Dansk Veterinær Konsortium

KØBENHAVNS UNIVERSITET | STATENS SERUM INSTITUT

Surveillance of swine influenza A virus in Denmark

Annual report 2024

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Kort sammenfatning af nøgletal

Der blev i 2024 modtaget 463 indsendelser (1591 prøver) til undersøgelse for svineinfluenza virus (swIAV). I alt indsendte 391 besætninger med forskellige CHR-numre prøver til overvågningen, og 248 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 32 % (79/248 besætninger), hvilket er et markant fald i forhold til 2023, hvor 40 % testede positive. HA- og NA-kombinationen (defineret som subtypen) blev bestemt for 230 indsendelser i 2023, hvilket svarer til 85 % (230/269) af de swIAV-positive indsendelser. Detaljeret genetisk karakterisering af hovedparten af de positive indsendelser viste en stor diversitet af swIAV i danske svin med en blanding af gener fra både grise og mennesker. Tilmed blev én ny genotype påvist. Antigen karakterisering af udvalgte virusisolater viste begrænset kryds-reaktion til antisera rejst mod især den ene af de nuværende tilgængelige swIAV vacciner til grise og mod humane sæsoninfluenzavacciner.

Vigtigste fund:

- Stabil prævalens af swIAV positive svinebesætninger men markant fald på 8 % points i H1pdm positive indsendelser
- Stigning på 6 % points andelen af H1avN2sw swIAVs, hvoraf de fleste bærer på en komplet pandemisk intern genkassette "PPPPPP". Disse virus tilhører oftest den genetiske clade 1C.2.4, som har en meget begrænset kryds-reaktion til den nuværende swIAV vaccine
- Fordobling af H1avN1av swIAV og højere prævalens af denne end H1pdmN1pdm swIAV
- Påvisning af fem reverse-zoonotiske tilfælde af human sæsoninfluenzavirus i grise, hvoraf fire var af H1N1pdm09 oprindelse og én var af H3N2 oprindelse
- Fund af E627K mutationen i PB2 proteinet i en dansk swIAV, som stammede fra human sæsoninfluenzavirus H3N2
- Lav kryds-reaktion mellem alle testede swIAV isolater og fritte antisera rejst mod de human sæson IAV-vaccine stammer, hvilket indikerer lav populationsimmunitet ved et spillover event af swIAV

Summary

In 2024, 463 submissions were received, which is a slight reduction of the number of submissions compared to 2023, but markedly lower than the number obtained earlier in the surveillance. The proportion of swIAV positive submissions was 58 %, which is a small decrease compared to 2023. However, on a herd-level, the proportion was the same as in 2023. A significant decrease in the proportion of submissions positive for the HA gene of H1N1pdm09 origin from 40 % in 2023 to 31 % in 2024 was observed, potentially representing a saturation of the herds driven by herd-immunity after several years with rapid spread of the reassortant H1pdmN1av first discovered in 2018. For the second consecutive year the prevalence of the H1avN2sw subtype increased which followed a continuous decrease from 2020-2022 overlapping the period where the H1pdmN1av subtype increased.

In the 2024 surveillance, 183 whole genome sequences were obtained providing a detailed insight into the genotype constellations, genetic markers and evolution. Since 2022 efforts have been made to subject all submission with a reasonable amount of swIAV to whole genome sequencing, which now makes it possible to evaluate changes in genotype patterns over time. Corresponding with the decrease in submissions positive for H1pdmNx swIAVs of which a great proportion carry the PPPPPA internal gene cassette (P= pandemic H1N1pdm09 and A = avian-like swine H1N1) a decrease in swIAVs carrying the PPPPPA internal

gene cassette was observed. Additionally, several novel gene cassettes at low frequency have been detected this year along with one novel genotype “H1pdmN2sw-5”.

The phylogenetic analyses revealed that the 1C.2.4 (H1av) clade is dominating in Denmark and constitute 74 % of all swIAV of 1C.2 origin. The 1A.3.3.2 (H1pdm09Nx) viruses were again as seen since 2020 divided into three major clusters; swine adapted H1pdmN1pdm, human seasonal H1pdmN1pdm and reassortant H1pdmN1av. However, two H1pdmN1av-2 viruses were present in the H1pdmN1pdm cluster suggesting that reassortment events between the HA surface genes of the two clades are also occurring. Evidence of different reassortment events including the HA surface genes of the different clusters is further confirmed by H1pdmN2sw swIAVs present in both the swine adapted H1pdmN1pdm cluster and the reassortant H1pdmN1av cluster. 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 antisera raised against human seasonal H1 vaccine strains. For the trivalent swine vaccine there was a low level of cross-reaction to the H1avNx (1C.2) 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 was observed, whereas cross-reaction was present to the ferret antisera raised towards the most recent human seasonal H3 strain.

The whole genome sequencing allowed for detailed genetic characterization of the different proteins on 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 potentially impact the pathogenesis and resistance to host defense mechanisms thereby enhancing the zoonotic potential. In addition, for the first time the E627K mutation in the PB2 protein was observed in a Danish swIAV. This was however, from one of the reverse-zoonotic cases where a human seasonal H3N2 had spilled into a pig herd, but if the virus continue to circulate among pigs it could potentially introduce this mutation to other swIAVs emphasizing the importance of genetic characterization of the whole genome, but also underscoring that additional phenotypic test are needed to test the implication of a range of these molecular markers.

In conclusion, the annual Danish swIAV surveillance performed in 2024 gave valuable insights into the swIAV distribution, circulation and evolution in Denmark and was essential for discovering novel genotypes, molecular markers and traits that can be selected for further phenotypic testing.

Key findings

- Stabile high prevalence of swIAV positive pig herds but marked 8 % points decrease in submissions positive for H1pdm (1A.3.3.2)
- An increase of 6 % points in the proportion of H1avN2sw viruses of which most carry a complete pandemic internal gene cassette “PPPPPP” and are of 1C.2.4 origin, which show very limited cross-reaction to the current swIAV vaccine
- Doubling of the H1avN1av swIAVs which now has a higher prevalence than H1pdmN1pdm swIAVs
- Detection of five reverse zoonotic events of human seasonal IAV in pigs, four of which being of H1N1pdm09 origin and one being of H3N2 origin.

- Identification of the E627K mutation in the PB2 protein in a Danish swIAV originating from human seasonal H3N2
- Low cross-reaction between all tested swIAV isolates and ferret antisera raised against the human seasonal IAV vaccine strain indicating low population immunity in the human population upon swIAV spillover events

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 human 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 pneumonia mainly 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 alters 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 of 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 with pigs is 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, 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. 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 lineages 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, 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 seasons the gene it is most related to.

H3hu#: Viruses carrying the specific HA gene of human seasonal origin, where ”#” indicates to which human influenza seasons 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 2024.

