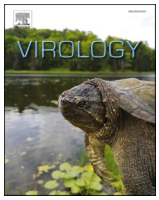
Virology 600 (2024) 110223

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology



Detection and subtyping of influenza A virus in porcine clinical samples from Spain in 2020

Alfredo A. Benito [a], Luis V. Monteagudo [b,d], Sofía Lázaro-Gaspar [a], Luna Mazas-Cabetas [a], Joaquín Quílez [c,d,*]

[a] Exopol S.L., Pol Río Gállego D/14, San Mateo de Gállego, 50840, Zaragoza, Spain
[b] Department of Anatomy, Embriology and Genetics, Faculty of Veterinary Sciences, University of Zaragoza, 50013, Zaragoza, Spain
[c] Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, 50013, Zaragoza, Spain
[d] Agrifood Institute of Aragón (IA2), University of Zaragoza-CITA, 50013, Zaragoza, Spain

ARTICLE INFO

Handling Editor: Dr. Jasmine Tomar

Keywords:
Influenza A virus
Pigs
Occurrence
Subtypes
Antigenic sites
Spain

ABSTRACT

A total of 1019 samples collected on 726 Spanish swine farms suffering from outbreaks of respiratory disease were screened for influenza A viruses (IAVs) using a RT-qPCR method. A subset of positive samples was further analyzed using a subtype-specific RT-qPCR method (n: 142) and Sanger sequencing (n: 64). A total of 19.4% samples from 23% farms tested positive, with infection being most common in suckling (53.6%) and weaning pigs (30.2%). Viruses belonging to four HA subtypes (H1av, H1hu, H1pdm, H3) were detected, with subtypes H1avN2, H1huN2 and H1avN1 accounting for over half of the specimens. An optimized protocol with newly designed primers allowed the detection of H3 viruses in a significant number of samples (21%). A comparison of antigenic positions revealed that circulating strains exhibited mutations with vaccine strains in a significant percentage of amino acid residues, both in the NA protein (27.8–43.3%) and particularly in the HA protein (51–75.3%).

1. Introduction

Swine influenza is a highly contagious viral respiratory infection of pigs causing significant economic losses for pig producers worldwide. Morbidity can reach as high as 100% within a herd and mortality can lead to 10–15% in naïve pigs (Ma, 2020). The etiological agents are influenza A viruses (IAVs) which are enveloped virus belonging to the family *Orthomyxoviridae*. IAVs contain eight segments of negative-sense single stranded RNA as its genome, with subtypes being defined by the hemagglutinin (HA) and neuraminidase (NA) spike-like glycoproteins. These surface glycoproteins frequently undergo minor amino acid changes, or antigenic drift, which results in the emergence of new variants (Van Reeth and Vincent, 2019). Additionally, genome reassortment or antigenic shift can occur as a consequence of the segmented genome of IAVs, which results in multiple lineages circulating globally (Ryt-Hansen et al., 2020).

In addition to their economic impact for the swine sector, IAVs are of public health concern as swine may provide an intermediate host for adaptation of avian influenza strains to humans, which can lead by reassortment to the generation of viruses with epidemic and pandemic potential (Krammer et al., 2018). The emergence in pigs of 2009 H1N1 pandemic virus (H1N1pdm), containing viral segments from avian, swine, and human viruses, highlighted the key role of pigs in contributing to IAV reassortment and evolution (Smith et al., 2009). Three subtypes, H1N1, H1N2, and H3N2 are simultaneously circulating in swine populations worldwide, but lineages may vary within each subtype depending on the region, i.e. North America, Europe and Asia (Bonin et al., 2018; Mancera Gracia et al., 2020). The established clade classification system for the HA gene of H1 subtype IAVs recognizes three major lineages—1A, 1 B, and 1C—and their sub-lineages (Anderson et al., 2016).

Immunodominant epitopes on HA and NA glycoproteins are the target of neutralizing antibodies elicited during influenza infection or vaccination, which is the main tool to control swine IAV infections. As the main antigen of influenza virus, mapping of hemagglutinin (HA) from H1N1 and H3N2 strains using variants selected by monoclonal antibodies has revealed the existence of six (Sa, Sb, Ca2, Ca1, Cb and Pa) and five (A – E) major antigenic sites, respectively (Wiley et al., 1981;

* Corresponding author. Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, 50013, Zaragoza, Spain.
E-mail address: jquilez@unizar.es (J. Quílez).

https://doi.org/10.1016/j.virol.2024.110223
Received 30 April 2024; Received in revised form 21 August 2024; Accepted 3 September 2024
Available online 8 September 2024
0042-6822/© 2024 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Caton et al., 1982; Skehel et al., 1984; Matsuzaki et al., 2014). Antigenic epitopes of NA glycoprotein are not well characterized but amino acid residues critical for monoclonal antibodies binding in both N1 and N2 subtypes have also been reported (Venkatramani et al., 2006; Wan et al., 2015).

Swine influenza is widespread in Europe. A serological study in the period 2006–2008 in farms with a history of respiratory problems from the most swine-dense European regions in Belgium, France, Italy and Spain showed that 90% farms were classified as positive for swine IAVs, with an individual level seroprevalence of 62% (Kyriakis et al., 2013). A subsequent large-scale surveillance molecular study conducted between 2015 and 2018 in 17 European countries revealed 30.5% of samples and 56.6% of swine farms with respiratory disease to be positive at the matrix (M) gene (Henritzi et al., 2020). Four main sublineages of IAVs circulate in the swine population in Europe, including the "avian-like" swine H1N1 lineage (H1avN1; clade HA-1C) which emerged in 1979 following transmission of H1N1 from duck to pig and replaced the "classical" human 1918-derived H1N1; the "human-like" H3N2 lineage originating in 1984 from the reassortment of human-like swine H3N2 and H1avN1; the "human-like" H1N2 (H1huN2; clade HA-1B) that appeared in 1994 from a reassortment between the swine H3N2 and a seasonal human H1N1; and the "pandemic 2009"- origin H1N1 (H1pdmN1pdm; clade HA-1A) which was transmitted from humans and is circulating in the swine population since 2009 (Chiapponi et al., 2021). The relative proportions of these four main lineages in the swine population in Europe remains largely stable but with very different relative levels of incidence in several countries (Simon et al., 2014). Hence, reassortant viruses combining genes from these different enzootic IAVs have emerged from the co-circulation among the swine population such as H1avN2, H1huN1av, and H1pdmN2 (Henritzi et al., 2020).

