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Two years of surveillance of influenza a virus infection in a swine herd. Results of virological, serological and pathological studies

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ABSTRACT

Swine farms provide a dynamic environment for the evolution of influenza A viruses (IAVs). The present report shows the results of a surveillance effort of IAV infection in one commercial swine farm in Argentina. Two cross-sectional serological and virological studies (n = 480) were carried out in 2011 and 2012. Virus shedding was detected in nasal samples from pigs from ages 7, 21 and 42-days old. More than 90% of sows and gilts but less than 40% of 21-days old piglets had antibodies against IAV. In addition, IAV was detected in 8/17 nasal swabs and 10/15 lung samples taken from necropsied pigs. A subset of these samples was further processed for virus isolation resulting in 6 viruses of the H1N2 subtype (δ2 cluster). Pathological studies revealed an association between suppurative bronchopneumonia and necrotizing bronchiolitis with IAV positive samples. Statistical analyses showed that the degree of lesions in bronchi, bronchiole, and alveoli was higher in lungs positive to IAV. The results of this study depict the relevance of continuing long-term active surveillance of IAV in swine populations to establish IAV evolution relevant to swine and humans.

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1. Introduction

Influenza A virus (IAV) infection is one of the major causes of acute respiratory disease outbreaks in pigs [1]. IAVs of H1N1, H3N2, and H1N2 have been commonly detected in commercial swine populations around the world including Argentina [1–3]. Within each of these subtypes, numerous antigenic and reassortant variants are found. Reassortment is frequent among not only IAVs of swine but also with IAVs from other sources, particularly human and occasionally avian origin. Perpetuation of these viruses in the pig population is accompanied by further reassortment, antigenic shift and/or drift [4,5].

Introduction of a new IAV in a swine herd typically produces an epidemic [2] followed by endemic and/or subclinical infections that can persist for long periods of time [3,6,7]. The pandemic H1N1 virus in 2009 (H1N1p) likely originated in swine and contained a constellation of gene segments derived from multiple reassortment events involving swine-, human-, and avian-origin IAV strains. The subsequent spillover of such virus back into pigs, had led to more intensive swine influenza surveillance efforts worldwide, particularly in commercial swine operations [1,8]. Several studies have suggested that influenza infection is far more common than suggested by confirmed clinical outbreaks [3,6,7,9]. IAV persistence in endemically infected herds is not well understood [7]. Most studies have evaluated IAV in swine focusing on dynamics of infections or detection of new reassortant strains. Few studies have considered the status of IAV infection at a single farm level. There are no reports (to our knowledge) of studies aimed at understanding the endemic nature of IAV infection in pigs over time [10,11]. Such studies allow for a better understanding of virus evolution in a defined setting [12]. The present report represents a two-year IAV surveillance

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effort in a commercial swine farm in Argentina and from which virological, serological, and pathological findings are described.

2. Materials and methods

2.1. Farm description

The farm is a closed, all-in-all-out operation with three sites and 6000-sow herd located in Buenos Aires Province, Argentina. Pigs were moved from the farrowing barns to the nursery at a mean weaning age of 21 days. Each nursery barn was filled in a week, with an average of 3000 pigs each. At 70 days-old, pigs were moved to the finishing facilities located about 1 km away. The farm has remained free of Aujeszky disease virus and *Actinobacillus pleuropneumoniae* infections. Argentina is free of Porcine Reproductive and Respiratory Syndrome virus. Vaccines against influenza were not licensed for use in Argentina at the time of the study.

2.2. Cross-sectional studies

Two cross-sectional serological and virological studies were implemented, one in April 2011 and the other in December 2012. Sample number was defined using the Epi Info™ software package (CDC, Atlanta, GA, USA). The sample number allows for detection of at least 1 IAV positive sample and was calculated for a population of ≥1000 animals with an estimated prevalence between 5 and 20% (95% confidence). Thus, for each cross-sectional study, 240 blood samples and nasal swabs were obtained from sows (n = 15), gilts (n = 15) and pigs from defined ages (n = 30 each from 7, 21, 42, 63, 77, 100 and 140-days old). With the aim of increasing the likelihood of IAV detection in each group, clinically affected animals were sampled. When less than 30 clinically affected animals were identified in a group, random sampling of clinically healthy animals was performed to achieve the target sample size. Anti IAV responses were evaluated by ELISA against nucleoprotein (ID Screen® influenza A Antibody Competition Multi-Species, Montpellier, France).