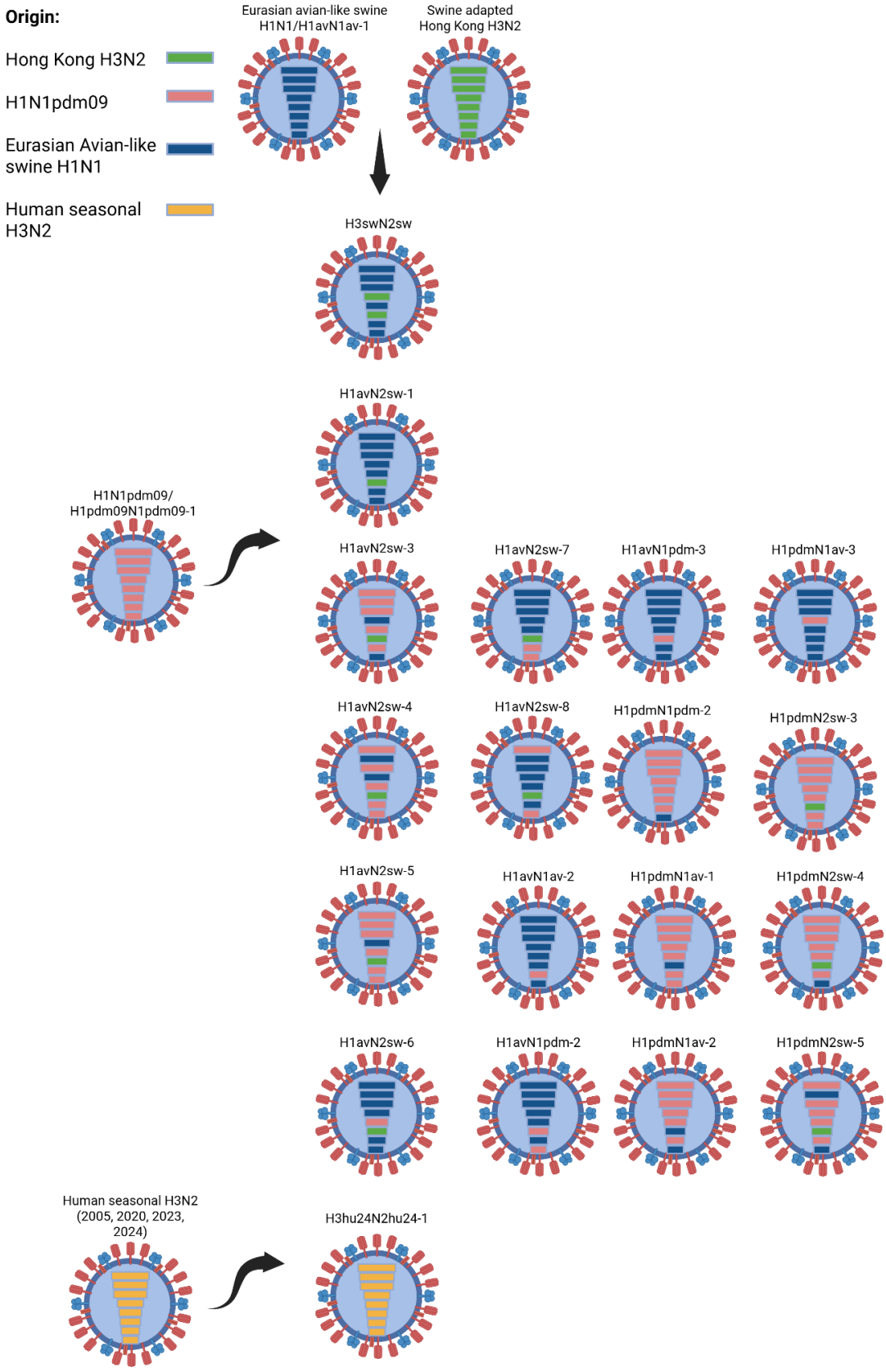

Figure 1. Overview of the swIAV introductions and circulating genotypes in Denmark 2024

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 can 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 spillovers occur

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

In 2024, the surveillance program encompassed one main objective: *passive surveillance of swIAV* with genetic characterization. However, an antigenic characterization of selected virus isolates was also included to provide valuable information of cross-protection between the contemporary swIAVs and the available swIAV and human IAV vaccines.

Passive surveillance of swIAV

The objective of this year's surveillance 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. The surveillance encompasses submissions to Statens Serum institut (SSI) and Veterinary Laboratory Kjellerup (VLK) requesting influenza diagnostics, often based on a history of respiratory disease in the herd.

Submissions

As illustrated in Figure 2, 463 submissions were received in 2024 representing only a slight reduction of submissions compared to 2023 (478), but markedly lower than the number obtained from 2019-2021 ranging from 586 in 2019 to 857 in 2021. A higher number of submissions were obtained between September-April, which is similar to the pattern observed previous years, except for 2021, where special efforts were applied to obtain more submissions in the first semester.

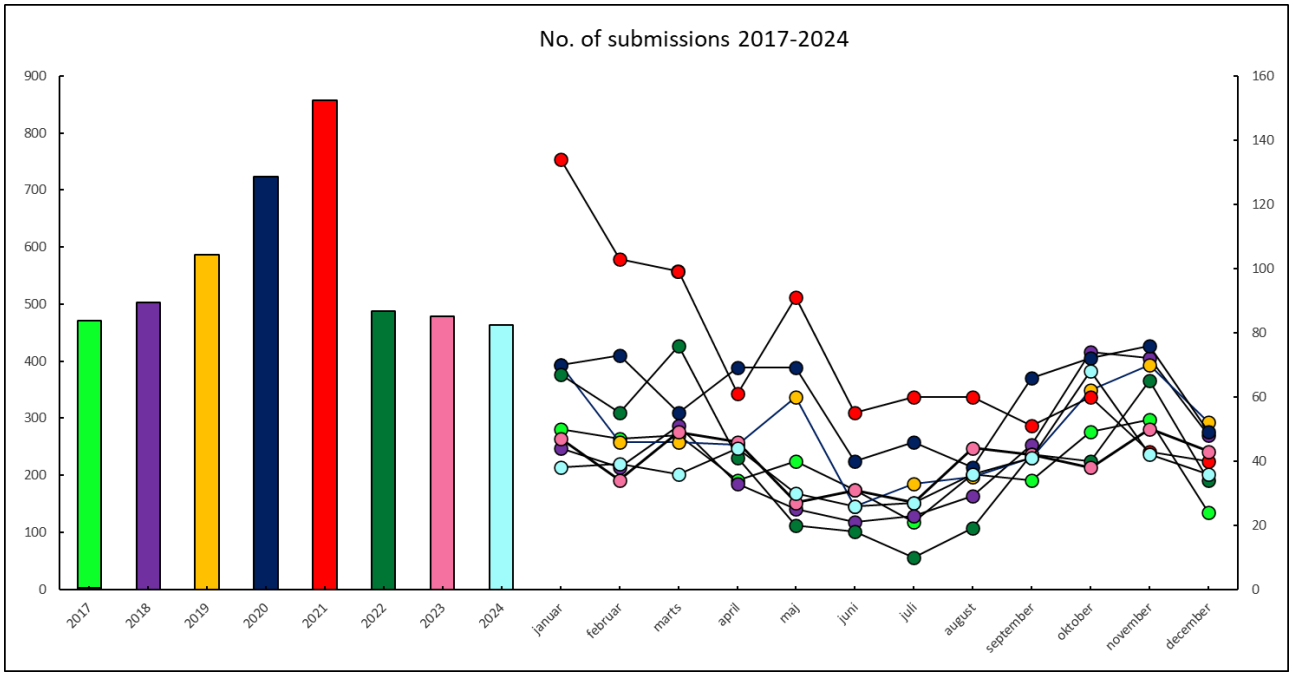

Figure 2. Distribution of the number of submissions for the swIAV surveillance program 2017-2024 divided over years (columns on the left) and months (graphs on the right). Data since 2012 is available but to simplify the figure only the last eight years of surveillance is included.

In total, 1591 samples were received in 2024 from the two laboratories "SSI" and "VLK". The SSI laboratory received on average 4.7 samples per submission (1102/236), whereas VLK received on average 2.1 samples per submission (489/227). In total, 391 herds with different CHR numbers submitted samples for the surveillance, and 248 herds tested positive at least once during the year corresponding to a proportion of swIAV positive herds of 63 %, equal to the proportion observed in 2023. The proportion of swIAV positive herds testing positive for H1pdm (1A.3.3.2) in at least one sample over the year was 32 % (79/248 herds) which is markedly lower than in 2023 (44 %).

The monthly proportion of swIAV positive and negative submissions varied over the year. The highest number of swIAV positive submissions was observed in January, February, May and October, whereas the lowest number of swIAV positive submissions were observed in March, June and July (Figure 3).

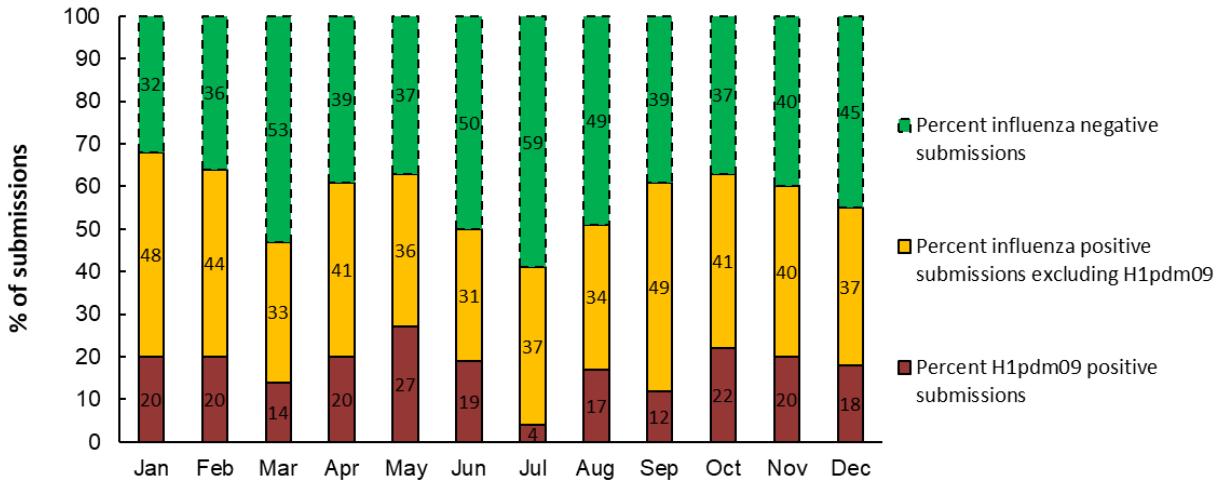

Figure 3. The 2024 monthly proportion (%) of swIAV negative and positive submissions with the proportion of H1pdm09 (1A.3.3.2) positive submissions indicated.

A small decrease in the percentage of swIAV positive submissions was observed compared to last year from 61 % in 2023 to 58 % in 2024 (269/463) (Figure 4). In addition, a marked decrease in the proportion of H1pdm (1A.3.3.2) positive submissions was observed compared to last year from 40 % in 2023 to 31 % in 2024. Since 2020 an increase in the proportion of H1pdm (1A.3.3.2) positive submissions has been observed underlining a clear shift in the swIAV dynamics in 2024.

