**Quantifying the zoonotic risk profile of European influenza A viruses in swine from 2010 to 2020 inclusive.**

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**Abstract**

H1 and H3 influenza A viruses (IAVs) circulating in European pigs differ markedly from most viruses circulating in other global swine populations. Since the 2009 pandemic, periodic interspecies transmission of IAVs from humans into European pigs has expanded the genetic diversity of these viruses, occasionally leading to sustained transmission in pigs. There have been multiple animal-origin IAV infections in people in Europe, particularly associated with the 1C lineage of H1 IAVs. For over 30 years the 1970.1 H3 lineage predominated in European pigs, but introduction of human seasonal viruses from the early 2000s into pig populations has been detected in several countries in Europe. Due to the predominance of H1 detections in pigs and their association with human zoonotic cases, we quantified antigenic evolution of H1 viruses among IAVs in European pigs and assessed diversity relative to swine IAV vaccine strains. We compared evolutionary patterns characterised with both natural host antisera (pigs) and ferret antisera raised to human seasonal vaccines or pre-pandemic candidate vaccine viruses (CVVs). The H1 1A.3.3.2 clade viruses antigenically drifted after reverse zoonoses into European pigs leading to loss in cross-reactivity to human seasonal vaccine strains. The H1 1B.1.2.2 clade viruses did not have cross-reactivity to older human seasonal influenza viruses nor to a CVV derived from an evolutionarily similar virus. The H1 1C.2.1 and 1C.2.2 variant strains had variable cross-reactivity against the tested 1C CVVs. We demonstrated that two clades of viruses in the 1C lineage, 1C.2.4 and 1C.2.5, have rapidly diversified. To further assess pre-pandemic risk, we used age-stratified human serum panels to measure potential cross-protection to contemporary IAVs in European pigs. Age-stratified sera showed poor cross-reactivity and suggest that several of the IAVs in European pigs have a high zoonotic risk to human populations. Our data objectively quantifies the genetic and antigenic diversity of co-circulating lineages of IAV in swine in Europe and identifies how specific groups of viruses represent a greater risk to animal and public health and can be used to inform future pre-pandemic preparedness efforts.

**Introduction**

. IoverThree predominant lineages of swine IAV H1 viruses (1A, 1B, and 1C) and multiple lineages of H3 from human seasonal IAV which have established over several decades continuously circulate in global pig populations (Anderson *et al.*, 2021). Following the spread of the 2009 H1N1 swine-origin pandemic (pdm09) in humans, the annual introduction of human seasonal H1N1pdm09 virus into pigs has led to over a decade of co-circulation, reassortment, and diversification of the hemagglutinin (HA), neuraminidase (NA), and the other six viral genes in endemic swine IAV lineages (Watson *et al.*, 2015; Lewis *et al.*, 2016; Rajão *et al.*, 2017; Gao *et al.*, 2017; Chastagner *et al.*, 2020; Henritzi *et al.*, 2020; Chepkwony *et al.*, 2021; Hufnagel *et al.*, 2023). This diversity has important implications for both pig health and control of IAV using vaccines and poses a challenge for pre-pandemic preparedness for the global public health community.

A coordinated European swine IAV surveillance network identified 23 distinct gene constellations of enzootic 1A lineage (pdm09/1A.3.3.2 clade), H1N1 Eurasian avian-like 1C lineage, H1N2 human seasonal 1B lineage and human-like H3N2 subtypes in European pigs from 2010 to 2013 (Watson *et al.*, 2015). Many constellations involved internal gene cassettes from Eurasian avian-like or pdm09 viruses with various neuraminidase (NA) pairings. These viruses have continued to reassort with an additional 16 new genotypes described by (Henritzi *et al.*, 2020), many of which were associated with reassortment and the acquisition of pdm09 genes. Novel reassortants have been reported in geographically restricted areas reflecting the co-circulation of pdm09 with regionally enzootic swine IAV, in the Netherlands and Belgium (Chepkwony *et al.*, 2021), France (Chastagner *et al.*, 2020), Denmark (Krog *et al.*, 2017), and Germany (Starick *et al.*, 2012). These genetically diverse lineages and reassorted viruses have different phenotypes and antigenic characteristics that complicates control measures and poses a zoonotic and pre-pandemic concern given that all these viruses maintain human-origin genes.

A strategy to minimise the economic losses associated with disease burden in pigs, which also reduces the risk of IAV transmission from pigs to human, is the use of vaccines in pig populations. In Europe, most commercial vaccines against influenza A virus in swine are inactivated, multivalent (H1N1, H3N2, and/or H1N2) whole‐virus vaccines with an adjuvant. There is no formal system for recommending swine influenza vaccine strains, and the current diversity in IAVs in pigs complicates control by use of inactivated vaccines (Vincent *et al.*, 2017). Formulating effective vaccines is challenged by the difficulty in updating vaccine seed viruses given the emergence of novel lineages and clades, antigenic drift, the time needed to approve and license veterinary vaccine products, maternal antibody interference, and the lack of adequate mucosal and cell-mediated immune responses (Platt *et al.*, 2011). Further, contributing to observed evolution and subsequent antigenic diversity, positive selection has been demonstrated at antibody epitopes in swine IAV HA genes and may be associated with partially effective vaccines (Kitikoon *et al.*, 2013). Influenza vaccines are primarily used in adult sows to clinically protect the gestating sow and her suckling piglets or, less frequently, during the grow/finish phase of production to decrease IAV disease, lung lesions, and transmission (Beaudoin *et al.*, 2012; Vincent *et al.*, 2017; Deblanc *et al*., 2018; Mollett *et al.*, 2023). Vaccinating piglets may be desired in some clinical situations, but the presence of passive maternal antibodies interferes with efficacy of inactivated vaccines (Andraud *et al*., 2023).

