

Swine influenza A virus infection dynamics and evolution in intensive pig production systems

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Author Summary: Infection with influenza A virus is widespread in pigs and contributes towards poor health and loss of productivity. Effective infection control measures are necessary to limit the impact of influenza on farms. However, modern farming practices are increasingly characterised by larger herd sizes and higher stocking densities, utilising a range of different husbandry practices such as all-in/all-out versus continuous flow plus either farrow-to-finish or various combinations of breeding, rearing and finishing. It is unclear how the move towards increased intensification impacts infectious diseases such as influenza. Accumulating evidence suggests that long-term infection of herds may be common. How influenza A virus can persist through long-term circulation within pig herds is poorly understood. The aim of the current study was to monitor the infection status of pigs at each stage of the production process, measure the extent of the developing immune response and to characterise the evolutionary changes in the circulating influenza A viruses over time. Our findings provide insights into the dynamics of viral infection and evolution and may contribute to improved infection control strategies.

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Abstract

Swine influenza A virus (swIAV) is one of the main viral pathogens responsible for respiratory disease in farmed pigs. While outbreaks are often epidemic in nature, increasing reports suggest that continuous, endemic infection of herds is now common. The move towards larger herd sizes and increased intensification in the commercial pig industry may promote endemic infection; however, the impact that intensification has on swIAV infection dynamics and evolution is unclear. We carried out a longitudinal surveillance study for over 18 months on two enzootically infected, intensive, indoor, and multi-site pig production flows. Frequent sampling of all production stages using individual and group sampling methods was performed, followed by virological and immunological testing and whole-genome sequencing. We identified weaned pigs between 4 and 12-weeks old as the main reservoir of swIAV in the production flows, with continuous, year-round infection. Despite the continuous nature of viral circulation, infection levels were not uniform, with increasing exposure at the herd level associated with reduced viral prevalence followed by subsequent rebound infection. A single virus subtype was maintained on each farm for the entire duration of the study. Viral evolution was characterised by long periods of stasis punctuated by periods of rapid change coinciding with increasing exposure within the herd. An accumulation of mutations in the surface glycoproteins consistent with antigenic drift was observed, in addition to amino acid substitutions in the internal gene products as well as reassortment exchange of internal gene segments from newly introduced strains. These data demonstrate that long-term, continuous infection of herds with a single subtype is possible and document the evolutionary mechanisms utilised to achieve this.

Keywords: swine influenza A virus; infection dynamics; intensive pig production; viral evolution

Introduction

Swine influenza A virus (swIAV) is a major pathogen of pigs. In naïve animals, infection usually presents as an acute respiratory disease characterised by high morbidity and low mortality (Olsen et al. 2012). Primary swIAV infection can lead to pneumonia caused by opportunistic secondary pathogens, and along with porcine reproductive and respiratory syndrome virus (PRRSv), is one of the main viral factors in the development of porcine respiratory disease complex (PRDC) (Brockmeier, Halbur, and Thacker 2002). PRDC is a leading health concern for producers globally and has significant economic impact. Efforts to control the spread of swIAV are mostly limited to management practices that reduce mixing of animals from different sources, such as all in/all out,

and vaccination of pregnant sows to protect piglets via maternal-derived antibodies (MDA).

Immunity to swIAV is primarily mediated by the development of neutralising antibodies to the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA). For H1 subtypes, the major antigenic sites have been mapped to five regions on the globular head of HA, designated as Sa, Sb, Ca1, Ca2, and Cb (Gerhard et al. 1981; Caton et al. 1982). Although less studied, key antigenic regions on NA have also been identified (Laver et al. 1982; Webster, Brown, and Laver 1984; Air et al. 1985). Amino acid substitutions at antigenic sites in HA and NA leads to increased antigenic distances over time (antigenic drift), facilitating reinfection of previously immune hosts. The segmented nature of the

swIAV genome can also result in reassortment of genetic segments during co-infection with two or more strains (antigenic shift). Indeed, pigs have long been hypothesised to be important in the wider ecology of influenza A virus (IAV), acting as a mixing vessel for reassortment of avian, swine, and human-adapted strains, due to the presence of both avian-like and human-like IAV receptors in the porcine respiratory tract (Scholtissek et al. 1985; Ito et al. 1998).

Influenza is endemic in European swine, with a recent large-scale surveillance study estimating prevalence at 56.6 per cent at the herd level (Henritzi et al. 2020). The evolutionary history of swIAV is complex. Like the situation in humans, H1 and H3 subtypes with different combinations of N1 and N2 circulate globally; however, the origin, antigenicity, and internal gene constellations are markedly different between North American, European, and Asian lineages, and a global nomenclature system has been proposed based on HA phylogeny (Anderson et al. 2016). In Europe, classical swine H1N1, a descendent of the virus that emerged in humans in 1918, circulated exclusively until the 1970s (Taubenberger et al. 2001). In 1979, classical H1N1 was replaced by an avian-like H1N1 (lineage 1C), which continues to circulate and is the most frequently detected lineage in European swine herds (Pensaert et al. 1981; Henritzi et al. 2020). A seasonal human H3N2 strain entered the European swine population in 1984 and reassorted with H1N1 lineage 1C to produce swine H3N2 (swH3N2) (de Jong et al. 2007). A decade later, human-like H1N2 (lineage 1B) emerged as a result of reassortment between human seasonal H1N1, swH3N2, and H1N1 lineage 1C (Brown et al. 1998). With the emergence of pandemic H1N1 in 2009 (pH1N1 clade 1A.3.3.2), reverse zoonosis into pigs occurred and pH1N1 clade 1A.3.3.2 strains are now widespread (Welsh et al. 2010). In addition, pH1N1 clade 1A.3.3.2 have contributed gene segments to other contemporary lineages via reassortment (Moreno et al. 2011). The diversification and evolution of swIAV lineages therefore represent a significant challenge to the efforts that aim to control influenza via vaccination.

Under the European Union Integrated Pollution Prevention and Controls (IPPC) Directive (<https://eur-lex.europa.eu/eli/dir/2008/1/oj>), pig farms with 750 or more sows or 2,000 or more production pigs >30 kg are considered intensive. In Northern Ireland, as in the rest of the UK, the pork industry has moved towards increased intensification over the last two decades. Agricultural census data for 2022 show that 87.5 per cent of the 738,540 pigs registered in Northern Ireland were present on only 73 intensive farms (https://www.daera-ni.gov.uk/sites/default/files/publications/daera/Agricultural%20Census%202022%20Publication_1.pdf). Intensification presents a challenge for the control of infectious diseases such as swIAV. Under traditional, non-intensive pig rearing systems, swIAV outbreaks followed a typical epizootic pattern, with onset of clinical signs throughout the herd, followed by rapid recovery and the development of strain-specific herd immunity (Olsen et al. 2012). However, the move to intensification may promote long-term infection of herds, due to high turnover rates and the continuous introduction of swIAV antibody-naïve animals maintaining a large population of susceptible pigs in which the virus can circulate continuously. Modelling-based estimates of the minimum population size necessary for continuous infection are relatively modest, at around 3,000 pigs (Pitzer et al. 2016). Cohort studies (following batches of pigs from birth to slaughter or through specific production stages) and longitudinal studies, (where specific populations of animals are sampled repeatedly over time) have provided valuable insights into swIAV infection dynamics in a variety of production systems (Loeffen et al. 2009;

Takemae et al. 2011; Simon-Grifé et al. 2012; Kyriakis et al. 2013; Rose et al. 2013; Diaz et al. 2017a, 2017b; Mine et al. 2019; Ryt-Hansen et al. 2019b; Bhatta et al. 2020). However, the impact of intensification on swIAV infection dynamics and evolution is unclear. We therefore carried out longitudinal surveillance on two intensive swine production flows over an 18-month period in order to understand: (i) which production stage(s) are most susceptible to infection, (ii) whether infection is epizootic or enzootic in nature, and (iii) if maintaining a large population of swIAV antibody-naïve animals lessens selection pressures that normally drive antigenic drift and shift.

