på øget zoonotisk potentiale.
- Lav kryds-reaktion mellem danske swIAVs og antisera rejst mod de humane sæson influenza vaccinestammer, hvilket kan indikere en ringe immunitet i den humane befolkning overfor de swIAV, der cirkulerer i danske svinebesætninger.

Summary

In 2023, 478 submissions were received, which is a slight reduction of the number of submissions compared to 2022, but markedly lower than the number obtained from 2019-2022. The proportion of swIAV positive submissions revealed a small decrease compared to 2022, but it was still over 60 %, which is significantly higher than all previous years (2012-2021). The proportion of submissions positive for the HA gene of H1N1pdm09 origin increased reaching 40 % of swIAV positive submissions, most likely driven by the vast detection of the H1pdm09N1av genotypes and a small increase in the H1pdm09N2sw subtype.

For the first time, an active surveillance in form of a screening for swIAV and avian influenza virus (AIV) in free-range and organic Danish swine herds was included. The results showed a significantly lower proportion (28 %) of swIAV positive herds in this production system compared to the passive surveillance program, where mainly conventional herds are included. The detected swIAV genotypes did not deviate from those detected in the passive surveillance program and - importantly no AIV were detected.

In the 2023 surveillance, the number of whole genome sequenced submissions was increased resulting in a total of 192 genotyped Danish swIAVs, which provided an important dataset for investigation of the genotype constellations, genetic markers and the evolution. It was evident that the internal cassette “PPPPPA” (P = pandemic H1N1pdm09 and A = avian-like swine H1N1) became increasingly prevalent in all the different HA and NA combinations detected. Even a human seasonal H3N2 virus that was detected in 2023, had gained a PPPPPA cassette, resulting in a novel triple-reassortant virus. The increased proportion of Danish swIAV carrying the PPPPPA internal gene cassette indicate, that these viruses have an advantage that potentially is related to the replication and/or the virus’ ability to modulate the host response post infection. In 2023, novel in-vitro assays to biologically characterize selected Danish swIAV were established, and the preliminary results revealed important differences in the different genotypes, that can aid in explaining the success and demise of Danish swIAVs in the herds, and additionally generate important results in relation to evaluating the zoonotic potential. In specific the in-vitro assay testing the growth of swIAVs in different cell cultures, revealed that a swine H1pdmN1pdm-2 virus similar to the first Danish zoonotic case, grew better than a human seasonal IAV in human alveolar cells. In addition, another in-vitro assay testing the ability of different swIAV to reduce the antiviral host response, showed that the H1pdmN1av-1 genotype could reduce the production of two important antiviral proteins in human alveolar cells and maintain efficient replication.

The phylogenetic analyses revealed that the 1C.2.4 (H1av) clade have successfully spread among Danish pigs. For the 1A (H1pdm09Nx) viruses, a clear division of the 1A.3.3.2 clade into two major clusters, representing H1pdmN1pdm viruses in one cluster and the H1pdm09N1av viruses in another cluster, was seen. The N1 phylogeny showed clear clustering according to the HA pairing, whereas the N2 phylogeny was more scattered with no clear clustering according to the HA pairing or genotype.

Antigenic characterization by hemagglutination-inhibition test revealed a general lack of cross-reaction between the Danish swIAVs and ferret sera raised against human seasonal H1 vaccine strains. For the trivalent swine vaccine there was a low level of cross-reaction to the H1avNx (1C) viruses, especially for the viruses belonging to the clade 1C.2.4. However, a greater level of cross-reaction was observed for the H1pdm09Nx viruses to the monovalent swine vaccine against H1N1pdm09 virus. For the detected H3N2 virus of human seasonal origin, a lack of cross-reaction was observed to the trivalent swine vaccine, whereas cross-reaction was seen to the human H3 ferret antisera panel.

The whole genome sequencing allowed for detailed genetic characterization of the different proteins in regards to molecular markers previously described to play a role in the resistance against antiviral drugs, virulence or zoonotic potential of swIAVs. Several markers were again discovered in the Danish swIAV, specifically in the PA and NP proteins that could enhance the zoonotic potential. In addition, for the first time a PB2 mutation was observed in one H1avN2sw-1 genotype in a previously defined to be important determinant for the host-range of AIV, however the amino acid change was different from the ones previously described. Another worrying finding was that for the first time the presence of the 275Y residue linked to resistance against the antiviral agent oseltamivir was detected in one of the swIAV of the H1pdm09N1av-2 genotype. These two viruses have been selected for further biological characterization, which this year was possible due to the extra funding provided for the surveillance to establish the above-mentioned in-vitro assays. Another method for biological characterization of swIAV in-vivo is to infect ferrets. Ferrets are a well-known model for human influenza infections. Two Danish swIAV of the H1pdm09N1av-1 and H1pdm09N1av-2 genotypes were evaluated for their transmission in ferrets. Both genotypes effectively infected the ferrets, but the H1pdm09N1av-1 genotype was also able to transmit among ferrets by aerosols, which further underlines its zoonotic potential.

In conclusion, the annual Danish swIAV surveillance performed in 2023 gave valuable insights into the swIAV distribution, circulation and evolution in Denmark and was essential for discovering swIAV with specific molecular markers and traits that potentially enhance their zoonotic risk.

Key findings

- > 60 % swIAV positive submissions and an increase in the proportion of submissions carrying an HA of H1N1pdm09 (1A) origin
- Increased occurrence of the PPPPPA internal gene cassette in all HA and NA combinations
- A human seasonal H3N2 was detected in Danish swine and this virus had gained the PPPPPA internal gene cassette indicating reassortment with swIAV strains. There was no data to support the spread of this virus to other herds.
- Domination of the 1C.2.4 clade among the Danish swIAVs, which for selected isolated viruses showed a limited cross-reaction to the H1av component of the current trivalent swine vaccine.
- Genetic markers in the PB2 and NA proteins related to host range and neuraminidase inhibitor (Tamiflu) resistance, respectively, were detected for the first time
- H1pdmN1pdm- and H1pdm09N1av genotypes were assessed using in vivo- and in-vitro assays revealing several traits that could indicate enhanced zoonotic potential

- Low level of cross-reaction between Danish swIAVs and antisera raised against human-seasonal influenza vaccine strains

Introduction

Swine Influenza A virus (swIAV) is not only a major pathogen causing health and welfare problems in pigs globally, but also an important zoonotic pathogen that in 2009 led to the last major influenza pandemic.

SwIAV mainly causes respiratory disease as the virus targets the epithelial cells of the respiratory tract such as the nasal, tracheal and bronchial epithelium causing destruction of the epithelium and mucosa and interstitial pneumoniamainly affecting the cranial and apical lung lobes. The clinical signs observed constitutes signs of respiratory disease similar to that observed in humans infected with influenza A virus (IAV) and also includes fever, lethargy, loss of appetite, nasal secretions, coughing and sneezing (1,2). SwIAV is also part of what is known as the Porcine Respiratory Disease Complex, where circulating bacteria and viruses infecting the pigs at the same time can lead to enhanced disease and higher mortality than expected for an infection with a single pathogen (3). As swIAV destroys the epithelium and alter the cytokine response, swIAV infected pigs can be more susceptible to other viral or bacterial infections causing severe pneumonia. Therefore, swIAV is also a significant factor in promoting antibiotic usage in the Danish swine herds.

Since the virus (H1N1) that caused the Spanish flu in 1918 were detected in pigs, swIAV has been regarded as a major concern in regard to seeding a virus into humans that could spark a new influenza pandemic (4). In 2009, this concern became real, as a novel pandemic in humans was due to a reassortant Mexican swine H1N1 virus (H1N1pdm09) carrying genes both related to the Spanish flu, an American swine H3N2 virus and a European swine virus with origin in birds (Eurasian avian-like swine H1N1) (5). During the 2009 pandemic in humans, the virus was re-introduced into the global pig population. Awareness on the importance of monitoring swIAV circulation arose, and several countries implemented surveillance systems for swIAV as a result.

Systematic swIAV surveillance data have been obtained in Denmark since 2011 (6). Globally, zoonotic infections with swIAV occur every year, with most cases registered in the USA with H3N2 viruses infecting participants in animal fairs, where close contact to pigs are prudent. In Denmark, two zoonotic cases of swIAV were registered in 2021, during the SARS-CoV-2 lock-down, which limited the normal human IAV season. The first case was with a swine adapted H1N1pdm09 virus causing normal influenza-like illness (7) and the second was with an swine H1pdm09N1av virus causing severe disease with convulsions (8). Several factors are important for evaluating the zoonotic potential of a given swIAV and no single determinant have been identified. Such factors include receptor binding specificity, replication efficiency in human cells, polymerase activity, ability of airborne transmission and existing population immunity in humans.

To understand the evolution of Influenza A viruses (IAVs) it is important to know the virus genome. IAVs have a negative sense RNA genome that is divided into eight segments. This makes them capable of evolving through two different mechanisms; viral drift and viral reassortment (9). The viral drift is caused by point mutations due to IAV being an RNA virus that does not have a proof-reading capacity when generating novel genomic RNA for virus progeny. The viral reassortments, on the other hand, is possible due to the segmented nature of the IAV genome, which can be mixed in novel combinations during co-infections of a host. Novel gene segment combinations will give rise to novel genotypes, and sometimes novel subtypes if a new combination of the HA and NA surface genes are generated (10). All influenza pandemics since the Spanish Flu have been caused by a reassortant IAV.

The subtype of an IAV is determined by the HA and NA segments, which encodes the main surface proteins on the surface of the IAV particle. Overall, four different subtypes are present in the swine population - H1N1, H1N2, H3N2 and more rarely, H3N1. H1Nx subtypes can be further classified into different lineages based on the origin of the HA gene segment for example avian (1C), human (1B) or pandemic H1N1pdm09 (1A) origin, and in this report the different combinations of surface genes of different lineages are referred to as HA and NA combinations. When the lineage of all eight gene segments are available the genotype can be determined based on the origin of each gene segment.

Abbreviations and overview on circulating Danish subtypes and genotypes

To help the reader, a short introduction to the nomenclature of the circulating swIAVs is presented in the following section, in addition to a historical background for each swIAV introduction.

H1avN1av/Eurasian avian-like swine H1N1 (1C.2 viruses according to BV-BRC): This virus was introduced as a whole virus from birds to pigs in the end of the seventies or beginning of the eighties. The first detection of this virus in Denmark was in 1981 and it quickly became enzootic.

H3swN2sw/swine adapted Hong Kong H3N2: This virus originated from the human pandemic initiating in Hong Kong in 1968. Following the human pandemic, it adapted to swine, and in 1984 it reassorted with the above-mentioned Eurasian avian-like swine H1N1, where it retained its surface genes but gained an avian-like internal gene cassette. This virus was first detected in Denmark in 1990 and has not been detected in the swIAV surveillance since 2014.

H1avN2sw (1C.2): This virus has the H1 of Eurasian avian-like swine origin and the N2 of the H3swN2sw virus. The initially documented H1avN2sw had the internal gene cassette of Eurasian avian-like origin, and was first detected in Denmark in 2003. This virus is still enzootic and has been the most prevalent HA and NA combination observed in Denmark since the surveillance was initiated in 2011. This virus has reassorted with the H1N1pdm09 virus to gain several different internal gene cassettes, with a full internal gene cassette of H1N1pdm09 origin being the most prevalent.

H1N1pdm09/H1pdm09N1pdm09 (1A.3.3.2): This virus caused the human influenza pandemic in 2009 and originates from Mexican swine. HA, NA and the internal genes are different from the other enzootic subtypes, and this virus is also considered enzootic in Denmark.

H1pdm09N1av (1A.3.3.2): This virus was first detected in the surveillance in 2018 and is a reassortant carrying the HA gene of H1N1pdm09 origin and the NA gene of Eurasian avian-like H1N1 origin. Since its first introduction, H1pdm09N1av has become highly prevalent being currently the second most common subtype.

H1huN2sw (1B): This virus was first detected in England in 1994 and is a reassortant carrying an HA gene of the human seasonal flu and the NA gene of H3swN2sw origin. This virus has never been detected in Denmark, but circulates in the majority of Europe.

H1pdm09: Viruses carrying the specific HA gene of H1N1pdm09 origin.

N1pdm09: Viruses carrying the specific NA gene of H1N1pdm09 origin.

H1av: Viruses carrying the specific HA gene of Eurasian avian-like swine/H1avN1av origin.

N1av: Viruses carrying the specific NA gene of Eurasian avian-like swine/H1avN1av origin.

N2sw: Viruses carrying the specific NA gene of swine adapted Hong Kong H3N2/H3swN2sw origin.

N2hu#: Viruses carrying the specific NA gene of human seasonal origin, where ”#” indicates to which human influenza season the gene it is most related to.

H3hu#: Viruses carrying the specific HA gene of human seasonal origin, where ”#” indicates to which human influenza season the gene it is most related to.

Internal gene cassette: To describe the origin of the six gene segments (PB2, PB1, PA, NP, M, NS) making out the internal genes of the swIAV genotypes the abbreviation “P” is used for H1N1pdm09 origin (1A.3.3.2) and “A” for Eurasian avian-like H1N1 (1C) origin, and one letter for each gene, so that “PPPPPP” for example indicate a complete internal gene cassette of H1N1pdm09 origin.

On the following page Figure 1 provides an overview on the different introductions of IAV into the Danish swine population is presented along with the subsequent reassortant genotypes that are currently circulating in Danish swine in 2023.