2.3. Pathological studies

Necropsies were performed in 163 pigs of nursery, growing, and fattening stages submitted for post-mortem diagnosis to the *Laboratorio de Patología Especial, Facultad de Ciencias Veterinarias, La Plata, Argentina,* between April 2011 and December 2012. From those cases with pneumonic lesions (n = 49) lung lesions were categorized based on morphologic changes as suppurative bronchopneumonia, pleuritis, embolic pneumonia or edema [13,14].

2.4. Histopathology and immunohistochemistry

In addition to the suspected cases received in 2011 and 2012, a retrospective histopathological study was performed in another 46 lung samples with pneumonic lesions processed since 2008. Lung lesions were always examined microscopically by the same pathologist. In each slide, pleura, connective tissue, bronchi, 10 randomly selected bronchioli and 5 fields of alveoli at 20X magnification were analyzed. Severity was assessed based on the degree of lesion (from 0 to 3) observed at each structure. Grade 0 represents no lesions; grade 1, only mild inflammatory changes (occasional necrosis and small amounts of neutrophils and mucus); grade 2, moderate inflammatory cells and focal necrosis; grade 3, severe inflammatory changes, complete epithelial necrosis and thrombi. In addition, histopathological diagnosis was made according to the morphologic pattern in: bronchitis/bronchiolitis, suppurative bronchopneumonia, fibrinous bronchopneumonia, fibrino-suppurative bronchopneumonia, interstitial pneumonia, bronchointerstitial pneumonia, embolic pneumonia, congestion and edema or pleuritis [13,14]. Immunohistochemistry (IHC) against nucleoprotein of IAV was carried-out as described previously [15] in 25 selected cases. Selection criteria were based on histopathological diagnosis and presence of necrotizing bronchiolitis suggestive of IAV.

2.5. IAV detection by rRT-PCR and virus isolation

Nasal samples were individually collected with Dacron swabs and stored in viral transport medium (1 ml of phosphate buffered saline plus penicillin 10,000 IU/ml, streptomycin 10,000 μg/ml, and albumin 25 mg/ml). Pooled nasal swabs samples (n ≤ 6) from pigs from a single age group were used for virus detection by rRT-PCR.

Lung samples were collected in sterile plastic containers and processed individually. Nasal swabs were collected and processed as was above mentioned. In both cases viral RNA (vRNA) was extracted from pooled nasal swabs and lung macerate supernatant using a QIAamp® Viral RNA Mini kit (Qiagen, Hilden, Germany). Purified vRNA was subjected to rRT-PCR to amplify 60 base pairs of the matrix (M) vRNA segment using the primer pair InfAfw (5′-GACCRATCCTGTCACCTCTGAC-3′) and InfArv (5′-AGGGCATTYTGGACAAAKCGTCTA-3′) and the probe InfA TGCAGTCCTCGCTCACTGGGCACG. The rRT-PCR was performed in an ABIPrism® 7500 SDS apparatus (Applied Biosystems™, Foster City, CA, USA). Samples corresponding to the rRT-PCR positive pools were further processed for virus isolation in Madin-Darby Canine Kidney cells (MDCK) as described previously [3]. vRNA was extracted from the positive culture supernatant and used to PCR amplify the HA, NA and M gene segments. Sequencing was performed using a BigDye® Terminator Kit (Applied Biosystems™, Foster City, CA, USA) on an ABI 3500 (Applied Biosystems™) using primers described by Hoffman [16]. Sequences were edited and analyzed with BioEdit© (Ibis Biosciences, Carlsbad, CA, USA). The HA, NA and M gene segments of each isolate were used for BLAST analyses (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to identify the most closely related IAV for each segment.