Materials and methods

Farm selection, organisation, and vaccination status

Three multi-site, indoor, intensive commercial pig production flows located in Northern Ireland were visited in January 2021 and tested for the presence of swIAV by real-time reverse-transcription polymerase chain reaction (RRT-PCR). All production stages including suckling pigs (sucklers), weaned pigs (weaners), finishing pigs (finishers), and gilts were sampled using nasal wipes, udder wipes, and oral fluid sampling. A reduced number of samples were collected during this initial visit compared to subsequent visits (46 versus 55 samples per production flow). Two of the three flows tested positive for swIAV by RRT-PCR in at least one production stage and were selected for inclusion in the longitudinal study, with a total of 12 visits over 18 months.

The organisation and herd details for each flow are shown in Fig. 1. Lactating sows were kept in farrowing crates. Piglets remained with the sows until 4 weeks of age, after which they were transferred to the nursery facilities on the weaning units. The weaning units had first and second stages (i.e. 7–15 kg and 15–30 kg). Pigs remained on the weaning unit until they reached 30 kg at ~12 weeks old, after which they were transported to the finishing facilities where they remained until reaching slaughter weight at ~24 weeks. Breeding, weaning, and finishing sites were all geographically separate sites and had independent temperature and ventilation systems with separated air intake and exhaust vents. Gilts (female pigs that have not yet produced a litter, raised to replace breeding sows), weaners and finishers on the three units were housed in pens across multiple rooms. Replacement gilts were obtained from a separate nucleus herd and were brought onto each individual breeding site from 4- to 8-week-old every month. For both flows, management practices included the use of all in/all out at the room level and vaccination of gilts and pregnant sows for swIAV. Gilts received inactivated trivalent swIAV vaccine (RespiPorc FLU3, IDT Biologika) at 6 and 3 weeks before service. Sows were vaccinated 3 weeks prior to farrowing.

Ethical statement

In this study, only non-invasive samples (nasal wipes, udder wipes, and oral fluids) were collected and therefore, did not require a specific licence according to UK Law. A trained veterinarian was involved in the sampling and consent of the farmer was obtained before sample collection.

Sample collection and processing

An overview of the sampling strategy used in this study is shown in Fig. 1. Sample size was determined based on a recent

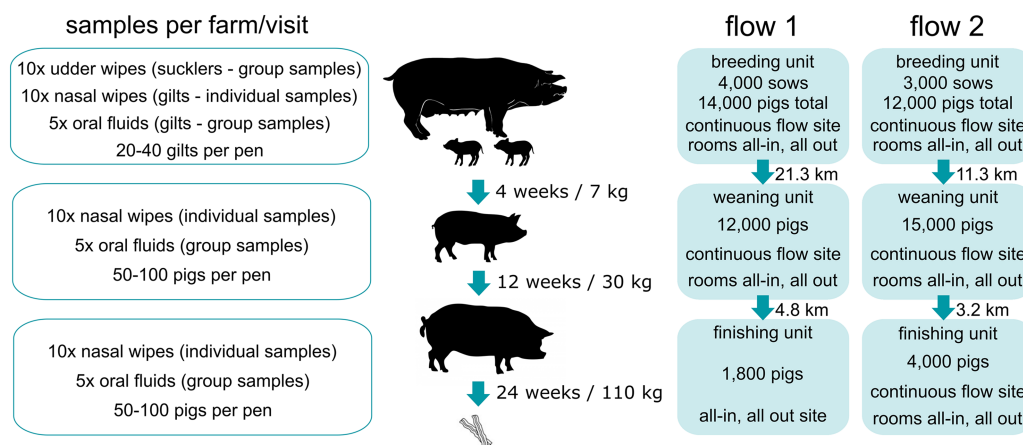


Figure 1. Overview of sampling strategy, production flow organisation, and management practices. (Left) Sample types and number of samples collected per production stage per visit. (Middle) Production stages showing progression milestones from farrow to slaughter. (Right) Production flow organisation including population at each production stage, distances between sites and management practices.

European-wide surveillance study that found 30.5 per cent of samples tested positive for swIAV by RRT-PCR (Henritzi et al. 2020). At the national level, 14.6 per cent of UK samples and 25.1 per cent of Republic of Ireland samples tested positive. Based on these data, we assumed that the prevalence in NI herds would be between 14.6 per cent and 30.5 per cent. Therefore, taking 10 individual samples (nasal wipes) per production stage per time-point would yield between 1 and 3 positive samples. Group samples (oral fluids and udder wipes) were included to increase the breadth of surveillance. Frequency of sampling was determined by available veterinary resource. Nasal wipes were collected as described previously (Edwards et al. 2014). Ten nasal wipes each were collected from gilts, weaned pigs, and finishing pigs per production flow per visit. Nasal wipes were collected by wiping a sterile 5 cm × 5 cm cotton gauze (MediSupplies Ltd #PMC5771) across the pig's snout which was placed into a sterile Nalgene Wide-Mouth HDPE 30 ml Bottle (ThermoFisher Scientific #2104-0001) containing 5 ml Brain-Heart Infusion Broth (Merck #53286) supplemented with antibiotics/antimycotics (1000IU penicillin G; 10 µg/ml amphotericin B; 1 mg/ml gentamicin). Udder wipes were collected as described previously (Garrido-Mantilla et al. 2019). Ten udder wipes were collected from sows/suckling pigs per farm per visit using the same reagents as used for nasal wipes. A single cotton gauze was used to collect the nasal/oral secretions deposited on the udders by the litter during or immediately after suckling and placed in bottles containing transport media as above. Oral fluids were collected as described previously (Garrido-Mantilla et al. 2019) using Oral Fluid Collection Kits (IDEXX #98-0002094-00). Undyed cotton, three-strand twisted ropes were placed hanging from pens and pigs allowed to chew for ~30 mins. Ropes were squeezed into plastic bags and the recovered fluids were transferred to 15 ml centrifuge tubes. A single oral fluid sample was collected per pen, with five pens each sampled for gilts, weaned pigs, and finishing pigs per farm per visit.

For all sample types, gloves were worn and changed between each sample collection. Pens, litters, or individual pigs were selected whenever possible based on the presence of clinical signs suggestive of swIAV infection, such as coughing or sneezing, and randomly when clinical signs were not observed. Following collection, samples were stored at 2–8°C for up to 4 days before transporting to the laboratory. Upon receipt, nasal/udder wipes (both gauze and unabsorbed transport media) were transferred

to 10 ml centrifuge tubes and centrifuged at 2,683 g for 10 min at 4°C. Following centrifugation, media present above the pelleted gauze was collected, aliquoted, and stored at –80°C. Oral fluid samples were centrifuged at 2,683 g for 10 min at 4°C to remove debris and supernatants aliquoted and stored at –80°C.

RNA extraction and RRT-PCR

Nucleic acids were extracted from 0.2 ml of clinical material or allantoic fluid using the IndiMag Pathogen Kit (Indical Bioscience #SP947457) on a KingFisher Flex purification system with 96 deep-well head (ThermoFisher Scientific #5400630), according to the manufacturers' instructions. Extracted material was tested for the presence of influenza virus RNA by RRT-PCR using primers and probe that detect a conserved region in the viral matrix gene, as described previously (Nagy et al. 2021). RRT-PCR analysis was carried out using AgPath-ID One Step RT-PCR Reagents (ThermoFisher Scientific #4387391) on a LightCycler 480 (Roche). Two replicates per sample were tested, with samples returning threshold cycle (ct) values <38 in duplicate considered positive.