The potential for another swine-origin IAV pandemic is reinforced by relatively frequent swine-to-human transmission events. Between February 2020 and September 2021, 38 zoonotic cases of swine IAV H1 and H3 viruses (referred to as “variants”) were reported to the World Health Organization (WHO) (WHO, 2020, 2021a, 2021b). Seven of these cases occurred in Europe and were caused by diverse H1 viruses of H1N1 and H1N2 subtypes, of different lineages and genome constellations, representing the complexity of currently circulating IAV in pigs in Europe. These zoonotic spillovers were predominantly caused by the Eurasian avian 1C lineage. Two H1N1 1C.2.1 clade viruses were reported in humans in the Netherlands (Parys *et al.*, 2021) and Germany (Heider *et al.*, 2024) respectively and one H1N2 1C.2.4 clade virus was reported in France (Simon *et al.,* 2022). In 2021 there was also a report of a variant virus from the H1N1 1A.3.3.2 clade in Denmark (Andersen *et al.*, 2022) and finally of a subtyped, but unknown clade, of H1N1 virus detected in Austria.

The risk of a swine-to-human transmission event is likely dependent on animal production systems that could differ in the relative degree of human-pig exposure, type of animal-human interface (e.g., live animal markets, exhibition practices, large commercial farms), viral ecology, the immune profile of the individual, and virus factors (Karesh *et al.*, 2012). Given the increase in genomic surveillance for IAV, it is now possible to address which viruses are circulating in pigs, how this genetic diversity influences antigenic profiles, and determine whether there are adequate pandemic-preparedness strategies. Currently, most swine IAVs significantly differ from the current H1 and H3 components of human IAV vaccines (Anderson *et al.*, 2021). Few of the genetic clades detected in swine globally include isolates that are genetically matched to a WHO pre-pandemic-preparedness candidate vaccine virus (CVV) and the available CVVs might not provide protection given observed genetic and antigenic differences in circulating swine viruses. Here we characterise the evolution of the major swine influenza A virus lineages in Europe. We assess the diversity relative to vaccine strains used in pigs and human seasonal and CVVs to evaluate pre-pandemic risk using human serology panels. Our data demonstrate that swine IAVs in Europe continue to diversify, and we suggest that a comprehensive understanding of the current evolutionary diversity is required to provide assessments on whether current vaccine strains match circulating diversity and how this diversity impacts pre-pandemic risk.

**Materials and methods**

**Viruses and sequence data**

Viruses from circulating H1 and H3 strains in European pig populations from 2010 to 2020 inclusive were shared by European collaborators within the joint WOAH-FAO network of expertise on animal influenza (OFFLU) swine influenza virus technical activity group. Further sequences from Global Initiative on Sharing All Influenza Data EpiFlu Database (www.gisaid.org) and Genbank (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database) were added to the dataset along with human variant sequence data. Geographical maps with the country of sampling and genetic information were generated in MicroReact (Argimón *et al.*, 2016). Viruses (Supplementary data 1) were propagated in Madin-Darby canine kidney (MDCK) cells or embryonated fowls’ eggs. Cell culture supernatant or allantoic fluid was harvested and clarified by centrifugation. Viruses were then ultracentrifuged and pellets were resuspended overnight at 4°C in sterile phosphate buffered saline at pH 7.4 and stored at -70°C.

**Phylogenetic analysis and strain selection**

Sequence alignments were generated for H1 and H3 segments using default settings in MAFFT v7.490 (Katoh and Standley, 2013). Maximum Likelihood (ML) phylogenetic trees were inferred for the H1 segments using IQ-Tree v2.2.6 (Nguyen *et al.*, 2015). Phylogenetic trees were visualised in RStudio 4.3.0. using the ggtree package (Yu, 2020). The lineage of the H1 and H3 viruses was assessed using the swine H1 Clade Classification system (Anderson *et al.*, 2016) and internal gene segments were classified using the octoFLU pipeline (Chang *et al.*, 2019). For each of the identified H1 and H3 contemporary clades, an HA1 protein consensus sequence was generated. Tabular comparisons of amino acid similarity between current candidate vaccine viruses (CVVs) or human seasonal vaccines and representative swine strains were generated using flutile v0.13.0 (<https://github.com/flu-crew/flutile>). Swine viruses that represent H1 clades that circulated within European pig populations within this study period were selected for further antigenic characterization.

**Antisera production**

Swine antisera to H1 viruses were generated as previously described in Chastagner *et al.*, (2018) at the Animal and Plant Health Agency (APHA), UK. Additional European-specific antisera raised against French strains collected between 1982 and 2015 were provided by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) (Supplementary data 1). Briefly, pathogen-free pigs (9 weeks-old) were inoculated intranasally with live virus (4mL 107-108 ELD50). Three weeks later, the animals were inoculated intramuscularly with same dose of virus diluted in the adjuvant Montanide ISA206 (3mL). Two weeks later, monovalent swine antisera were collected.

Ferret antisera produced against CVV strains (A/Ohio/09/2015, A/Michigan/383/2018) were provided by the Virology, Surveillance and Diagnosis Branch, Influenza Division, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Ferret antisera produced against human seasonal vaccine strains, variant strains and CVVs (A/California/4/2009, A/Brisbane/02/2018, A/Brazil/11/1978, A/Pavia/65/2016, A/Michigan/45/2015, A/Netherlands/3315/2016) were provided by the Worldwide Influenza Centre at the Francis Crick Institute. Ferret post-infection monovalent antisera produced against European viruses (A/Swine/Germany/AR2749/2015, A/Swine/Netherlands/AR647/2015, A/Swine/Netherlands/AR647/2015, A/Swine/Germany/AR2749/2015) were provided from the National Reference Laboratory for Avian Influenza, FLI, Germany (Henritzi *et al.*, 2020). The remaining ferret post-infection monovalent antisera were generated at USDA National Animal Disease Center, Ames, Iowa by live exposure inoculation of pathogen-free ferrets. Ferrets were exsanguinated and sera was collected 2-3 weeks post infection (Supplementary data 2).