2.6. Statistical analysis

The degree of histopathological lesion related to IAV infection was analyzed in 37 lung cases. Based on IHC or rRT-PCR results cases were classified in positive (n = 21) or negative (n = 16) to IAV. A non-parametric test was applied due to lack of normal distributions. Differences in degree of lung lesions between positive and negative cases were analyzed using the Kolmogorov-Smirnov test. The relationship between histopathological diagnosis and presence of necrotizing bronchiolitis and IAV positive cases were analysis by Chi-square test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Variations in incidence of exposure to IAV in pigs based on aged and year of study

The swine farm under study had a prior history of exposure and circulation of IAV. In 2008, the farm was positive for IAV where a wholly human-origin H3N2 virus was isolated from 40 to 50 days old pigs [8]. In October 2009, a novel IAV was identified, a reassortant with HA and NA gene segments from an H1N1 of the δ2 cluster and internal gene segments from an H1N1p virus [17]. Thereafter, recurrent influenza-like illness were observed, particularly in pigs in the post-weaning period. These observations triggered the two-cross sectional studies presented in this report. The first study was performed in April 2011 and the second study was performed in December 2012 (Fig. 1). It must be noted that IAV in commercial swine does not follow the type of seasonality seen with IAV

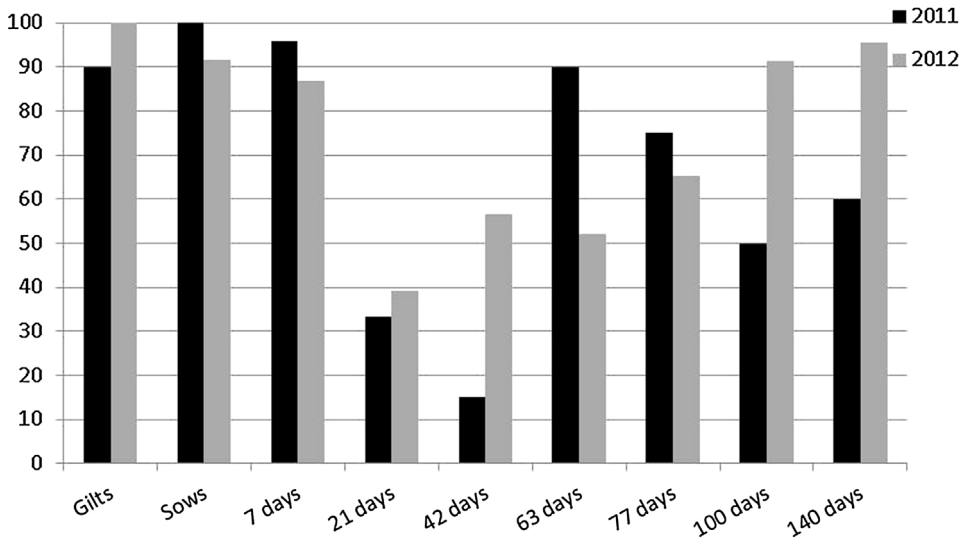

Fig. 1. Percentage of positive pigs to IAV at each sampled age in both cross-sectional serological studies.

Table 1
Results of two cross-sectional studies for detection of IAV in swine by serology and virus detection by rRT-PCR.

Year	Sample type	Site 1				Site 2		Site 3			Total
		Gilts	Sows	7[a]	21	42	63	77	100	140	
		NP ELISA results in pig sera – N° positive/total per age group (% positive)									
2011	Sera	14/15 (93)	15/15 (100)	29/30 (96)	10/30 (33)	5/30 (17)	27/30 (90)	23/30 (77)	15/30 (50)	18/30 (60)	156/240 (65)
2012	Sera	15/15 (100)	14/15 (93)	26/30 (87)	12/30 (40)	17/30 (57)	16/30 (53)	20/30 (67)	28/30 (93)	29/30 (97)	177/240 (74)
		rRT-PCR results in pig nasal swabs – N° positive/total per age group (% positive)									
2011	Nasal swabs	0/15 (0)	0/15 (0)	0/30 (0)	6/30[b] (20)	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	6/240 (2.5)
2012	Nasal swabs	0/15 (0)	0/15 (0)	8/30[c] (27)	0/30 (0)	3/30[d] (10)	0/30 (0)	0/30 (0)	0/30 (0)	0/30 (0)	11/240 (4.5)