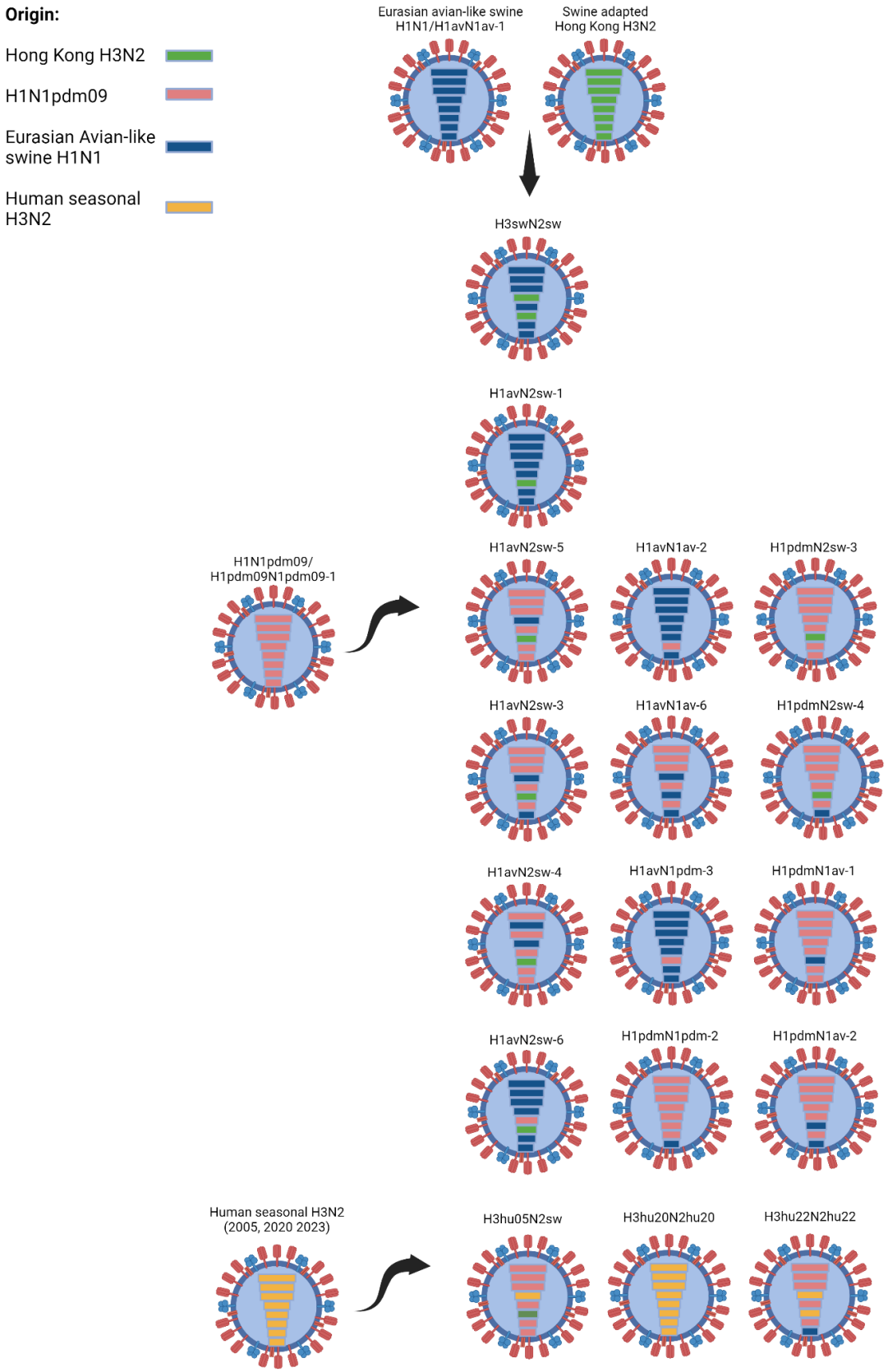

Figure 1. Overview of the swIAV introductions and circulating genotypes in Denmark 2023

Objectives and results

The overall aim of the Danish passive surveillance program is to investigate the contemporary circulating swIAV subtypes and genotypes in Danish swine herd and aid in identifying the cause of disease in Danish pig herds, which in turn can aid in reducing the antibiotic usage. The surveillance program is focused on several veterinary and zoonotic aspects;

The veterinary aspects include:

- To obtain a better understanding of the complex epidemiology of swIAV under Danish conditions.
- To ensure continuously updated virus stocks for the fast production of vaccines that result in enhanced disease in swine
- To ensure that the diagnostics assays used in Denmark are able to identify all circulating swIAV
- To document to export markets, which swIAV are present in Denmark.
- To contribute to a common european and global overview of the circulating swIAVs.

The zoonotic aspects include:

- Early detection and isolation of novel swIAV with increased zoonotic potential
- Early detection of molecular markers that have been related to increased risks for humans
- Early detection of swIAVs carrying molecular markers related to antiviral resistance
- Identification of genetic changes in circulating swIAV, which can aid in the development of proper diagnostic assays and vaccines for humans if spill-overs happens.

The extent of the Danish passive swIAV surveillance differs from year to year.

In 2023, the surveillance encompassed three main objectives: *passive surveillance of swIAV*, *screening of IAV including both swIAV and avian influenza (AI) in outdoor (free range and organic) swine herds and Biological characterization of novel/divergent swIAV, that are* presented in the following.

Objective 1 – passive surveillance of swIAV

The first objective was to obtain a real-time insight into the contemporary circulating swIAV in Denmark including detailed genetic characterization to map the evolution of enzootic and emerging strains.

Submissions

As illustrated in Figure 2, 478 submissions were received in 2023, which is a slight reduction of the number of submissions compared to 2022, but markedly lower than the number obtained from 2019-2022. A higher number of submissions were obtained between August-April, which is similar to the pattern observed previous years, with the exception of 2021, where special efforts were applied to obtain more submission in the first semester.

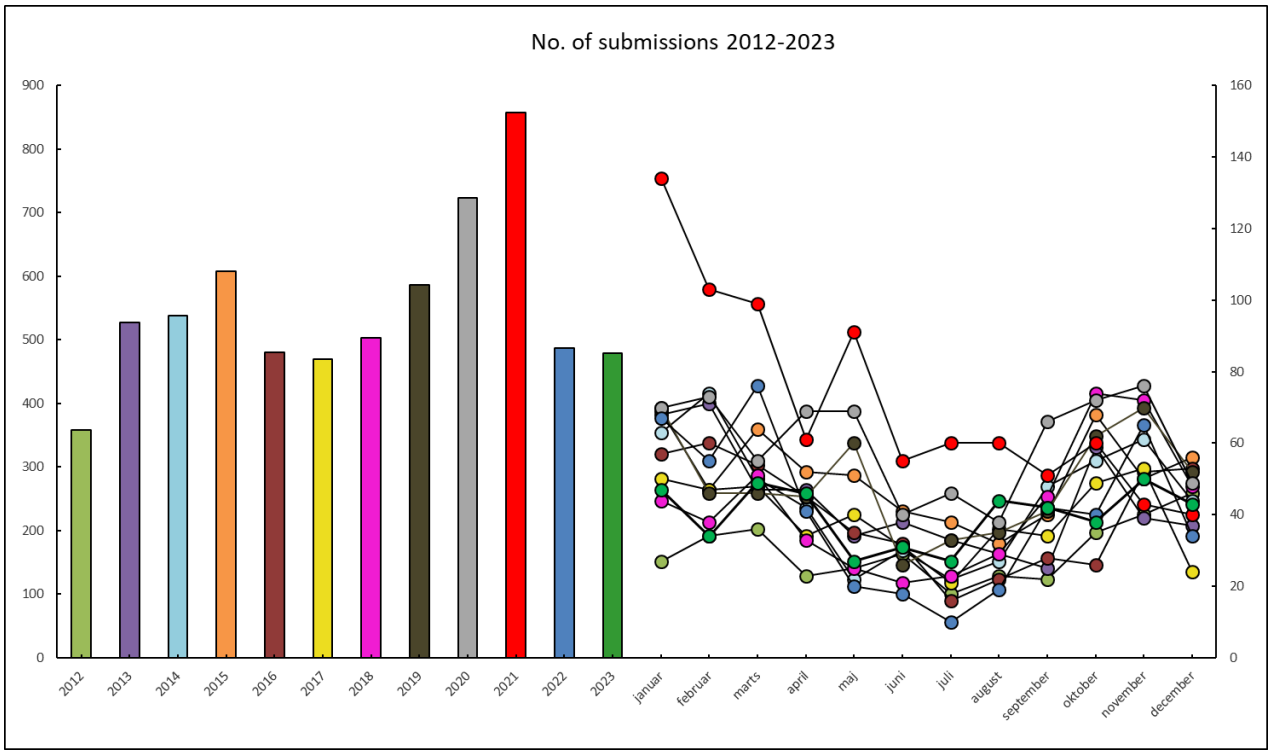

Figure 2. Distribution of the number of submissions for the swIAV surveillance program 2012-2023 divided over years (columns on the left) and months (column on the right).

In total, 1612 samples were received in 2023 in the two laboratories “SSI” and “L&F”. The SSI laboratory received on average 4.6 samples per submission (1139/245), whereas L&F received on average 2 samples per submission (473/228). In total, 378 herds with different CHR numbers submitted samples for the surveillance, and 239 herds tested positive a least once during the year corresponding to a proportion of swIAV positive herds of 63 %. The proportion of swIAV positive herds testing positive for H1pdm (1A.3.3.2) in at least one sample over the year was 44 % (106/239 herds).

The monthly proportion of swIAV positive and negative submissions varied over the year. The highest number of swIAV positive submissions was observed in February and May, whereas the lowest number of swIAV positive submissions were observed in April and November. The largest proportion of H1pdm09 positive submissions was observed in May and December (Figure 3).

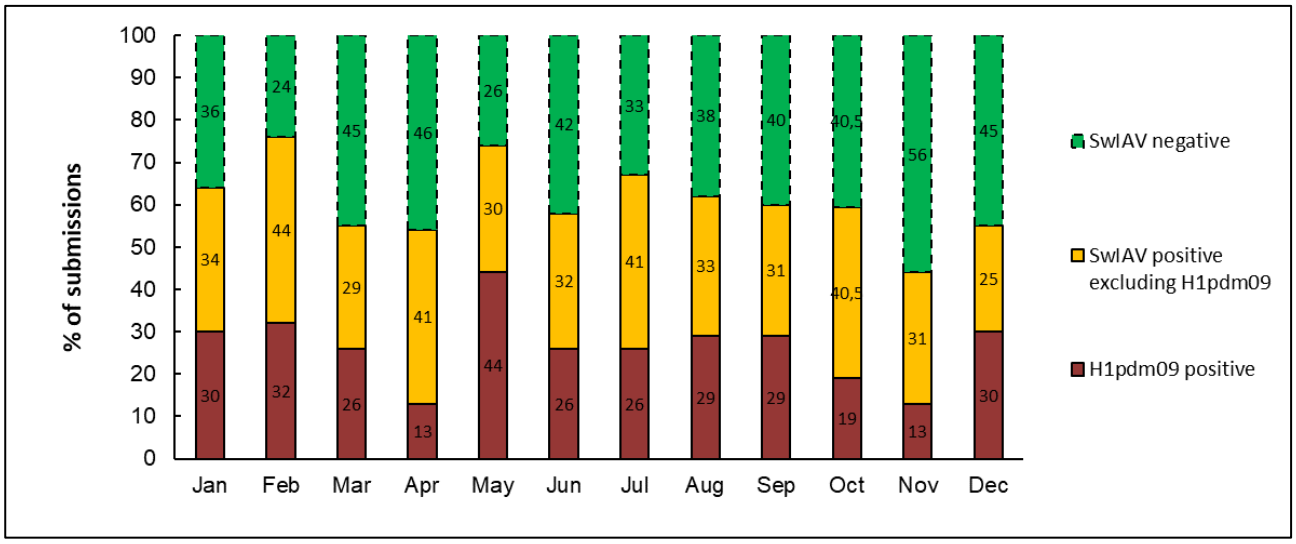

Figure 3. The 2023 monthly proportion (%) of swIAV negative and positive submissions with the proportion of H1pdm09 (1A.3.3.2) positive submissions indicated.

Considering the numbers of swIAV positive and negative submissions and the proportion of H1pdm09 (1A.3.3.2) positive submission over the years of the surveillance, a small increase in the percentage of swIAV positive submission have been observed from 2017-2021. However, a significant jump in the proportion was observed in 2022, where 67 % of the submissions were swIAV positive. In 2023, a decrease was observed reaching 61 % swIAV positive submissions (293/478), which is still significantly higher than the proportion observed previous year 2012-2021 (Figure 4). The proportion of H1pdm (1A.3.3.2) positive submissions of the swIAV positive submissions was 40 % in 2023, representing a 4 % percentage point increase compared to 2022, but similar to the level observed in 2021.

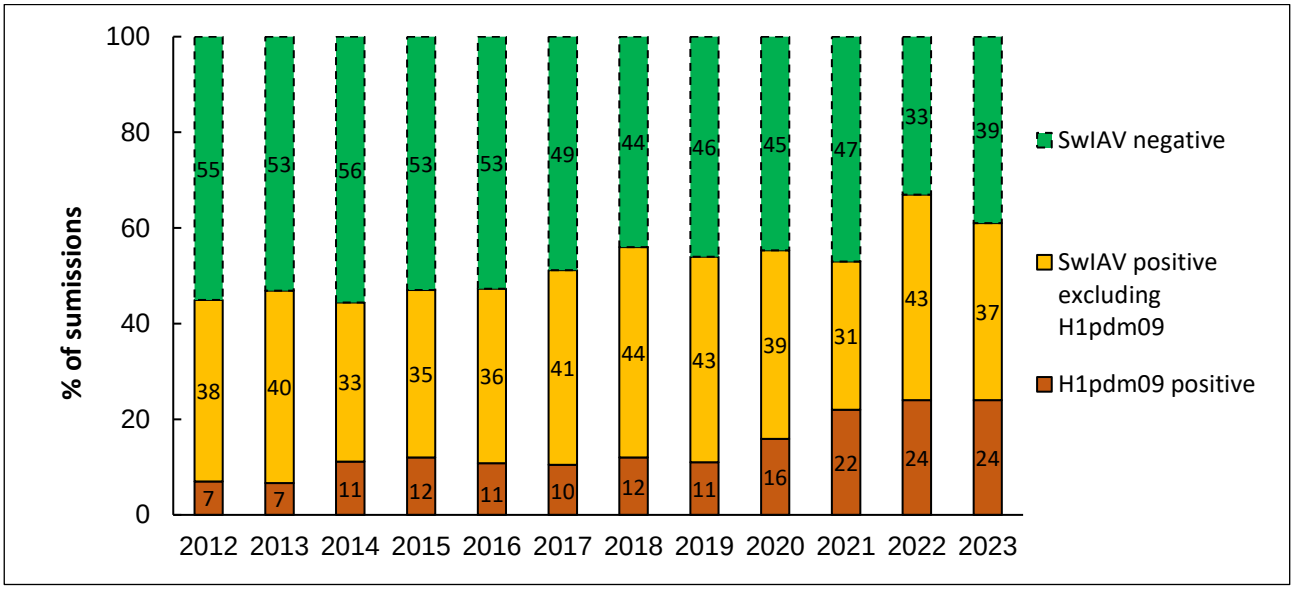

Figure 4. The annual proportion (%) of swIAV negative and positive submissions with the proportion of H1pdm09 positive submissions indicated.