**Haemagglutination inhibition assays**

Hemagglutination inhibition assays were generated according to standard methods previously described (Lorusso *et al.*, 2011). Swine and human antisera were treated with receptor-destroying enzyme (RDE) and heat inactivated at 56°C for 30min and adsorbed with 50% turkey red blood cells (RBC) to remove nonspecific inhibitors of haemagglutinin. Ferret antisera were heat inactivated at 56°C for 30min, treated with 20% kaolin, and adsorbed with 0.75% guinea pig red blood cells to remove nonspecific inhibitors of haemagglutinin. Serial 2-fold dilutions starting at 1:10 were tested for their ability to inhibit the agglutination of 0.5% turkey RBC with 4-8 hemagglutinating units of virus. HI assays were performed according to standard techniques as described in the World Organization for Animal Health manual of diagnostic tests using ferret and human antisera with guinea pig RBC and turkey RBC respectively to measure hemagglutination in the presence of 20nM of oseltamivir carboxylate as described in Anderson *et al.,* (2020).

**Antigenic cartography, vaccination assessment and antibody landscaping**

Antigenic cartography was performed using HI assay data with both swine and ferret antisera to generate 2 and 3-dimensional (2D and 3D) antigenic maps (one using ferret and one using swine antisera) to visualise and quantify the antigenic inter-relationships between the H1 influenza A strains as previously described (Smith *et al.*, 2004; Lewis *et al.*, 2014). Antigenic maps were created using RStudio v4.3.0 and Racmacs v1.2.9. The strains in the antigenic maps were coloured by genetic lineage. One antigenic unit (AU) is equivalent to a two-fold difference in titre in the HI assay.

Antibody landscapes were generated using RStudio 1.4.1106 as previously described (Fonville *et al.*, 2014; Souza *et al.*, 2018). The HI titres were plotted in the third dimension as a smooth landscape over the ferret 2D antigenic map. As the minimum dilution used in the HI assay was 1:10, undetectable titres were given a set value of <10. The X and Y axis of the antibody landscape represent the antigenic distance between the different antigens while the Z axis (height of the landscape) represent the antibody titres (geometric mean value of each respective virus strain). The antibody landscapes visually represent the breadth of the antibody responses of each host (or hosts) to infection. The antibody landscapes were statistically compared using a t-test in Excel.

To assess vaccination in pigs, the antigenic distance between sera raised in ferrets against swine vaccine seed strains and the European test viruses were inferred using antigenic cartography. The antigenic distance between sera and test strains were visualised by plotting in numbers. Three AU difference between vaccine sera and test antigen are considered antigenically different enough to trigger an update to the human influenza vaccine (Ampofo *et al.*, 2015).

**Human sera cohorts**

Human cohort sera representing different geographic and demographic populations (Ursin *et al.*, 2020; Kuo *et al.*, 2021) were tested against contemporary European swine H1 and H3 IAV, human seasonal virus reference strains and CVVs to assess human seasonal influenza-derived antibody recognition of swine strains and to identify IAV diversity that would likely not be covered either by human seasonal strain immunity or by putative pandemic-preparedness CVV strain responses. Two cohorts of human sera were tested against the selected swine H1 and H3 viruses. A convalescent cohort was composed of influenza-infected individuals (*n=10*) from Taiwan and Hong Kong (season 2016-17), with sera collected at 28 days post-infection. Sera from this cohort were previously tested and demonstrated HI titres against the H1 vaccine strain A/Michigan/45/2015 (Venkatesh *et al.*, 2022) and the H3N2 vaccine strain A/Hong Kong/4801/2014 (Souza *et al.*, 2022). A post-vaccination cohort consisted of subjects from Johns Hopkins Hospital (Baltimore, MD, U.S.) vaccinated in the autumn of 2017 with a quadrivalent influenza vaccine. Age and gender metadata were provided, so female and male samples were grouped and stratified by decade of age: 1940-50s, 1960s, 1970,1980s and 1990s. Five subjects per age group per gender were randomly selected for the final cohort panel (*n=50)*.

The human subjects’ protocol was approved by the Johns Hopkins School of Medicine Institutional Review Board (IRB90001667) and National Institutes of Health Division of Microbiology and Infectious Diseases (protocol 15-0103). Patients were enrolled at the Johns Hopkins Medical Institute (JHMI) Department of Emergency Medicine or on inpatient floors.

**Results**

**Genomic epidemiology of SIV in pigs**

Geographic analysis of the circulation of swine H1 viruses from 2010 to 2020 inclusive, showed different lineage distributions across time and between different countries within European pigs (Figure 1). The co-circulation of all 1A, 1B and 1C lineage viruses in any given year was observed between 2010 and 2020 in France, Germany and Italy, 2011-2013 in the United Kingdom, 2011-2017 in the Netherlands, 2016-2020 in Spain and in 2019 in Belgium. The 1C lineage viruses were the most frequently detected from 2010-2020. The 1A.3.3.2 (pdm09) viruses were the only clade detected in Finland, Hungary, Iceland and the Republic of Ireland whereas no 1A.3.3.2 viruses were detected in Austria, Czech Republic or Switzerland. Since 2015 several larger genetic clades of 1A.3.3.2 (pdm09) viruses that have persisted in swine have been observed in Germany, Italy and Denmark, with subsequent detections in other countries. These clades have longer branch lengths and circulation within pigs of greater than one year.

The 1B viruses in Europe arose from the introduction of human seasonal influenza virus strains with ancestral strains genetically similar to the human seasonal vaccine A/Chile/1/83. Since their first detection in Europe in 1994 (Brown *et al.,* 1994), 1B lineage viruses have diversified into multiple clades (1B.1.1, 1B.1.2, and 1B.1.2.1, 1B.1.2.2, and 1B.1.2.3) some of which appear spatially restricted to a particular country’s swine population. The 1B.1.1 have only been detected in the UK except for one strain detected in Spain in 2016. The 1B.1.2 clade was only detected in Spain. The 1B.1.2.1 clade viruses have been detected in multiple countries in continental Europe and have long branch lengths suggesting that they are diversifying. The 1B.1.2.3 clade was only detected in France and the 1B.1.2.2 clade was only detected in Italy.