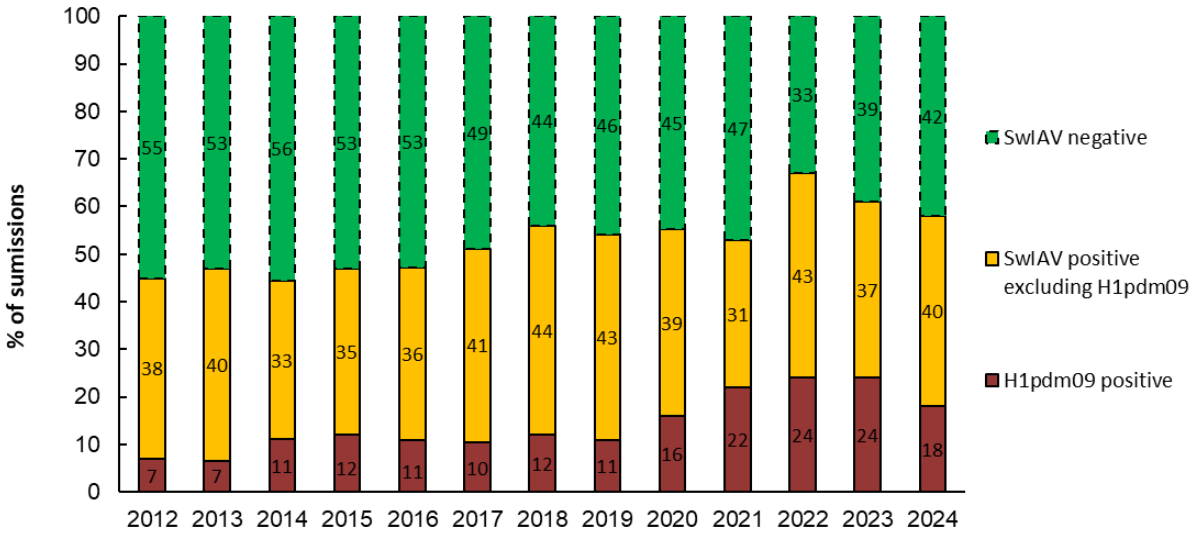

Figure 4. The annual proportion (%) of swIAV negative and positive submissions with the proportion of H1pdm09 positive submissions indicated.

Combinations of HA and NA genes (subtyping)

Through the passive swIAV surveillance program, 230 submissions had the HA and NA combination, defined as the subtype, determined in 2024, which corresponds to 85 % (230/269) of the swIAV positive submissions. As illustrated in Figure 5, H1avN2sw (1C.2) viruses still represent the largest proportion of the identified swIAVs with 58 % (133/230) of the subtyped submissions, revealing a 5 %-point increase

compared to 2023. The second most prevalent swIAV was the H1pdm09N1av (1A.3.3.2) virus constituting 17 % (39/230). The proportion of H1pdm09N1av was 4 %-point lower than the previous year. However, the proportion of H1pdm09N1av could be slightly underestimated since 3 % of the subtyped submissions 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. However, this was also the case in 2023. For the first time since 2019, the proportion of the H1avN1av was higher than the proportion of H1N1pdm09, and thereby the third most prevalent virus constituting 10.4 % of the submissions and a doubling in number compared to 2023. H1N1pdm09 and H1pdm09N2sw were detected at similar proportions (4,8 % and 5,2 %, respectively). H1avN1pdm09 was found in two submissions, whereas a human seasonal 2024 influenza virus (H3huN2hu) and a H1avNx were each detected in single submissions.

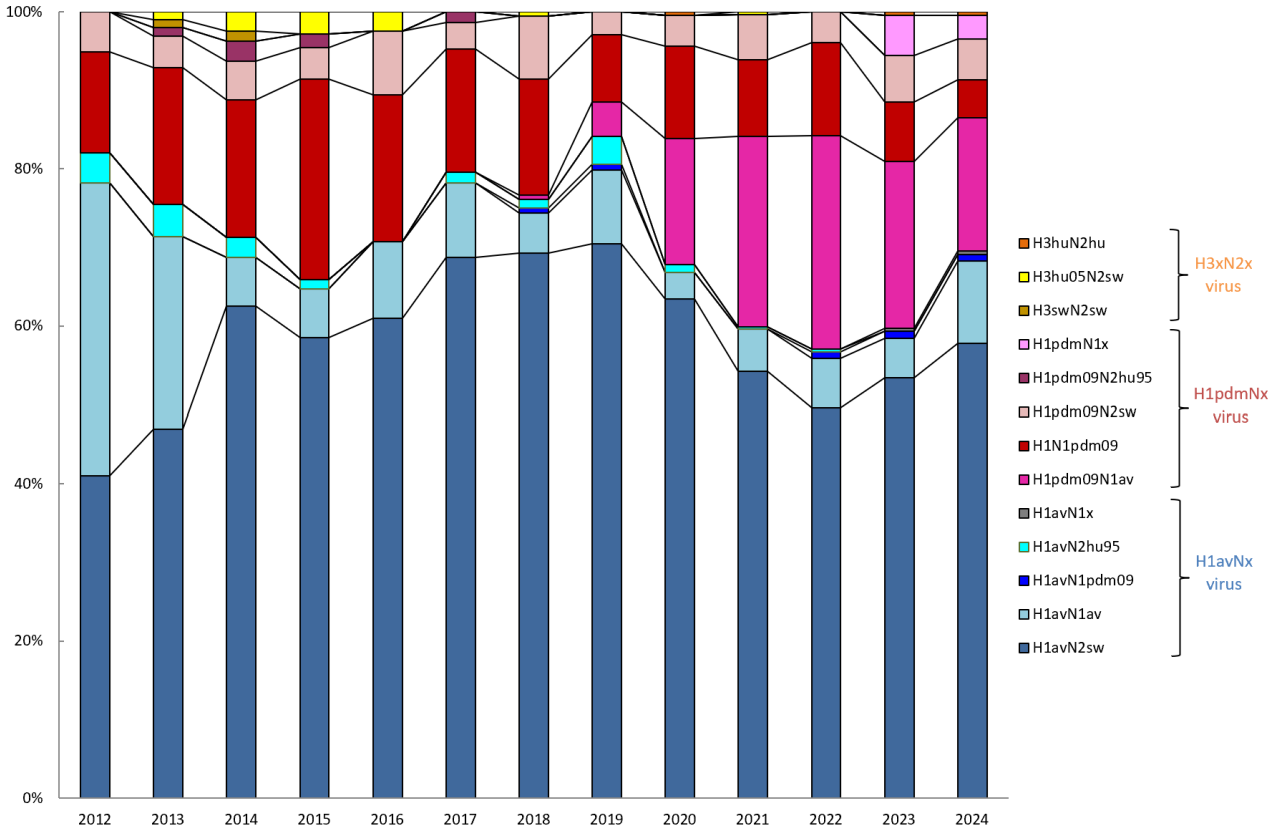

Figure 5. HA and NA combinations (subtypes) in Denmark 2012-2024.

In total, eight submissions showed several swIAVs in the same sample 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 3.5 % of the total number of submissions, where the HA and NA combinations were determined.

Fourteen 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 3/14 (21 %) of these cases a change in virus was observed over time (from H1pdm09N1av to H1avN2sw, from H1pdm09N2sw to H1av, H1pdm09, N1x, N2sw and from H1avN1av to H1avN2sw).

Genotyping

Since 2022, whole genome sequencing has been attempted on all positive swIAV submissions (with Ct values < 30). In 2024, 183 swIAV viruses were genotyped including 127 H1avNx viruses, 55 H1pdmNx viruses and one H3N2 virus determining the origin of each internal gene segment by NGS. The proportions of the different H1avNx genotypes detected from 2022-2024 are presented in Figure 6.

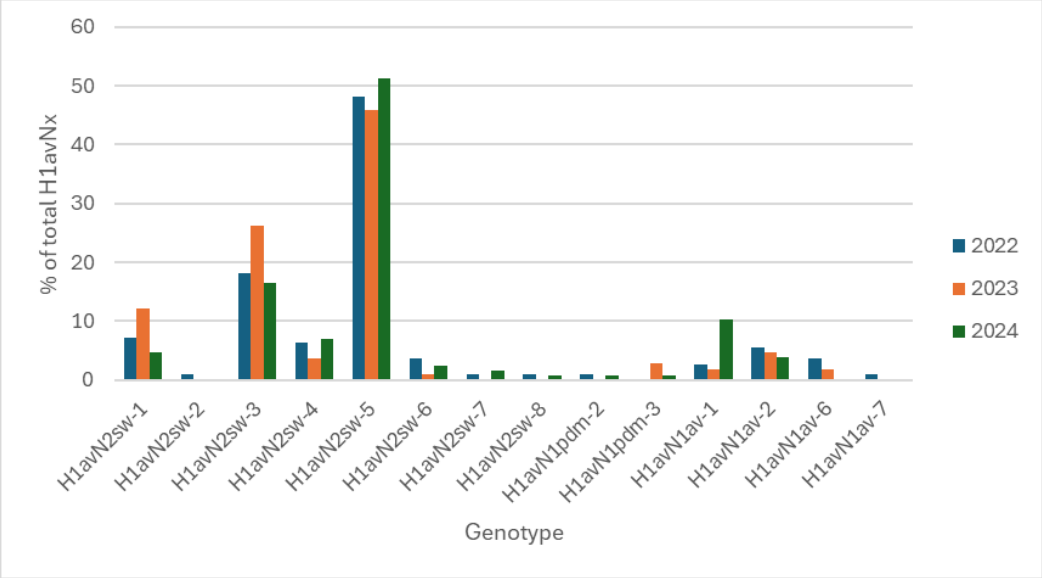

Figure 6. Genotypes of the H1avNx viruses in Denmark 2022-2024.