Combinations of HA and NA genes (subtyping)

Through the passive swIAV surveillance program, 236 submissions had the HA and NA combination (previously defined as subtype) determined in 2023, which corresponds to 80 % (236/293) of the swIAV positive submissions. As illustrated in Figure 5, H1avN2sw (1C) viruses still represented the largest proportion of the detected swIAVs (53 %) and a small increase was observed compared to 2022. The second most prevalent swIAV was the H1pdm09N1av (1A.3.3.2) virus (21%)The proportion of H1pdm09N1av was slightly lower than last year. However, it should be noted that in 2023 several submissions (5 %) were deemed to be "H1pdm09N1x" in the multiplex qPCR (determining the HA and NA combination), which most likely represent some additional H1pdm09N1av viruses, where the viral load was not high enough to confirm the HA and NA lineage by NGS. H1N1pdm09 was the third most prevalent virus constituting only 7.6 % of the submissions, which is the lowest proportion of H1N1pdm09 viruses ever observed in the surveillance. A similar low proportion of H1avN1av (5 %) and H1pdm09N2sw (5.9 %) were observed this year and finally two H1avN1pdm09 viruses, one H1avN1x and a full human seasonal influenza virus (H3hu20N2hu20) were detected in swine.

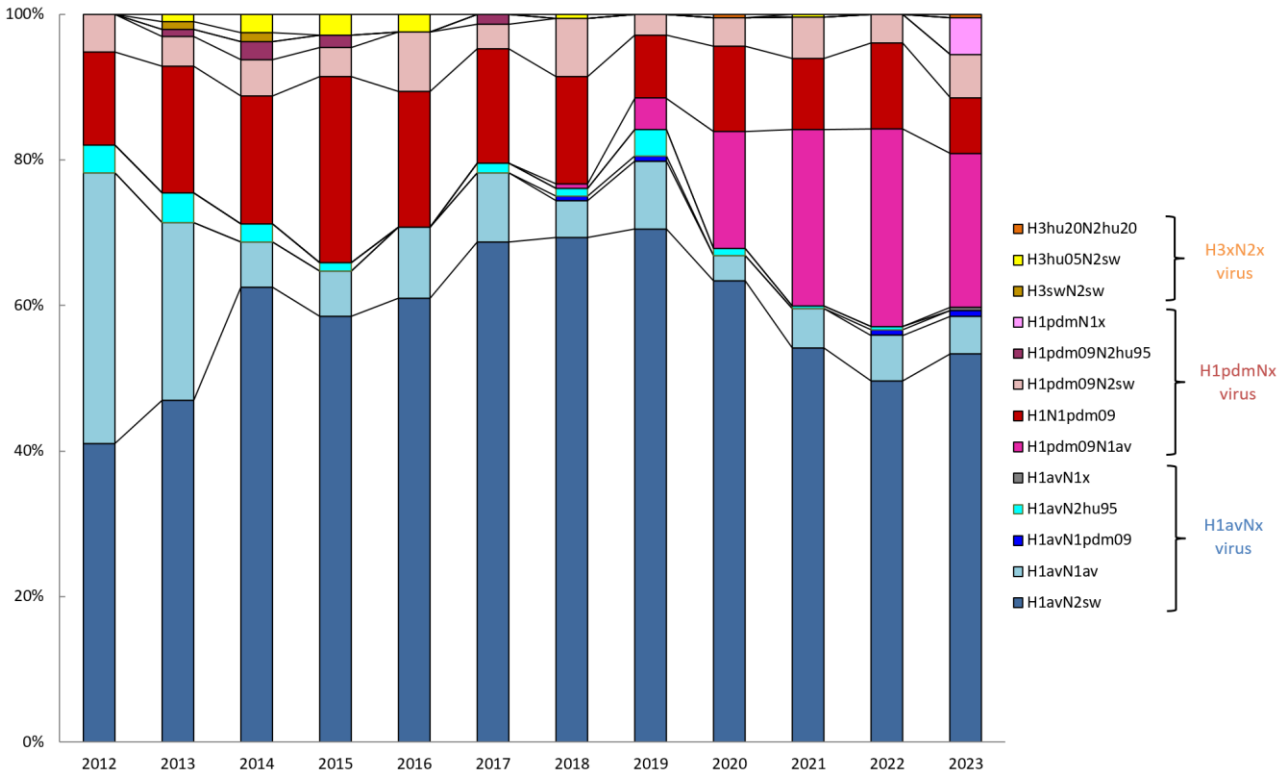

Figure 5. HA and NA combinations in Denmark 2012-2023.

In total, eight submission showed several swIAVs in the same sample and ten submission showed different swIAVs in the different samples of the same submission, in both cases indicating that several swIAVs were circulating within the same herd (defined by CHR no.) at the same time. The number of submissions where multiple swIAVs were present corresponded to 7 % of the total number of submissions, where the HA and NA combinations were determined.

Thirty-one herds submitted samples for the swIAV surveillance more than once, where it was possible to determine the HA and NA combination at each time point, providing an overview on the potential change in HA and NA combination over time. In 9/31 (29 %) of these cases a change in virus was observed over

time. The specific changes are detailed in Table 1. No clear tendency towards one HA and NA combination dominating over another was observed. However, in 8/9 cases a change from an H1avNx to or from H1pdm09Nx was observed indicating the lack of cross-reactive antibodies between the H1 of the two lineages. However, in one case the change was from H1N1pdm09 to H1pdm09N1av.

	1st submission	2nd submission	3rd submission	4th submission
Herd 1	H1pdm09N1av	H1avN1av		
Herd 2	H1pdm09N1av	H1avN2sw		
Herd 3	H1avN2sw	H1avN2sw	H1avN2sw	H1pdm09N1av
Herd 4	H1avN2sw	H1N1pdm09		
Herd 5	H1avN2sw	H1pdm09N1av		
Herd 6	H1pdm09N1av	H1avN2sw		
Herd 7	H1pdm09N2sw	H1avN2sw		
Herd 8	H1N1pdm09	H1pdm09N1av		
Herd 9	H1avN2sw	H1pdm09N1av		

Table 1. Change in swIAVs (HA and NA combination) over time (different submissions) in individual herds.

Genotyping

From the passive swIAV surveillance, eighty nine (89) H1avNx viruses, fifty six (56) H1pdmNx viruses and one H3N2 virus were genotyped determining the origin of each internal gene segment by NGS. In addition, eighteen (18) H1avNx (1C) and 28 H1pdmNx (1A.3.3.2) viruses from the private company "Aerocollect", the active swIAV screening in free-range and organic herds (objective 2) and a KU-CEVA project were genotyped resulting in a total of 107 H1avNx, 84 H1pdmNx and one H3N2 virus (total = 192) being genotyped in 2023. The additional samples were included to obtain a wider selection of Danish swIAVs for the genetic characterization. The private company Aerocollect performed an active screening of Danish swine herds from a specific veterinary clinic in 2023 and it was a selection of these samples that were included. The proportion of the different H1avNx and H1pdm09Nx genotypes found in 192 swIAV samples is presented in Figure 6.

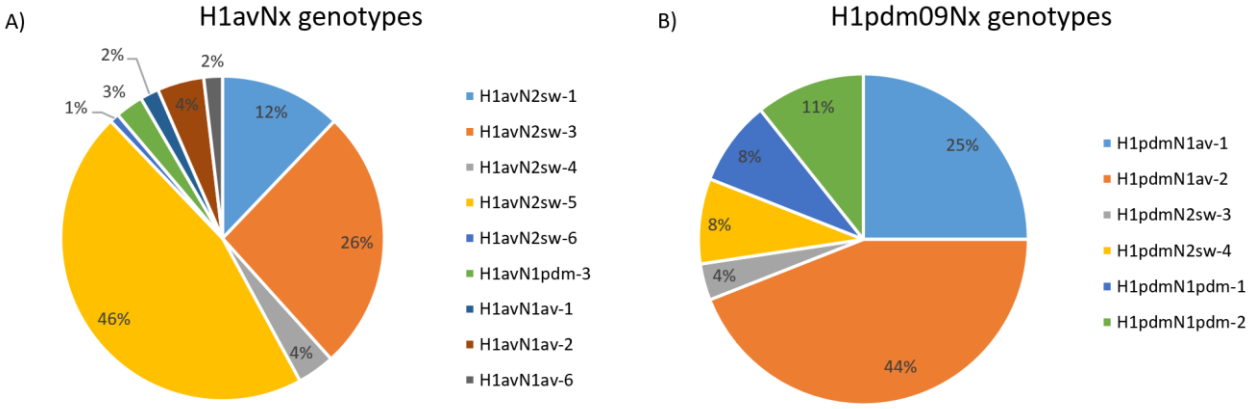

Figure 6. Genotypes of the H1avNx and H1pdm09Nx viruses in Denmark 2023, from both passive surveillance (objective 1), Aerocollect, KU-CEVA project and the active surveillance in free range and organic herds (objective 2).

For the H1avNx viruses, the H1avN2sw-5 genotype dominated and constituted 46 % of the genotyped H1avNx (1C) viruses. This genotype has a complete internal gene cassette of H1N1pdm09 (1A.3.3.2) origin

(PPPPPP). The second most commonly detected genotype was the H1avN2sw-3 genotype (26 % of the genotyped H1avNx viruses), where all internal gene segments are of H1N1pdm09 (1A.3.3.2) origin with the exception the NS gene segment being of Eurasian avian-like H1N1 (1C) origin (PPPPPA). This genotype was first detected in 2020 in two herds. It is interesting that also the H1avN1av (1C) virus is detected with a similar internal gene cassette (PPPPPA) in the H1avN1av-6 genotype constituting 4 % of the genotyped H1avNx viruses. The initially first documented H1avN2sw-1 genotype in Danish swine carrying a complete internal gene cassette of Eurasian avian-like H1N1 origin (AAAAAA) was still significantly represented constituting 12 % of the genotyped H1avNx viruses.

For the H1pdm09Nx (1A.3.3.2) viruses, it is clear that the H1pdmN1av-2 genotype (PPPPPA) was dominating, constituting 44 %. However, the initially detected H1pdmN1av-1 virus (PPPPPP) still constituted 25 % of the H1pdm09Nx viruses. A more equal distribution was observed between H1pdmN1pdm-1 (PPPPPP) and H1pdmN1pdm-2 (PPPPPA), with 8 % and 11 %, respectively. Interestingly, a novel H1pdmN1av genotype (H1pdmN1av -3) was detected this year carrying a complete internal gene cassette of Eurasian avian-like H1N1 (1C) origin.

In 2023, a single H3N2 virus was detected. This virus was a novel reassortant carrying the surface HA and NA segments of human seasonal origin from 2022, whereas all internal gene segments were of H1N1pdm09 origin with the exception the NS gene segment being of Eurasian avian-like H1N1 origin (PPPPPA).

Details on the specific gene constellation of the different genotypes detected in 2023 are presented in Figure 1.

When relating the internal gene cassette to the 1A and 1C clades defined by the annotation tool (https://www.bv-brc.org/app/SubspeciesClassification) of the H1xNx viruses, it is seen that the some clades have a higher proportion of a specific internal gene cassette constellation, but the different gene cassettes are represented in most clades (Table 2). The total portion of the different gene cassette constellations across all HA and NA combinations identified, confirms that the PPPPPA and PPPPPA are the most common with an almost 50/50 distribution (Table 2).

Internal cassette:	**Total:**	**H1avN2sw**				**H1avN1av**		**H1avN1pdm**	**H1pdmN1pdm**	**H1pdm09N1av**	**H1pdmN2sw**
AAAAAA	9.4 % (18/191)	1.06 % (1/95)	1.06 % (1/95)	7.45 % (7/95)	4.26 % (4/95)	0%	22.2 % (2/9)	100 % (3/3)	0%	0%	0%
PPPPPA	**43.5%** (83/191)	1.06 % (1/95)	5.32% (5/95)	21.28 % (20/95)	2.13 % (2/95)	0%	22.2 % (2/9)	0%	56.25 % (9/16)	63.8% (37/58)	70 % (7/10)
PAPPPP	2.1% (4/191)	0%	0%	4.26 % (4/95)	0%	0%	0%	0%	0%	0%	0%
PPPPPP	**41.9 %** (80/191)	2.13 % (2/95)	0%	45.74 % (44/95)	3.19 % (3/95)	0%	0%	0%	43.75% (7/16)	36.2% (21/58)	30 % (3/10)
AAAPAA	0.5% (1/191)	0%	0%	1.06 % (1/95)	0%	0%	0%	0%	0%	0%	0%
AAAAPA	2.6% (5/191)	0%	0%	0%	0%	55.6 % (5/9)	0%	0%	0%	0%	0%
Clade:		1C.2	1C.2.2	1C.2.4	1C.2.5	1C.2	1C.2.2	1C.2.4	1A.3.3.2	1A.3.3.2	1A.3.3.2

Table 2. Proportion of the different internal gene cassette constellations in the different HA and NA combinations and H1 clades, and the total proportion of these cassettes across all HA and NA combinations.

Phylogeny

The phylogeny of the Eurasian avian-like H1 (1C) of the Danish swIAV of 2023 is presented in Figure 7. In total, 146 HA sequences from the passive surveillance, Aerocollect, the Organic and Free-range screening and KU-Ceva project were included in the tree from 2023, along with selected reference sequences.