[a] Pig age in days.
[b] A/swine/Argentina/CIP051-A160/2011 (H3N2). Genbank #: KC876550; KC876547; KC876544.
[c] A/swine/Argentina/CIP051-C_{02}·M_1.1/2012 (H1N2) (δ2). Genbank #: KR863479; KR863480; KR863481.
[d] A/swine/Argentina/CIP051-C_{02}·M_1.5/2012 (H1N2) (δ2). Genbank #: KR863420; KR863421; KR863422.

in humans. Instead, IAV activity in swine is typically associated with changes in the production cycle and the transition of pigs from nursery to growing and fattening sites. In both cross-sectional, serological studies showed that more than 90% of gilts (14/15) and sows (14/15) had prior exposure to IAV (Table 1). In 7 days old piglets the percentage of seropositives remained high (>85%, 29/30 in 2011 and 26/30 in 2012). Immediately after weaning, 21 day old piglets, <40% (10/30 in 2011 and 12/30 in 2012) had maternal antibodies against IAV. Contrasting observations were made in 42 days old pigs with <20% (5/30) showing IAV antibodies in 2011, whereas >50% (17/30) were positive in 2012. From 63 to 140 days old, end of nursery and growing and fattening periods, the percentage of positive pigs ranged from 50% to 95%. In the study of 2011, the number of seropositive pigs was highest in the 63 days old group (27/30, 90% of positive pigs), progressively decreasing with age (50–60% in 100 and 142 days old pigs, respectively). In contrast, in the study of 2012, seroconversion progressively increased with age from 53% (63 days old pigs, 16/30) to 97% (142 days old pigs, 29/30).

3.2. No correlation between clinical signs, serological status, and IAV detection

No correlation between clinical signs, serological status, and virus detection was observed (Table 1). In the study of 2011, IAV was detected by rRT-PCR only in the 21 days old pigs (20%, 6/30). Despite the fact that the 42 days old pigs showed the more severe clinical respiratory signs and the fewer pigs with anti-IAV antibodies, no virus was detected in this group. The results of the study of 2012 revealed virus detection in the group of 7- (8/30) and 42-days (3/30) old pigs, with the latter group once again showing the most clinical respiratory signs. Virus characterization revealed a H3N2 subtype in 2011 and an H1N2 δ2 subtype in 2012.

In addition to the nasal swab samples from the two cross-sectional studies, from May 2011 to December 2012, additional samples from suspected IAV cases observed at nursery stage were received and characterized from the same farm: 8/17 nasal swabs and 10/15 lung samples processed were positive by rRT-PCR. From these samples 4 viruses were isolated and characterized as a H1N2 δ2 related to non-contemporary human IAV (Table 2).

BLAST analysis revealed that the HA gene of these viruses were similar to IAV of human lineages previously isolated in the farm under study: H3 (A/swine/Argentina/CIP051-A2/2008) and H1 (A/swine/Argentina/CIP051-BsAs76/2009). The NA gene analysis showed more than 99% of nucleotide identity with human N2 (A/swine/Argentina/CIP051-A2/2008), whereas the M gene of all isolates were related with pandemic IAV (A/Singapore/GP875/2009 and A/Finland/728/2010).

Table 2
summary of necropsy, histopathological, immunohistochemical and rRT-PCR results.

	2008–2011	2011	2012	Total
Macroscopic Lung lesions				
Bronchopneumonia	NA	17	16	33
Circulatory changes	NA	10	0	10
Pleuritis	NA	2	2	4
Embolic pneumonia	NA	2	0	2
Histopathology				
Suppurative bronchopneumonia	14	3	8	25
Bronchitis/bronchiolitis	5	8	1	14
Circulatory changes	5	6	0	11
Interstitial pneumonia	5	2	2	9
Pleuritis	4	2	2	8
Fibrinosuppurative bronchopneumonia	4	1	1	6
Others	9	9	4	22
Immunohistochemistry				
Positive/total	7/15	1/3	3/7	11/25
IAV detection (rRT-PCR)				
Lung (positive/total)	NA	5/13[a]	3/4[b,c,d]	8/17
Nasal swab (positive/total)	NA	2/7	8/8	10/15