Pigs represent the largest livestock category in Spain, which is the second top pig-producing country in the EU, and the fourth largest producer of pork worldwide after China, the United States and Germany (https://ec.europa.eu/info/food-farming-fisheries/animals-and-animal-products_en, accessed on November 30th, 2023). Two northeastern Spanish regions are among the most swine dense regions in Europe and concentrate more than a half of population of pigs in Spain: Aragon (28.6%) and Catalonia (23.3%) (https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/sectores-ganaderos/porcino/default.aspx, accessed on November 30th, 2023). Influenza is endemic in intensive production systems of white pigs in Spain, with IAVs being commonly found by RT-qPCR in nasal swabs from both farms with respiratory disease episodes and apparently subclinical randomly selected farms (Sosa Portugal et al., 2020). Serological surveillance studies have reported a farm-level seroprevalence of 94–100% and an animal level seroprevalence of 62.3–80% (Van Reeth et al., 2008; Simon-Grifé et al., 2011; Kyriakis et al., 2013). In Iberian swine, these percentages were 58% and 33.4%, respectively (Encinas et al., 2021). Serological studies in the period 2002–2003 demonstrated that subtype H1N1, H3N2 and H1N2 IAVs co-circulated in densely populated pig areas in Spain (Maldonado et al., 2006; Van Reeth et al., 2008). A subsequent molecular study in the period 2010–2013 revealed that subtypes H1avN1 and H3N2 circulated widely in pig populations in Spain but H1N1pdm was not detected (Simon et al., 2014). In the period 2017–2019, the most prevalent subtypes were H1avN2hu, H1avN1av and H1huN2hu, followed by H3huN2hu and H1pdmN1pdm, revealing that this genetic diversity is continuously expanding with new viral variants and highlighting the need of continuous surveillance of IAVs in this host (Sosa Portugal et al., 2020). The current study aimed to investigate the occurrence and genetic diversity of IAVs from swine farms with outbreaks of respiratory disease distributed throughout Spain. For this purpose, cases submitted to a veterinary diagnostic laboratory in 2020 were analyzed. Additionally, amino acid residues in antigenic sites within the HA and NA proteins were analyzed to explore the similarity between circulating strains and IAV strains contained in the two commercial vaccines available in Spain.

2. Materials and methods

2.1. Ethics approval statement

Samples were collected from pigs by veterinary surgeons for diagnostic purposes after the permission of farm owners, with no specific permits being required by the authority for the specimen collections. Animal care and use committee approval was not necessary for this study. Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes does not apply to non-experimental clinical veterinary practices.

2.2. Sample collection

A total of 1019 samples collected between January 1st and December 31st, 2020 in swine farms with outbreaks of respiratory disease were delivered for diagnostic purposes to a specialized veterinary laboratory (Exopol S.L., San Mateo de Gállego, Spain). Specimens were received in the form of lung tissues (42%), oral fluids (29%), nasal swabs (26%) and bronchoalveolar lavages (3%) and originated from 726 farms distributed in 43/50 Spanish provinces. Most samples corresponded to pools of specimens and were collected from suckling piglets (n: 28), weaning pigs (n: 265), fattening pigs (n: 498) and sows (n: 92). The age of pigs was unknown for 136 samples. Most farms submitted either a single (n: 546), two specimens (n: 116) or three specimens (n: 38) with the remaining farms submitting four to six samples. The sample size selection from different farms, age groups, or time periods could not be controlled, because the samples were specifically submitted for diagnostic purposes under the criteria of the veterinary surgeons in response to an unusual increase in the number of cases of respiratory disease.

A subset of samples positive for swine influenza A virus (n: 142) was selected for genotyping based on both the cycle threshold (Cq) value $<$ 32 against IAVs and their origin from farms located in different regions with the purpose of covering a wide geographical area. The selection was also proportional to the number of specimens submitted from each province. Namely, these selected samples originated from 126 farms located in 22 provinces distributed throughout Spain, with most of them corresponding to weaning pigs (n: 61) and fatteners (n: 33) while a much smaller number were samples from suckling piglets (n: 12) and sows (n: 4). Half of specimens originated from three provinces while between 1 and 9 samples were analyzed from the remaining provinces. The age of pigs was not available for a total of 22 samples. On six farms, multiple submissions corresponding to independent outbreaks on different dates were analyzed. Data about IAV vaccination in the involved farms were not available in this study.

2.3. Nucleic acid extraction, identification and subtyping of swine IAV by RT-qPCR

The commercial kit, MagMAX™ Core Nucleic Acid Purification (Thermo Fisher Scientific, Waltham, MA, USA) with an automated magnetic particle processor (KingFisher Flex; Thermo Fisher Scientific, Waltham, MA, USA) was used for extraction of nucleic acids according to the manufacturer's instructions. A quantitative reverse transcription-PCR (RT-qPCR) assay based on the amplification of the conserved segment of the matrix (M) gene was performed using a commercial kit (EXOone Influenza A virus, Exopol S.L., San Mateo de Gállego, Spain) to confirm the presence of influenza A virus in all 1019 samples. Samples with Cq value $<$ 38 were considered positive. A subset of 142 positive samples with Cq value $<$ 32 were further investigated to identify the influenza A subtype by hemagglutinin (HA) and neuranimidase (NA) genes amplification targeting H1av, H1pdm, H1hu, H3hu, N1 (including N1all and N1pdm), and N2. Subtype-specific real-time PCRs (RT-qPCR) as described by Henritzi et al. (2016) were used.

A novel RT-qPCR assay was developed in this study to increase the sensitivity of the procotol described by Henritzi et al. (2016) for detection of H3 subtypes. This assay was optimized based on preliminary findings that provided a low detection rate of H3 subtypes. The new primers H3exoFW (5′-GGGGACCCTCABTGTGA-3′); H3exoRV (5′-UAMTCCGGCACATCATAAGG-3′) and the probe H3exoS (5′-TTGAACGCAGCAVRGCTTWCAGCA-3′) were designed by alignments of SIV H3 sequences retrieved from GenBank and Spanish sequences obtained in the current study, using the unipro UGENE and Primer Blast software. The RT-qPCR amplification was carried out using the SensiFAST Probe No-ROX One-Step Real-time PCR kit (Bioline Reagents Ltd, London, United Kingdom) in a 20 μL reaction mixture containing 10 μL of 2 × One Step RT-PCR Buffer, 500 nM of each primer, 250 nM probe, 0.4 μL of Ribosafe RNase inhibitor, 0.2 μL of Reverse transcriptase and 5 μL extracted RNA. The reaction was run in a QuantStudio 5 Real-time PCR machine (Applied Biosystems, Marsiling, Singapore), following the manufacturer's instructions; briefly: a reverse transcription step at 45 °C for 20 min; then heated at 95 °C for 5 min and a three-step cycle (15 s at 95 °C, 60 s at 58 °C, 30 s at 72 °C) was repeated for 40 cycles.