The Eurasian avian 1C lineage has substantially diversified since their introduction into pigs to form multiple clades with complex spatial dynamics The 1C.1 viruses were only detected in the United Kingdom. Between 2020 and 2020, the 1C.2 viruses diversified so rapidly that this clade required two new subclade designations from analysis of data within the study period, i.e. the 1C.2.4 and 1C.2.5 viruses (Anderson *et al*., 2021). Italy, the Netherlands, Germany, Belgium, France and Spain have multiple co-circulating 1C viruses alongside the 1C.2.1 clade. Until 2012 the 1C.2.2 viruses were exclusively detected in Germany however they have since been detected in multiple other countries in Europe. Two phylogenetically similar variants of the 1C.2.2 clade were detected in the Netherlands and Germany in 2019 and 2020 respectively despite the relatively broad geographical and genetic diversity among other co-circulating viruses of this clade (Figure 1). The 1C.2.4 viruses have diverged from other 1C.2.X viruses, cluster phylogenetically by country and have been detected broadly across Europe. This clade of viruses is genetically diverse with long branch lengths relative to other 1C.2 clade viruses co-circulating in different regions. The 1C.2.5 viruses have been detected in Italy since 2010 and sporadically in Germany and Denmark, viruses cluster by country on long branch lengths. The 1C.2.6 was only detected in Spain, and this clade was only detected since 2015.

H3 lineage viruses were less commonly detected in European pigs and were more spatially restricted than the H1 subtype viruses and have decreased in detection frequency since 2013 (Figure 2). H3 viruses were co-detected with H1 viruses in Denmark, the Netherlands, Belgium, France, Spain, Italy, Hungary, Serbia and Russia but were not detected in any countries which did not also have H1 viruses. The H3 1970.1 lineage viruses have circulated in European pigs since their introduction from humans associated with the 1968 H3N2 pandemic (Figure 2). Between 2010 and the end of 2020, the 1970.1 lineage was the most predominant except in Denmark, and Russia where it was not detected. Within Italy however, genetic heterogeneity of these viruses was observed. A genetic clade of these viruses circulated in Italy between at least 2012 and 2019 which did not cluster with viruses detected elsewhere. Spain, Germany, the Netherlands and Denmark have detected both the H3 1970.1 and a more recent lineage of H3 that emerged from a human-to-swine spillover in the early 2000s (2000.3 lineage) and in Germany, and the Netherlands between 2014-2016 these lineages co-circulated. The 2000.3 lineage was also detected in Russia and Spain. Other unique human-to-swine spillovers without evidence for continued transmission were detected Denmark in 2010 and in Russia in 2014 (Other-Human-2010) and a novel H3 Other-Human-2000 was detected in Russia in 2018, with sequences situated on a long branch length.

**Surface protein-encoding genes reassortment dynamics of swine IAVs**

We generated a tanglegram to visualise HA-NA reassortment dynamics within a subsection of the collected European H1 swine IAVs (Supplementary data 3). Phylogenetic trees and respective tanglegram lines were coloured by HA clade. We observed exchange of the HA of the 1C lineage of European viruses (pink/purple) with other N1 and N2 NA segments represented by the crossing of the tanglegram lines. This provides evidence for reassortment in the HA and NA pairings of these viruses, specifically within the 1C.2.1 and 1C.2.5 clades and of sequence data collected from Italy. In contrast, the 1A (blue), 1B (green), and 1C.2.2 viruses (dark pink) displayed sets of parallel lines that suggest preferential HA and NA pairings with limited HA and NA reassortment despite both N1 and N2 subtypes being detected. The observed reassortment of the 1C lineage HA and NA segments and pairing of the 1C HA with both N1 and N2 NAs could affect antigenic phenotype and the transmission of different groups of viruses in the 1C lineage.

**Antigenic evolution in European pigs**

We constructed antigenic maps from data using swine sera in HI assays to characterise the antigenic properties of H1 viruses circulating in European pigs (Figure 3 A-C). Here we observed that genetic clade did not always correspond to antigenic positioning in the map. The 1A.3.3.2 (pdm09) viruses were antigenically similar to the human seasonal pdm09 vaccine strain (A/California/4/09) but there were two outlying swine strains from Germany and Belgium (Figure 3A). The outlying strains from Germany and Belgium were 5 AU from A/California/4/09 (Supplementary data 1).

The 1B lineage viruses showed greater antigenic variability than the 1A viruses (Figure 3B). The earliest strains in the lineage were antigenic outliers, defined as >3 AU from other viruses. The H1N1 and H1N2 1B.1.2 strains isolated from pigs in Spain which were tested were located in the centre of the antigenic map but were antigenically distinct (2-3 AU) from the tested 1B.1.2.1 strain, also from Spain. The most recent 1B.1.2.1 strains from Belgium were located as outliers on the opposite side of the map from the earliest strains we characterized and 3 AU from the 1B.1.2.1 strain from Spain. The two 1B.1.2.2 clade H1N2 strains from Italy were 3 AU apart from each other and between 1.5 and 3.4 AU from the 1B.1.2 strains from Spain, illustrating that not only does genotype not always correlate with antigenic phenotype but that within-clade antigenic diversity is a challenge in terms of both vaccine strain selection/match for use in pigs and for potential pandemic preparedness.