Similar to the previous two years it is evident that the H1avN2sw-5 carrying the PPPPPP internal gene cassette and constituting 51 % of all genotyped H1avNx viruses is the most prevalent genotype in Denmark. The second most prevalent genotype is the H1avN2sw-3 genotype constituting 16.5 % of the H1avNx viruses and carrying the PPPPPA internal gene cassette. However, a decline in this genotype has been observed compared to last year. Interestingly an increase in the H1avN1av-1 genotype occurred in 2024 reaching 10 % of the H1avNx viruses compared to 2023 where it constituted 2 %. The genetic constellation of this virus with all genes of Eurasian avian-like origin is similar to the first complete Eurasian avian-like H1N1 that was introduced in 1981.

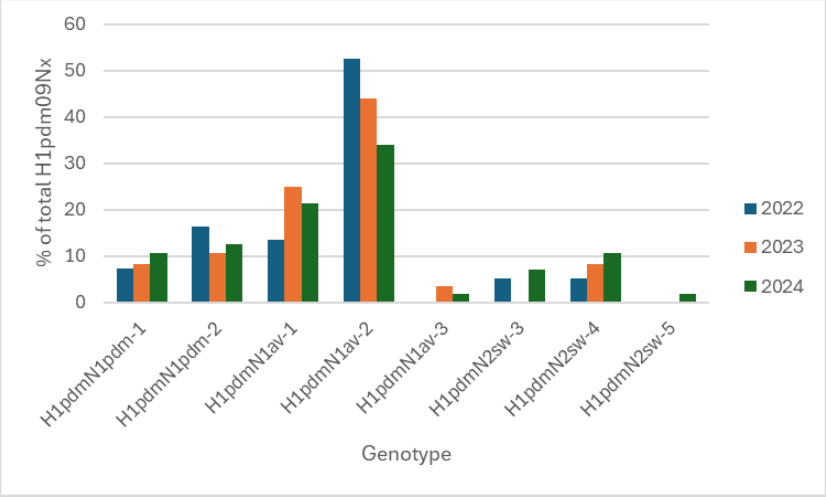

Figure 7. Genotypes of the H1pdm09Nx viruses in Denmark 2022-2024.

For the H1pdm09Nx (1A.3.3.2) viruses, the H1pdmN1av-2 genotype (PPPPPA) was dominating even though a decrease in the proportion was observed, now constituting 33 % (Figure 7). A similar pattern was observed for the H1pdmN1av-1 virus (PPPPPP) constituting 22 % of the H1pdm09Nx viruses. A more equal distribution was observed between H1pdmN1pdm-1 (PPPPPP) and H1pdmN1pdm-2 (PPPPPA), with 11 % and 13 %, respectively. A slight increase in the proportion of H1pdmN2sw-4 (PPPPPA) genotypes was observed.

Another previously undetected genotype, H1pdmN2sw-5, was detected in 2024, carrying the internal gene cassette "PAPPPA".

In 2024, a single H3N2 virus was detected with all genes of human seasonal 2024 origin.

Details on the specific gene constellation of the different genotypes detected in 2024 are presented in Figure 1.

When relating the internal gene cassette to the 1A.3.3.2 and 1C.2 clades defined by the annotation tool (https://www.bv-brc.org/app/SubspeciesClassification) of the H1xNx viruses, it is seen that some clades have a higher proportion of a specific internal gene cassette constellation (Table 1).

Internal cassette:	**Total (%):**	H1avN2sw	H1avN2sw	H1avN2sw	H1avN2sw	H1avN1av	H1avN1av	H1avN1av	H1avN1pdm	H1pdmN1pdm	H1pdmN1av	H1pdmN2sw
AAAAAA	12,1	1		5	1	1	10	2	1		1	
PPPPPA	30,2		6	15	2				1	7	18	6
PAPPPP	4,9			9								
PPPPPP	46,7	1	3	59						6	12	4
AAAPAA	1,7			2	1							
AAAAPA	2,7						5					
PPPPAA	0,5				1							
APPPPA	0,5				1							
PAPPPA	0,5											1
H1 clade:		1C.2	1C.2.2	1C.2.4	1C.2.5	1C.2	1C.2.2	1C.2.4	1C.2.4	1A.3.3.2	1A.3.3.2	1A.3.3.2

Table 1. The number of viruses with the different internal gene cassette constellations in the different HA and NA combinations and H1 clades, and the total proportion (n/183) of these cassettes across all HA and NA combinations.

The total portion of the different gene cassette constellations across all HA and NA combinations, reveals that three internal gene cassettes are represented in the majority (89 %) of the Danish swIAVs. The PPPPPP has this year increased compared to the PPPPPA gene cassette this year and a smaller increase is also observed in the AAAAAA internal gene cassette. It is also evident that a higher diversity in total number of internal gene cassettes is observed this year. Interestingly, the PPPPPP internal gene cassette is mainly carried by swIAV of the 1C.2.4 clade (74 % of the 1C.2 swIAVs) and the 1A.3.3.2 clade (40 % of the 1A.3.3.2 swIAVs), whereas the PPPPPA is found in a more similar proportion among swIAV of both the 1C.2.2 and 1C.2.4 clade. Interestingly, for the H1avN1av swIAV only the AAAAAA internal gene cassette is observed in viruses belonging to both the 1C.2, 1C.2.2 and 1C.2.4 clade.

Phylogeny

The phylogeny of the Eurasian avian-like H1 (1C.2) of the Danish swIAV of 2024 is presented in Figure 8. In total, 127 HA sequences were included in the tree from 2024, along with selected reference sequences.

