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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 ($\delta$2 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.



## 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