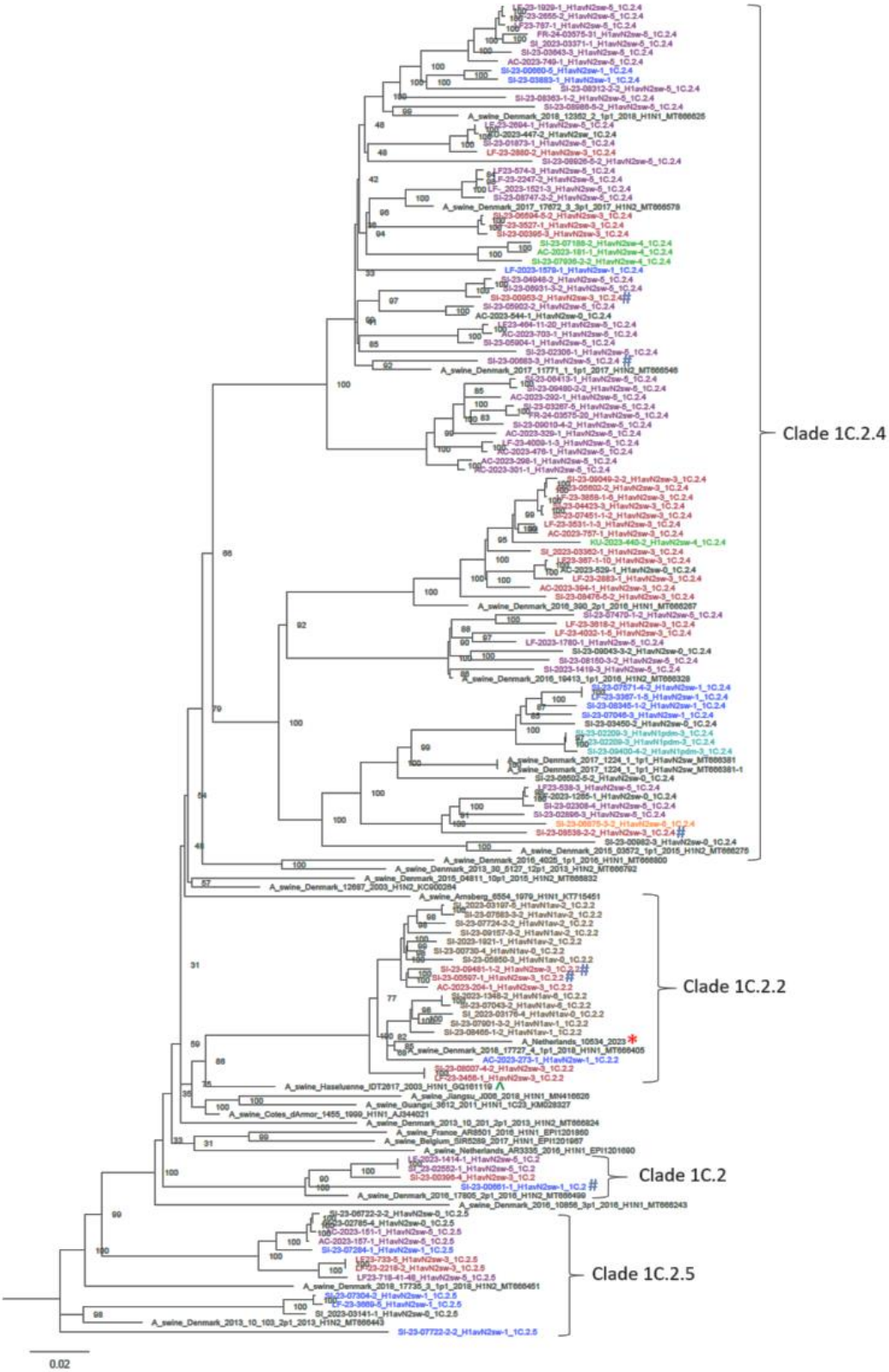

Figure 7. Maximum likelihood tree of Danish H1av from 2023 and selected reference sequences. The abbreviation "SI", "LF", "AC", "FR" and "KU" in the taxon indicate if the sample comes from SSI, L&F, Aerocollect, the free-range and organic screening or KU-Ceva project, respectively. "A_swine_Arnsberg_6554_1970_H1N1_KT715451" was used as an outgroup. "*" indicate zoonotic case swIAVs from 2023, "^" indicate the vaccine strains of Respiporc FLU3, Ceva animal health and "#" indicate that the virus was used for the HI-test. The H1avNx viruses are colored according to the genotype; H1avN2sw-1 (blue), H1avN2sw-3 (red), H1avN2sw-4 (green) H1avN2sw-5 (purple), H1avN2sw-6 (orange), H1avN1av (brown) and H1avN1pdm09 (turquoise).

For the H1avNx viruses it was observed that the Danish swIAVs cluster according to the 1C.2 clades defined by the BV-BRC (https://www.bv-brc.org/app/SubspeciesClassification), and that four major clades are represented including 1C.2, 1C.2.2, 1C.2.4 and 1C.2.5. The majority of the sequences belonged to the 1C.2.4 clade, which was further divided into two major "undefined" subclades. The upper subclade mainly contained H1avN2sw-5 viruses with a complete internal gene cassette of H1N1pdm09 origin (PPPPPP), whereas the lower subclade was divided further into two clusters, with one mainly representing the H1avN2sw-3 genotype that has the PPPPPA internal gene cassette. In other clusters, the more recently discovered H1avN1pdm09 viruses are situated along with H1avN2sw-1, one H1avN2sw-3, a few H1avNsw-5 and one H1avN2sw-6. Interestingly, all the H1avN1av viruses sequenced in 2023, belonged to the 1C.2.2 clade, along with one H1avN2sw-1 and three H1avN2sw-3. In the smaller 1C.2 and 1C.2.5 clades a mix of H1avN2sw-1, H1avN2sw-3 and H1avN2sw-5 were found.

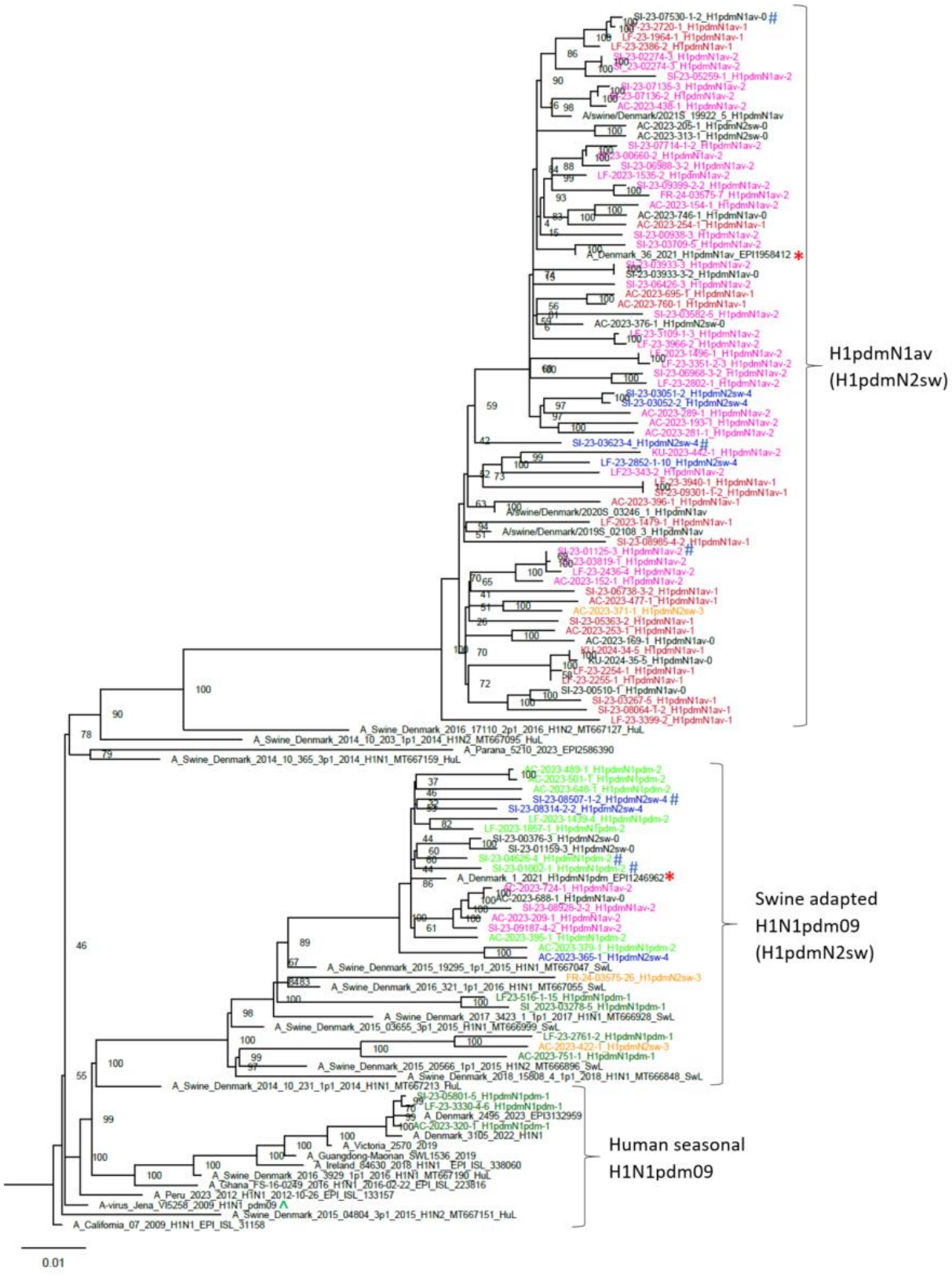

Figure 8. Maximum likelihood tree of Danish H1pdm09 (clade 1A.3.3.2) from 2023 and selected reference sequences. The abbreviation “SI”, “LF”, “AC”, “FR” and “KU” in the taxon indicate if the sample comes from SSI, L&F, Aerocollect, the free-range and organic screening or KU-Ceva project, respectively.

“A_California_07_2009_H1N1_EPI_ISL_31158” was used as an outgroup. “*” indicate zoonotic case swIAVs from 2023, “^” indicate the vaccine strains of Respiporc FLUpan H1N1, Ceva animal health and “#” indicate that the virus was used for the HI-test. The H1pdm09Nx viruses are colored according to the genotype; H1pdm09N1av-1 (red), H1pdm09N1av-2 (pink), H1pdmN1pdm-1 (dark green), H1pdmN1pdm-2 (light green), H1pdmN2sw-3 (orange) and H1pdmN2sw-4 (blue).

For the H1pdm09Nx (1A.3.3.2) viruses, it was observed that three major clades of H1pdm clade 1.A.3.3.2 were present in the tree, with one mainly constitutes of the H1pdm09N1av reassortant viruses but also some H1pdm09N2sw viruses of both the H1pdm09N2sw-3 and -4 genotype (Figure 8). The clustering of H1pdm09N1av-1 and H1pdm09N1av-2 genotypes in two separate clusters, as observed in previous years was no longer present. Within the swine-adapted H1N1pdm09 cluster it was clearly seen that the H1pdmN1pdm-2 viruses clustered in one separate upper cluster, whereas the H1pdmN1pdm-1 genotype were present outside the cluster. It was also observed that four H1pdm09N1av-2 viruses also clustered close to the H1N1pdm09-2 viruses, which could suggest novel reassortant events. Finally, three H1pdmN1pdm-1 viruses were located in the “human seasonal H1N1pdm09” cluster along with recent human seasonal H1N1pdm09 from 2022 and 2023, indicating recent reverse-zoonotic events.

In 2023, one H3N2 virus was detected, and the H3 belonged to the H3 clade 3C.2a1b.2a.2a.1b (2a.1b) defined by BV-BRC and clustering close to sequences of contemporary human seasonal 2021-2022 IAVs (Figure 9), whereas the previously detected Danish H3huN2sw viruses belonged to a separate subcluster with older H3 human seasonal viruses. The human seasonal cluster showed large genetic diversity to the swine adapted Hong Kong H3N2 cluster that also includes the current H3 vaccine strain available for swine in Europe.

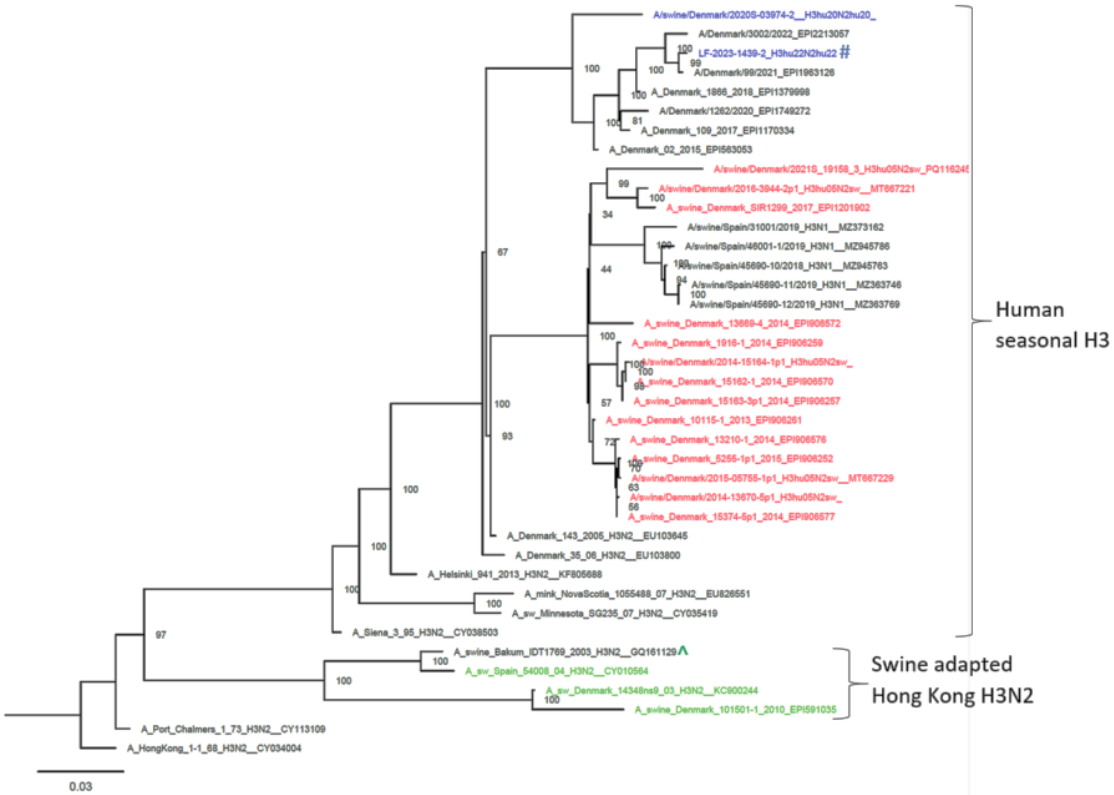

Figure 9. Maximum likelihood tree of Danish H3 and selected reference sequences. “A_HongKong_1-1_68_H3N2_CY034004” was used as an outgroup. “^” indicate the vaccine strains of Respiporc FLU3, Ceva

animal health and "#" indicate that the virus was used for the HI-test The Danish H3huN2sw reassortant viruses from previous years of the surveillance have a red taxon, the Danish H3huN2hu viruses of 2020 and 2023 have a blue taxon and the Danish H3N2 swine adapted Hong Kong viruses from previous years of the surveillance have a green taxon.