The 1C viruses have circulated in European pigs since the late 1970’s. They have also been associated with human variant infections in Italy, the Netherlands, Germany, France, Switzerland and Austria. In the antigenic maps there was no clear positioning defined by the 1C clade (Figure 3C). However, the ancestral strain A/swine/France/Finistere-2899/1982, early clade strains from 2005 (e.g. A/swine/France/Morbihan-0070/2005), along with more recent strains from 2015 (A/swine/France/65-150242/2015 and A/swine/Belgium/Gent-173/2015) were antigenically distinct from the main group. We identified key amino acid substitutions that might be responsible for the antigenic variation that we observed in these strains. The prototype virus A/swine/France/Finistere-2899/1982 had 10 amino acid differences (positions 107, 132, 134, 138, 142, 185, 190, 195, 196 and 197 (H1 numbering) when compared to the 1C clade consensus sequence. We identified only one key amino acid change for the virus A/swine/Belgium/Gent-173/2015 (position 129) and for A/swine/France/Morbihan-0070/2005 (position 168) when we compared them with the consensus sequence of the 1C HA genes. The H1N2 virus A/swine/France/65-150242/2015 had 8 amino acids that differed from the main 1C consensus (positions 124, 125, 126, 138, 155, 156, 163, 166). Notably the main antigenic cluster demonstrated significant antigenic diversity and occupied a space 8 AU by 3AU, and this likely makes selection of one strain to cover all current 1C antigenic diversity unlikely.

**Antigenic characterization of European swine viruses by ferret antisera**

We constructed antigenic maps from data using ferret sera raised against swine vaccine seed strains, candidate vaccine viruses and human seasonal vaccines in HI assays to characterise the antigenic properties of 1A and 1C European swine viruses as this is relevant for assessment of candidate vaccine viruses (CVV) for pre-pandemic preparedness. No human variant cases of 1B viruses had been observed in Europe up to the end of 2020. We observed that within the 1A lineage (Figure 4A), an antigenically similar group of viruses were 2-4 AU distance to a recent 1A.3.3.2 (pdm09) human seasonal vaccine strain, A/Brisbane/02/2018. A swine strain from Germany from the 1A.3.3.2 clade was an antigenic outlier at more than 7AU from the human seasonal A/Brisbane/02/2018.

The 1C lineage viruses (Figure 4B) had more defined positioning in ferrets compared to swine and we observed two separate antigenic clusters as shown on the 3D antigenic map. One cluster was comprised of European swine strains that circulated between 2017 and 2019 which were antigenically similar to the 1C.2.1 CVV strain A/Netherlands/3315/2016 and the 1C.2.2 human variant strain A/Netherlands/Gent-193/2019. The other main cluster contained swine viruses from 2019 and 2020 that were antigenically similar to the 1C.2.1 CVV A/Netherlands/10370-1/2020. There were outlier swine strains collected in Italy (clade 1.C.2.4), Germany (clade 1.C.2.4) and Belgium (clade 1.C.2.1) that were located around the antigenic map. The isolates from Italy and Belgium were located at 3 AU from the main clusters, while A/swine/Germany/AR313/2015 was located at 4 and 6 AU from the two main antigenic clusters.

**Comparison of immune response characterised using swine or ferret antisera**

Swine antisera raised against influenza A viruses from pigs are routinely used in HI assays to characterise within-host influenza evolution. However, vaccine strain assessments for humans are commonly undertaken using polyclonal ferret antisera raised to vaccine strains or candidate vaccine viruses for human pre pandemic risk assessment. Here we compared the antigenic relationships among a subset of strains using both swine and ferret polyclonal antisera to carry out antibody landscaping (Figure 5). Our focus was the serological response of these hosts to influenza A virus, whether through vaccination (swine) or infection (ferret) and we interpolated the surfaces over the antigenic map positioned with ferret antisera.

There are two measures that can be analysed from antibody landscapes: the magnitude (level of antibody titer and height of the landscape) and breadth (span of the smooth surface) of the antibody response. The magnitude and breadth of the antibody response were very similar in both animal models, demonstrating the same level of cross-reaction across the European H1 strains whether detected by ferret (Figure 5A) or swine (Figure 5B) sera. There was no statistical difference between the antibody response of the two animal models after immunisation with European H1 strains.

**Vaccination in pigs**

Ferret derived sera are sometimes raised against swine vaccine seed strains in Europe to determine the likely antigenic vaccine match to contemporary viruses. Figure 6 shows the antigenic distances from the 1A and 1C strains to the ferret-derived sera raised against vaccine seed strains used in European pigs from European isolates collected between 2014 and the end of 2020, human seasonal strains and CVVs. We observed that clade 1A.3.3.2 H1N2 strains from Germany and Denmark were over 3 AU and up to 5 AU away from the 1A.3.3.2 swine vaccine seed strain A/Jena/Msv-Vi5258/2009 (Figure 6A). No European 1B test strains were titrated against the ferret-derived sera raised against the 1B vaccine seed strain, A/Wsv/1832/2000, however a clade 1B CVV (A/Michigan/383/2018) and an ancestral strain (A/Brazil/11/1978) were used as surrogates and were 1.6 and 5.9 AU away from the within-clade vaccine seed strain (Figure 6B). 1C viruses also showed heterogeneity in cross-reactivity to the 1C.2.2 ferret serum raised against the vaccine seed strain A/Swine/Haselunne/Idt2617/2003. Four of eight H1N1 and one of two H1N2 1C.2.1 clade strains were over 3 AU from this vaccine seed strain. One H1N1 clade 1C.2.2 strain was over 3 AU from the within-clade vaccine seed strain. All 1C.2.4 strains were over 3AU from the vaccine seed strain, up to 6 AU, and the H1N2 1C.2.5 was 2.7 AU from the vaccine see strain (Figure 6C).

**Human serology**

Based on our phylogenetic analysis of the European swine H1 lineages, we selected strains to test in HI assays using two human antisera cohorts (post-vaccination and post-infection) to characterise potential immunity to swine strains and to assess the relative risk of incursion into humans (Figure 7). We also tested the cohort antibody response to human vaccine strains (boxplots coloured in grey) to determine if any current seasonal vaccine strains would offer cross-neutralisation against swine strains and to ensure that the cohorts included ‘low’, ‘medium’ and ‘high’ responders to the seasonal vaccine (Figure 7).

