# Co-infection of classic swine H1N1 influenza virus in pigs persistently infected with porcine rubulavirus

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## A R T I C L E   I N F O

## A B S T R A C T

Porcine rubulavirus (PorPV) and swine influenza virus infection causes respiratory disease in pigs. PorPV persistent infection could facilitate the establishment of secondary infections. The aim of this study was to analyse the pathogenicity of classic swine H1N1 influenza virus (swH1N1) in growing pigs persistently infected with porcine rubulavirus. Conventional six-week-old pigs were intranasally inoculated with PorPV, swH1N1, or PorPV/swH1N1. A mock-infected group was included. The co-infection with swH1N1 was at 44 days post-infection (DPI), right after clinical signs of PorPV infection had stopped. The pigs of the co-infection group presented an increase of clinical signs compared to the simple infection groups. In all infected groups, the most recurrent lung lesion was hyperplasia of the bronchiolar-associated lymphoid tissue and interstitial pneumonia. By means of immunohistochemical evaluation it was possible to demonstrate the presence of the two viral agents infecting simultaneously the bronchiolar epithelium. Viral excretion of PorPV in nasal and oral fluid was recorded at 28 and 52 DPI, respectively. PorPV persisted in several samples from respiratory tissues (RT), secondary lymphoid organs (SLO), and bronchoalveolar lavage fluid (BALF). For swH1N1, the viral excretion in nasal fluids was significantly higher in single-infected swH1N1 pigs than in the co-infected group. However, the co-infection group exhibited an increase in the presence of swH1N1 in RT, SLO, and BALF at two days after co-infection. In conclusion, the results obtained confirm an increase in the clinical signs of infection, and PorPV was observed to impact the spread of swH1N1 in analysed tissues in the early stage of co-infection, although viral shedding was not enhanced. In the present study, the interaction of swH1N1 infection is demonstrated in pigs persistently infected with PorPV.

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## 1. Introduction

Respiratory diseases in pigs are considered a primary health problem and are responsible for great economic losses in the worldwide swine industry (Sørensen et al., 2006). Viruses that cause respiratory disease and pneumonia in growing pigs include the porcine reproductive and respiratory syndrome virus

(PRRSV), swine influenza virus (SIV), pseudorabies virus (PRV), porcine rubulavirus (PorPV), porcine circovirus type-2 (PCV-2), and porcine respiratory coronavirus (PRCV). All these viral agents can act individually or through interaction with each other, and associations with other infectious agents of bacterial origin can also occur (Choi et al., 2003; Deblanc et al., 2012; Grau-Roma and Segales, 2007; Kirkland and Stephano, 2006; Morin et al., 1990; Rivera-Benitez et al., 2013a; Segales et al., 1997). PorPV is the etiological agent of blue-eye disease (BED) in pigs. This disease remains endemic in Mexico and has only been diagnosed in that country (Escobar-Lopez et al., 2012; Stephano et al., 1988). The virus genome is composed of single-stranded negative-sense RNA. The virus belongs to the family *Paramyxoviridae* (Wang et al., 2011). In growing pigs, the infection becomes established predominantly in the respiratory tract and the central nervous system. In lungs, interstitial pneumonia has been described, and an increase in respiratory signs was observed in experimental infection (Reyes-Leyva et al., 2004; Reyes-Leyva et al., 2002; Rivera-Benitez et al., 2013a; Stephano et al., 1988). Acute swine influenza virus infection causes interstitial pneumonia and bronchiolitis, with cough, dyspnea, fever, and lethargy as clinical manifestations, although recovery is usually rapid. The common subtypes circulating in swine have been characterised as H1N1, H1N2, and H3N2 (Brookes et al., 2009; Olsen et al., 2006). SIV has been associated with porcine respiratory disease complex (PRDC) in growing or fattening pigs (10–22 weeks of age), while interactions with *Mycoplasma hyopneumoniae*, PRRS, and PCV-2 (Deblanc et al., 2012; Van Reeth et al., 1996, 2001 Yazawa et al., 2004) have been widely studied. There are no previous studies of experimental or natural co-infection of PorPV and SIV. However, in the swine farms in the central and western-central regions of Mexico, seropositivity (circulating antibodies specific against SIV and PorPV) in growing pigs is common. These regions are the most important swine production regions in Mexico (Avalos et al., 2011; Bobadilla et al., 2010; Escobar-Lopez et al., 2012). Primary infection with PorPV is common in pigs under field conditions, and it may become persistent (Cuevas et al., 2009; Wiman et al., 1998). As a consequence, persistently infected pigs have greater susceptibility to secondary infections, and these infections may become exacerbated. The objective of this study was to analyse the pathogenicity of experimental co-infection with porcine rubulavirus and classic swine H1N1 influenza virus in growing pigs.