Influenza anti-NP blocking ELISA

The detection of swIAV anti-NP antibodies in oral fluid samples using a modified, commercially available ELISA (Influenza A Ab Test, IDEXX #99531-01) has been described previously (Panyasing et al. 2014; Gerber et al. 2017). Modifications to the manufacturer's instructions include increasing the volume of sample loaded to 0.2 ml and incubating plates at 21°C for 16 h. Following incubation, optical densities were measured at 650 nm and sample-to-negative (S/N) ratios calculated according to the manufacturer's instructions. Two replicates were tested for each sample and the percentage coefficient variation (%CV) was calculated as instructed. Samples with %CV >15 were repeated. Repeat samples returning %CV >15 were considered invalid and were excluded. Valid samples with mean S/N ratios ≤0.6 were considered positive.

Virus isolation

Ten-day-old embryonated specific-pathogen-free chicken eggs (VALO Biomedica) were used to isolate swIAV from RRT-PCR

positive clinical samples. Undiluted nasal/udder wipe fluid (0.1 ml) was inoculated into the allantoic cavity using a 1-ml syringe fitted with a 26 G needle. Oral fluid samples were supplemented with antibiotics/antimycotics (1000IU penicillin G; 10 µg/ml amphotericin B; 1 mg/ml gentamicin) prior to inoculation. Three eggs per sample were inoculated and allantoic fluid harvested and pooled following 4 days incubation at 37°C. Debris was pelleted at 800 g for 10 mins at 4°C and aliquots stored at -80°C. Successful virus isolation was confirmed either by hemagglutination of 0.5 per cent chicken blood cells (Envigo #S.B-0008) or by RRT-PCR.

Influenza whole-genome sequencing

The selected samples for whole-genome sequencing (WGS) were extracted using the QIAamp® Viral RNA Mini Kit (Qiagen #52906) with the addition of carrier RNA. A multiplex RT-PCR assay was designed to amplify the whole segmented influenza RNA genome using primers with complementary sequence to the IAV genome universal conserved termini regions (Zhou et al. 2009) and additional adapted primers with sequence specific to the 3'-end complementary of PA, PB1, and PB2 genes enabling the amplification of the larger polymerase gene segments (Supplementary Table S1). The amplification reaction used the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen #12574018). The generated amplicons were assessed for correct amplicon base pair size on a 1 per cent agarose gel. The library was prepared with the Nextera XT DNA Library prep kit (Illumina #FC-131-1096) following the manufacturer's instructions with changes, as described previously (Fusaro et al. 2019). The underrepresented distal ends of the amplicon coverage are due to the nature of the transposon's double-stranded DNA cleavage mechanism; however, this was resolved by concatenating overhangs of Nextera transposase adapter sequences to each primer set as either a full length or as a truncated version (Supplementary Table S1). The quality of data was screened using Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) while selecting a high Q ≥33 score and then reviewed by the MultiQc programme (Ewels et al. 2016). Paired reads were aligned using the Burrows-Wheeler Aligner maximum exact matches (BWA-MEM) (v0.7.17-r1188) read aligner to selected indexed reference genomes A/swine/Northern Ireland/2005-22/2021 (H1N2) and A/swine/Northern Ireland/2004-30/2021 (pH1N1) in SAMtools (v1.10) (Li 2013) creating a sequence alignment map (SAM) file (Li et al. 2009). The SAM files were indexed to binary sequence map (BAM) files, and the read depth and breadth of coverage were determined using SAMtools before visualising, generating consensus sequences and single-nucleotide polymorphisms (SNP) calling in Geneious R10 (Biomatters). WGS data are available under GISAID accession numbers EPI_ISL_18221301 to EPI_ISL_18221395.

HA/NA protein structure homology modelling

HA/NA protein structure homology models of representative flow 1 and flow 2 strains were generated using Swiss-Model (<https://swissmodel.expasy.org/>). The HA amino acid sequence of A/Northern Ireland/2012-08/2021 (pH1N1) was modelled using the H1 of A/Michigan/45/2015 (<https://doi.org/10.2210/pdb7KNA/pdb>) as template, with a final Global Model Quality Estimate of 0.76. The HA of A/Northern Ireland/2006-10/2021 (H1N2) was modelled using H1 of A/Hickox/JY2/1940 (<https://doi.org/10.2210/pdb6ONA/pdb>) as template, with a final Global Model Quality

Estimate of 0.77. The NA of A/Northern Ireland/2006-10/2021 (H1N2) was modelled using the N2 of A/Moscow/10/1999 (https://www.wwpdb.org/pdb?id=pdb_00007u4f) as template, with a final Global Model Quality Estimate of 0.79.

Phylogenetic analysis of swIAV

The complete coding sequences of PB2, PB1, PA, HA, NP, NA, NS1, NEP, M1, and M2 were aligned by MUSCLE in MEGA version 11.0.13 (<https://www.megasoftware.net/>). The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model, with phylogeny tested using 1,000 bootstrap replications. Clade designation was performed using the Swine H1 Clade Classification Tool at the BV-BRC (<https://www.bv-brc.org/app/SubspeciesClassification>).

Results

Utility of nasal wipes, udder wipes, and oral fluids for swIAV surveillance

Effective surveillance for swIAV requires a balance between obtaining sufficient sample numbers to detect a representative cross-section of circulating strains alongside consideration of the downstream diagnostic methods to be utilised versus cost and ease of sampling. Nasal swabs are considered as the gold-standard for virological detection of swIAV. However, pigs must be suitably restrained to obtain nasal swabs, making collection of large numbers labour-intensive. Alternative sampling approaches for swIAV surveillance have received attention in recent years, including nasal wipes, udder wipes, environmental swabbing, aerosol sampling, and oral fluids (Goodell et al. 2013; Edwards et al. 2014; Garrido-Mantilla et al. 2019). Of these, nasal wipes and udder wipes have been shown to perform equivalent to nasal swabs for the detection and isolation of swIAV while being relatively easy to obtain. Oral fluids are an increasingly popular sample type for routine viral surveillance, allowing groups of pigs to be sampled with ease. In addition to detection of shed viruses, oral fluids have also been successfully utilised to measure secreted swIAV antibodies (Panyasing et al. 2014; Gerber et al. 2017). However, virus isolation rates and WGS data quality from oral fluids are generally low and obtaining samples from suckling pigs is difficult due to lack of interest in the cotton ropes. In this study, we primarily utilised nasal wipes to obtain individual samples suitable for downstream applications including virus isolation, and oral fluids to obtain group samples to expand the breadth of surveillance. Litters of suckling pigs were sampled using udder wipes, overcoming the limitations of oral fluids for this age group.