2.4. Sequence analysis of HA and NA genes

The subtype of selected IAV isolates (n: 64) previously identified by RT-qPCR was also determined by Sanger sequencing of the HA and NA genes. These isolates were selected based on the absence of co-infections by mixed subtypes and the origin of the samples, with the aim of covering the widest geographical area. The RT-PCR amplification was carried out using the same commercial mastermix used in step 2.3 in a 50 μL reaction mixture containing 25 μL of 2 × One Step RT-qPCR Buffer, 500 nM of each primer, 1 μL of Ribosafe RNase inhibitor, 0.5 μL of Reverse transcriptase and 15 μL extracted RNA. The reaction was run in a QuantStudio 5 Real-time PCR machine (Applied Biosystems, Marsiling, Singapore), following the manufacturer's instructions; briefly: a reverse transcription step at 45 °C for 15 min, denaturation at 95 °C for 10, followed by 40 cycles of 95 °C for 5 s, 55–58 °C for 30 s and 72 °C for 60 s. The annealing temperatures and primers for each target gene are described in Table 1. The PCR products were subjected to electrophoresis in 1.5% w/v agarose gels and visualized with a UV transilluminator. PCR products of expected amplicons size were purified and sequenced in both directions with the forward and reverse primers used for amplification at STABvida laboratories (Caparica, Portugal).

A preliminary nucleotide query step was performed in the Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on May 13th, 2023) to identify closely related sequences. Reference sequences of swine IAVs from Spain and other European countries (Denmark, Germany and Netherlands) were retrieved from the Genbank influenza virus resource database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database; accessed on August 17th, 2023). Alignment of the consensus sequences against each other and with reference sequences was done using ClustalW and edited with BioEdit version 7.2.5 (https://bioedit.software.informer.com; accessed on May 13th, 2023). Neighbor-joining phylogenetic trees using the Kimura's two parameter model were constructed by means of the MEGA software (https://www.megasoftware.net; accessed on https://www.megasoftware.net). The robustness of branching patterns was tested by 1000 bootstrap replicates. Tree drawing was performed online by means of the iTOL v5 tool (Letunic and Bork, 2021). Nucleotide diversity among IAV strains allocated to different subtypes (H1, H3, N1 and N2) was evaluated by means of DNAsp software (Rozas et al., 2017).

The amino acid sequence of neutralization epitopes previously described within HA and NA was deduced for all IAVs strains with complete sequences available from this study and compared with the amino acid composition of the same antigenic epitopes in the vaccine strains included in the three vaccines licensed in Spain: A/Port Chalmers/1/1973 (H3N2), A/swine/Hasselunne/2617/2003 (H1avN1), A/swine/Bakum/1769/2003 (H3N2), A/swine/Bakum/1832/2000 (H1huN2), and A/Jena/VI5258/2009(H1N1pdm09) (Wiley et al., 1981; Caton et al., 1982; Skehel et al., 1984; Matsuzaki et al., 2014). Translation was performed by means of the EMBL-EBI translation tool (Madeira et al., 2019).

2.5. Nucleotide sequence accession numbers

Sequences of IAV strains from this study were submitted to Genbank (accession numbers OQ363327 to OQ363376, OQ411283 to OQ411295 and OQ439192).

3. Results

3.1. Detection and subtyping of swine IAV by RT-qPCR

A total of 198 samples (19.4%) from 167 farms (23%) tested positive with M gene RT-qPCR. Most of these positive samples (83.8%) showed a Cq value below 34. Age distribution of IAV positive pigs showed higher infection rates in suckling (15/28, 53.6%) and weaning pigs (80/265; 30.2%) than fattening pigs (62/498, 12.4%) and adults (6/92, 6.5%). The monthly percentage of positive submissions fluctuated throughout the year, although the highest percentages were reported in July–August (28.6–29%) and the lowest rates in January–February and September–October (10–16%).

Of the 142 samples with a Cq value below 32 selected for subtyping, the HA and NA subtypes were successfully identified in 136 and 139 samples, respectively (Table 2). A total of 22 samples tested negative for all H types in a preliminary analysis using the protocol described by Henritzi et al. (2016). A subsequent sequence analysis of 12 of these samples showed that they belonged to the H3 type. This finding prompted the development of a novel approach. The optimized RT-qPCR assay with newly designed primers developed in this study significantly increased the detection of H3 subtypes as compared with the protocol by Henritzi et al. (2016), which only allocated four samples to H3 subtypes. Namely, the optimized protocol provided Cq values remarkable lower for these four samples (23.5–28.3 *versus* 31.8–34) and identified a total of 30 samples as belonging to H3 subtypes.

The subtypes of the IAVs could be determined by RT-qPCR in 108/142 samples (76%). H1avN2 was by far the most common subtype as

Table 1
Primers used for Sanger sequencing of HA and NA genes of swine IAVs.

Target	Primer	Sequence 5′ to 3′	Ta[a] (°C)	Amplicon size (bp)	References
HA región 5′	SZHA+	CTCGAGAGCAAAAGCAGGGG	55	985	Zou, 1997
	HA_979	GTGATGGGATGTACATTCTG			Martín-Valls et al. (2014)
HA región 3′	H1exoFw	ATAACDTTYGAGGCCAMTGGRAA	58	990	This study
	HARK	GTATTAGTAGAAACAAGGGTGTTTT			Bragstad et al. (2005)
NA región 5′	SZANA+	AGCAAAAGCAGGAGTTTAAAATG	55	869	Zou, 1997
	NA880Rv	AGGATAACAGGAGCAYTCCTC			This study
NA región 3′	NA_680	TGAGAACACAAGAGTCTGAATGTG	55	773	Martín-Valls et al. (2014)
	NARK	GTATTAGTAGAAACAAGGAGTTTTTT			Bragstad et al. (2005)

[a] Annealing temperature.

Table 2
Age distribution of IAV subtypes detected by RT-qPCR from January to December 2020 in swine farms from 22 provinces in Spain.