We selected A/Brisbane/02/2018 (1A.3.3.2) human vaccine strain as the human representative for the H1 swine virus analyses. An HI titre of 1:40 is generally considered the putative threshold that would confer protection against a future influenza infection. We plotted the HI titres obtained against the reference human seasonal vaccine and the representative swine influenza strains (Figure 7) to characterise the distribution of population immunity. For the human seasonal vaccine strain and a contemporary 1A.3.3.2 swine strain (A/swine/Germany/R2279/2016), which was still antigenically similar to the putative introduction strain from humans, we saw that the majority of the cohort had titres > 1:40 and indeed a median response of 4 GMT or 1:160. The A/swine/Republic of Ireland/AR6853/2016 (1A.3.3.2) – another strain antigenically similar to the putative strain from humans also showed a distribution of titres in the cohort which was generally higher than the 1:40 threshold, but with a median value below 4 GMT. Two additional swine 1A.3.3.2 strains, A/swine/Denmark/SIR1570/2017 and A/swine/Italy/241572/2020, showed an even higher distribution of titres with all over 1:40 and a median of 5 GMT for both strains. However, A/swine/Germany/BAK36/2017 was poorly recognised by the majority of the cohort antisera, the median was below 1:40 and some antisera showed no cross-reactivity. When we measured the antigenic distance from the A/Brisbane/02/2018 ferret antiserum to the tested swine strains we found that three were ~4 AU away, and the A/swine/Germany/BAK36/2017 was 6 AU away from the antiserum. This suggests that viruses that have drifted >4 AU from any seasonal or pre-pandemic preparedness vaccine may present a higher risk of incursion relative to population immunity. Given the demonstrated antigenic heterogeneity of circulating European swine 1A viruses, this should warrant close monitoring.

For the human cohort immunity assessment of the human seasonal 1B lineage, we selected two vaccine strains: A/Brazil/1/1978 – a human seasonal strain and closest ancestor to the putative introduction into swine and A/Michigan/383/2018 (1B.2.1) – a 1B lineage CVV, as references, together with contemporary European 1B viruses from pigs. The distribution of titres against A/Brazil/1/1978 were spread widely, with a median of 2 GMT, but with some individuals showing no cross-reactivity and others high cross-reactivity. This distribution likely relates to previous influenza exposure or vaccination with pre-2009 human seasonal viruses and low reactors likely represent younger individuals who might be at risk of infection. We included A/Michigan/383/2018 as a reference as it was the only available CVV proposed from this lineage. All individuals had titres above 2 GMT (range 1-8 GMT) against A/Michigan/383/2018, A/swine/Italy/202244/2019 (1B.1.2.2) and A/swine/France/29-200240/2020 (1B.1.2.3). The other four strains had titres varying between 0-8 GMT though the median of each was above 2 GMT.

The 1C lineage viruses which circulate in pigs have caused variant infections in humans in a number of European countries and in China. To analyse the cross-reactivity of human sera to Eurasian avian-like 1C lineage viruses we selected CVVs A/Hunan/42443/2015 (1C.2.3) and A/Netherlands/3315/2016 (1C.2.1). We also selected the three variant strains A/Netherlands/Gent-193/2019 (1C.2.2), A/Pavia/65/2016 (1C.2.1), and A/Netherlands/10370/2020 (1C.2.1) from which a CVV has been proposed. Given the demonstrated antigenic heterogeneity and genetic diversity of the 1C lineage viruses, we selected twelve swine 1C viruses isolated from various European countries and from various clades. This includes 1C.2.1 strains (n=7) from France, Germany, Spain, Italy and Belgium; 1C.2.2 strains (n=3) from Germany, Spain and the Netherlands; a 1C.2.4 strain from Germany; and a 1C.2.5 strain from Italy.

The median antibody response against most 1C lineage European viruses, CVVs and variants was over 2 GMT and most European viruses had greater recognition from the cohort sera than the 1C.2.3 CVV A/Hunan/42443/2015. The exception was A/Pavia/65/2016, an antigenic outlier (Figure 4B), which had a median GMT of 1.5 with GMT ranging from 0-6. For three viruses tested (1C.2.2s A/Netherlands/Gent-193/2019 and A/swine/Netherlands/Gent-193/2019 and the 1C.2.4 A/Swine/GermanyR1696/2014) the entire cohort had GMT >2. Interestingly, these three viruses were positioned more centrally in the antigenic map (Figure 4B). However, for the other nine viruses tested, there was heterogeneity in cohort titre and a proportion of individuals had titres of <2 GMT, suggesting a variable infection risk amongst the cohort. Given the antigenic heterogeneity of 1C lineage viruses shown, the number of antigenically distinct isolates and the proportion of cohorts with insufficient titres for protection against 1C lineage viruses, when triaging viruses for risk evaluation, viruses which are >3 AU from putative seasonal or candidate vaccine virus antisera should be prioritised.

**Discussion**

Influenza A viruses in swine have consistently posed a challenge to animal production by contributing to swine respiratory disease complex and reducing productivity. Although vaccine is used in some European countries to attempt to control disease, it is not used in all, and in some EU Member States or production systems there has been evidence of loss of vaccine efficacy. Factors which influence vaccine efficacy include the age of the animal and stage of the production cycle, the potential confounding effect of maternally derived antibody and less than optimal strain match between circulating and vaccine strains. Here we showed that the heterogeneity of circulating virus in European pigs is significant at the geographical, genetic, and antigenic levels.

We found that different HA lineages are co-detected in different countries, in combination with different neuraminidases and at different times. Genetic diversity has increased throughout our study timeframe, resulting in the need to update nomenclature to include newly detected and expanding lineages. Of the 1A lineage viruses, only the 1A.3.3.2 viruses have been maintained and the previously circulating classical-like 1A viruses have not been detected since the early 1990’s. Interestingly, introductions of human seasonal influenza A virus into swine did not always result in onwards transmission for more than a year, contrasting data from the USA and Brazil (Junqueira *et al.*, 2023; Markin *et al.*, 2023). H3 viruses are diverse, a result of multiple reverse zoonoses events and some long branch lengths indicated undersurveilled maintenance in pig populations. The spatial distribution of the swine virus lineages we observed through time was likely a result of discrete pig production systems and relative lack of animal movement within Europe.