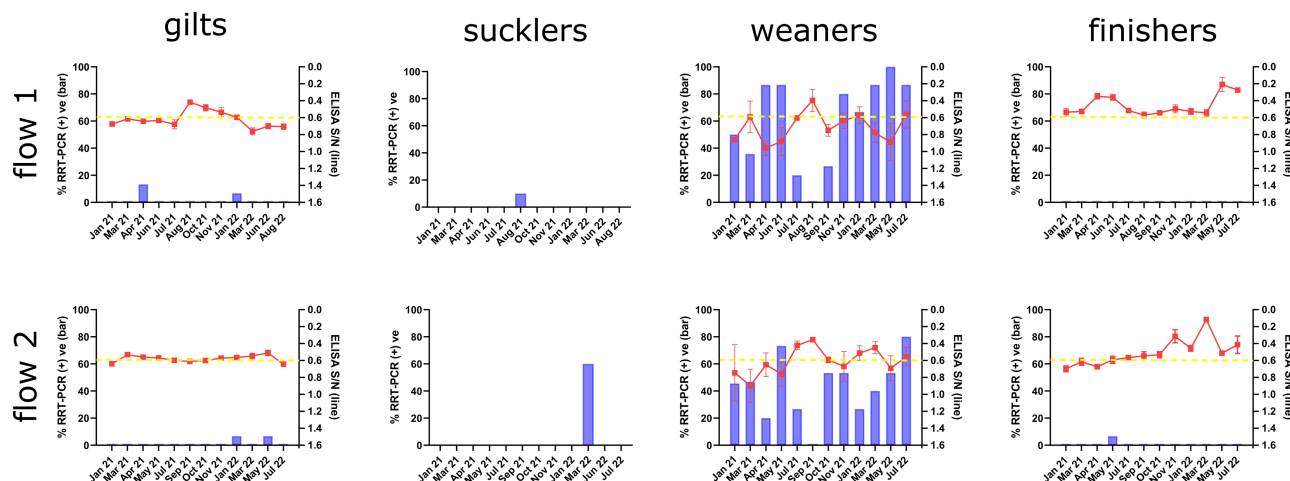
In the current study, nasal wipes and oral fluids were found to be comparable in terms of detection of swIAV by RRT-PCR (Table 1), having positivity rates of 18.1 per cent and 17.3 per cent, and mean ct values of 31.4 and 30.8, respectively. However, viral isolation rates in embryonated eggs were low for oral fluids (10.9 per cent) compared to nasal wipes (52.3 per cent). The utility of udder wipes was less clear due to the apparent low prevalence of swIAV on the breeding units under investigation. During the 18-month study period, only 7 of 240 (2.9 per cent) udder wipes tested positive and only 1 viral isolate was obtained.

Prevalence of swIAV in different production stages

Figure 2 summarises the proportion of samples testing positive for swIAV by RRT-PCR from each production stage. Associated

Table 1. Molecular detection and viral isolation rates for swIAV from nasal wipes, udder wipes and oral fluids

Sample type	No. of samples	No. of swAIV positive	RRT-PCR			Virus isolation in eggs		
			swAIV positive (%)	Average ct	ct Range	Virus isolation attempted	Virus isolation achieved	Success rate (%)
Udder wipe	240	7	2.9	32.7	25.9–37.6	7	1	14.3
Oral fluid	341	59	17.3	30.8	25.0–36.8	55	6	10.9
Nasal wipe	718	130	18.1	31.4	20.3–37.7	130	68	52.3
Total	1299	196	15.1	31.30	20.3–37.7	192	75	39.1

**Figure 2.** Comparison of viral prevalence with antibody status. Nasal wipes, udder wipes, and oral fluids were tested for the presence of swIAV by RRT-PCR and plotted as percentage positivity (left y-axis, blue bars). Each data point for gilts, weaners, and finishers represents 15 samples (10 nasal wipes and 5 oral fluids), except for the initial January 2021 time-point that represents 12 samples (10 nasal wipes and 2 oral fluids). For sucklers, each data point represents 10 udder wipes (10 litters). Oral fluid samples were also tested for the presence of influenza NP antibodies by a blocking ELISA and plotted as S/N ratios on the same graph (right y-axis, red line). S/N ratios ≤ 0.6 are considered antibody positive. The dashed yellow line indicates the 0.6 S/N ratio cut-off value.

ct values of positive samples are included in [Supplementary Fig. S1](#). On both production flows, the vast majority of positive samples (183 of 196) were obtained from weaned pigs between 4 and 12 weeks old. Virus was detectable throughout the 18-month study period on the weaning units apart from a single time-point (August 2021 for flow 1 and September 2021 for flow 2). Two discrete waves of infection could be discerned. For flow 1, the first wave of infection peaked during April 2021 (86.7 per cent positivity, mean ct 29.3), followed by a decline and eventual disappearance in August 2021. The virus re-emerged in September 2021, resulting in a second wave of infection that peaked in May 2022 (100 per cent positivity, mean ct 26.6). A similar pattern was observed for flow 2, with the initial wave peaking during May 2021 (73.3 per cent positivity, mean ct 29.5), followed by a subsequent decline and disappearance in September 2021. The virus re-emerged in October 2021 and was maintained within the herd until the end of the study, reaching peak prevalence in July 2022 (80 per cent positivity, mean ct 31.0).

For all other production stages, swIAV was either absent or present at a very low prevalence. Where present, detections by RRT-PCR on these units were sporadic and associated with ct values >31 . An exception to this observation occurred on the flow 2 breeding unit. Following 14 months without detection, 60 per cent of samples obtained from the breeding unit tested positive for swIAV in March 2022, with ct values ranging from 25.9 to 34.9. Subsequent sampling points in June and July 2022 tested negative by RRT-PCR.

swIAV antibody status in different production stages

Of the 341 oral fluid samples collected during this study, 11 had insufficient material remaining following molecular testing to allow testing by ELISA or failed ELISA quality control due to high %CV values of replicates and so weren't included in subsequent analysis. ELISA data from the remaining 330 oral fluid samples analysed for the presence of swIAV specific antibodies revealed different patterns of antibody positivity for each production stage ([Fig. 2](#) and [Supplementary Fig. S1B](#)). Antibody levels in gilts were relatively uniform, with ELISA S/N values trending around the assay cut-off value of 0.6 for most of the study period. A spike in antibody positivity occurred in flow 1 gilts in August 2021 followed by a return to baseline values.

Weaned pigs displayed the most variability in antibody levels, ranging from high S/N values (indicating antibody-naïve pigs) to low S/N values (indicating antibody positive pigs). At the herd level, mean S/N values in oral fluid samples from weaned pigs on flow 1 inversely correlated with viral prevalence data obtained by RRT-PCR. Peak swIAV antibody levels were recorded on flow 1 during August 2021, which coincided with the disappearance of the virus between the first and second waves of infection. Conversely, the lowest antibody values were recorded in April 2021 and May 2022, coinciding with peak viral prevalence of the first and second waves, respectively. For flow 2, the relationship between swIAV antibody levels and viral prevalence data was less clear. Similar to flow 1, antibody levels in flow 2 weaned pigs peaked in

September 2021 and this coincided with the disappearance of the virus between the first and second waves of infection. However, a second peak in antibody levels in March 2022 occurred during a period of increasing viral prevalence.

Antibody levels in finishing pigs were characterised by long periods of uniformity around the S/N 0.6 cut-off value punctuated with spikes indicating increasing antibody levels in the herd. For flow 1 finishing pigs, these increases in antibody levels occurred during April 2021 and May 2022, whereas for flow 2 finishing pigs, antibody levels peaked in November 2021 and March 2022.

Genetic analysis of swIAV subtypes

The WGS method employed in this study consistently produced high quality sequence data when the starting material had ct values ≤ 28 . Of the 196 swIAV positive samples obtained, only 27.6 per cent (54 of 196) were suitable for direct sequencing from the original clinical material. In order to maximise the number of viral sequences available, viral isolation in embryonated chicken eggs was attempted for all RRT-PCR positive samples, regardless of ct value. Using this approach, 75 viral isolates were obtained and complete genomic sequences determined. WGS was attempted for a subset of samples with ct values > 28 where virus isolation was unsuccessful; however, these resulted in only partial genomic sequences.

Analysis of viral WGS data revealed that two swIAV subtypes were in circulation (Fig. 3). For flow 1, sequence data were only obtained from weaned pigs and identified that pH1N1 clade 1A.3.3.2 was present for the entire 18-month study period (Fig. 3A).

During the first wave of infection, only pH1N1 was detected on the weaning unit. During the second wave, pH1N1 predominated but co-circulated with H1N2 clade 1B.2 between November 2021 and May 2022. H1N2 clade 1B.2 is rarely detected in European swine and is generally associated with herds in North and South America. For flow 2, the majority of sequence data was obtained from weaned pigs, although a single complete genome was obtained from a suckling pig during the March 2022 outbreak in the breeding unit and a partial genome sequence was determined from gilt in May 2022. H1N2 clade 1B.2 was the dominant strain present on flow 2 and was exclusively present on the weaning unit for the entire duration of the study. The single gilt sequence was also identified as H1N2 clade 1B.2. However, the strain responsible for the suckling pig outbreak on the flow 2 breeding unit was identified as pH1N1 clade 1A.3.3.2.