Subtype	Age group					
	Suckling piglets (1–4 w. of a.) [a]	Weaning pigs (5–8 w. of a.) [a]	Fatteners	Sows	Other [b]	Total
H1avN2	1	29	7	1	7	45
H1huN2	6	4	6		1	17
H1avN1	2	2	3	1	4	12
H3N2	1	7			4	12
H3N1	1	6	2		2	11
H1pdmN2		5				5
H1pdmN1			2	1	1	4
H1pdmN1pdm			1	1		2
Co-infections						
H1avH1huN1		1	4		3	8
H1avH1huN2		1	3		2	6
H1avH1huN1N2			2			2
H1avH3N1N2	1	3				4
H1huN1N2		1				1
H1pdmN1pdmN1N2			1			1
H1pdmH3N2		2				2
H1avH1huNx			1			1
H1avNx					2	2
H3Nx			1			1
HxN1		2			2	4
HxN2		1			1	2
Total	**12**	**64**	**33**	**4**	**29**	**142**

[a] w. of a.: week of age.
[b] Age unknown.

was detected in almost half of these fully subtyped specimens (42%) followed by H1huN2 (16%). Subtypes H1avN1, H3N2, H3N1 were each found in 10–11% samples and the remaining specimens (10%) were allocated to three H1pdm subtypes (H1pdmN2, H1pdmN1, H1pdmN1pdm). Co-infections with more than a single subtype were detected by RT-qPCR in a total of 24/142 (17%) samples mostly from weaners and fatteners that tested positive for more than a H and/or N, and it was not possible to precisely establish their subtypes. In 10 samples (7%), only the H or the N lineage could be determined (Table 2). The distribution of subtypes by age categories showed that H1avN2 was the most common subtype in weaning pigs (29/64) while H1huN2 virus predominated in suckling piglets and both subtypes were seen in a similar number of fatteners. The presence of different subtypes co-circulating in the farm was confirmed in three farms submitting several specimens on the same date. Our results also showed that pigs were infected with different subtypes in 4/6 farms suffering repeated outbreaks of respiratory disease throughout the study.

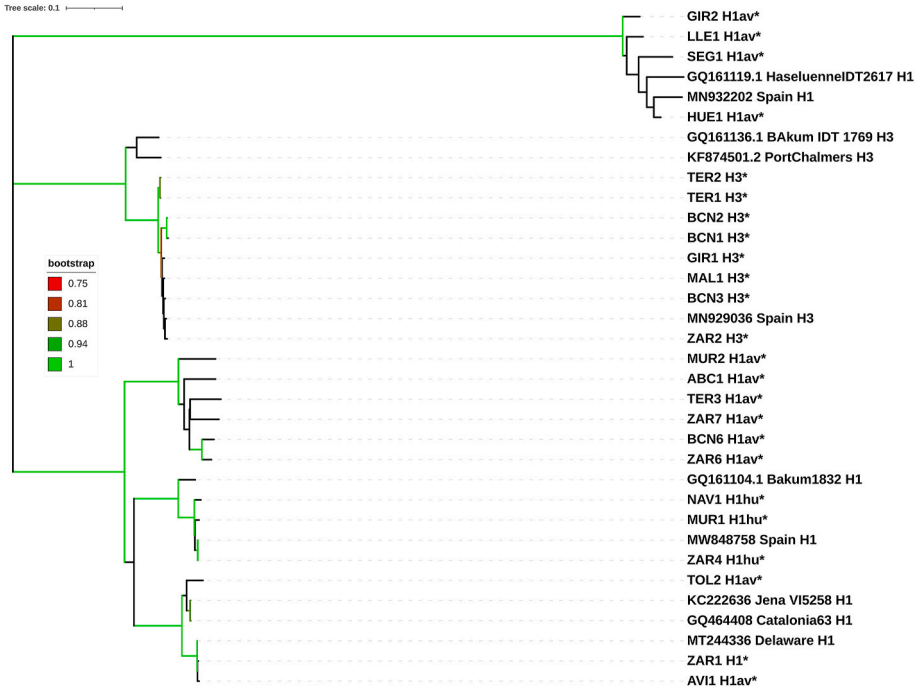


Fig. 1. Neighbor-Joining tree for hemagglutinin (HA) nucleotide sequences of IAVs collected from Spanish swine farms in 2020 (marked with *) compared to representative swine IAV strains, and subtypes contained in the vaccines available in Spain. Only branches having bootstrap support values over 0.23 are represented in colors different from black.

3.2. Sequencing and phylogenetic analysis

The phylogenetic analysis involved a total of 25 H A and 39 NA sequences, respectively, which were analyzed separately according to the subtype. IAV strains belonging to different subtypes segregated into distinct branches and clustered with porcine strains from Spain and other countries (Figs. 1 and 2). The nucleotide diversity index for the H1 subtype sequences was Pi = 0.32191, and the mean number of nucleotide differences between all pair of sequences was k = 254.955 in the 815 nucleotides segment conserved in all the isolates, indicating a high sequence variability. The H1 nucleotide sequences were dispersed into four different clusters. Four sequences belonging to H1av subtypes clustered separately from other H1av sequences and showed similarity to the vaccine strain A/swine/Haseluenne/IDT2617/2003 (H1N1) and the previously described sequence from Spanish swine with GenBank accession number MN932202. When these four sequences were removed from the genetic variability analysis, the nucleotide diversity in the remaining set of sequences reduced to Pi = 0.19194 and k = 152.067. The genetic diversity was noticeably lower among the H3 sequences, which clustered closely together and were similar to the H3N2 vaccine strains A/Port Chalmers/1/1973 and A/swine/Bakum/IDT1769/2003. Specifically, the H3 nucleotide sequences showed an average pairwise sequence difference per site (Pi) of 0.01712, and the mean number of nucleotide differences was k = 14.464 in the 845 nucleotides segment obtained for all the isolates. DNAsp analysis for the NA gene showed greater genetic diversity for the N1 sequences compared to the N2 sequences. The nucleotide diversity index (Pi) for N1 was calculated as 0.18978, and the average number of nucleotide differences between pairs of sequences (k) was found to be 137.022 in the 722 nucleotides segment conserved in all the isolates. The nucleotide diversity index (Pi) for N2 was determined to be 0.11729, with an average of 161.036 nucleotide differences (k) calculated between pairs of sequences in a segment of 1373 nucleotides.