The phylogeny of the N1 and N2 sequences are available in Appendix 1 and 2. For the N1 sequences two major clusters were observed; one containing all the N1pdm09 sequences and one containing all the N1av sequences. The N1pdm09 were divided into smaller clusters, with the NA of H1pdmN1pdm-1 and two genotypes being in one subcluster and the NA of the H1avN1pdm09 being in another subcluster. For the N1av sequences, two subclusters were also present with the genotype H1pdmN1av-1 and 2 being in one subcluster, and the H1avN1av-1, 2 and 6 genotypes being in another subcluster. For the N2 sequences no?? clear clustering according to genotypes were observed. However, the N2 of the H3N2 virus of human seasonal origin detected in 2023, clustered separately from the other N2 of swine origin.

Objective 2 – screening of IAV including both swIAV and avian influenza (AI) in outdoor (free range and organic) swine herds

In 2023, 40 organic or free-range swine herds with minimum 100 sows existed in Denmark. In the current screening, twenty five herds volunteered to participate. In each herd, nasal swab samples were obtained in the farrowing field, at weaning and approx. 2-3 weeks after weaning. Seven of the 25 herds tested positive for IAV corresponding to 28 % of the herds. Four of these herds were organic herds, whereas three were free-range herds. Four of the seven herds tested positive at weaning, whereas one tested positive in the farrowing field and two tested positive 2-3 weeks after weaning. Five of the seven IAV positive herds showed clinical signs of respiratory disease. Several different HA and NA combinations were detected with high level of similarity to viruses found in the passive swIAV surveillance program (Figure 10) and avian influenza viruses were not detected in any of the herds.

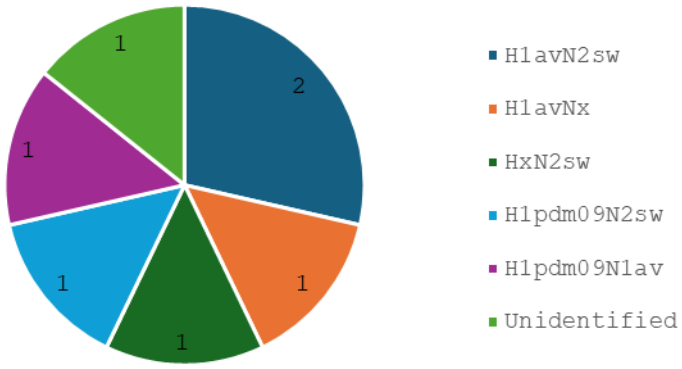

Figure 10. HA and NA combinations in IAV positive organic and free-range pig herds in Denmark 2023.

Objective 3 – Biological characterization of novel/divergent swIAV

Molecular markers

Previous research studies have identified several molecular markers/mutations of interest, when evaluating the virulence, zoonotic potential and antiviral resistance of swIAV. The presence of these markers were all examined in the sequences of the Danish swIAV from 2023. As mentioned under objective 1, 192 whole genome sequences (WGS) of swIAV from 2023 were available for further investigations. However, even

though the quality of the sequences were high enough to determine the genotype, not all sequences resulted in translatable proteins, that could be examined for molecular markers.

The PB2 protein

In total, 141 PB2 gene segments had a sufficiently high sequence quality for the assessment of the presence of the E627K mutation in the deduced protein sequence. This mutation is highly important for the host range of AIV, and is a marker for mammalian adaptation (11). None of the proteins carried the E627K mutation but one virus (LF-23-3669-5_H1avN2sw-1) had an E627A mutation. This mutation has only been described previously for an H5N8 avian influenza virus infecting an ostrich in 2021 and the impact of this mutation is presently unknown (12).

The PA protein

In total, 172 PA gene segments had a sufficiently high sequence quality for the assessment of the presence of the four V100I, N321K, I330V and A639T previously described to increase the pathogenicity and transmission of Eurasian avian-like H1N1 in ferrets (13), which are used as a model for human influenza infections. Eleven PA proteins (6.4 %) had all four mutations. The genotypes carrying these four mutations included H1pdmN1pdm-1, H1pdm09N1av-1, H1pdm09N1av-2, H1avN2sw-3 and H1avN2sw-5, all having a PA of H1N1pdm09 origin. In addition, seven PA proteins (4 %) also showed mutations at all sites, however with the V100I being a V100S/A and these all included genotypes with a PA protein of Eurasian avian-like H1N1 origin (H1avN1pdm-3 and H1avN2sw-1). One additional H1pdmN1av-2 also had V100L, with a PA protein of H1N1pdm09 origin. Overall, 48.6 % of all the PA proteins observed in 2023 carried 3/4 mutations in these sites. Whereas 20 % carried 2/4 mutations and another 20 % carried 1/4 mutations. Only two PA proteins (1 %) had none of the four mutations.

The NP protein

In total, 191 NP gene segments had a sufficiently high sequence quality for the assessment of mutations related to the MxA escape. The mutations includes 48Q, 53D, 98K, 99K, 100I/V and 313V (14). Position 48, 98 and 99 are important for the NP proteins of avian like H1N1 origin, while position 53, 100 and 313 are important for the NP proteins of H1N1pdm09 origin (14). Only 22/191 (11.5 %) available NP proteins were of Eurasian avian-like H1N1 origin and these generally had 48Q, 53E, 98K, 99K, 100R and 313F. All the NP proteins of Eurasian avian-like H1N1 carried the 48Q and 98K mutations, whereas only 14 proteins had the 99K mutation. The remaining 169 NP proteins were of H1N1pdm09 origin. In total, only 15/169 (9 %) carried the 53E mutation, whereas 120/169 (71 %) carried the 100I/V mutation and all the NP proteins of H1N1pdm09 origin carried the 313V mutation. These results indicate that a high prevalence of the NP mutations conferring MxA resistance are present in the Danish swIAVs.

The NA protein

In total, 189 NA gene segments (85 N1 and 104 N2) had a sufficiently high sequence quality for the assessment of the presence of neuraminidase inhibitor resistance, being H275Y and N295S in N1 and E119G/D/A/V and R292K in N2. All, were examined for For the first time in the surveillance one of the N1 proteins of Eurasian avian-like H1N1 origin of an H1pdm09N1av genotype had the 275Y residue. However, none of the N1 proteins had the 295S residue. None of the N2 proteins had any mutations in the two positions encoding for neuraminidase inhibition resistance.

The NS1 protein

The NS1 protein plays an important role in the regulation of the host innate immune response to IAV infections. As described in the 2022 report, the two zoonotic Danish swIAVs had a special NS1 constellation (8,15), and therefore all NS1 proteins were investigated for truncations and origin. In total, 192 NS1 gene segments had a sufficiently high sequence quality for the assessment. One hundred and eight (56 %) NS1 proteins were of Eurasian avian-like H1N1 origin and 84 were of H1N1pdm09 origin (44 %). All the NS1 proteins of H1N1pdm09 origin had the classical H1N1pdm09 truncation resulting in a protein of 219 amino acids. The majority of the genotypes carrying this NS1 protein were the H1avN2sw-5 (58 %), H1pdmN1av-1 (25 %), H1pdmN1pdm-1 (8.3 %), H1avN2sw-4 (4.8 %) and H1pdmN2sw-3 (3.6). However, the Eurasian avian-like H1N1 origin NS1 protein were present in five different lengths; 217, 219, 220, 228 and 230 amino acids, where 230 is the classical full length NS1 protein. In total, 27 (25 %) of the Eurasian avian-like H1N1 origin NS1 proteins had a length of 217 amino acids and included the genotypes H1avN2sw-3 (14.8 %), H1pdmN1av-2 (33.3 %), H1pdmN1pdm-2 (33,3 %),H1pdmN2sw-4 (14.8 %) and H3hu22N2hu22 (3.7 %). Only six viruses had an NS1 protein of Eurasian avian-like H1N1 origin of 219 amino acids, and included H1avN2sw-1 (16.7 %), H1avN1pdm-3 (16.7%), H1avN2sw-3 (50 %) and H1pdmN1av-2 (16.7 %) genotypes. In addition, five viruses had an NS1 protein of Eurasian avian-like H1N1 origin of 220 amino acids and included the H1avN2sw-3 (80 %) and H1pdmN2sw-4 (20 %) genotypes. Two H1avN1av-2 viruses carried an NS1 protein of Eurasian avian-like H1N1 origin of 228 amino acids. The full length NS1 protein of Eurasian avian-like H1N1 origin of 230 amino acids included 63 % of the total Eurasian avian-like H1N1 origin NS1 proteins. The genotypes carrying this NS1 protein included H1avN1av-1 (2.9 %), H1avN1av-2 (4.4 %), H1avN1av-6 (2.9 %), H1avN2sw-1 (17.6 %), H1avN2sw-3 (27.9 %), H1avN2sw-6 (1.5 %), H1pdmN1av-2 (39.7 %) and H1pdmN2sw-4 (2.9 %).

Isolation of swIAV in cell culture

In total, 20 viruses from 2023 were successfully isolated in MDCK-SIAT cells.

Antigenic characterization

To evaluate the cross-reaction to sera raised against both the human seasonal and swine H1 and H3 vaccine viruses, hemagglutinin inhibition (HI) tests was performed on a selection of Danish swIAV isolates from 2023 including six H1avNx, six H1pdmNx and one H3hu22N2hu22.

H1xNx	A/Victoria/4897/2022	A/Michigan/45/15	A/California/07/09	Respiporc FLUpan H1N1	Respiporc FLU3 (H1av)
Reference:					
Hamburg/1580/2009 3.SIAT 11-10-2024_H1pdmN1pdm-1	20	320	320	640	20
Deurne/IDT19038/2013 2.SIAT 14-10-2024_H1avN1av	40	40	40	80	640
Isolates:					
2023-08538-2 3.SIAT 22-01-2024_H1avN2sw-3 (1C.2.4)	<20	<20	20	20/40	20
2023-00661-1 3. SIAT 21-08-2023_H1avN2sw-1 (1C.2)	40	<20	<20	20	20
2023-00597-1 3. SIAT 21-08-2023_H1avN2sw-3 (1C.2.2)	20	80	80	20	160
2023-00683-3 3. SIAT 21-08-2023_H1avN2sw-5 (1C.2.4)	<20	<20	<20	20	20/40

2023-09481-1 2.SIAT 16-02-2024_H1avN2sw-3 (1C.2.2)	40	320	320	20/40	160
2023-00953-2 3.SIAT 14-12-2023_H1avN2sw-3 (1C.2.4)	<20	<20	20	<20/20	20
2023-07530-1 3.SIAT 22-01-2024_H1pdmN1av-1	<20	<20	<20	320	20
2023-01125-3 2.SIAT 14-12-2023_H1pdmN1av-2	<20/20	<20/20	20	80	20
2023-08507-1 3.SIAT 22-01-2024_H1pdmN2sw-4	<20	<20	20	80/160	20
2023-03623-4 3.SIAT 19.10.2023_H1pdmN2sw-4	<20/20	40	40/80	80	20
2023-04626-4 2.SIAT 30.11.2023_H1pdmN1pdm-2	<20	<20	20	320	20/40
2023-01002-1 3. SIAT 21-08-2023_H1pdmN1pdm-2	<20/20	<20	20	160	20

H3xN2x	**A/Thailand/8/2022**	**A/Switzerland/8060/2017**	**Respiporc FLU3 (H3)**	**Respiporc FLU3 (all)**
Reference:				
Warendorf/IDT22506/2015 3.SIAT 07-10-2024	<20	20	1280/2560	160/320
Isolates:				
2023-02552-3 3.SIAT 23-06-2023_H3hu22N2hu22-1	160	20	40	<20

Table 2. HI-titers of H1xNx and H3xN2x Danish swIAV isolates from 2023 to human and swine H1xNx and H3xN2x vaccine strains. HI cross-reaction between virus and sera was regarded as significant/positive if the titer was ≥40 (16). Red figures indicate that no significant cross-reaction was observed.

The results revealed that 50 % (3/6) of the H1avNx cross-reacted (minimum titer ≥40) to hyperimmune monovalent sera raised against the H1av strain included in Respiporc FLU3. Interestingly, the two viruses showing highest cross-reaction belonged to the 1C.2.2 clade. As the human seasonal H1 strain is of H1N1pdm09 origin, great genetic differences are expected compared to the H1avNx strains, also reflected in lack of cross-reaction observed between the sera raised against the most recent human H1pdm09 vaccine strain A/Victoria/4897/2022 and all the H1avNx viruses. Two viruses showed a titer of 40 and the remaining viruses were negative. All the H1pdm09Nx viruses, reacted with the hyperimmune sera raised against the swine Respiporc FLUpan H1N1 vaccine with a minimum titer of 80. However, all six viruses had no cross-reaction to the most recent human seasonal vaccine strain A/Victoria/4897/2022 and only 1/6 viruses showed cross-reaction to the older A/California/07/09 strain, which have high genetic similarities to strain included in the swine vaccine. The H3hu22N2hu22 virus showed cross-reaction to the monovalent sera raised against the H3 component of the Respiporc FLU3 vaccine with a titer of 40, but not to the hyperimmune sera rasied against the full Respiporc FLU3 vaccine. However, a higher level of cross-reaction was seen to the recent human H3 vaccine strain A/Thailand/8/2022, which was expected since this virus was a reassortant between human seasonal H3N2 and swIAV, with human seasonal surface genes.