Phylogenetic analysis of pH1N1 HA sequences indicated that all strains detected on the flow 1 weaning unit were closely related (≥ 99.36 per cent nucleotide identity) and were distinct from the pH1N1 detected on the flow 2 breeding unit (≤ 91.18 per cent nucleotide identity with flow 1 strains; Fig. 3B). In contrast, all H1N2 strains were closely related regardless of flow origin (≥ 98.66 per cent nucleotide identity). The six internal gene segments of all strains identified in this study were derived from the pH1N1 clade 1A.3.3.2.

Antigenic drift of swIAV HA and NA

As the major antigenic proteins, HA and NA accumulate amino changes in response to immune pressure, resulting in antigenic

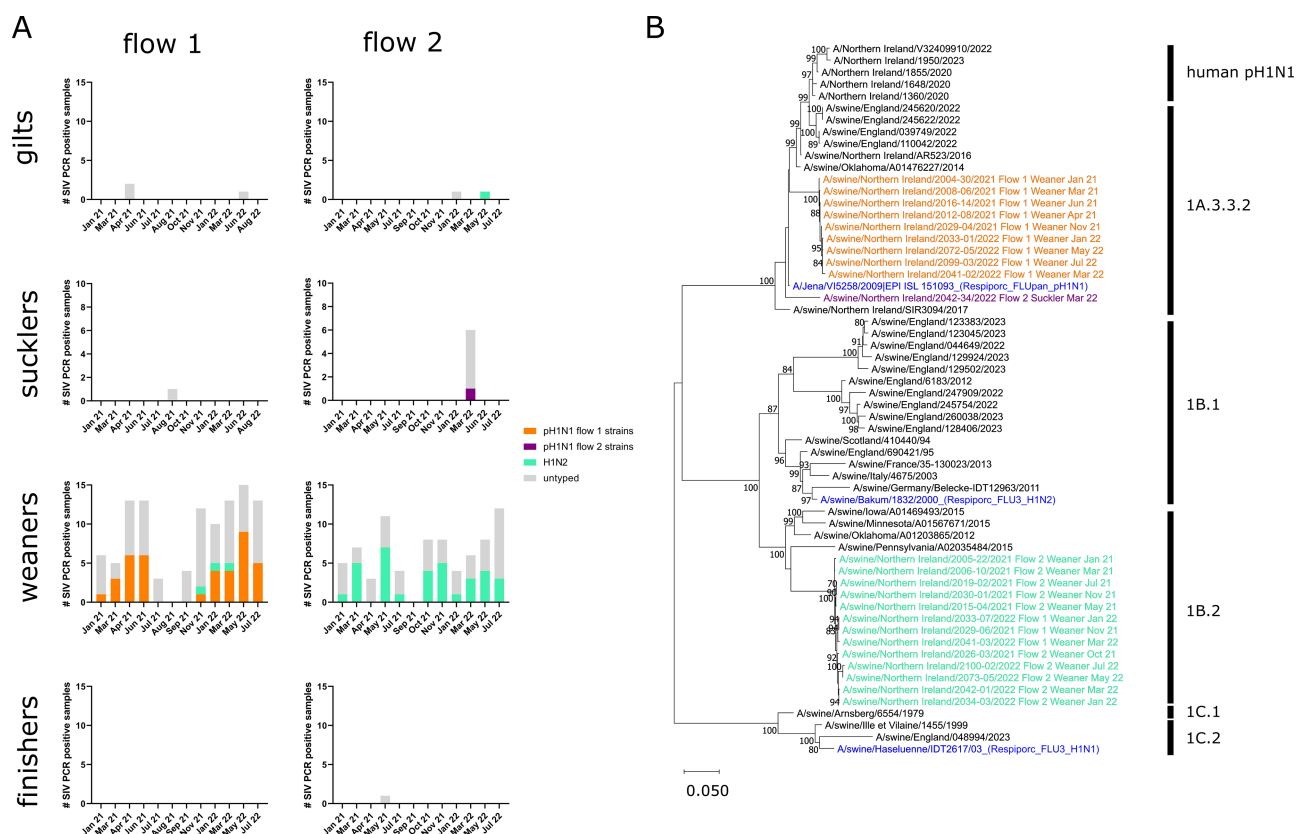


Figure 3. Subtyping and phylogenetic analysis of swIAV. (A) Stacked bar charts showing the proportional contribution of different swIAV subtypes to the overall prevalence for each production stage on both flows: pH1N1 flow 1 strains (orange), pH1N1 flow 2 strain (purple), H1N2 (green), and untyped (grey). (B) Maximum likelihood phylogenetic tree of representative pH1N1 and H1N2 sequences obtained based on complete HA coding sequences, with 1,000 bootstrap replications. Trees colour-coded according to the subtype/ strain: pH1N1 flow 1 strains (orange), pH1N1 flow 2 strain (purple), H1N2 (green), reference swine and human sequences (black), swine vaccine strains (blue).

drift and immune escape. The observation of rising swIAV antibody levels on the weaning units, followed by the disappearance and subsequent re-emergence of virus, led us to speculate that increasing exposure within the herd exerted selection pressure that favoured the emergence of antigenically drifted strains. To investigate this, predicted HA and NA amino acid sequences from viral isolates obtained from the weaner units spanning the entire 18-month study period were aligned and substitutions recorded to infer antigenic changes. This revealed a similar pattern of evolution over time on both production flows (Fig. 4). For flow 1, the pH1N1 HA and NA proteins were remarkably stable over the initial 6-month period of the first wave of infection (Fig. 4A). Of the 16 strains isolated from 4 sampling time-points during this period, 12 had identical HA and NA sequences. Four strains had 1 or 2 amino

acid changes in HA, all of which were only detected in a single isolate at a single time-point. With the re-emergence of the virus during the second wave of infection, several mutations in HA and NA became fixed, with strains carrying these changes displacing previously circulating strains. Mutations emerging at the start of wave 2 included S83P and V345I in HA and I10V in NA, respectively, with an additional HA S45N mutation emerging in January 2022. A variant with two additional substitutions in NA (V62I and I106V) emerged in May 2022 and co-circulated with the original wave 2 variant until the end of the study in July 2022. For flow 2, the H1N2 HA and NA protein sequences were similarly static during the 6-month period of the initial infection wave (Fig. 4D). Of the 12 strains isolated over 4 sampling time-points, most differed only at two variable positions (30 and 309) in HA. Three isolates