Such genetic diversity has led to a complex antigenic profile in pigs, where some viruses have not substantially evolved phenotypically from their putative human seasonal ancestors, whereas others within the same lineage in other countries or even within the same country, have markedly drifted. Despite the observations presented here, we still do not have a fulsome picture of the true diversity as many pig populations in Europe remain poorly sampled or data are not publicly available. Anecdotally, some production units have substantial longitudinal problems with influenza whereas others detect disease infrequently. The underlying factors contributing to this complex epidemiology are poorly understood. However, this onward and divergent evolution in different swine populations reflects a challenge for control in pigs, i.e., one pdm09 antigen may not be sufficient to cover observed antigenic diversity in Europe but should be tailored to appropriate regions based on pig production. In humans, these viruses are likely antigenically drifted from immunity which would be derived from vaccination or natural exposure to the pdm09 in human populations.

Phenotypic evolutionary assessment of influenza viruses in pigs has commonly focussed on using swine antisera in HI assays. This is entirely logical as the pig serves as a natural host model for the evolution of the viruses within pig populations. However, assessment of influenza in humans commonly relies on responses to influenza viruses generated by ferrets as does some vaccine strain selection in other animals (e.g., horses). To date there has not been a quantitative and comparative assessment of the two hosts immunological responses to influenza and whether one or other of the models (pig or ferret) are more appropriate to assess phenotypic evolution in different scenarios. Here we showed, despite slightly varied immunisation regimes, with matched viruses and antisera raised in both hosts, that the breadth and relative magnitude of the immunological response was equivalent using antibody landscaping. This is critical as it allows triage of viruses circulating in pigs to be validly undertaken using either model system, and extrapolation between the two in selecting viruses of interest for further assessment such as antigenic variance from vaccine strain or for pandemic preparedness. This also indicates that either vaccination (in pigs) or infection (in ferrets) as the antigen presentation route is equivalent, demonstrating a level of tolerance between different immunisation regimes, for example when comparing two doses of vaccine in pigs and one dose of live virus in ferrets.

Since swine influenza also impacts animal health and production, we assessed the distance from currently circulating strains to vaccine strains used in European pigs. We found variability in putative strain match based on HI assay data and ferret antisera, with some 1C lineage strains close to the within-lineage antiserum whereas others including 1A.3.3.2, 1C.2.4, 1C.2.5 clade viruses were distant (>3 AU). Such large antigenic distances are likely to impact vaccine efficacy in the field, as reported by Deblanc *et al.,* (2024) on the emergence of the H1N2 clade 1C.2.4 virus in France, despite adjuvant use to broaden a response (Souza *et al.*, 2018). Subsequent work should focus on defining correlates of cross-protection for the observed antigenic diversity in pigs to provide a quantitative basis for when vaccine might need updating. Furthermore, the impact of using poorly matched vaccine upon future virus evolution is unknown at population level and requires evaluation, as though vaccination protects against disease, it may not reduce shedding, nor transmission between nor within herds. This may contribute to virus selection and thus change the rate of antigenic evolution.

Since 2017 there have been several instances of variant cases (human infections with swine influenza A viruses) in Europe. These human variant cases have not been limited to a particular swine HA lineage or country (WHO, 2017; WHO, 2018; WHO, 2020; WHO 2021a; WHO 2021b; WHO 2021c). Rather they have been sporadically detected primarily through influenza-like illness and subsequent international health regulation requirements. We analysed representatives of the sampled diversity in European pigs along with reference human seasonal or candidate vaccine viruses to quantify the immunological profile of two human cohorts to these strains and assess the potential for these viruses to infect humans and spread. Vandoorn *et al.,* (2020) found age and lineage dependant cross reactive human antibodies against swine strains tested in their study. In our study, as with the antigenic analyses with swine and ferret anti-sera, lineage did not reliably predict immunological profile with human sera. Within lineages, there were some strains that could be considered ‘riskier’ as there was poor immunological recognition by human cohorts, particularly for some of the tested 1A.3.3.2 (pdm09) and 1C lineage strains.

There are likely other factors that contribute to interspecies transmission and subsequent spread in human populations – this includes the risk-interface, immunological status of the host based on prior exposure and vaccination, and virus factors such as HA-NA combinations and transmission efficiency. In some cases, there may be a ‘putative protective’ effect from a human seasonal N1 rather than an N2 combination that was detected in swine viruses that had antigenically drifted at the HA level in swine. In general, prior studies have demonstrated significantly less antigenic diversity in swine NA than in the HA gene (Kaplan *et al.*, 2021; Hufnagel *et al.,* 2023) and extending pandemic preparedness strategies to consider the neuraminidase may overcome the challenge represented by significant diversity in the HA gene in swine in Europe and globally. This effect may not be revealed by assessment with the HI assay, and as such, human cohort serological analyses should be incorporated into a formal pandemic preparedness risk-assessment pipeline with further downstream steps to assess virus neutralisation, human receptor binding, and other potential risk factors such as anti-viral resistance.

**Data availability**

The data associated with this study are available as supplemental material and are posted at https://github.com/acoggon3/Euro-Swine-Risk

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**Figure/Table legends**

**Figure 1: Maximum likelihood phylogenetic tree of influenza A H1 viruses circulating in pigs in Europe in 2010-2020.** Sequences from pigs in European countries between January 2010 until December 2020 are coloured by genetic lineage as shown in the colour key, strains names with identical sequences are concatenated (n=1563). Variant strains, CVVs and human seasonal vaccine strains are included as reference strains and are coloured grey (n=55). The proportions of each lineage are plotted by country on a MicroReact geographical map and the frequency of each genetic lineage detected per year is shown on the timeline (n=1697).

**Figure 2: Maximum likelihood phylogenetic tree of influenza A H3 viruses circulating in pigs in Europe in 2010-2020.** Sequences from pigs in European countries between January 2010 until December 2020 are coloured by genetic lineage as shown in the colour key (n=231). Variant strains, CVVs and human seasonal vaccine strains are included as reference strains (n=53). The proportions of each lineage are plotted by country on a MicroReact geographical map and the frequency of each genetic lineage detected per year is shown on the timeline (n=231).