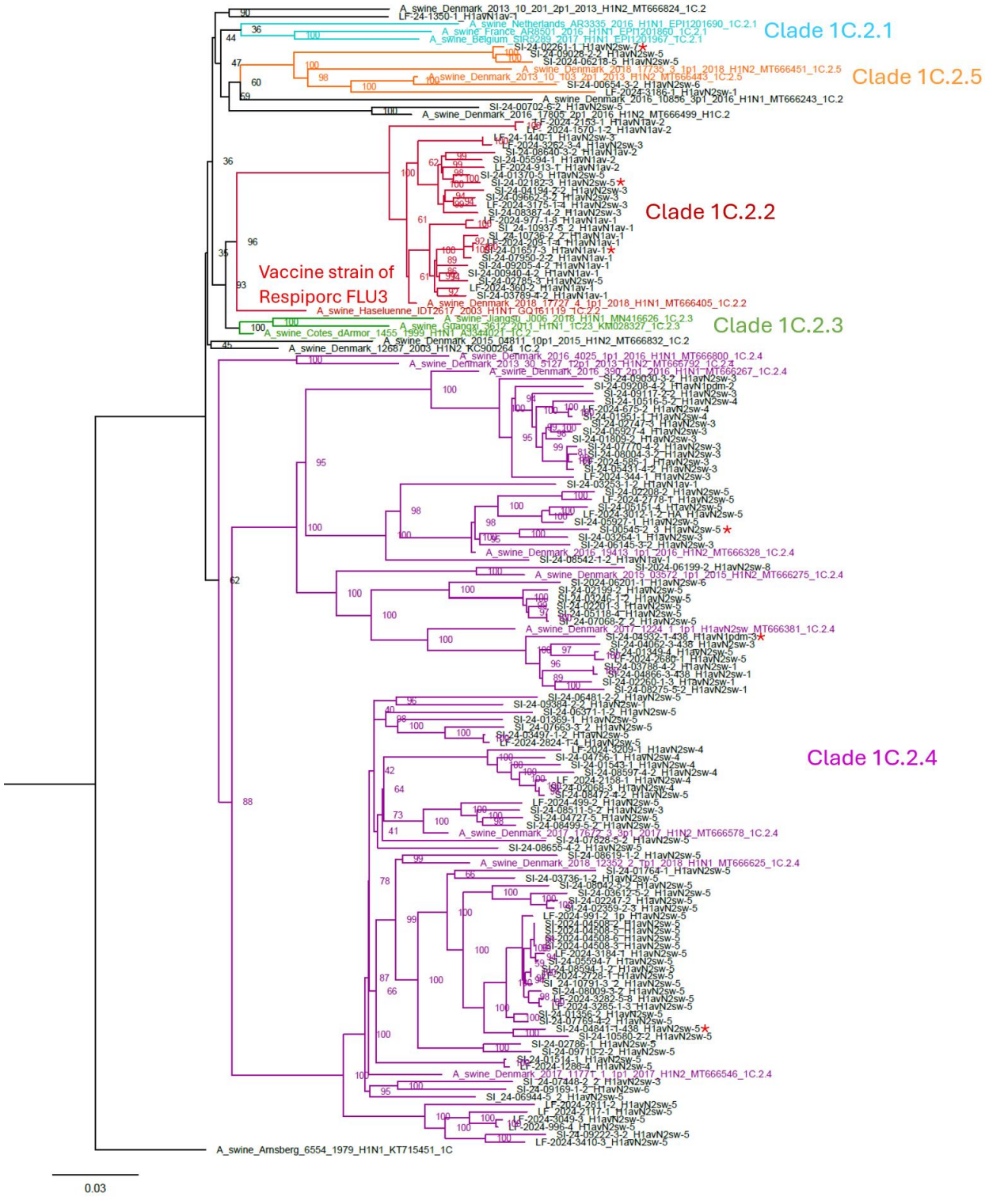

Figure 8. Maximum likelihood tree of Danish H1av from 2024 and selected reference sequences. The abbreviation "SI" and "LF" in the taxon indicate if the sample comes from SSI or L&F. "A_swine_Arnsberg_6554_1970_H1N1_KT715451" was used as an outgroup. "*" indicate that the virus was tested in the HI-test (Table 2).

The phylogeny of Eurasian avian-like H1 (1C.2) Danish swIAVs from 2024 is shown in Figure 8, including 127 HA sequences and reference strains. Danish H1avNx viruses mainly clustered within BV-BRC-defined 1C.2 clades (1C.2.2, 1C.2.4, and 1C.2.5). A few sequences grouped with undefined 1C.2 references, suggesting possible new subclades. Most viruses belonged to 1C.2.4, which divided into two main clusters: one containing H1avN2sw-5 viruses with a full H1N1pdm09 gene cassette (PPPPPP), and another including H1avN2sw-3, H1avN2sw-5, and H1avN1pdm. Like in 2023, H1avN1av viruses mainly clustered in 1C.2.2 (which includes the Respiporc FLU3 vaccine strain), except for a few found in 1C.2 or 1C.2.4. Five viruses formed the 1C.2.5 clade, covering four H1avN2sw genotypes.

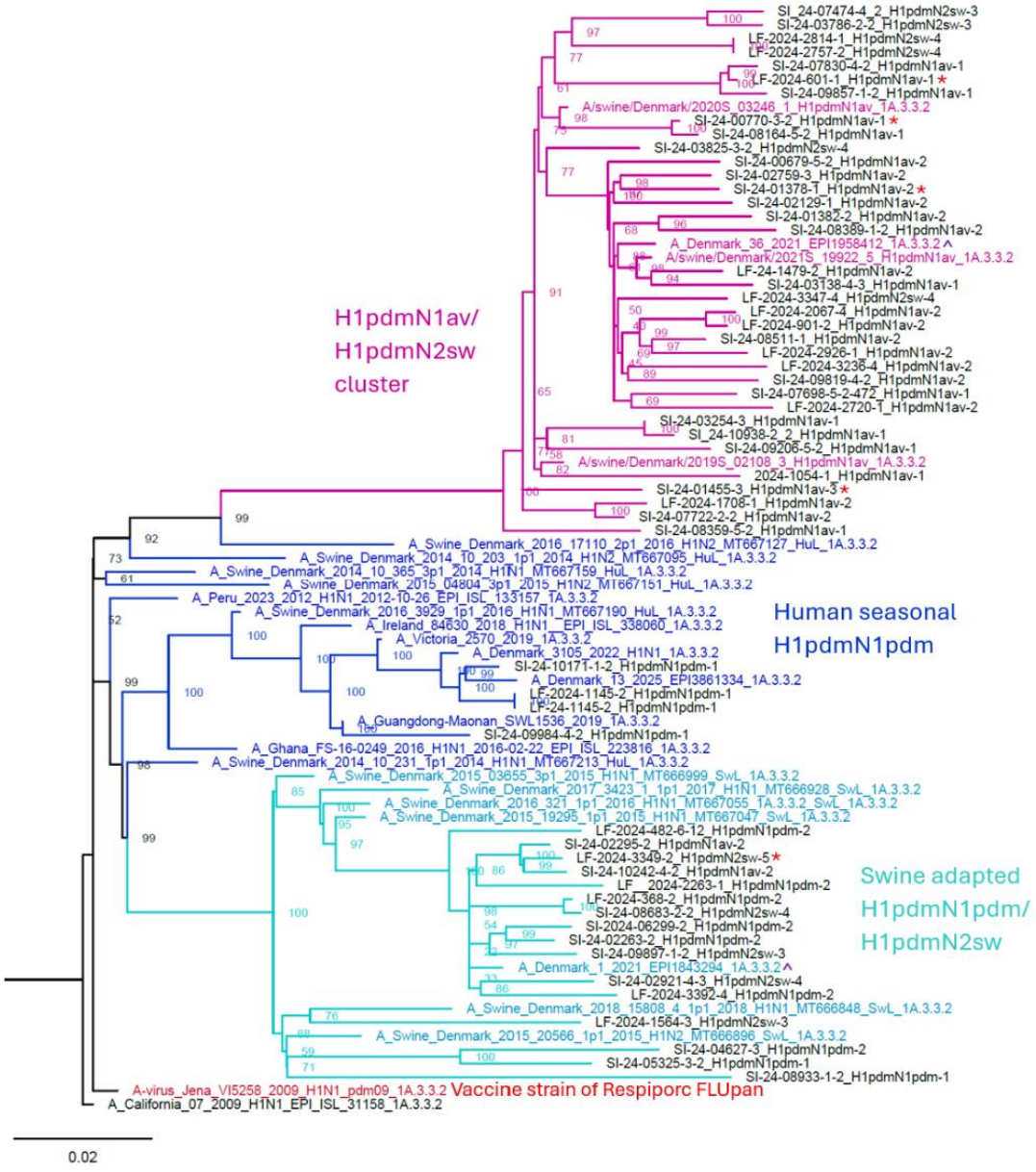

Figure 9. Maximum likelihood tree of Danish H1pdm09 (clade 1A.3.3.2) from 2024 and selected reference sequences. The abbreviation "SI" and "LF" in the taxon indicate if the sample comes from SSI or L&F. "A_California_07_2009_H1N1_EPI_ISL_31158" was used as an outgroup. "*" indicate that the virus was tested in the HI-test (Table 2) and "^" indicate the previous Danish zoonotic cases.

Fifty-five H1pdm HA sequences were analyzed (Figure 9). As in 2023, three main 1A.3.3.2 clusters appeared: human seasonal H1pdmN1pdm (reverse zoonoses), swine-adapted H1pdmN1pdm/H1pdmN2sw, and reassortant H1pdmN1av/H1pdmN2sw. H1pdmN1av-1, -2, and -3 grouped in the reassortant cluster, with most H1pdmN1av-2 near the Danish zoonotic case A_Denmark_36_2021, though two were also found in the swine-adapted cluster. H1pdmN2sw viruses appeared in both the reassortant and swine-adapted clusters. The H1pdmN1pdm-1 and -2 genotypes formed the swine-adapted cluster, while only H1pdmN1pdm-1 appeared in the human seasonal group, reflecting recent spillovers from humans.

In 2024, one H3N2 virus was detected clustering close to sequences of contemporary human seasonal 2024 IAVs (Figure 10), indicating a recent spill-over event from humans to pigs. Previously detected Danish reassortant 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.