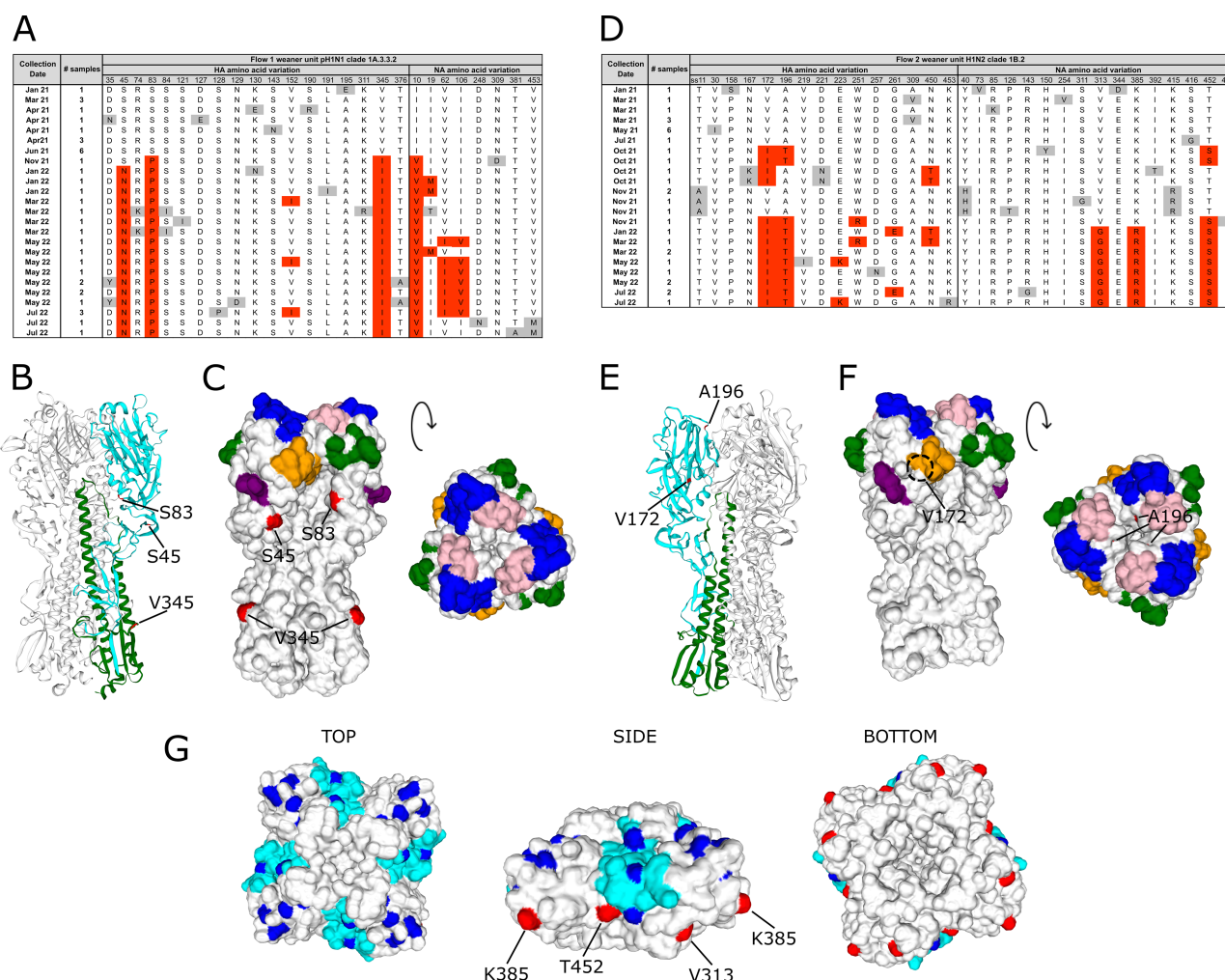


Figure 4. Amino acid substitutions in the swIAV surface glycoproteins over time. (A) Amino acid substitutions in HA of flow 1 weaner unit pH1N1 clade 1A.3.3.2 strains. Consensus sequences were derived from strains isolated during the first 6 months of the study. Deviations from the consensus are highlighted. Grey highlights indicate substitutions that were detected during a single time-point. Red highlights represent substitutions detected during two or more time-points. HA numbering is according to HA0, following cleavage of the 17-residue signal sequence. (B) HA homology model of A/swine/Northern Ireland/2012-08/2021 (flow 1 weaner unit pH1N1 clade 1A.3.3.2, collection date April 2021). HA1 domain (cyan) and HA2 domain (green) from a single monomer is highlighted with locations of major substitutions indicated in red. (C) Surface rendering of A/swine/Northern Ireland/2012-08/2021 HA homology model. Antigenic sites indicated: Sa (blue), Sb (pink), Ca1 (orange), Ca2 (green), and Cb (purple). Locations of major substitutions highlighted in red. (D) Amino acid substitutions in HA of flow 2 weaner unit H1N2 clade 1B.2 strains. (E) HA homology models of A/swine/Northern Ireland/2006-10/2021 (flow 2 weaner unit H1N2 clade 1B.2, collection date March 2021). HA1 domain (cyan) and HA2 domain (green) from a single monomer is highlighted with locations of major substitutions indicated in red. (F) Surface rendering of A/swine/Northern Ireland/2006-10/2021 HA homology model. Antigenic sites indicated: Sa (blue), Sb (pink), Ca1 (orange), Ca2 (green), and Cb (purple). Locations of major substitutions highlighted in red. The location of buried residues, not visible in surface renderings, is indicated by a dashed circle. (G) HA homology model of A/swine/Northern Ireland/2006-10/2021. Mem5 epitope (cyan), monoclonal antibody escape mutations (blue). Locations of major substitutions indicated in red.

had one or two additional substitutions in HA or NA that were only detected on one occasion. The dominant strain that emerged during the second wave of infection contained the substitutions V172I and A196T in HA and T452S in NA. A variant with an HA sequence identical to the original strain (except for a single substitution in the HA signal sequence) but with additional mutations in NA (Y40H and K415R) emerged briefly during the second wave of infection but was not maintained. The dominant H1N2 variant acquired two additional mutations in NA (V313G and K385R) in January 2022 that became fixed and displaced previous circulating strains for the remainder of the study.

Isolation of IAV in eggs can result in egg adaptation mutations, making subsequent sequence interpretation difficult (Robertson 1993). In order to assess the impact of egg adaptation on the HA and NA sequences of the swIAV strains described here, WGS was performed on a subset of samples directly from original clinical material and compared with sequences from the corresponding egg isolates (Supplementary Table S2). Of the 18 paired samples sequenced (8 pH1N1 and 10 H1N2), 13 had identical egg/clinical-derived HA and NA amino acid sequences. Five strains (4 pH1N1 and 1 H1N2) had between 1 and 3 amino acid positions in HA that differed between the original clinical material and egg isolate. However, there was no evidence of positive selection for egg-adapted variants after a single passage in eggs at any position in HA or NA for either subtype, indicating that the changes observed over time were a result of antigenic drift.

We next examined the observed substitutions in HA and NA in the context of their 3D protein structures. Homology models were constructed using representative sequences of the pH1N1 and H1N2 strains identified in this study and the locations of the major substitutions mapped. For pH1N1, positions 45 and 83 are surface exposed and located in the globular head of HA1, but not within the five classical antigenic regions (Fig. 4B and C). Both positions are adjacent to antigenic site Cb, with position 83 close to the conserved N-linked glycosylation site at position 87. Position 345 (HA2 position 18) is located in the fusion peptide, a highly conserved 23-residue sequence responsible for membrane fusion. The NA I10V substitution is located in the N-terminal transmembrane domain (homology models for pH1N1 NA were not produced as the N-terminus is removed by Pronase prior to NA structure determination). For H1N2, the two major substitution sites in HA are located on the globular head, close to the antigenic regions. Position 172 is buried beneath antigenic site Ca1, whereas position 196 is only partially exposed and located at the top of the globular head, in a pocket close to the junction of antigenic sites Sa and Sb (Fig. 4E and F). The three major substitution sites in the NA of H1N2 (313, 385, and 452) are all surface-exposed and located on the side of the molecule (Fig. 4G). Position 452 is at the bottom of the Mem5 epitope and immediately adjacent to position 253, previously identified in monoclonal antibody escape experiments, whereas positions 313 and 385 are not located in currently mapped antigenic sites (Air et al. 1985; Lee and Air 2006; Venkatramani et al. 2006).