3.3. Amino acid composition of antigenic epitopes

A comparison of amino acids identified in the antigenic sites of hemagglutinin (HA) and neuraminidase (NA) proteins between strains from this study and IAV strains from the three vaccines licensed in Spain is presented in Tables S1–S4. No gene sequences are made publicly available for the vaccine strain A/swine/Olot/1984. The comparison of 39 amino acid residues within six antigenic sites (Sa, Sb, Ca2, Ca1, Cb and Pa) in two circulating H1 strains showed identity with three vaccine strains at 10 amino acid positions (25.6%) and differences with one or more of the vaccine strains at 29 amino acid residues (75.3%) (Matsuzaki et al., 2014). Furthermore, a comparison within five antigenic sites (A – E) in two H3 strains revealed identity with two vaccine strains at 24/49 (49%) amino acid residues but found differences with one or both vaccine strains at 25/49 (51%) amino acid positions (Underwood, 1982). Comparing the antigenic amino acid positions reported in N1 and N2 strains by Wan et al. (2015) and Venkatramani et al. (2006), respectively, revealed that circulating strains exhibited mutations with the vaccine strains at 13/30 (43.3%) and 5/18 (27.8%) amino acid residues, respectively.

4. Discussion

Influenza A virus (IAV) is one of the most important viral pathogens in swine herds globally and a major cause of acute respiratory disease outbreaks in pigs. A number of studies in the last few years have shown that infections are frequently subclinical, with most farms in Europe being endemically infected by one or more IAV (Van Reeth and Vincent,

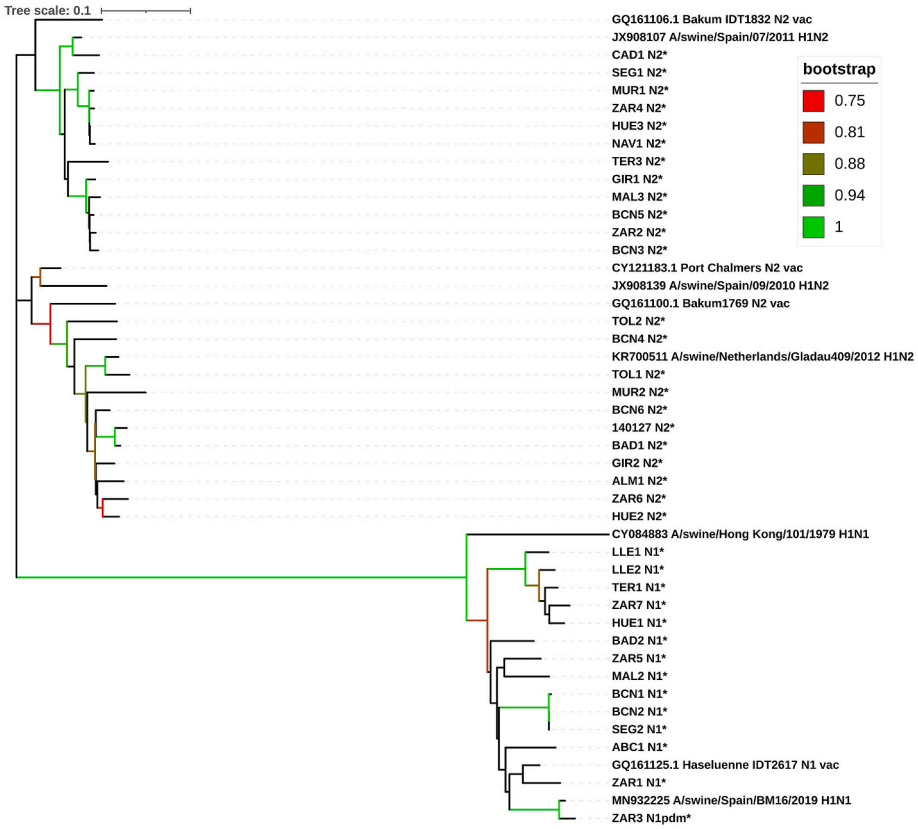


Fig. 2. Neighbor-Joininig tree for neuraminidase (NA) sequences of IAVs collected from Spanish swine farms in 2020 (marked with *) compared to representative swine IAV strains, and subtypes contained in the vaccines available in Spain. Only branches having bootstrap support values over 0.3 are represented in colors different from black.

2019; Li and Robertson, 2021). Results of this study have showed that infection by IAV is prevalent in Spanish swine farms suffering from outbreaks of respiratory disease, since almost 20% samples from 23% farms were reported to be positive using RT-qPCR in a veterinary diagnostic laboratory. A limitation of the study was related to the sampling design and the representativeness of results from different age groups or time intervals, as the samples were directly submitted by veterinarian surgeons from the farms for diagnostic purposes during outbreaks of respiratory diseases. Most submissions corresponded to weaning and specially fattening pigs but IAV infection was much more common before the fattening period, with one in two and one in three samples from suckling and weaning pigs testing positive, respectively. Declining of maternally-derived antibodies has been related to the increase of the circulation of the virus in nurseries although IAV infection can occur in piglets in the presence of colostral-derived antibodies against the subtypes circulating in the farm (Simon-Grifé et al., 2012; Rose et al., 2013). In fact, piglets can be already IAV positive during the first week of life and have been reported to play a central role in maintaining influenza infections in breeding herds and spreading IAVs to other farms if transported to off-site facilities at weaning (Ryt-Hansen et al., 2019; Garrido-Mantilla et al., 2021).

The percentage of positive samples exhibited fluctuations throughout the 12-months period of study, although highest rates of positivity were reported in the hottest months. Previous studies have assessed the seasonality of influenza in swine using samples submitted to diagnostic laboratories in different geographical areas; evidence of seasonal variations of positive IAV submissions was found from some laboratories but not from others (Ferreira et al., 2018). The current study was not designed to determine the prevalence of IAV infection and comparisons with previous surveillance programs is difficult. A large-scale surveillance study conducted between 2015 and 2018 with M-gen RT-qPCR in 17 European countries reported high prevalence values both at animal level (30.5%) and farm level (56.6%), with active IAV infection being not seasonally restricted (Henritzi et al., 2020). A more recent cross-sectional study performed with specimens from pigs aged up to 9 weeks from 12 European countries reported 37.9% samples from 78.6% farms to be IAV-positive with M gen RT-qPCR, with samples from weaners aged 4–6 weeks being more likely to be positive compared to samples from nursery pigs aged 7–9 weeks (Lillie-Jaschniski et al., 2022). A previous study in Spain has shown that more than 93% farms have at least one IAV seropositive animal, with more than 62% pigs being seropositive (Simon-Grifé et al., 2011). Another Spanish study using RT-qPCR with nasal swabs confirmed the presence of IAVs in 68.7% pig farms suffering respiratory disease outbreaks, with most positive submissions corresponding to nurseries (36.5%) (Sosa Portugal et al., 2019).