a: A/swine/Argentina/CIP051-A199/2011 (H1N2 δ2). Accession numbers: KR863473; KR863474; KR863475. b: A/swine/Argentina/CIP051-A241.2/2012 (H1N2 δ2). Accession numbers: KR863426; KR863427; KR863428. c: A/swine/Argentina/CIP051-A241.9/2012 (H1N2 δ2). Accession numbers: KR863423; KR863424; KR863425. d: A/swine/Argentina/CIP051-C_{05}·M_{21}/2012 (H1N2 δ2). Accession numbers: KR863393; KR863394; KR863395.

3.3. Association between lung lesions and IAV infection

Necropsies were performed in 163 pigs found dead at different stages of the production cycle (nursery, growing and fattening stages), during Fall, Spring and Summer seasons of 2011 and 2012. From these, 49 (30%) had macroscopic lung lesions. The most common pattern of lung lesion was bronchopneumonia (n = 33, 67%). Other lung lesion patterns observed were edema (n = 10, 20%), pleuritis (n = 4, 8%), and embolic pneumonia (n = 2, 4%). By rRT-PCR and IHC, 11 samples (22%) were positive to IAV. Histopathological studies of these 49 lung samples were complemented with similar studies on 46 lung samples obtained from the same farm for the period 2008–2010. Histopathological analyses of both sets of samples revealed that the most common patterns of lesions were suppurative bronchopneumonia (n = 25, 26%) followed by bronchitis/bronchiolitis (n = 14, 15%). Others patterns of lesions observed included circulatory changes (n = 11, 12%), interstitial or bronchointerstitial pneumonia (n = 9, 10%), pleuritis (n = 8, 7%), and fibrinosuppurative bronchopneumonia (n = 6, 6%). Necrotic bronchiolitis characteristic of viral lung infection was more frequently observed in IAV positive pigs (n = 20, 54%) than in IAV negative pigs (n = 10, 27%). Likewise, suppurative bronchopneumonia was more commonly observed in cases associated with positive IAV detections (n = 24, 50%) than in cases with negative IAV results (n = 7, 20%). Statistical associations ($p < 0.05$) between necrotizing bronchiolitis (χ^2, 7.49) and suppurative bronchopneumonia (χ^2, 5.51) with IAV positive cases were detected.

Results of the Kolmogorov-Smirnov test (Table 3) detected statistical differences between positive and negative groups at each lung structure analyzed. The degree of lesion observed in bronchi, bronchioli and alveoli was higher in positive IAV cases while pleura and connective tissue were less compromised than in IAV negative cases ($p < 0.05$).

Distribution of viral antigen in lung samples differed depending on the IAV strain identified during infection (Fig. 2). In H1N2 δ2-associated infections, viral antigen positive cells were observed in the epithelium of small and medium size bronchioli with little staining observed in the large bronchioli and bronchi. In the alveoli, both walls and lumen showed moderate viral antigen staining in macrophages. H3N2-associated infections showed positive immunostaining mainly in the epithelium of the small bronchioles and cells shed into the lumen. In bronchi, few positive cells were detected and were slightly more frequently observed in cells found in the airway lumen. Epithelial cells in damaged submucosal glands were intensely marked. Occasionally, neumocytes and alveolar macrophages were positive for viral antigen in H3N2-associated samples. Whether such differences are strain-specific differences or simply related to distinct NA subtypes in each of these virus infections, it remains to be elucidated.

4. Discussion

In Argentina, recurrent IAV outbreaks have been frequently reported by swine practitioners and owners since 2009. In these farms, IAV infections are considered a nursery problem mostly affecting successive batches [3]. Previous serological and virological characterization studies showed that influenza virus strains that circulate in pigs in Argentina are not related to those reported in pigs elsewhere in the world with the exception of the H1N1p virus [3,8,17]. Upon independent multiple introductions of the H1N1p virus in pig populations around the world, multiple reassortment events have been and continue to take place with previously circulating swine IAV strains [17–20]. These novel reassortant viruses have been most commonly reported in single sampling occasions with limited studies looking at their perpetuation over time [15,17,18,21]. Co-circulation of different IAV subtypes has been reported [7,11], which increases the probability of emergence of novel reassortant viruses that can be either more virulent to swine or carry zoonotic potential [11]. Long-term surveillance studies in swine farms have not been consistently performed and thus it was the goal of this present report.