Genetic drift of the six internal gene segments

In order to gain a more complete understanding of the evolution of swIAV over time, predicted amino acid sequences from the six internal gene segments of all weaner unit isolates were also aligned and substitutions occurring in the major gene products recorded. Similar to HA and NA, the six internal gene segments were relatively static during the first wave of infection (Fig. 5). For flow 1, amino acid substitutions in PB2, PB1, PA, and NP were detected during this period; however, these were mostly limited

to a single sampling time-point and none of the substitutions became fixed (Fig. 5A). With the re-emergence of pH1N1 during the second wave of infection, strains containing N350K and K716N in PA displaced previously circulating strains. This strain subsequently acquired M473I in PB2 and R189K in PB1. For H1N2, amino acid substitutions were observed in all internal gene segments during the first wave of infection, but similar to those observed for pH1N1, most were only detected at a single time-point. An exception was M112I in NS1, which first emerged early in wave 1 but did not become fixed until the end of that wave, after which it dominated. Similarly, S82N in M2 emerged at the very end of wave 1 and became fixed thereafter and T257A in PB1 emerged at the start of wave 2, displacing previously circulating variants.

Reassortment of the six internal gene segments

Sequence analysis of H1N2 isolates obtained from the flow 2 weaning unit indicated that an antigenic shift occurred in March 2022, with a reassortment event involving segments 1, 2, 3, 5, and 8 (Fig. 5B). To investigate further, nucleotide sequence alignment of all segments from all available production stages was performed (Fig. 5C shows the result of this analysis for segment 1 as an example). This revealed that the internal gene segments formed three distinct clusters (I, II, and III). Clusters I, II, and III had internal gene segments derived from pH1N1 flow 1 strains, pH1N1 flow 2 strains and H1N2 strains, respectively. Five internal gene segments of the reassorted H1N2 originated from the pH1N1 flow 2 strain that first emerged in March 2022 on the flow 2 breeding unit and detected in a suckling pig (Fig. 5D). On the flow 2 weaning unit, the reassorted H1N2 displaced the previous strain and was the only strain detected for the remainder of the study, which included the peak of the second infection wave. Interestingly, the partial H1N2 sequence obtained from flow 2 gilt in May 2022 contained all six internal gene segments from the pH1N1 flow 2 strain, indicating the occurrence of a second reassortment event in flow 2.

An independent reassortment event was also detected on flow 1. As described earlier, pH1N1 was the sole subtype detected on the flow 1 weaning unit from January 2021 until November 2021, when H1N2 entered the unit and co-circulated until March 2022. Analysis of the six internal gene segments from all flow 1 strains revealed that the internal gene constellation of pH1N1 was unchanged throughout the entire study period. In contrast, a single isolate of H1N2 obtained in March 2022 was shown to contain the six internal gene segments from the co-circulating pH1N1 (Fig. 5C (indicated with arrow) and Fig. 5D). H1N2 was not detected on the flow 1 weaning unit after March 2022.

Discussion

The study reported here had two major objectives. Firstly, to investigate the infection dynamics of swIAV in intensive pig production systems. Secondly, to characterise the evolution of swIAV in large, infected herds over time. With regard to the first objective, we found that prevalence of swIAV across the different production stages varied considerably. On the breeding units under investigation, prevalence was generally very low. This may be partly explained by the presence of MDA, as levels would be high in piglets <4 weeks of age due to sow vaccination. However, studies looking at protection offered by MDA have produced conflicting reports in relation to the extent of protection afforded (Blaškovič et al. 1970; Mensík, Valíček, and Pospíšil 1971; Renshaw 1975). RespiPorc FLU3 does not contain a pH1N1 component

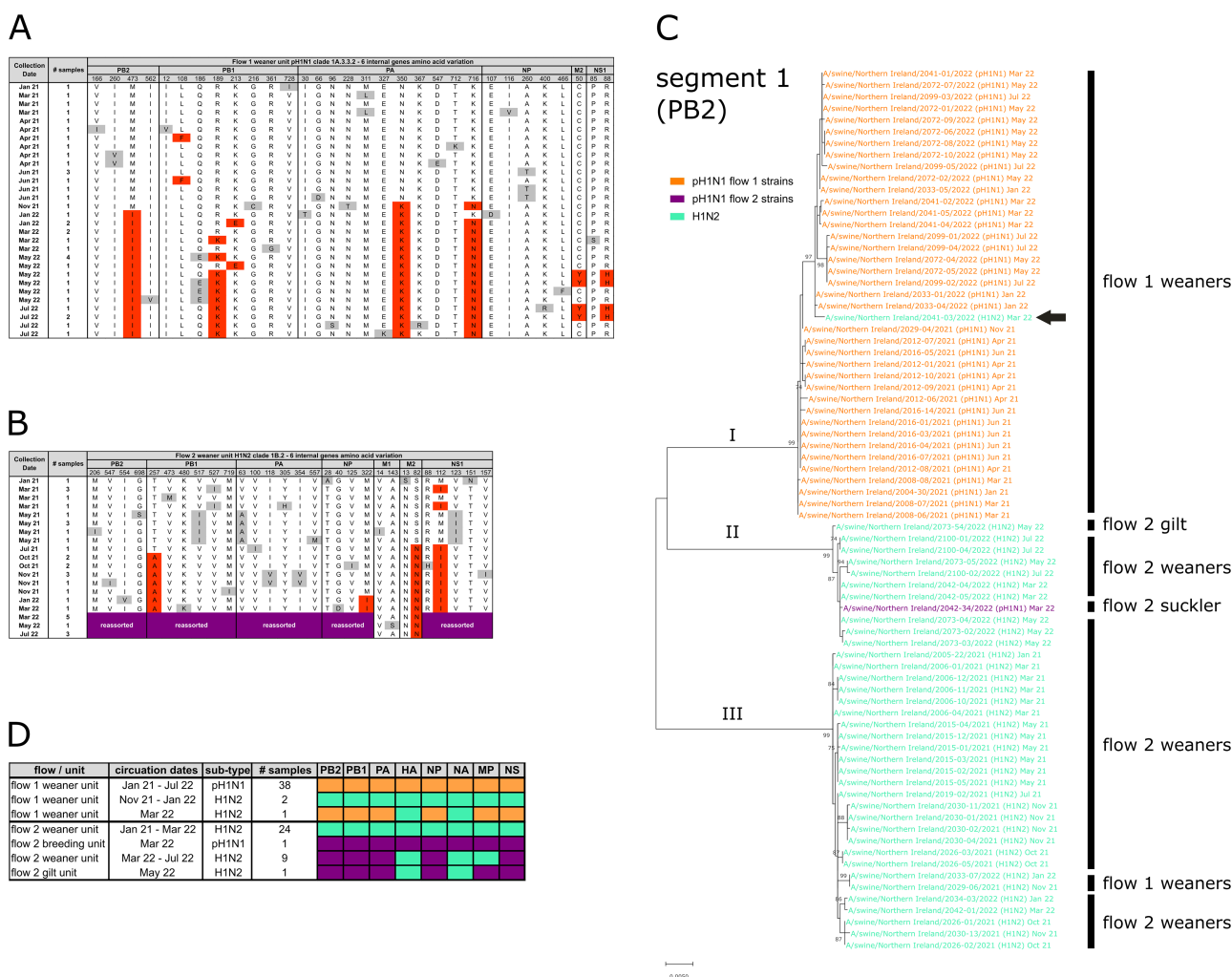


Figure 5. Evolution of the six internal gene segments. Amino acid substitutions in the major products of the six internal gene segments of (A) flow 1 weaner unit pH1N1 strains and (B) flow 2 weaner unit H1N2 strains. Consensus sequences were derived from strains isolated during the first 6 months of the study. Deviations from the consensus are highlighted. Grey highlights indicate substitutions that were detected during a single time-point. Red highlights represent substitutions detected during two or more time-points. Purple blocks indicate reassortment of segments. (C) Maximum likelihood phylogenetic tree of all pH1N1 and H1N2 sequences obtained based on complete PB2 coding sequences, with 1,000 bootstrap replications. Trees colour-coded according to the sub-type/strain: pH1N1 flow 1 strains (orange), pH1N1 flow 2 strain (purple), and H1N2 (green). Arrow indicates a reassortant H1N2, containing internal gene segments from pH1N1 flow 1 strains. (D) Schematic of the internal gene constellations of all sequenced strains.

and so MDA produced by sows that receive this vaccine would be unlikely to prevent infection of piglets upon exposure. This may partially explain the outbreak of pH1N1 on the flow 2 breeding unit in March 2022. Interestingly, although widespread pH1N1 infection was detected, this was epizootic in nature and limited to a single time-point, clearly indicating that infection dynamics on the breeding units are markedly different from those on weaning units, likely due to the different husbandry practices utilised. Similarly, Respiorc FLU3 contains H1 strains from clades 1B.1 and 1C.2, as these are the most prevalent, non-pandemic H1 strains circulating in European herds. The finding that the H1N2 strains circulating in Northern Ireland belong to clade 1B.2 may mean that current vaccines have reduced effectiveness due to increased antigenic distance. Experiments measuring the degree of cross-protection between 1B.1 and 1B.2 are currently planned.