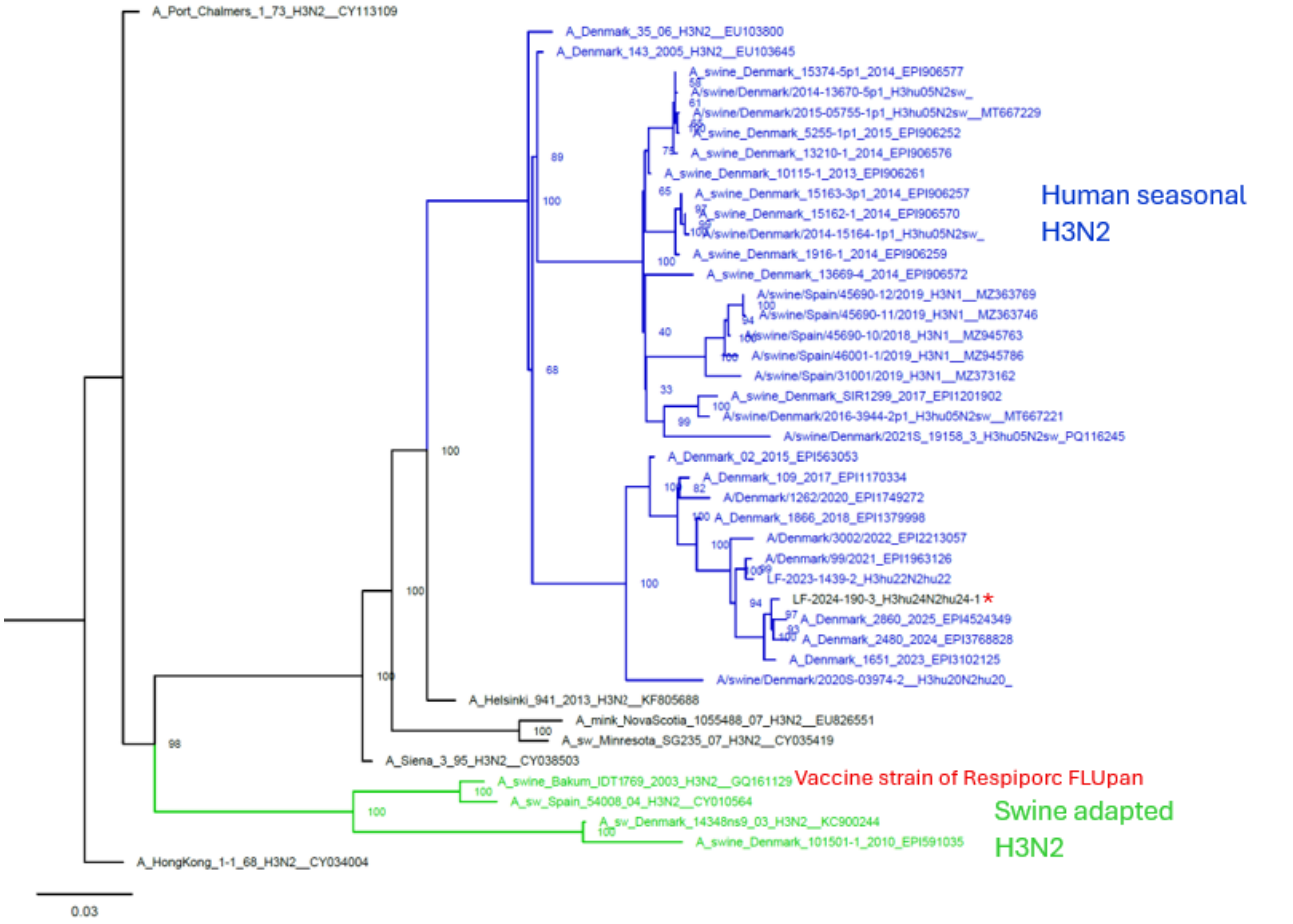

Figure 10. Maximum likelihood tree of Danish H3 and selected reference sequences. The abbreviation "SI" and "LF" in the taxon indicate if the sample comes from SSI or L&F. "A_HongKong_1-1_68_H3N2_CY034004" was used as an outgroup. "*" indicate that the virus was used for the HI-test (Table 3).

The phylogeny of the N1 and N2 sequences are available in Appendix 1 and 2. For the N1 sequences three major clusters were observed; one containing all the H1pdmN1pdm swIAVs, one containing all the N1av

sequences of H1avN1av swIAVs and one containing all the reassortant H1pdmN1av swIAVs. However, three NA sequences from H1avN1av (H1avN1av cluster-2) were clustering more closely to the H1pdmN1av reassortants than the H1avN1av cluster-1, potentially representing similar genetic drift in a proportion of the H1avN1av swIAVs. The H1pdmN1pdm cluster was divided into smaller clusters based on genotypes. For the N2 sequences it was observed that most H1avN2sw-3 and all H1avN2sw were in separate cluster, but otherwise no clear clustering according to genotypes were observed.

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 was all examined in the sequences of the Danish swIAV from 2024. As mentioned under objective 1, 184 whole genome sequences (WGS) of swIAV from 2024 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, 140 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). For the first time the E627K mutation was observed in one but this virus was a H3hu24N2hu24 virus and most likely a reverse-zoonotic case of the 2024 human seasonal H3N2 into a pig herd. All the human H3N2 viruses of that season carried the same 627K residue. However, two additional viruses showed two other mutations at this site. One virus was a H1avN1av-2 and had the 627V and the other virus was a H1avN2sw-1 and had the 627A mutation, like the mutation observed in a single virus last year. Interestingly, these two viruses originate from the same herd, meaning that the virus has maintained the same mutation for over one year. 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, 176 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. In total, 150 of the PA proteins were of H1N1pdm09 origin, and 26 were of Eurasian-avian like origin. Of the PA proteins of Eurasian origin, eight carried three of the four mutations, five carried two mutations, 12 carried one and one virus had none of the four mutations. For the PA proteins of H1N1pdm09 origin, six carried all four mutations, 76 carried three mutations, 28 carried two mutations, 39 carried one mutation and one PA protein had none of the mutations. The viruses with a PA protein carrying all four mutations were of the H1pdmN1av-2, H1avN2sw-5 and H1pdmN1pdm-1 genotypes.

The NP protein

In total, 182 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 Eurasian avian like H1N1 origin, while position 53, 100 and 313 are important for the NP proteins of H1N1pdm09 origin (14). Only 25/182 (13.7 %) available NP proteins were of Eurasian avian-like H1N1 origin and these generally had 48Q, 98K and 99K. At position 98 only one of the Eurasian avian-like NP proteins had an R instead of a K, whereas this was the case of seven

NP proteins on position 99. The remaining 157 NP proteins were of H1N1pdm09 origin. In total, only 7/157 (4.5 %) carried the 53D mutation, whereas 121/157 (77 %) 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, 175 NA gene segments (65 N1 and 119 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. None of the N1 or N2 proteins carried any of the mutations.

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.

All NS1 segments were recovered resulting in 183 proteins for the analysis. Eighty-eight (47.5 %) NS1 proteins were of Eurasian avian-like H1N1 origin, 95 (52 %) were of H1N1pdm09 origin and 1 (0.5 %) was of human seasonal H3N2 origin. All the NS1 proteins of H1N1pdm09 origin had the classical H1N1pdm09 truncation resulting in a protein of 219 amino acids. Most of the genotypes carrying this NS1 protein were the H1avN2sw-5 (65.6 %), H1pdmN1av-1 (12.5 %), H1avN2sw-4 (11.4 %), H1pdmN1pdm-1 (6.3 %), and H1pdmN2sw-3 (4.2). However, the Eurasian avian-like H1N1 origin NS1 protein were present in three different lengths; 217, 219 and 230 amino acids, where 230 is the classical full length NS1 protein. In total, 20 (23 %) of the Eurasian avian-like H1N1 origin NS1 proteins had a length of 217 amino acids and included the genotypes H1avN2sw-3, H1avN2sw-8, H1pdmN1av-2, H1pdmN1pdm-2, H1avN1av-1, H1pdmN2sw-4 and H1pdmN2sw-5. Only five viruses had an NS1 protein of Eurasian avian-like H1N1 origin of 219 amino acids, and included H1avN2sw-1, H1avN2sw-7, H1pdmN1av-2 and H1pdmN1av-3. The full length NS1 protein of Eurasian avian-like H1N1 origin of 230 amino acids included 71 % of the total Eurasian avian-like H1N1 origin NS1 proteins. The genotypes carrying this NS1 protein included H1avN1av-1, H1avN1av-2, H1avN2sw-1, H1avN2sw-2, H1avN2sw-3, H1avN2sw-6, H1pdmN1av-2, H1pdmN1av-3 and H1pdmN2sw-4.

Isolation of swIAV in cell culture

In total, 28 viruses from 2024 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 2024 including six H1avNx, five H1pdmNx and one H3hu24N2hu24.

	A/Norway/25089/2022	A/Michigan/45/15	A/California/07/09	Respiporc FLUpan	Respiporc FLU3 (H1av)	Respiporc FLU3 (all)
Deurne/IDT19038/2013 (H1av control)	<20	20/40	20/40	40	320	40
SV194 24-00545-2 (H1avN2sw, clade 1C.2.4)	20	<20	<20	20	20	<20
SV204 24-01657-3 (H1avN1av-1, clade 1C.2.2)	<20	40/80	40	20	80	20
SV209 24-02261-1 (H1avN2sw-7, clade 1C.2.5)	<20	<20	<20	20	20	<20
SV210 24-04841-1 (H1avN2sw-5, clade 1C.2.4)	20	20	<20/20	20	20	<20
SV211 24-02182-3 (H1avN2sw-5, clade 1C.2.2)	<20	40/80	40/80	20	80	20
SV219 24-04932-1 (H1avN1pdm-3, clade 1C.2.4)	20	<20	<20	20	20	<20
Hamburg/1580/2009 (H1pdm control)	20	320	320/640	1280	20	20
SV200 24-01378-1 (H1pdmN1av-2, clade 1A.3.3.2)	20	<20	<20	80	20	20
SV201 24-01455-3 (H1pdmN1av-3, clade 1A.3.3.2)	<20	<20	<20	80	<20	<20/20
SV203 24-01607-2 (H1pdmN1av-1, Clade 1A.3.3.2)	<20	40/80	80	40	<20	<20
SV215 24-00770-3 (H1pdmN1av-1, clade 1A.3.3.2)	<20	<20	<20	160/640	20	<20
SV221 24-10780-4 (H1pdmN2sw-5, clade 1A.3.3.2)	<20	<20	<20	80	20	<20/20

Table 2. HI-titers of H1xNx Danish swIAV isolates from 2023 to human and swine H1xNx 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 of the HI-test revealed that of the 1C.2 swIAVs (Table 2), only the two isolates of the 1C.2.2 clade showed cross-reaction to the Respiporc FLU3 hyperimmune monovalent sera raised towards the 1C.2 strain of the vaccine, whereas none of the 1C.2 viruses of any clade showed cross-reaction to the trivalent sera raised against all three vaccine strains. The same two viruses of the 1C.2.2 clade also showed cross-reaction to the two ferret vaccine sera raised against the two older human 1A.3.3.2 strains, but otherwise no cross-reactions to the ferret sera raised against the human seasonal vaccine strains were observed. All 1A.3.3.2 swIAVs from 2024 showed cross-reaction to the hyperimmune sera raised towards the 1A.3.3.2 vaccine strain included in Respiporc FLUpan H1N1 (Table 2). However, only two of these swIAVs showed cross-reaction to ferret sera raised towards the two older human seasonal vaccine strains and none of the 1A.3.3.2 viruses showed cross-reaction to the ferret sera raised against the most recent human seasonal 1A.3.3.2 strain.