Replication in human cells

In 2023, the surveillance included a characterization of two selected 2023 swIAVs of the H1pdmN1pdm-1 and H1pdmN1pdm-2 genotypes (A/swine/Denmark/SI-23-03391-2/2023 (swH1N1pdm-1) and A/swine/Denmark/SI-23-04626-4/2023 (swH1N1pdm-2)) in immortalized cell cultures of human and swine origin representing different parts of the respiratory tract (A549: human lung cells, Calu-3: human bronchial cells, RPMI-2650: human nasal cells and NPTr: swine tracheal cells) (Figure 11). A human seasonal H1N1pdm09 virus (huH1N1_2023) from 2023 was used as a reference. The preliminary results revealed that all three viruses were able to grow in the four cell lines, but to varying degrees. The H1pdmN1pdm-1(swH1N1pdm-1 in Figure 11) did not grow in the human cell lines, as it either plateaued or decreased in titer over time. However, it was able to grow in the NPTr swine cell line, reaching a peak titer of 2.2*10^4 at 72 hours post infection (hpi). This indicates that it would not be able to replicate in the human airways. The H1pdmN1pdm-2 (swH1N1pdm-2 in Figure 11) and human seasonal virus on the other hand exhibited similar growth kinetics in the Calu-3 cell line. Interestingly, the human seasonal virus reached higher titers in the NTPr swine cell line compared to the swine viruses, peaking at 3.1*10^6 at 72 hpi. Among the viruses tested, only H1pdmN1pdm-2 was able to replicate in the alveolar A549 cell line and it replicated to higher tiers than the human seasonal H1N1pdm09 in the human RPMI-2650 cell line.

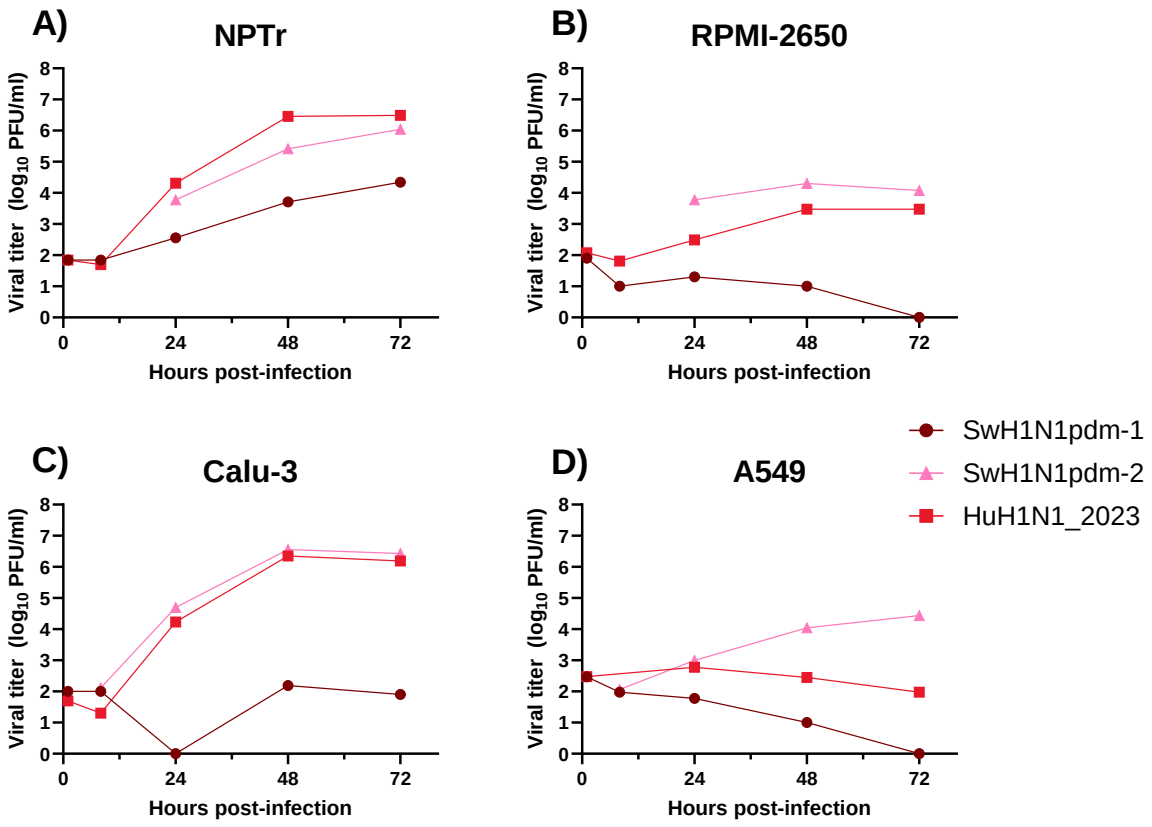

Figure 11. Replication kinetics of A/Denmark/67/2023 (huH1N1_2023), A/swine/Denmark/SI-23-03391-2/2023 (swH1N1pdm-1) and A/swine/Denmark/SI-23-04626-4/2023 (swH1N1pdm-2) IAV in NPTr (A), RPMI-2650 (B), Calu-3 (C) and A549 (D). Cells were infected at a MOI of 0,01 PFU/cell in triplicate wells. The supernatant was collected at the indicated timepoints, and virus was quantified by plaque assay run in duplicates.

Innate host response to swIAV

Cells defend against viral infection by activating the innate immune response. Recognition of viral features inside the cell triggers the secretion of type I interferon (IFN) cytokines and downstream synthesis of antiviral proteins, such as Myxovirus resistance protein A (MxA) that obstructs viral replication. Counteractively, IAVs can disrupt the cellular innate immune pathways to enhance viral replication and transmission. Therefore, assessing the capability of swIAVs to modulate the innate immune system in the human host cell environment is valuable for zoonotic risk predictions.

To detect the innate immune response to viral infection, a basic in vitro cell-based and quantitative PCR (qPCR) method can measure the transcription of cellular genes involved in innate immunity and evaluate whether the genes were upregulated (suggestive of ineffective viral modulation) or downregulated (suggestive of effective viral modulation). A preliminary experiment was performed by infecting human lung cells (A549) with five IAVs representing human seasonal H1N1pdm09 (A/Denmark/238/2020), swIAV H1pdm09N1av-1 (2020-15063-1), reverse zoonotic human seasonal-like H1N1pdm09 (2020-3114-3), swIAV H1pdmN1pdm-1 (2017-10298-4) and the first Danish zoonotic case with a H1pdmN1pdm-2 (A/Denmark/1/2021).

The preliminary results indicated that all five IAVs induced the transcription of IFN-β and MxA genes to varying levels in the A549 human lung cells after 24 hours (Figure 12). The highest standardized gene expression was observed in cells infected with A/Denmark/1/2021 (IFN-β = 7.2, MxA = 5.5) and 2017-10298-4 (IFN-β = 5.5, MxA = 4.6), while the lowest was measured in cells infected with A/Denmark/238/2020 (IFN-β = 2.6, MxA = 0.6) and 2020-3114-4 (IFN-β = 2.8, MxA = 1.8). Cells infected with 2020-15063-1 expressed the IFN-β gene to an intermediate level between swIAV and human seasonal H1N1pdm09 (IFN-β = 4.3), but the expression of the MxA gene was comparable to the lower levels observed in human seasonal H1N1pdm09 infected cells (MxA = 2.1).

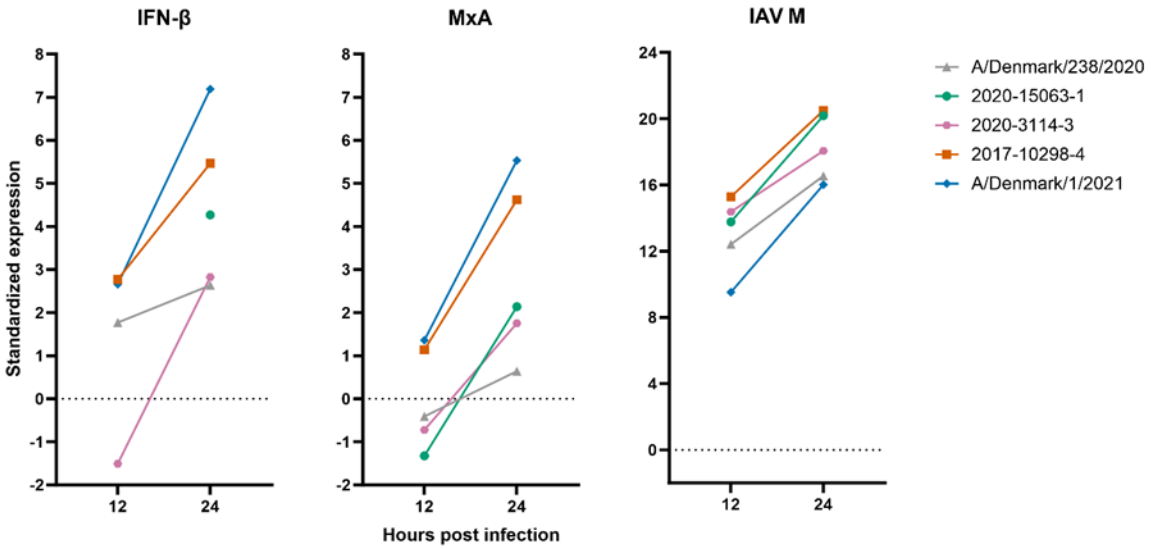

Figure 12. Expression of cellular (IFN-β and MxA) and influenza A virus (IAV M) genes in A549 cells after 12 and 24 hours post infection with selected influenza A viruses. Gene expression in infected cells was standardized by the gene expression in uninfected control cells (indicated by dotted line).

In-vivo characterization in ferrets

A/swine/Denmark/15063-1/2020, an H1pdm09N1av-1 genotype virus (and also used in the in-vitro transcription assay) and A/swine/Denmark/19922-5/2021 an H1pdm09N1av-2 genotype (showing high nucleotide identities (99-99.9 %) to the second Danish zoonotic case (A/Denmark/36/2021)) were inoculated intranasally into ferrets at a concentration of 10^5 50% tissue culture infectious dose (TCID50)/mL. The results of the nasal washes revealed that all inoculated donor ferrets were positive for swIAV at 2 days post inoculation (DPI) (Figure 13A and B). All donor ferrets inoculated with A/swine/Denmark/19922-5/2021 had tested negative for swIAV by 7 DPI (Figure 13B), whereas 2/3 ferrets inoculated with A/swine/Denmark/15063-1/2020 were still positive at 7 DPI (Figure 13A). All direct contact ferrets, introduced into the same cage as the donor ferrets on 1 DPI, also became IAV positive at 2 DPI and were all positive at 7 DPI and 4/6 ferrets had cleared the infection by 9 DPI. In addition, to the contact ferrets, airborne contacts were also introduced on 1 DPI. The airborne contacts of the A/swine/Denmark/19922-5/2021 infected donors displayed no clear signs of viral replication with only 1/3 ferrets testing weakly positive at 2 DPI (Figure 13B). On the contrary, 2/3 the airborne contacts of A/swine/Denmark/15063-1/2020 donors showed clear evidence of transmission at 7 DPI and at 9 DPI 3/3 ferrets were positive (Figure 13A). Subsequent HI-tests confirmed that all donor and direct contact ferrets had positive HI-titers (320-1280) at 14 DPI (17).

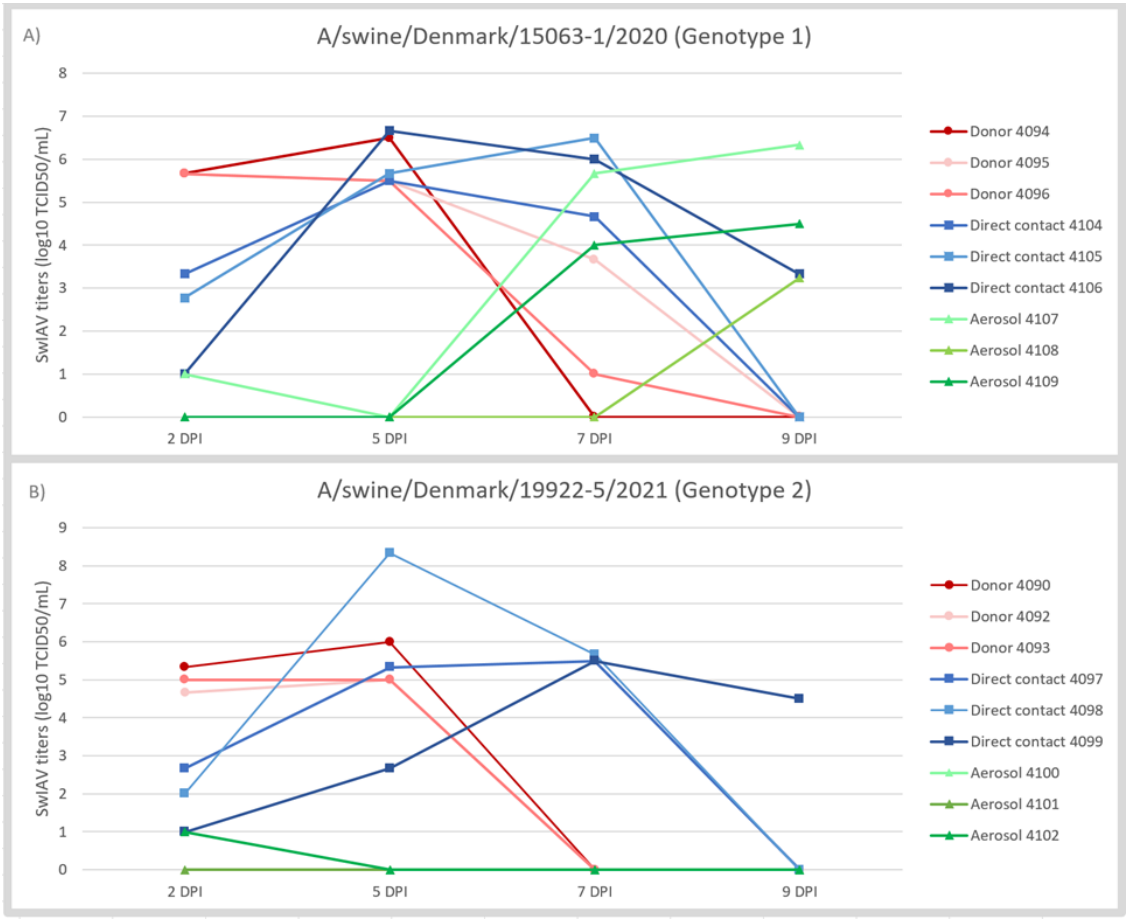

Figure 13. Viral titers of the nasal washes of the donors, direct- and aerosol contacts ferrets inoculated with the two different H1pdm09N1av strains; Genotype 1 (A) and Genotype 2 (B).