The pattern of seropositive animals observed in both cross-sectional serological studies was different, in agreement with previous studies [22]. The presence of an immunologically heterogeneous swine population in farms with age segregated systems, as in this report, could lead to persistence IAV infection. The detection of IAV in piglets at 7 and 21 days old suggest susceptibility to IAV infection despite presence of maternal antibodies, which is in agreement with previous studies [11,23]. Infections in young piglets could be due to either suboptimal levels of maternal antibodies and/or challenge with heterologous IAV viruses [10,24–27]. IAV-infected pigs can shed virus for long periods of time even in the presence of maternal antibodies [23].

Table 3
Kolmogorov-Smirnov test applied at positive and negative IAV cases in order to detect differences in degree of lesion at different lung structures.

Lung structure	N° Positive	N° Negative	Rank average positive	Rank average negative	Kolmogorov-Smirnov
Bronchi	53	36	53.90	31.88	3.41*
Bronchioli	210	160	219.11	141.31	6.64*
Alveoli	105	80	103.85	78.75	4.58*
Pleura	21	16	17.47	21	1.72*
Connective tissue	21	16	15.71	23.31	2.34*

* $p < 0.05$.

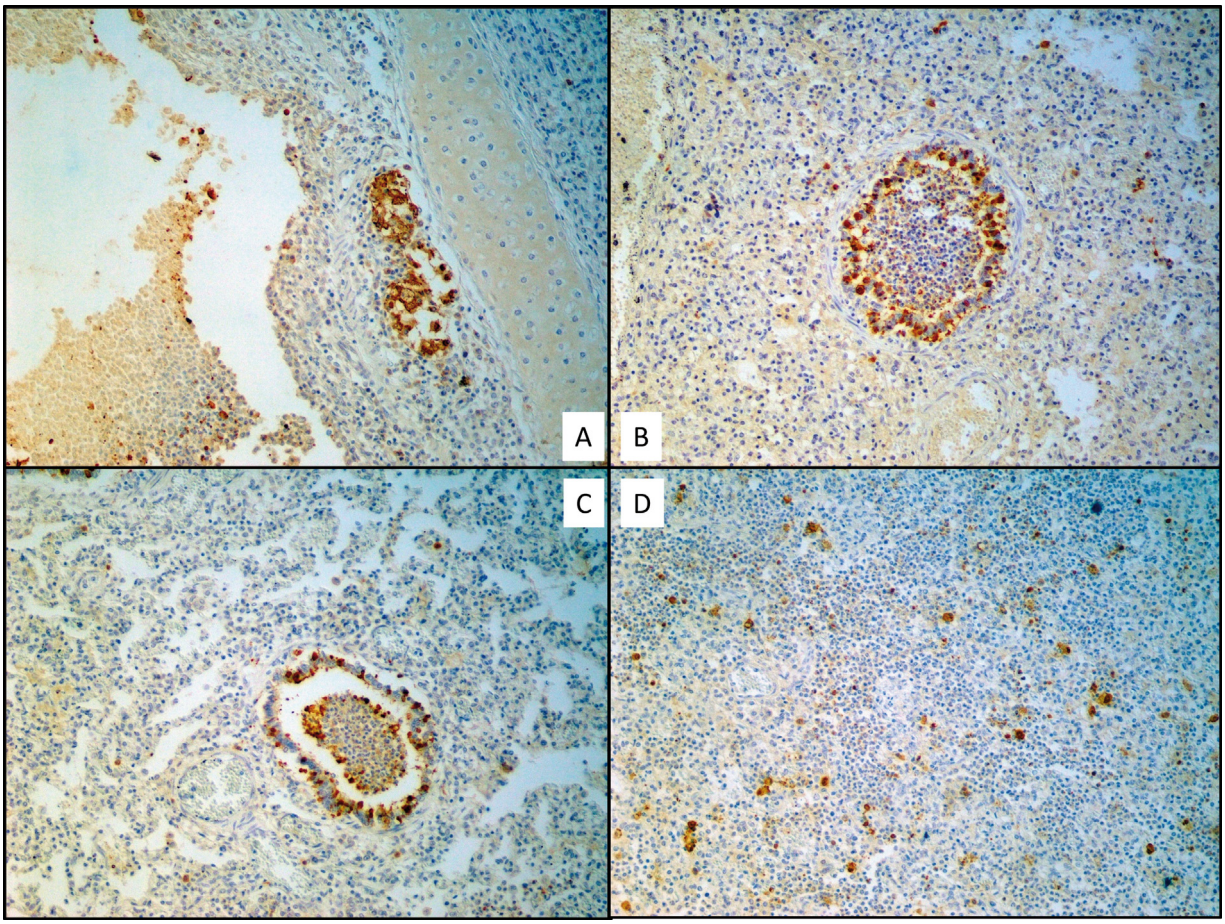

Fig. 2. Immunohistochemical images of lungs positive to H3N2 and δ2H1N2 subtypes.
A) High number of epithelia cells of submucosal gland of large bronchi stained for viral antigen. Subtype H3N2. Obj. 20X B) Intense immunostaining observed in lining epithelium and necrotic debris in airway lumen. Subtype δ2H1N2. Obj. 20X C) Positive cells observed in the epithelium of medium size bronchioli and necrotic debris. Subtype: H3N2. Obj. 20X D) Few positive cells detected in the alveolar spaces and septum walls. Subtype H3N2. Obj. 20X.

The predominance of bronchopneumonia was consistent with previous studies [29]. Suppurative pneumonia could be the result of high levels of cytokines secreted by IAV-infected epithelial and/or macrophages cells [30,31]. Studies have described the histopathological lesions in IAV-infected swine, either naturally or experimentally [3,8,15], and in which a consistent pattern of bronchial and bronchiolar epithelia were shown to be the main targets of IAV [30,32,33]. However, it must be noted that we cannot rule out the possibility that other concomitant bacterial or viral factors may have contributed to the lesion patterns observed under field conditions.