	A/Thailand/8/2022	A/Switzerland/8060/2017	Respiporc serum (H3sw)	Respiporc FLU3 all
Warendorf/IDT22506/2015 (H3sw control)	<20	20	1280/2560	320
SV188 24-00720-2 (H3hu24N2hu24)	160	20	20	20

Table 3. HI-titers of H3xNx Danish swIAV isolates from 2024 to human and swine 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.

For the single H3hu24N2hu24 detected in this year surveillance, the HI-test revealed that while the strain showed great cross-reaction with ferret sera raised towards recent human seasonal H3N2 strain, no cross-reaction was observed with the hyperimmune monovalent or trivalent sera against the H3N2 component or all three vaccine strains, respectively (Table 3).

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" (17) 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.

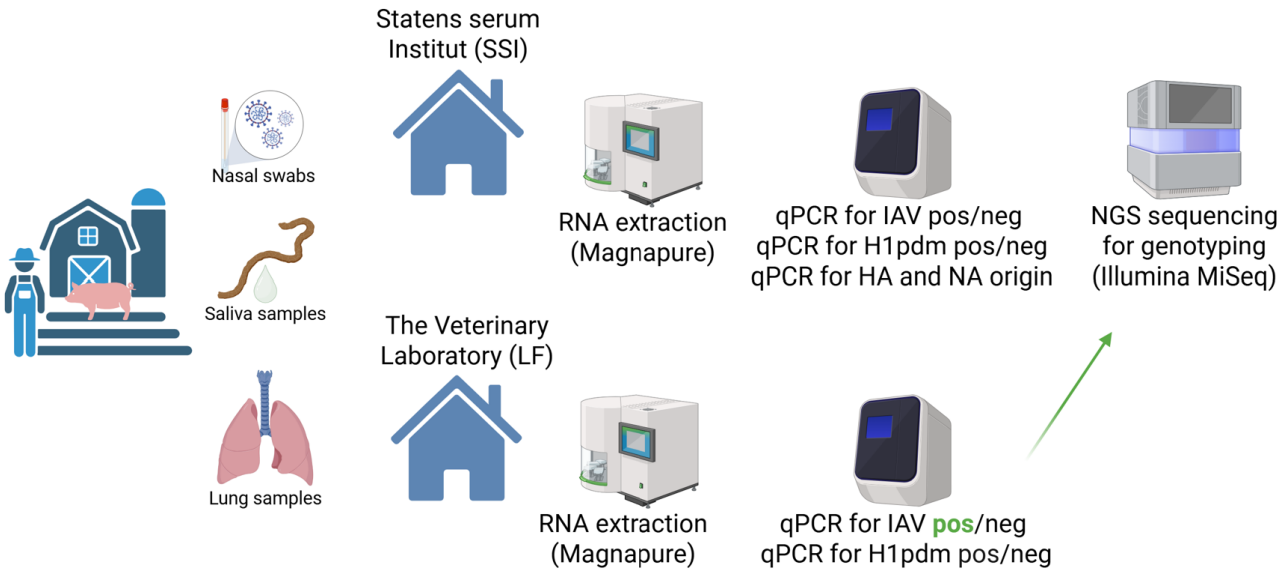

Figure 11. Sample origin and tests performed on samples included in the annual report.

The NGS data were trimmed and mapped to a reference panel of swIAV strains, and the resulting consensus sequences of the eight segments were extracted and used for genotyping using an in-house pipeline at SSI, specifically developed for genotyping Danish swIAVs. The subsequent genetic and phylogenetic analyses were performed at Copenhagen University using CLC main workbench, version 22.0.1 and IQ-Tree (18).

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 was 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 on 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 specific H1av strain used in the vaccine, 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 ≥ 40 used was considered positive. In humans a titer of 40 is recognized as "protective" as a result of a 50% reduction in disease in healthy adults.

Discussion and conclusion

The last two years a slight annual reduction in the number of submissions for the Danish passive swIAV surveillance program was observed and efforts should be made to secure that this tendency does not continue as the it will impact the possibility to detect if new more concerning variants of Influenza A viruses arises. A small decrease in the proportion of swIAV positive submissions was observed, but the proportion of swIAV positive herds was identical to 2023, indicating a steady endemic state of swIAV in Denmark. The proportion of submissions positive for H1pdm decreased significantly in 2024 compared to 2023. This decrease followed three consecutive years of increase in the H1pdm proportion that was likely a result of the introduction and rapid spread of reassortant H1pdmN1av swIAVs, which could indicate that some level of herd immunity on an overall level has been reached. It could also be explained by more herds using the Respiporc FLUpan H1N1 vaccine in their vaccination programs. Finally, swIAVs genotypes that have increased numbers since last year, particularly the H1avN2sw-5 and the H1avN1av-1, could have gained some traits that could result in competitive advantages. However, additional tests are needed to test this and were beyond the scope of the 2024 surveillance program.

As an explanation for the decrease in 1A.3.3.2 swIAVs in 2024, a lower proportion of the PPPPPA internal gene cassette was also observed this year. This gene cassette is mainly present in the H1pdmN1av-2 and H1pdmN1pdm-2 genotypes and especially the H1pdmN1av-2 showed a decrease in proportion this year. Additionally, the H1avN2sw-3 genotype carrying the PPPPPA internal gene cassette declined in 2024 further explaining the lower proportion of the PPPPPA internal gene cassette. In 2024, one H1pdmN2sw-5 novel genotype was discovered introducing a novel internal gene cassette into the Danish swIAV gene pool. The constantly expanding number of swIAV genotypes can be problematic, as these novel gene constellations can result in swIAV that have new traits, that potentially increase their zoonotic potential.

In addition to the massive genetic diversity of swIAVs on a gene segment level, there is also a great diversity within the single gene segment as illustrated by the phylogenetic trees of the HA gene segments. It is evident that the three major 1C.2 and 1A.3.3.2 clades are present in Denmark, and from the 1C.2 swIAVs some sequences are outside the defined clades requiring an update of the current global swIAV nomenclature. The specific clade of the 1C.2 swIAVs seems to play a great role in the level of cross-reaction to the swine vaccine strain, with the vast majority of Danish swIAVs belonging to the 1C.2.4 clade, which show no cross-reaction to the vaccine in the HI-test. However, for the 1A.3.3.2 swIAVs, which has not been circulating in Denmark for as long as the 1C.2 swIAV, there was a higher cross-reaction to the swine vaccine. However, from a zoonotic perspective it was evident that both the 1C.2 and 1A.3.3.2 swIAVs were not cross-reacting to the ferret sera raised against the human seasonal IAV strains, and therefore limited antibody cross-reactivity is expected if a person becomes infected with the contemporary swIAVs. Additionally, we have also published a scientific paper about the Danish swIAV genotypes H1pdmN1av-1 and H1pdmN1av-2 (19), both showing very limited population immunity further emphasizing that if a Danish swIAV starts transmitting from human to human the pre-existing immunity will be very limited.