Methods

Objective 1

The samples for the Danish swine influenza A passive surveillance program included nasal swabs, salvia samples or lung samples (Figure 14). Two Danish laboratory receives samples for the surveillance program, State Serum Institute (SSI), Copenhagen and The Veterinary Laboratory (LF), Kjellerup. At both laboratories the RNA is extracted from the sample using the MagNA Pure 96 DNA and Viral NA Small Volume Kit automated on the Magna Pure 96 (Roche, Switzerland). The extracted RNA was subsequently tested using real-time reverse transcriptase PCR for determining if the sample was positive or negative for swIAV and when positive if the HA gene was of H1N1pdm09 origin. These PCRs were performed at the individual laboratories. SSI had two additional multiplex real-time reverse transcriptase PCR applied on their samples to further characterize the lineage of the HA and NA genes. All swIAV positive samples from both laboratories with a ct-value ≤ 30 in the initial PCR, where subsequently whole genome sequenced (WGS) using NGS to determine the genotype. This was done using the previously published universal influenza primers ""MBTuni-12R" and "MBTuni-13" (18) for an initial conventional PCR, where after the resulting PCR products was sequences using the Nextera XT library prep kit on the Illumina MiSeq sequencing platform. In addition, to obtain a higher number of samples for full genome sequencing than what was collected during the annual passive swIAV surveillance program, 38 samples from an active screening for swIAV in Denmark from the private company "Aerocollect", four project samples (KU) and four samples from the screening of swIAV in free-range and organic herds were included.

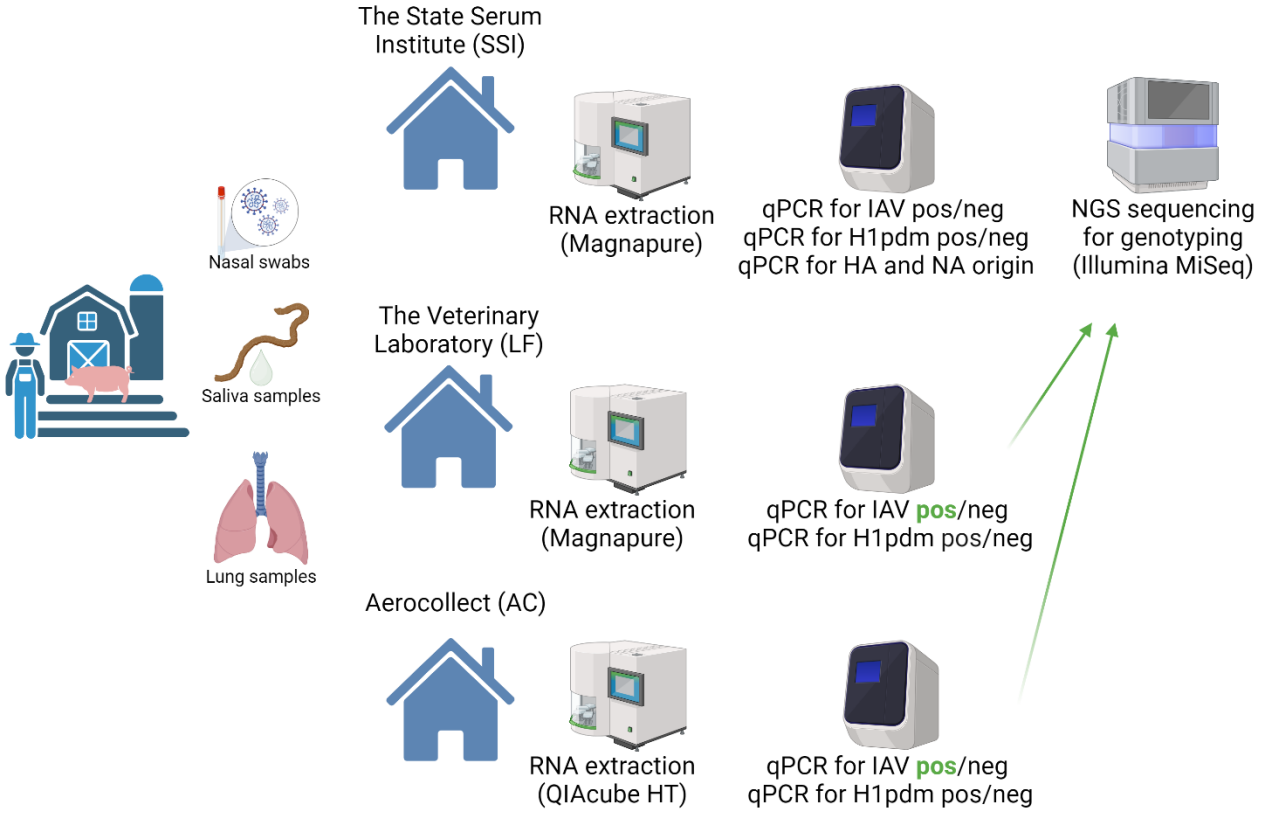

Figure 14. Sample origin and tests performed on samples included in the annual report.

The resulting NGS data were trimmed and mapped to a reference panel of swIAV strains and the following consensus sequences the eight segments extracted of for genotyping using an internal pipeline at SSI. The subsequent genetic and phylogenetic analyses were performed at Copenhagen University using CLC main workbench, version 22.0.1 and IQ-Tree (19).

Objective 2

For the screening of swIAV in Danish free range and organic swine herds, 40 herd with over 100 sows were identified and offered to be part of the screening. In total, 25 herds participated. In each herd thirty nasal swab samples were obtained: in the farrowing field (n= 10), at weaning and approx. 2-3 weeks (n=10) after weaning (n=10). Samples collected from the free range and organic swine herds were subjected to the same analyses as the samples being received at SSI for the passive swine influenza A surveillance. The only difference was that the resulting sequences were also assembled de novo (without references) to investigate if any segments were of Avian Influenza origin.

Objective 3 – Biological characterization of novel/divergent swIAV

Molecular markers

Previously described molecular markers at specific amino acid positions related to specific properties (virulence, replication, zoonotic potential, resistance to neuraminidase inhibitors) of influenza A virus were investigated in the amino acid sequences deduced from the WGS for the PB2, PA, NA and NP proteins, and the prevalence of different truncations of the NS1 proteins were also examined. This was done by translating the different genes into proteins and aligning them for manual inspection.

Isolation of swIAV in cell culture

Thirty-eight samples were selected based on their genetic differences for viral isolation in MDCK-SIAT1 cells. Viruses were isolated by inoculation of MDCK-SIAT1 cells with clinical material using procedures described in the Manual for the laboratory diagnosis and virological surveillance of influenza, WHO Global Influenza Surveillance Network (22), with minor modifications (23). Briefly, the MDCK-SIAT1 cells were grown in T-75 flasks with Dulbecco's Modified Eagle's Medium (Sigma) containing 1% L-glutamine (200 mM (Sigma)), 1% penicillin/streptomycin (10000 µg/ml (Gibco)) and 5% fetal bovine serum (Gibco). When a confluent monolayer of cells were obtained 100µL of processed specimen was added to the flasks and inoculated for 60 min at 37°C and 5% CO2 and new growth media was applied. The cells were visualized for CPE daily and harvested at day 3-4 post inoculation.

Antigenic characterization

Thirteen of the viral isolates were chosen for antigenic characterization using hemagglutination inhibition (HI) assay, where the cross-reaction to antisera raised against both human seasonal vaccine strains (provided by WHO) and the current swine influenza vaccine strains (provided by Ceva Animal Health) were tested. It should be mentioned that the antisera raised against the human seasonal vaccine strains were produced in ferrets, whereas the antisera against the swine influenza A vaccine strains were so called "hyperimmune sera" raised in pigs. For the swine influenza A vaccines both a trivalent vaccine (Respiporc FLU3) and a monovalent vaccine (Respiporc FluPan H1N1) are available and for the trivalent vaccine both antisera raised against the H1av individual strain and the full vaccine were used. Briefly, inactivated sera were mixed with 50% erythrocytes to remove specific inhibitors of haemagglutination and agglutination factors. Two-fold serum dilutions were tested against the isolates, starting at a dilution of 1:20 followed by

the addition of 0.6% guinea pig red blood cells.A titer of $\geq$ 40 used was considered as positive. In humans a titer of 40 is recognized as “protective” as a result of a 50% reduction in disease in healthy adults.

Replication in human and swine cells

To evaluate the growth kinetics of swIAV in the four cell lines (A549: human lung cells, Calu-3: human bronchial cells, RPMI-2650: human nasal cells and NPTr: swine tracheal cells), the viruses were diluted to the same concentration (MOI of 0.01 based on plaque assay on MDCK cells) before being inoculated onto the different cell lines. After infection for 1 hour, the inoculum was removed and replaced with growth media containing trypsin. The supernatant was collected at different time points (1, 8, 24, 48 and 72 hours) and analyzed for viral genome content by RT-qPCR and infective particles by viral titration on MDCK cells. Growth kinetic curves were then generated and compared.

Innate host response to swIAV

An in vitro cell-based and qPCR method was used to evaluate the transcription of IFN-β and MxA genes involved in innate immunity during viral infection of A549 cells. Cells were infected with selected IAV. After 12 and 24 hours post infection, total RNA was extracted from the infected cells, messenger RNA was synthesized into copy DNA, and the expression of specific innate immunity related genes (type I IFN-β subtype (IFN-β) and MxA) was measured by qPCR using gene specific primers. In addition, quantification of IAV M gene in the cell culture confirmed viral replication.

In-vivo characterization in ferrets

The two ferret studies (one for each virus strain) were performed in Biosafety Level 2 facilities St. Jude Children's Research Hospital, Memphis, USA in separate cubicles. For each viral strain, three donor ferrets were inoculated on day 0 with 1mL of virus (10^5 $TCID_{50}$) and were housed in separate cages. At day 1 (24 hours later) both direct and aerosol contacts were added to the donor ferrets. The direct contacts were co-housed with the donors, whereas the airborne contacts were housed in the same cage but separated physically from the donors and the direct contacts. This study design resulted in nine ferrets per viral strain including one donor ferret, one direct transmission ferret and one aerosol contact per cage. At day 2, 5, 7 and 9 nasal washes were performed to evaluate the viral titer using $TCID_{50}$.

Discussion and conclusion

Objective 1.

The number of submissions and samples obtained were similar to 2022 and again provided a valuable insight into the circulating swIAV in Denmark. In 2022 and 2023 a significant increase in the proportion of swIAV positive submissions were observed, compared to all previous years of the surveillance, which highlights the vast distribution of swIAV in Denmark. From the results, it is again confirmed that swIAV is present all-year-round in all regions of Denmark. The current intensive Danish swine production systems with fewer herds and a higher number of animals, increased litter-sizes, weekly batches and early weaning, all favors the endemic state of swIAV circulation. The endemic state is worrying from several different perspectives as the constant circulation of swIAV enhance the viral evolution and risk of reassortment events creating novel swIAV, which makes it more difficult to control and also increases the risk of generating novel swIAV with enhanced zoonotic potential. That novel reassortants is frequently generated in Danish swine herds and subsequently spread, is shown by several of the results of the Danish swine influenza surveillance program. First, the H1pdm09N1av reassortant, which was detected in a single herd in 2018, in 2023 constituted at least 21 % of all submissions from which the HA and NA lineage was

determined, emphasizing the potential increased fitness of this virus, and illustrating how fast a novel swIAV can spread among Danish herds. Second, the internal gene cassette "PPPPPA" combining genes of H1N1pdm09 (P) and Eurasian avian-like H1N1 origin (A) was detected for the first time in 2020 in a single submission, and by 2023 all but one of the different circulating HA and NA combinations include a genotype with this internal gene constellation. In addition, the PPPPPA internal gene cassette constituted 43.5 % of all the internal gene cassette registered in 2023; again underlining the benefit for Danish swIAV to carry this cassette.

The massive viral evolution has also been documented in the phylogenetic analyses, where especially the H1av sequences display huge diversities within all of the pre-defined 1C clades, but especially evident for the 1C.2.4 clade, which could be divided into two novel clades. Most of the Danish H1avNx swIAVs belong to the 1C.2.4 cluster, which is genetically distinct from the older Eurasian avian-like sequences and the H1avNx strain included in the trivalent swine vaccine. The large genetic difference is also reflected antigenically, as very limited cross-reaction between the 1C.2.4 isolates and the swine vaccine strain was observed. However, the 1C.2.2 viruses similar to the strain included in the swine vaccine showed cross-reaction (titer $\geq$40). The limited cross-reaction can potentially result in a limited effect of the vaccine in some of the Danish swine herds, which can problematize the control of respiratory disease and thus negatively impact the antibiotic usage. However, in-vivo studies are needed to confirm the impact of a limited cross-reactivity to the vaccine. For the H1pdmNx swIAVs, great genetic differences were also observed, with especially the successful H1pdm09N1av genotypes clustering separately from other H1pdmNx viruses. However, from the results of the antigenic characterization of the H1pdmNx viruses it appears that all H1pdmNx viruses still have cross-reaction (HI titers $\geq$40) to the swine H1N1pdm09 vaccine strain.

An H3N2 virus with close relatedness to the H3 gene to human seasonal H3N2 virus was detected in a single swine herd. This virus likely arose by reassortment between a human H3N2 virus and an enzootic swIAV from which it gained the PPPPPA internal gene cassette. The human seasonal circulating H3N2 strains are very different from the regular swine H3N2 strain, which is both reflected in the genetic differences observed and lack of cross reaction between the swine vaccine strain and the reassortant H3N2 virus measured by HI test.

The results obtained from the 2023 surveillance highlight the importance of maintaining a national passive Danish surveillance program and securing a certain number of annual submissions to obtain a representative sample size to be able to evaluate patterns, tendencies and evolutions. However, the current sample size does not secure the finding of swIAV circulating at a low prevalence, which might impact the preparedness if a novel swIAV appears. It should also be noted that approx. 50 % (228/478) of the submissions were sponsored by the medical company Ceva Animal Health. There is a risk that their diagnostic samples will not be submitted to the Danish surveillance program in the future, and therefore considerations should be made on how to maintain a minimum number of submissions to secure the value of the surveillance program.