For subtyping of IAVs, specific primers need to be designed to detect different gene segments, mainly HA and NA, although efficiency of RT-qPCR relies heavily upon these specific primers having no mismatches with target amplicon sequence, with outdated primers resulting in low test sensitivity (Li and Robertson, 2021). In the RT-qPCR protocol by Henritzi et al. (2016), which has been widely used in previous epidemiological surveys, primers were designed specifically for subtyping of IAV strains circulating in Europe. However, our preliminary sequencing assays of RT-PCR-amplified fragments of the HA segment in some specimens suggested that this protocol underestimated the number of samples allocated to the H3 lineage. In this study, new primers with higher homology with target regions within the H3 sequences of Spanish IAVs were designed, and it provided the basis to improve the sensitivity to detect this lineage. Namely, a total of 30 samples were allocated to this lineage using the optimized protocol with these newly designed primers, that also provided a remarkable lower Cq value for the four samples identified as belonging to the H3 lineage with the protocol by Henritzi et al. (2016).

The study confirmed the circulation of viruses from the four main HA subtypes (H1av, H1hu, H1pdm, H3). The estimation of genetic variation within the viral populations showed noticeably higher genetic diversity among the H1 sequences, with the Pi value (average number of pairwise nucleotide differences per site) being approximately 19 times lower among the H3 sequences. H1av was by far the most common lineage, accounting for over half of 108 fully subtyped specimens (53%), with subtype H1avN2 being notably dominant over subtype H1avN1 (42% *versus* 11%, respectively). H1huN2 was the only subtype reported within the H1hu lineage (16%), and pandemic H1 reassortants (H1pdmN2, H1pdmN1, H1pdmN1pdm) showed a detection value of 10%. Finally, a significant proportion of specimens (21%) were identified as H3 viruses, with subtypes H3N2 and H3N1 occurring in a similar number of samples. Comparison with the results of a large-scale study in 17 European countries over a three-year observation period (2015–2108) showed remarkable differences in the distribution of certain lineages and subtypes. The surveillance program also reported H1av to be the most prevalent lineage in Europe with an overall prevalence of 54.2% at the farm level. However, subtype H1avN1av (39.2%) was clearly dominant over H1avN2, which occurred in only 12.6% farms and was sporadically reported in Spain (Henritzi et al., 2020). These authors noticed that subtype H1avN2 was even less detectable in a previous surveillance of the European network for influenza in pigs (ESNIP3) conducted between 2010 and 2013 (Simon et al., 2014). It is also noteworthy to mention differences in the frequency of H3 viruses, which were significantly common in our study (>21%), whereas they were rarely detected (3.9% at farm level) in the European study by Henritzi et al. (2020), with no cases found in Spain.

Monitoring the subtypes of IAVs circulating in the swine population provides valuable information not only for public health surveillance but also for vaccine selection. Vaccination is the most effective intervention against swine influenza, but protection is significantly impacted by antigenic mismatches between vaccine and circulating strains in the HA and, to a lesser extent, NA proteins, especially in inactivated vaccines (Sandbulte et al., 2015; Mancera Gracia et al., 2020). Some studies have shown that amino acid substitutions in the HA are associated with decreased levels of antibody and immune protection afforded by vaccination (Smirnov et al., 2004). The vaccines currently licensed in Spain contain inactivated whole virus of the most common circulating IAV subtypes in Europe: A/swine/Olot/1984 (H1avN1), A/Port Chalmers/1/1973 (H3N2), A/swine/Bakum/IDT1769/2003 (H3N2), A/swine/Haseluenne/IDT2617/2003 (H1N1), A/swine/Bakum/1832/2000 (H1N2), and A/Jena/VI5258/2009 (H1N1 pdm09). All samples analyzed in this study were submitted for the diagnosis of respiratory diseases, and no data about IAV vaccination on the farms were available, which is a limitation. Moreover, few samples from suckling piglets and sows were analyzed in this study. Although amino acid sequence similarity is not always predictive of cross-protection, the comparison of amino acid residues within antigenic positions between circulating and vaccine strains has revealed low similarity for both NA (60–66.6%) and especially HA amino acid residues (33.3–49%). However, further studies including hemagglutination-inhibition assays or neutralizing antibody assays could provide additional insights into the efficacy of commercial vaccines.

5. Conclusion

The results of this study have corroborated that IAVs are prevalent in Spanish swine farms suffering from outbreaks of respiratory disease, with infections being most common among suckling and weaning pigs. Three subtypes (H1avN1, H1huN2 and particularly the major subtype H1avN2) accounted for over half of the specimens, although the use of an optimized protocol with newly designed primers enabled the detection of a significant number of H3 viruses. The comparison of antigenic positions between a limited number of circulating strains and vaccine strains has revealed the presence of substitutions in a significant number of HA and NA amino acid residues, highlighting the need for more comprehensive studies to investigate whether these mutations influence

the level of protection provided by commercial vaccines.

Funding

This research received no external funding.

CRediT authorship contribution statement

Alfredo A. Benito: Methodology, Conceptualization. **Luis V. Monteagudo:** Writing – review & editing, Formal analysis. **Sofía Lázaro-Gaspar:** Methodology, Investigation. **Luna Mazas-Cabetas:** Investigation, Formal analysis. **Joaquín Quílez:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank the Government of Aragon (Spain) for the funding granted to the research group A16_23 R.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2024.110223.

References

Anderson, T.K., Macken, C.A., Lewis, N.S., Scheuermann, R.H., Van Reeth, K., Brown, I. H., Swenson, S.L., Simon, G., Saito, T., Berhane, Y., Ciacci-Zanella, J., Pereda, A., Davis, C.T., Donis, R.O., Webby, R.J., Vincent, A.L., 2016. A phylogeny-based global nomenclature system and automated annotation tool for H1 hemagglutinin genes from swine influenza A viruses. mSphere 1, e00275–16. https://doi.org/10.1128/mSphere.00275-16.