The IAV-antigen positive group showed higher degree of histopathological lesions in bronchi, bronchiole, and alveoli when compared to the IAV-negative group. Previous studies have suggested that the release of pro-inflammatory cytokines associated with viral infection play a role in the severity of lesions in the lung [30,31,33]. However, potential differences in virulence among different IAV subtypes and the low number of positive lung samples to each IAV subtype detected precludes further conclusions.

The viral antigen labelling of epithelium of submucosal glands in H3N2 positive lungs differs with previous reports in which a predominantly bronchiolar or alveolar localization was observed [30]. Differences in labelling related to H3N2 could be associated with early infection steps or the severity of the infection related to these field samples [33].

Persistence IAV infection in swine farms has been recently described in Spain and North American [7,9,34]. The farm under study in this report has shown persistent IAV infection since its first detection in 2008. Just like the report from Spain, IAV persistence is not due to a single IAV strain. Instead, multiple IAV subtypes were detected with evidence of underlying reassortment. The emergence of viruses containing gene segments from the H1N1p strain, particularly the matrix gene segment has been associated with high transmission efficiency [36]. Endemic IAV infection in a farm pro-

vides the base population to generate antigenic drift and/or shift and further IAV evolution. From a public health perspective, it is essential to determine the evolution of IAV in swine due to the risk of reintroduction of novel viruses into the human population [17,20,21]. This study further emphasizes the importance of maintaining continuous IAV surveillance systems.

Finally, the persistence of multiple lineages/subtypes of IAV in a single farm would undercut vaccination strategies if based on a single antigen/subtype. It reinforces the need for a comprehensive understanding of circulating viruses and the development of vaccines base exclusively on locally circulating strains.

Conflict of interest statement

The authors have no conflict of interest.

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