Objective 2

In 2023, Danish free range and organic herds were screened for circulation of IAV (swIAV and avian influenza virus, AIV). This is the first controlled test of the presence of IAV in these extensive, partly outdoor systems and the study provided novel data from this sub-population of Danish pigs, which might become important in the future where more out-door herds are expected to be established to fulfill demands from the consumers. It was striking that the proportion of swIAV positive herds was significantly lower (28%)

compared to the herds included in the passive surveillance. The difference can potentially be explained by the difference in surveillance. The screening of the organic and free-range herds represents an active surveillance element, whereas the passive surveillance is based on samples from pigs experiencing respiratory disease. However, it should be noted that five of the seven swIAV positive herds in the organic and free-range screening also experienced respiratory disease. One could speculate that the larger number of square meters per pig (lower pig density) in the free-range and organic pig herds could lead to a decreased risk of persistent swIAV circulation. In addition, an unpublished study describing an active surveillance in 148 Danish conventional herds in 2023, showed that 50 % of the included herds were positive for swIAV which is also significantly higher than the level observed in the free-range and organic herds. Finally, it was evident that the same swIAV strains circulate in the organic and free-range herds compared to the herds included in the passive surveillance program, which is in accordance with the free range and organic herds receiving breeding sows from the conventional herds. No AIV of contemporary bird origin was detected, but if the number of free range and organic herds increases in the future, it is highly relevant to maintain a screening for IAV, since outdoor pigs will have a higher likelihood of exposure to AIV, including HPAIV and since pigs are susceptible to both AIV and swIAV there is a risk of the pig serving as a mixing vessel for new re-assorted viruses.

Active surveillance is generally considered the most appropriate methodology for early detections of novel viruses (Eric Carlsson). Thus, to improve the power of the surveillance it should be considered to complement the passive surveillance with active surveillance including test of free range and organic farms where the animals are closer to the wild life reservoirs.

Objective 3

It is worrying that a large proportion of Danish swIAVs contain markers that could be related to an increased zoonotic potential, and there is a need for phenotypic assays to test the actual impact of these markers. In 2023, extra funding was given to further evaluate the zoonotic potential of selected Danish swIAV considered to possess some traits that could enhance the zoonotic potential. At the moment, several assays for biological characterization are being established at University of Copenhagen and SSI. One of these assays is the in-vitro cell replication assay, where the preliminary results indicate that the selected H1pdmN1pdm-2 strain, which was similar to the first Danish zoonotic case, was able to infect the human respiratory cell lines to a larger or similar extent as a human seasonal H1N1pdm09 virus. This result highlights that these H1pdmN1pdm-2 viruses could have an increased zoonotic potential compared to other Danish swIAV genotypes. However, a greater number of viruses needs to be tested to indicate if this trait is strain specific or genotype specific. Another in-vitro assay that is being implemented is the in-vitro transcription assay, where the expression of two important host antiviral proteins are being evaluated post cell culture infections with different swIAVs. The preliminary results of this assay highlighted that the H1pdm09N1av-1 (A/swine/Denmark/2020-15063-1), which was also used for the ferret study, was able to reduce the expression of antiviral proteins in human lung cells and replicate unperturbed emphasizing the zoonotic potential of this strain. The ferret studies testing the two different genotypes of H1pdm09N1av highlighted the ability of H1pdm09N1av-1 (A/swine/Denmark/2020-15063-1) in spreading through aerosols, implicating this virus as having an increased potential for human to human transmission. Additionally, one of our recent pre-prints describing these ferret studies (17), also included an evaluation of the level of pre-existing immunity in the human population, by testing 119 serum samples from people from all age-groups. These results revealed minimal pre-existing immunity in the population; further highlighting the risk of a successful and alarming swine-to-human spillover (17).

The research group at University of Copenhagen are currently working on setting up an additional in vitro assay to test the different swIAV polymerase genes and their effect on the replication efficiency. Several of the viruses identified in the 2023 surveillance carrying the specific PB2 and PA mutations will be selected for testing in this assay, with the single virus carrying the E627A being a high priority. For the first time in the Danish swIAV surveillance, a virus of the H1pdmN1av-2 genotype carried the 275Y residue linked to resistance against the antiviral agent oseltamivir (Tamiflu). This virus is currently being propagated in cells, and will be tested in the already established antiviral resistance phenotypic (20) assay at SSI to measure the exact level of susceptibility of this virus to oseltamivir to confirm or exclude the resistance. If the Danish swIAVs are carrying this mutation, it is highly worrying from a human health perspective, since such a mutation will enhance the impact of a future swine to human spillover, since oseltamivir is one of the few effective viral agents for treatment of severe influenza virus infections in humans. The fact that this mutation was observed in an H1pdmN1av-2 genotype, which has already caused one zoonotic infection (8), is again alarming. It is unclear how this mutation arose in a swIAV as it is normally only observed in the human seasonal influenza strains, where antiviral treatment with oseltamivir are used and drives the evolution of such mutations. The mutation could have occurred randomly, and fortunately the selection pressure to maintain the mutation should be low in the swine population.

In conclusion, several variants, subtypes and genotypes of swIAVs are circulating in Danish pigs and several of these harbor one or more traits that potentially can enhance the zoonotic potential, and at the moment both population immunity and the human seasonal IAV vaccine strains provide a sub-optimal protection. It is therefore important to investigate more swIAV strains in biological assays. In addition, more emphasis in establishment of better control strategies to limit the circulation of swIAV in Denmark should be prioritized as a better control of swIAV is a prerequisite for a lower risk to the general population.

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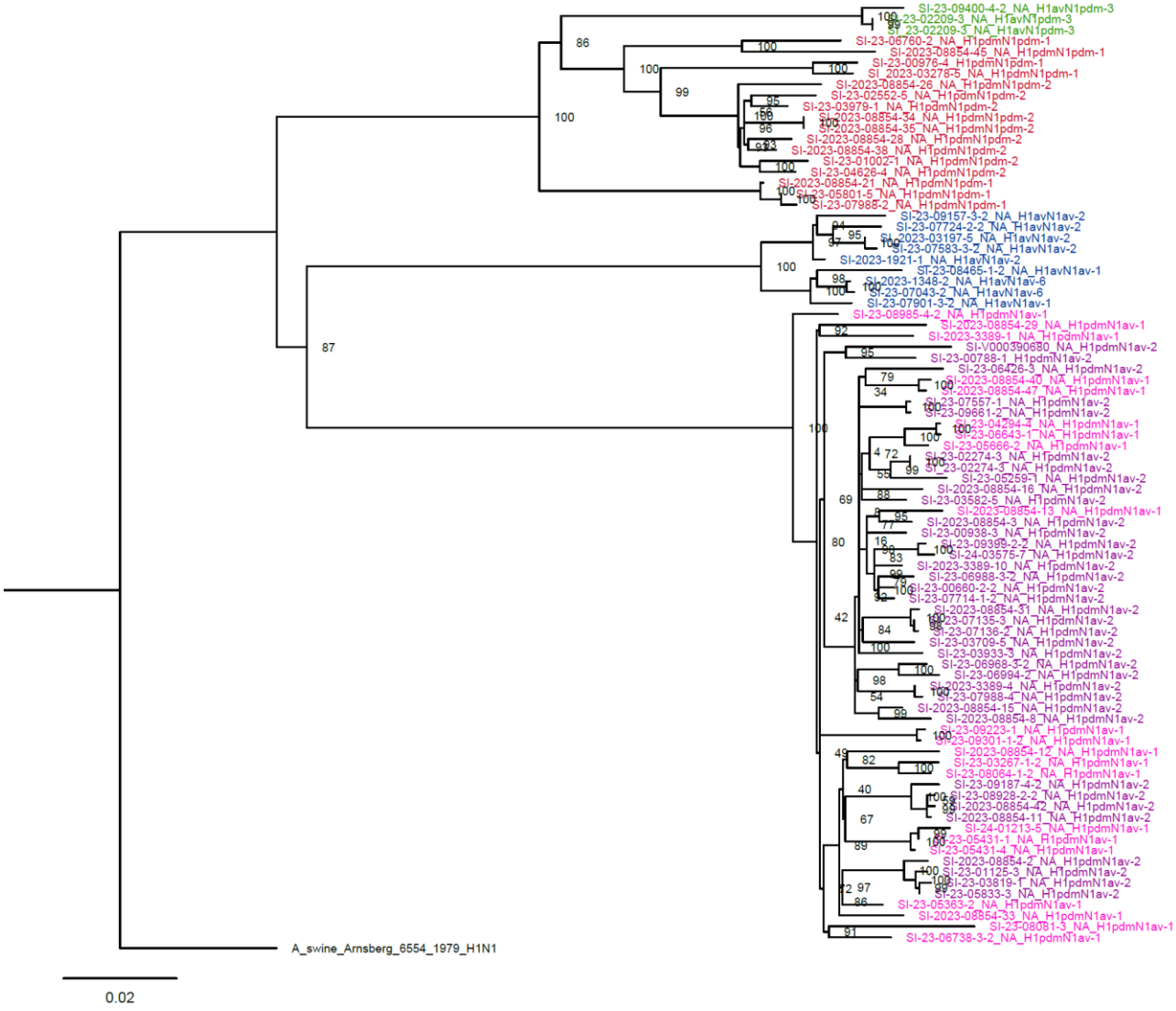
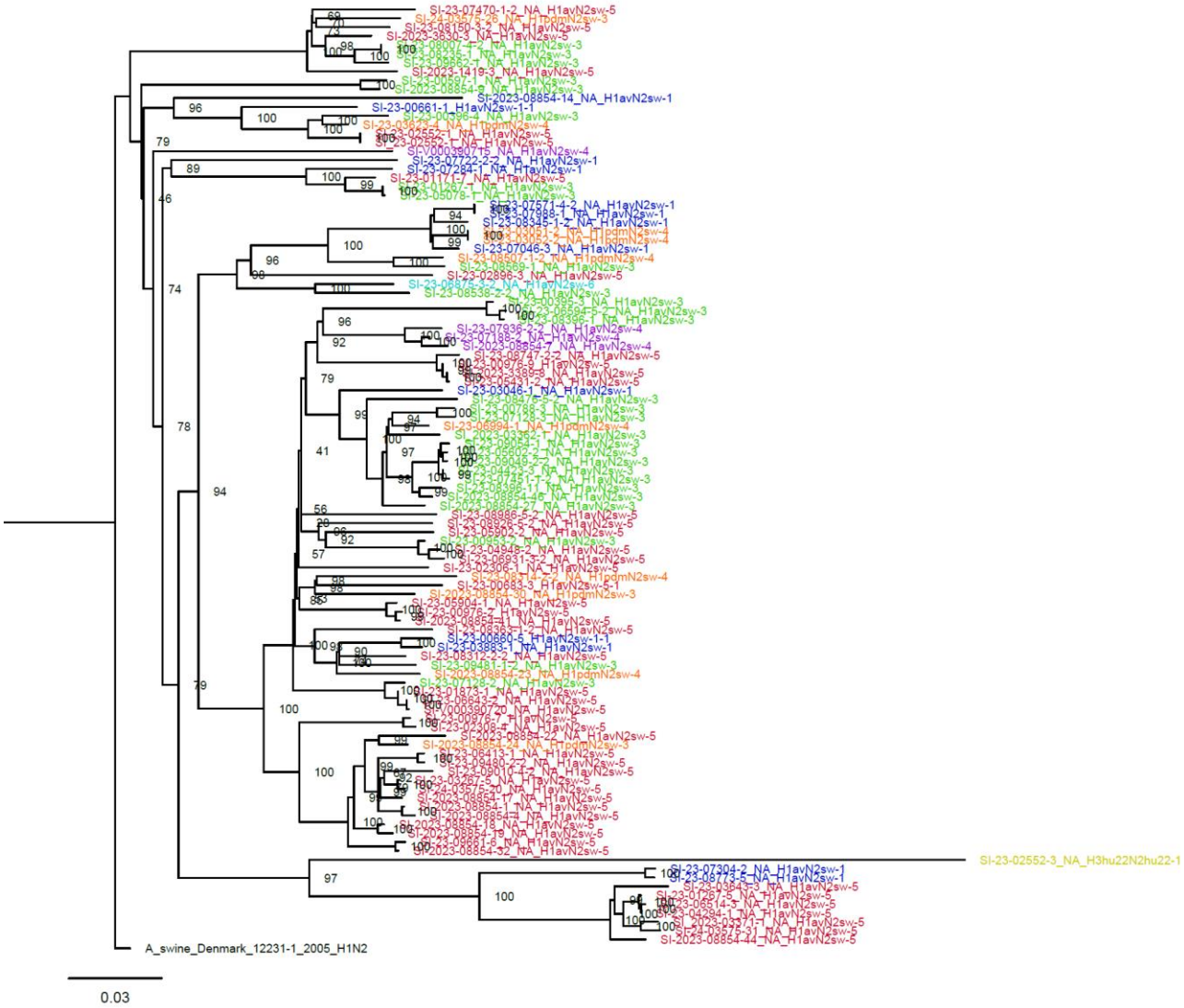
Appendix



Maximum likelihood tree of Danish NA genes of H1xN1x viruses from 2023.
"A_swine_Arnsberg_6554_1979_H1N1" was used as an outgroup. The taxon color indicate the HA and NA combination or the genotype; Blue indicate all H1avN1av viruses. Red indicate all H1pdmN1pdm viruses. Green indicate all H1avN1pdm viruses. Pink indicate all H1pdmN1av-1 genotypes and purple indicate all H1pdmN1av-2 genotypes.



Maximum likelihood tree of Danish NA genes of HxN2x viruses from 2023.

"A_swine_Denmark_12231-1_2005_H1N2" was used as an outgroup. The taxon color indicate the genotype. Blue indicate H1avN2sw-1, green indicate H1avN2sw-3, purple indicate H1avN2sw-4, red indicate H1avN2sw-5 and turquois indicate H1avN2sw-1. Orange indicate H1pdmN2sw viruses and yellow indicate the single human seasonal H3hu22N2hu22-1 virus.