Bonin, E., Quéguiner, S., Woudstra, C., Gorin, S., Barbier, N., Harder, T.C., Fach, P., Hervé, S., Simon, G., 2018. Molecular subtyping of European swine influenza viruses and scaling to high-throughput analysis. Virol. J. 15, 7. https://doi.org/10.1186/s12985-018-0920-z.

Bragstad, K., Jørgensen, P.H., Handberg, K.J., Mellergaard, S., Corbet, S., Fomsgaard, A., 2005. New avian influenza A virus subtype combination H5N7 identified in Danish mallard ducks. Virus Res. 109, 181–190. https://doi.org/10.1016/j.virusres.2004.12.004.

Caton, A.J., Brownlee, G.G., Yewdell, J.W., Gerhard, W., 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31, 417–427. https://doi.org/10.1016/0092-8674(82)90135-0.

Chiapponi, C., Prosperi, A., Moreno, A., Baioni, L., Faccini, S., Manfredi, R., Zanni, I., Gabbi, V., Calanchi, I., Fusaro, A., Beato, M.S., Cavicchio, L., Torreggiani, C., Alborali, G.L., Luppi, A., 2021. Genetic variability among swine influenza viruses in Italy: data analysis of the period 2017–2020. Viruses 14, 47, 10.3390/v14010047.

Encinas, P., Del Real, G., Dutta, J., Khan, Z., van Bakel, H., Del Burgo, M.Á.M., García-Sastre, A., Nelson, M.I., 2021. Evolution of influenza A virus in intensive and free-range swine farms in Spain. Virus Evol 7. https://doi.org/10.1093/ve/veab099 veab099.

Ferreira, J.B., Grgić, H., Friendship, R., Nagy, É., Poljak, Z., 2018. Influence of microclimate conditions on the cumulative exposure of nursery pigs to swine influenza A viruses. Transbound. Emerg. Dis 65, e145–e154. https://doi.org/10.1111/tbed.12701.

Garrido-Mantilla, J., Sanhueza, J., Alvarez, J., Culhane, M.R., Davies, P., Allerson, M.W., Torremorell, M., 2021. Impact of nurse sows on influenza A virus transmission in pigs under field conditions. Prev. Vet. Med. 188, 105257. https://doi.org/10.1016/j.prevetmed.2021.105257.

Henritzi, D., Zhao, N., Starick, E., Simon, G., Krog, J.S., Larsen, L.E., Reid, S.M., Brown, I. H., Chiapponi, C., Foni, E., Wacheck, S., Schmid, P., Beer, M., Hoffmann, B., Harder, T.C., 2016. Rapid detection and subtyping of European swine influenza viruses in porcine clinical samples by haemagglutinin- and neuraminidase-specific tetra- and triplex real-time RT-PCRs. Influenza Other Respir. Viruses 10, 504–517. https://doi.org/10.1111/irv.12407.

Henritzi, D., Petric, P.P., Lewis, N.S., Graaf, A., Pessia, A., Starick, E., Breithaupt, A., Strebelow, G., Luttermann, C., Parker, L.M.K., Schröder, C., Hammerschmidt, B., Herrler, G., Beilage, E.G., Stadlbauer, D., Simon, V., Krammer, F., Wacheck, S., Pesch, S., Schwemmle, M., Beer, M., Harder, T.C., 2020. Surveillance of European domestic pig populations identifies an emerging reservoir of potentially zoonotic swine influenza A viruses. Cell Host Microbe 28, 614–627. https://doi.org/10.1016/j.chom.2020.07.006 e6.

Krammer, F., Smith, G.J.D., Fouchier, R.A.M., Peiris, M., Kedzierska, K., Doherty, P.C., Palese, P., Shaw, M.L., Treanor, J., Webster, R.G., García-Sastre, A., 2018. Influenza. Nat. Rev. Dis. Prim. 4, 3. https://doi.org/10.1038/s41572-018-0002-y.

Kyriakis, C.S., Rose, N., Foni, E., Maldonado, J., Loeffen, W.L.A., Madec, F., Simon, G., Van Reeth, K., 2013. Influenza A virus infection dynamics in swine farms in Belgium, France, Italy and Spain, 2006-2008. Vet. Microbiol. 162, 543–550. https://doi.org/10.1016/j.vetmic.2012.11.014.

Letunic, I., Bork, P., 2021. Interactive Tree of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49 (W1), W293–W296. https://doi.org/10.1093/nar/gkab301.

Li, Y., Robertson, I., 2021. The epidemiology of swine influenza. Anim. Dis 1, 21. https://doi.org/10.1186/s44149-021-00024-6.

Lillie-Jaschniski, K., Lisgara, M., Pileri, E., Jardin, A., Velazquez, E., Köchling, M., Albin, M., Casanovas, C., Skampardonis, V., Stadler, J.A., 2022. New sampling approach for the detection of swine influenza a virus on European sow farms. Vet. Sci. 9, 338. https://doi.org/10.3390/vetsci9070338.

Ma, W., 2020. Swine influenza virus: current status and challenge. Virus Res. 288, 198118. https://doi.org/10.1016/j.virusres.2020.198118.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R.N., Potter, S.C., Finn, R.D., Lopez, R., 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res, 47, W636–W641. https://doi.org/10.1093/nar/gkz268.

Maldonado, J., Van Reeth, K., Riera, P., Sitjà, M., Saubi, N., Espuña, E., Artigas, C., 2006. Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet. J. 172, 377–381. https://doi.org/10.1016/j.tvjl.2005.04.014.

Mancera Gracia, J.C., Pearce, D.S., Masic, A., Balasch, M., 2020. Influenza A virus in swine: epidemiology, challenges and vaccination strategies. Front. Vet. Sci. 7, 647. https://doi.org/10.3389/fvets.2020.00647.

Martín-Valls, G.E., Simon-Grifé, M., van Boheemen, S., de Graaf, M., Bestebroer, T.M., Busquets, N., Martín, M., Casal, J., Fouchier, R.A., Mateu, E., 2014. Phylogeny of Spanish swine influenza viruses isolated from respiratory disease outbreaks and evolution of swine influenza virus within an endemically infected farm. Vet. Microbiol. 170, 266–277. https://doi.org/10.1016/j.vetmic.2014.02.031.