**Figure 3 A-C:** **Antigenic maps highlighting the antigenic relationships among 1A, 1B and 1C lineage viruses in European pigs respectively as defined by swine anti-sera.** Strains are represented by coloured annotated spheres with the colour denoting lineage as per Figure 1. Monovalent polyclonal swine sera raised to influenza A viruses in pigs are shown as cubes. The scale bar represents one antigenic unit (AU) or a two-fold difference in HI assay titre. Panel A shows the European 1A.3.3.2 lineage viruses from swine highlighted in blue. Panel B shows the European 1B lineage viruses from swine highlighted in greens. Panel C shows the European 1C lineage viruses from swine highlighted in pink/purples. CVVs and human seasonal vaccines are shown in cream colours.

**Figure 4 A-B: Antigenic map highlighting the antigenic relationships among the swine European 1A and 1C lineages respectively, as defined by ferret anti-sera.** Strains are represented by coloured spheres with the colour denoting lineage as per Figure 1. Polyclonal ferret sera raised to swine influenza A viruses are shown as cubes. The scale bar represents one antigenic unit (AU) or a two-fold difference in HI assay titre. Panel A shows the European 1A.3.3.2 clade viruses from swine highlighted in blue. Panel B shows the European 1C lineage viruses from swine highlighted in pinks/purples. CVVs and human seasonal vaccines are shown in cream colours.

**Figure 5: A-B: Antibody landscaping representing the immunological response to a range of European H1 strains in post-infection ferret antisera and post-vaccination polyclonal swine antisera.** The X and Y axis represent the coordinates of the virus strains derived from the 2D antigenic maps. The European H1 strains have been coloured green. The Z axis represents the geometric mean of the HI titres at each antigenic point for each host population. Panel A shows the results using the 2D ferret antigenic map and post-infection ferret antisera and panel B shows the results using the 2D swine antigenic map and post-vaccination polyclonal swine antisera.

**Figure 6 A-C: Antigenic distances between H1 test viruses and European swine influenza vaccine seed strains. Test viruses are coloured by lineage, CVV or vaccine seed strain.** PanelA against the 1A.3.3.2-clade A/Jena/Msv-Vi5258/2009, panel B against the 1B-lineage A/Wsv/1832/2000 and panel C against the 1C.2.2-clade A/Swine/Haselunne/Idt2617/2003 vaccine seed strains. The height of the bar represents the antigenic units between the test virus and the vaccine seed strain. Three AU difference between vaccine sera and test antigen are considered antigenically different enough to trigger an update to the human influenza vaccine (Ampofo *et al.*, 2015).

**Figure 7 A-C: Log2 of Geometric Mean Titre (GMT) of HI responses of human antisera against circulating European H1 swine viruses.** Boxplots depict the 25% (Q1) and 75% (Q3) quantile. The whiskers illustrate the variability outside the Q1 and Q3. Points positioned outside of the whiskers of the boxplot are outliers. Each dot represents the geometric mean titre (GMT) log2 (HI titre/10) of each human antiserum against each virus strain. The dotted line indicates the positive HI titre threshold (1:40 or 2GMT) purported to confer protection against infection. The boxplots for the human vaccine strains or CVVs have been coloured in grey. Panel A shows sera tested against 1A human vaccine virus A/Brisbane/02/18 and European Swine 1A.3.3.2 viruses a) A/Swine/Germany/R2279/2016, b) A/Swine/Republic Of Ireland/Ar6853/2016, c) A/Swine/Germany/Bak36/2017, d) A/Swine/Denmark/Sir1570/2017 and e) A/swine/Italy/241572/2020. Panel B shows sera tested against human vaccine virus A/Brazil/1/78, CVV A/Michigan/383/18, 1B.1.2.2 viruses a) A/Swine/Italy/126300/2019, b) A/Swine/Italy/202244/2019 and c) A/Swine/Italy/202244/2019, 1B.1.2.3 A/Swine/France/29-200240/2020 and 1B.1.1 strains a) A/Swine/England/208046/2018 and b) A/Swine/England/062058/2018. Panel C shows sera tested against the 1C.2.3 and 1C.2.1 CVVs a) A/Hunan/42443/15 and b) A/Netherlands/3315/2016, respectively which are coloured grey. Variant strains the 1C.2.2 a) A/Netherlands/Gent-193/2019 the 1C.2.1 b) A/Pavia/65/2016 and c) A/Netherlands/Gent-193/2019. The 1C.2.1 strains a) A/swine/Belgium/SIR5289/2017, b) A/swine/France/SIR3244/2017 c)A/swine/Germany/BAK63/2017, d) A/swine/Italy/6625-3/2019, e) A/swine/France/85-200222/2020, f) A/swine/Spain/45690-4/2018. The 1C.2.2 isolates a) A/swine/Germany/AR8941/2016, b) A/swine/Netherlands/Gent-193/2019 c) A/swine/Spain/6370-1/2018 and d) A/swine/Spain/6370-2/2018. The 1C.2.4 isolates a) A/swine/Germany/R1696/2014 and b) A/swine/Germany/AR313/2015. Finally, the 1C.2.5 A/swine/Italy/45510/2019.

**Supplementary data 1:** Swine antisera and test viruses used in this study including subtype and clade information where available. Antigenic distances as calculated using antigenic cartography between test antigens and antisera raised in swine.

**Supplementary data 2:** Ferret antisera and test viruses used in this study including subtype and clade information. Antigenic distances as calculated using antigenic cartography between test antigens and antisera raised in ferrets.

**Supplementary data 3:** Maximum likelihood phylogenetic trees of HA and NA segment pairings of H1 viruses circulating in European pigswhere sequence was available for both segments (n=1424)**.** Identical tips are connected with lines coloured according to HA genetic lineage as per figure 1 in order to visualise the diversity of HA and NA gene segment pairings among H1 European viruses. Parallel lines indicate genetically stable pairings and crossed lines indicate heterogeneity in gene pairings.