In 2024, five cases of reverse-zoonotic events with four being of H1pdmN1pdm human seasonal origin and one being of H3N2 human seasonal origin, were detected. The risk of introductions of human seasonal origin viruses into the Danish swine population should not be underestimated, as these viruses can continue circulating in the pig population and then serve as a reservoir of older human seasonal strains, that the younger human population will not have immunity towards. This has been seen previously in the surveillance, where gene segments of both 1995 and 2005 were circulating at the latest in 2022 and 2021, respectively, as the H1pdmN2hu95, H1avN2hu95 and H3hu05N2sw subtypes. Additionally, the pig population immunity, especially towards the human seasonal H3N2, is extremely limited as very few herds have tested positive for H3N2 during the entire period of the Danish swIAV surveillance. Additionally, the HI-test revealed no cross-reaction of the H3hu24N2hu24 virus isolated from pigs this year, so in a situation where this virus starts spreading effectively between pigs, the vaccine will most likely provide no or very limited cross-protective HA antibodies. Additionally, specific mutations can be introduced into the swine population by human IAVs. This year, for the first time, we identified the E627K in the PB2 gene, which is known to impact the host-range of avian IAVs. The effect that this mutation would have on swIAV is unknown, but having swIAVs with this mutation circulating in Denmark, would serve as an additional risk if reassortants between avian IAV and swIAV were to occur. Additionally, two swIAV also showed different mutations in this position and their effect on host range and replication efficiency needs to be evaluated in phenotypic assays that are not part of the basic surveillance program. On the molecular level, several risk markers are present in the majority of the sequences detected in 2024, underlining that a high number of "higher-risk" swIAVs are currently circulating in Denmark, but again phenotypic testing is needed to graduate the risk.

In conclusion, the annual surveillance of swIAV in Denmark is crucial for keeping track of the constantly evolving swIAV strains and identify higher risk viruses. SwIAV continue to negatively impact animal welfare, antibiotic use, CO_2 emissions and production economy and as illustrated by the antigenic characterization and the distribution of swIAV in Denmark, novel and better control measures are needed. The massive occurrence of swIAV in Danish pig herds present a risk for human health, as swIAV can sporadically infect humans and potentially adapt to spread between humans causing epidemics or in worst-case cause pandemics as observed in 2009.

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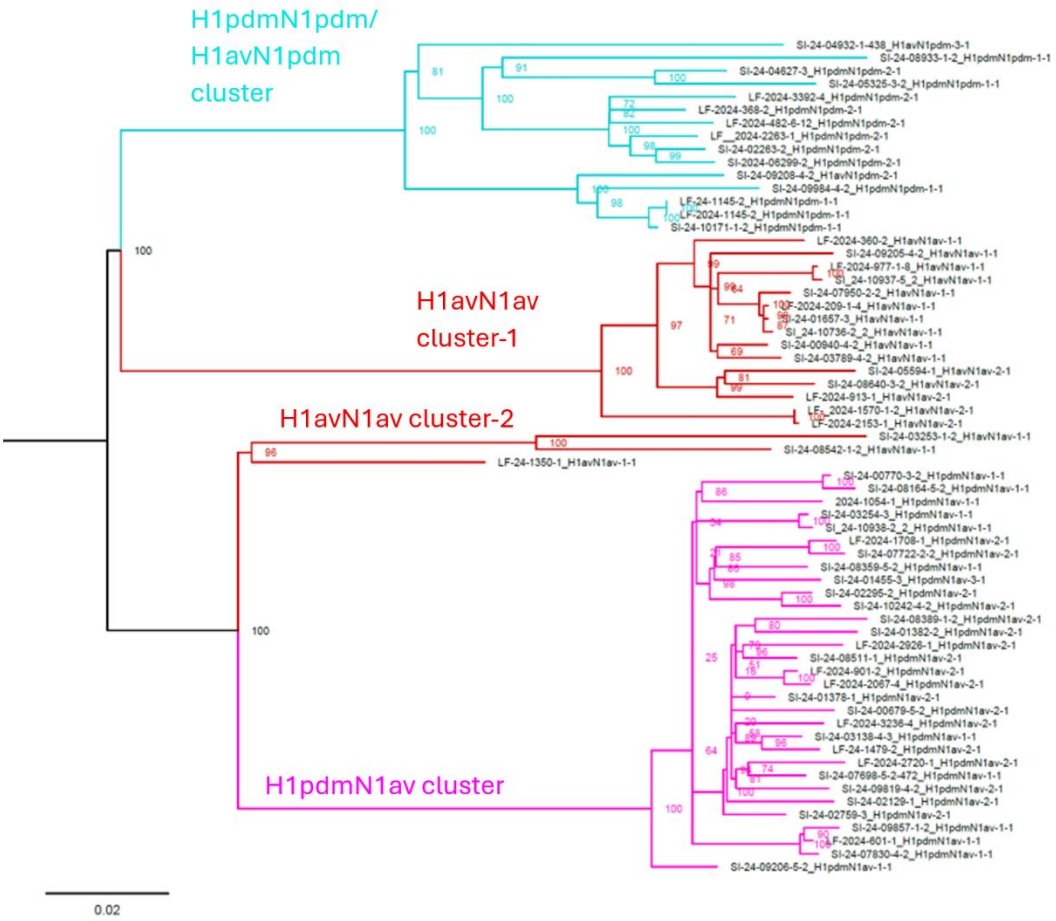
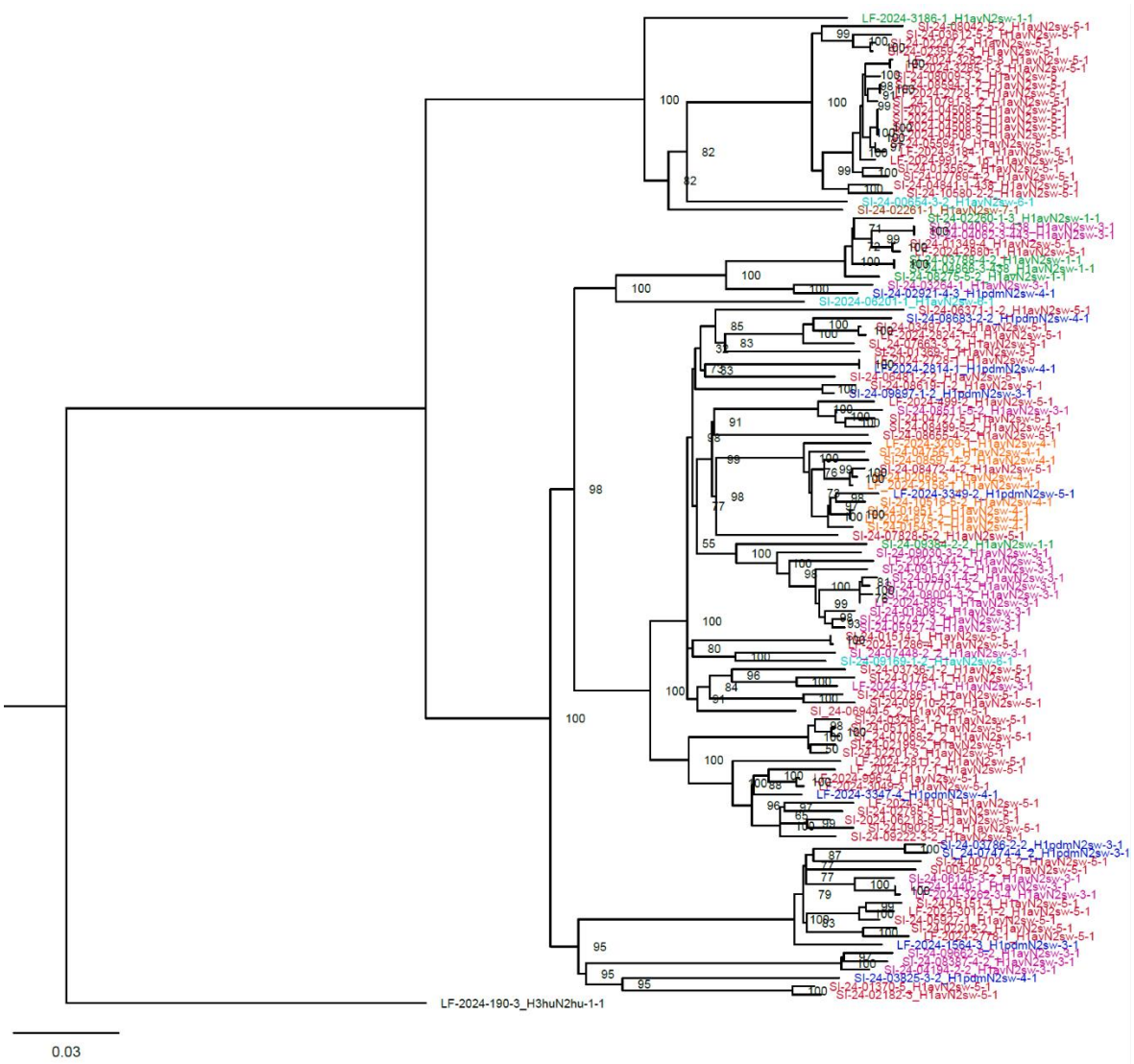
Appendix 1: Maximum likelihood tree of Danish NA genes of H1xN1x viruses from 2024



The N1 tree was midpoint-rooted and the clusters are defined by the branch color and indicated in the tree.

Appendix 2. Maximum likelihood tree of Danish NA genes of HxN2x viruses from 2024



A human seasonal N2 from LF-2024-190-3_H3huN2hu-1-1 was used as an outgroup. The taxon color indicates the genotype. Green indicate H1avN2sw-1, pink indicate H1avN2sw-3, orange indicate H1avN2sw-4, red indicate H1avN2sw-5, turquoise indicate H1avN2sw-6. Blue indicate H1pdmN2sw viruses.