Matsuzaki, Y., Sugawara, K., Nakauchi, M., Takahashi, Y., Onodera, T., Tsunetsugu-Yokota, Y., Matsumura, T., Ato, M., Kobayashi, K., Shimotai, Y., Mizuta, K., Hongo, S., Tashiro, M., Nobusawa, E., 2014. Epitope mapping of the hemagglutinin molecule of A/(H1N1)pdm09 influenza virus by using monoclonal antibody escape mutants. J. Virol. 88, 12364–12373. https://doi.org/10.1128/JVI.01381-14.

Rose, N., Hervé, S., Eveno, E., Barbier, N., Eono, F., Dorenlor, V., Andraud, M., Camsusou, C., Madec, F., Simon, G., 2013. Dynamics of influenza A virus infections in permanently infected pig farms: evidence of recurrent infections, circulation of several swine influenza viruses and reassortment events. Vet. Res. 44, 72. https://doi.org/10.1186/1297-9716-44-72.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E., Sánchez-Gracia, A., 2017. DnaSOv6: DNA sequence Polymorphism Analysis of Large Datasets. Mol. Biol. Evol. 34, 3299–3302. https://doi.org/10.1093/molbev/msx248.

Ryt-Hansen, P., Larsen, I., Kristensen, C.S., Krog, J.S., Wacheck, S., Larsen, L.E., 2019. Longitudinal field studies reveal early infection and persistence of influenza A virus in piglets despite the presence of maternally derived antibodies. Vet. Res. 50, 36. https://doi.org/10.1186/s13567-019-0655-x.

Ryt-Hansen, P., Pedersen, A.G., Larsen, I., Kristensen, C.S., Krog, J.S., Wacheck, S., Larsen, L.E., 2020. Substantial antigenic drift in the hemagglutinin protein of swine influenza A viruses. Viruses 12, 248, 10.3390/v12020248.

Sandbulte, M.R., Spickler, A.R., Zaabel, P.K., Roth, J.A., 2015. Optimal use of vaccines for control of influenza A virus in swine. Vaccines (Basel) 3, 22–73. https://doi.org/10.3390/vaccines3010022.

Simon, G., Larsen, L.E., Dürrwald, R., Foni, E., Harder, T., Van Reeth, K., Markowska-Daniel, I., Reid, S.M., Dan, A., Maldonado, J., Huovilainen, A., Billinis, C., Davidson, I., Agüero, M., Vila, T., Hervé, S., Breum, S.Ø., Chiapponi, C., Urbaniak, K., Kyriakis, C.S., Esnip3 consortium, Brown, I.H., Loeffen, W., 2014. European surveillance network for influenza in pigs: surveillance programs, diagnostic tools and Swine influenza virus subtypes identified in 14 European countries from 2010 to 2013. PLoS One 9, e115815. https://doi.org/10.1371/journal.pone.0115815.

Simon-Grifé, M., Martín-Valls, G.E., Vilar, M.J., García-Bocanegra, I., Mora, M., Martín, M., Mateu, E., Casal, J., 2011. Seroprevalence and risk factors of swine influenza in Spain. Vet. Microbiol. 149, 56–63. https://doi.org/10.1016/j.vetmic.2010.10.015.

Simon-Grifé, M., Martín-Valls, G.E., Vilar, M.J., Busquets, N., Mora-Salvatierra, M., Bestebroer, T.M., Fouchier, R.A., Martín, M., Mateu, E., Casal, J., 2012. Swine influenza virus infection dynamics in two pig farms, results of a longitudinal assessment. Vet. Res. 43, 24. https://doi.org/10.1186/1297-9716-43-24.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Douglas, A.R., Knossow, M., Wilson, I.A., Wiley, D.C., 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA 81, 1779–1783. https://doi.org/10.1073/pnas.81.6.1779.

Smirnov, Y.A., Gitelman, A.K., Govorkova, E.A., Lipatov, A.S., Kaverin, N.V., 2004. Influenza H5 virus escape mutants: immune protection and antibody production in mice. Virus Res. 99, 205–208. https://doi.org/10.1016/j.virusres.2003.11.012.

Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S., Peiris, J.S., Guan, Y., Rambaut, A., 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459, 1122–1125. https://doi.org/10.1038/nature08182.

Sosa Portugal, S., Cortey, M., Tello, M., Casanovas, C., Mesonero-Escuredo, S., Barrabés, S., Pineda, P., Wacheck, S., Martín-Valls, G., Mateu, E., 2019. 2021. Diversity of influenza A viruses retrieved from respiratory disease outbreaks and subclinically infected herds in Spain, 2017- Transbound. Emerg. Dis 68, 519–530. https://doi.org/10.1111/tbed.13709.

Underwood, P.A., 1982. Mapping of antigenic changes in the haemagglutinin of Hong Kong influenza (H3N2) strains using a large panel of monoclonal antibodies. J. Gen. Virol. 62, 153–169. https://doi.org/10.1099/0022-1317-62-1-153.

Van Reeth, K., Brown, I.H., Dürrwald, R., Foni, E., Labarque, G., Lenihan, P., Maldonado, J., Markowska-Daniel, I., Pensaert, M., Pospisil, Z., Koch, G., 2008. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002-2003. Influenza Other Respir. Viruses 2, 99–105. https://doi.org/10.1111/j.1750-2659.2008.00043.x.

Van Reeth, K., Vincent, A.L., 2019. Influenza virus. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W., Zhang, J. (Eds.), Diseases of Swine, 11th Ed. Wiley Blackwell, pp. 576–593.

Venkatramani, L., Bochkareva, E., Lee, J.T., Gulati, U., Graeme Laver, W., Bochkarev, A., Air, G.M., 2006. An epidemiologically significant epitope of a 1998 human influenza virus neuraminidase forms a highly hydrated interface in the NA-antibody complex. J. Mol. Biol. 356, 651–663. https://doi.org/10.1016/j.jmb.2005.11.061.

Wan, H., Yang, H., Shore, D.A., Garten, R.J., Couzens, L., Gao, J., Jiang, L., Carney, P.J., Villanueva, J., Stevens, J., Eichelberger, M.C., 2015. Structural characterization of a protective epitope spanning A(H1N1)pdm09 influenza virus neuraminidase monomers. Nat. Commun. 6, 6114. https://doi.org/10.1038/ncomms7114.

Wiley, D.C., Wilson, I.A., Skehel, J.J., 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289, 373–378. https://doi.org/10.1038/289373a0.

Zou, S., 1997. A practical approach to genetic screening for influenza virus variants. J. Clin. Microbiol. 35, 2623–2627. https://doi.org/10.1128/jcm.35.10.2623-2627.1997.