We identify weaned piglets as the major reservoir for continuous circulation of influenza in the intensive farms under investigation. Weaned piglets between 4 and 12 weeks old represent a

diverse population in terms of serological status and susceptibility to swIAV. Newly weaned piglets have high levels of MDA due to sow vaccination. However, the efficacy of MDA at this stage is uncertain, with several studies indicating incomplete protection (Ryt-Hansen et al. 2019a,b). Exposure to swIAV in the presence of MDA has also been associated with impaired immune responses that may facilitate recurrent infections of individual pigs (Rose et al. 2013). Despite this, MDA is thought to provide at least partial protection up to 10 weeks; therefore, progress through this production stage is characterised by waning immunity and increasing susceptibility to infection (Cador et al. 2016). The very high prevalence of swIAV on the weaning units suggests that exposure would be frequent and piglets would likely become infected at an early stage post-waning of immunity. Recovered piglets that mount an appropriate humoral response would reduce the pool of susceptible piglets available for infection by homologous strains. However, the high turnover rate—including removal of older exposed pigs to specialised finishing herds and the introduction of newly weaned piglets from the breeding units, means the proportion of pigs

susceptible to infection is likely maintained at a level sufficient to sustain continuous infection of the herd.

Measurement of swIAV antibodies in oral fluid samples demonstrated high seroprevalence in finishing pigs in this study. We presume seroconversion occurred following natural infection on the weaning units since swIAV was almost completely absent from both finishing herds for the entire duration of study (except for a single ~ct 35 detection on flow 2 in May 2022). Introductions into the finishing unit would be expected to occur occasionally but these do not appear to result in large outbreaks, most likely due to the high degree of pre-existing immunity within the compartment. A previous study of Dutch farrow-to-finish and specialised finishing herds found that swIAV incidences (based on seroconversion per period of time) mostly occurred at the start of the finishing period (12–16 weeks) on farrow-to-finish farms and at the end of the finishing period (16–22 weeks) on specialised finishing herds (Loeffen et al. 2009). This contrasts with our findings and may be explained by lower levels of circulating virus in the preceding production stages of the Dutch farms, resulting in a higher proportion of unexposed/susceptible animals entering the finishing units.

The second objective of this study was to characterise the evolution of swIAV over time in continuously infected intensive herds. Few studies have utilised WGS to study on-farm evolution of swIAV over extended periods. A yearlong study of five farrow-to-wean farms in the USA sequenced 123 complete genomes, identifying 3 subtypes that clustered into 7 distinct viral groups and revealed a complex picture of viral emergence, persistence and subsidence within pig herds (Diaz et al. 2017b). The same group also sequenced 92 complete genomes during a 15-week cohort study on a wean-to-finish farm, identifying 2 subtypes that clustered into 3 distinct viral groups that co-circulated at different proportions over time (Diaz et al. 2017a). These two studies differ from the findings in the present study, where only a single subtype was maintained for the entire duration of study on each farm. Mapping the pattern of amino acid substitutions in the viral surface glycoproteins on the weaner units over time revealed long periods of stasis punctuated by periods of rapid evolution. Such periods of rapid evolution occurred following peaks in swIAV antibody levels in the herd and dramatically reduced viral prevalence. We speculate that periodic reductions in the pool of susceptible animals below maintenance levels, caused by high rates of infection that outpace recruitment of susceptible animals, exerts immune pressure that drives antigenic drift. The location of several of the substitutions noted in HA and NA is suggestive of an antigenic drift. However, antigenic characterisation using hemagglutination/neuraminidase inhibition assays will be necessary to demonstrate this conclusively.

In addition to substitutions in the surface glycoproteins, we also detected changes in the other major viral gene products, suggesting that additional selection pressures beyond humoral immunity to HA and NA are involved. Most of these changes emerged in parallel with those in HA and NA and may be epistatic in nature. We also detected a number of reassortant events between established and newly introduced strains. While some reassortant viruses were only detected once, others rapidly displaced strains containing the original internal gene constellation, indicating increased allelic fitness of the new genetic segments. Taken together, these observations suggest a dynamic situation, whereby a single strain can be maintained within a herd for extended periods of time without significant antigenic evolution but can rapidly adapt once immune pressure increases due to flux in the size of the susceptible pool. Adaptation is via accumulation of amino acid substitutions in both the surface glycoproteins

and the internal gene products, and via exchange of internal gene segments from newly introduced strains. Further analyses are planned to determine the rate of viral evolution and examine positive and negative selection of genetic variants using modelling approaches.

We acknowledge the limitations of our study. Our data may not be representative of pig production in the region. The majority of swine farms in Northern Ireland wean piglets to nursery facilities on-site, and at the end of the growing stage either finish on-site (farrow-to-finish) or transport off-site to specialised finishing herds. Differences in husbandry practices and opportunities for viral transmission are likely to impact infection dynamics and evolution; therefore, extrapolation of our findings to other production systems must be done with care. The sequence data presented here may also not represent the true viral diversity in the production systems under study. The sampling approach was designed to maximise the opportunities to detect swIAV across the entire production flows. While we are confident that the predominant circulating strains were captured using this approach, minor variants circulating at lower prevalence were likely missed. The methodological choices made may also have introduced bias. For example, only viral sequences from egg isolates were included in the main analysis. While we have demonstrated that there are minimal differences between original and egg isolate-derived sequences, we have limited sequence data for those strains that were not successfully isolated in eggs. Of the 196 PCR positive samples obtained in this study, only 38 per cent were successfully isolated/sequenced. The immunological data obtained were solely from measurement of anti-IAV NP antibodies in aggregate oral fluid samples by ELISA. While these data give a general indication of swIAV exposure within the herd, definitive statements about swIAV immunity are not possible. Similarly, the ELISA cannot differentiate between exposure and vaccine-induced antibodies. As MDA can persist for up to 10 weeks in piglets, interpretation of ELISA data in animals under this age is difficult. Finally, we recognise that direct experimental evidence for antigenic drift is lacking in this study.

Despite these limitations, the findings of the present study may contribute to the design of improved infection control strategies. Alternative vaccination protocols that include weaned pigs should be explored to determine the optimum strategy for reducing swIAV prevalence in intensive pig production systems. Furthermore, this study highlights the importance of swIAV monitoring, including genetic characterisation, in order to select the most appropriate vaccines for deployment on farm. In addition, the use of the alternative sampling methodologies described herein should facilitate expanded routine surveillance to monitor herds for the emergence of novel variants, including those with increased zoonotic potential.

Data availability

Sequence data generated in this study have been shared via GISAID, the global data science initiative under the accession numbers EPI_ISL_18221301 to EPI_ISL_18221395.

Supplementary data

Supplementary data is available at VEVOLU Journal online.

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