## 2. Materials and methods

### 2.1. Viruses and cells

The PorPV PAC-3 strain was used (Jalisco/1992; GenBank access number: EF413173) (Ramirez-Mendoza et al., 1997). The viral stock was multiplied in the MDCK cell line (*Madin-Darby Canine Kidney*). The PAC-3 strain of PorPV has been shown to cause respiratory disease and clinical presentations in experimentally infected growing pigs (Rivera-Benitez et al., 2013a). In this study, for co-infection, the A/Swine/New Jersey/11/76 (H1N1) strain (swH1N1) (GenBank access number: K00992–M57477) was used. The swH1N1 viral stock was propagated in MDCK cell cultures and in the allantoic cavity of 9-day-old embryonated chicken eggs. The A/Swine/New Jersey/11/76H1N1 strain was isolated from an outbreak of swine influenza in the United States and is considered the classic North American prototype that infects pigs (Kendal et al., 1977). In both cases, the viral stocks were titrated in cell cultures, and the Reed and Muench method was used to calculate the titre; the obtained titres were expressed as the 50% tissue culture infectious dose (TCID$_{50}$).

**Table 1**
Experimental design.

| Group | No. of pigs | Inoculation | | Necropsy (day)[a] |
|---|---|---|---|---|
| | | Day 0 | Day 44 | |
| PorPV/Mock | 6 | PorPV | MEM | 46, 52 |
| PorPV/swH1N1 | 6 | PorPV | swH1N1 | 46, 52 |
| Mock/swH1N1 | 6 | MEM | swH1N1 | 46, 52 |
| Mock/Mock | 6 | MEM | MEM | 46, 52 |

[a] The necropsies were performed on 3 pigs each day. Pigs were 6 weeks old at day zero. Inoculations were made intranasally.

### 2.2. Animals and experimental design

Twenty-four 6-week-old crossbred conventional pigs were obtained from a PorPV- and SIV-free commercial farm. When the pigs arrived, their nasal swabs were subjected to real-time RT-PCR to confirm that the pigs were negative for PorPV and SIV infection. The pigs were housed in experimental isolation units at the *Departamento de Medicina y Zootecnia de Cerdos* of the *Facultad de Medicina Veterinaria y Zootecnia* at *Universidad Nacional Autónoma de México*. All pigs were fed a commercial diet and had access to water *ad libitum*. After a 3-day adaptation period, pigs were randomly distributed into 4 groups: PorPV/Mock (*n* = 6), Mock/swH1N1 (*n* = 6), PorPV/swH1N1 (*n* = 6) and Mock/Mock (*n* = 6). The experimental design described above is summarised in Table 1. All procedures and the experimental protocol were approved by the Institutional Experimental Animal Care Sub-committee of the *Universidad Nacional Autónoma de México*.

### 2.3. Clinical observation

The pigs were first evaluated clinically, and the observed clinical signs of respiratory disease were quantified using the Loeffen et al. (2003) model. The categories evaluated were as follows: activity (value 0: active pigs in an alert state, 1: reduced activity, 2: apathy), breathing frequency (value 0: normal, 1: slightly elevated, 2: clearly elevated), abdominal breathing (value 0: normal, 1: slight abdominal breathing, 2: abdominal breathing, jerking), and coughing (value 0: absent; 1: present). The scores for each observation were recorded for each time point and then arranged on a scale from 0 to 7 (Loeffen et al., 2003). Rectal temperature was measured, and samples were taken both pre- and post-infection.

### 2.4. Necropsy and sample collection

The samples collected were nasal and oral swabs (polyester swabs were placed in 1 mL of antibiotic supplemented culture medium) and blood samples from the jugular vein. The samples (blood and swabs) were collected both pre- and post-infection on day -2, 0, 1, 3, 7, 14, 28, 43, 46, 50, and 52. The pigs of all groups were euthanised at 2 different points during the experiment: 46 and 52 DPI (three pigs in each group) (Table 1). During necropsies, all macroscopic alterations of the respiratory tract were recorded, and a series of respiratory tissue (RT) and lymphoid tissue (SLO) sections were collected (RT: nasal mucosa, anterior and bronchial trachea, and lung; SLO: soft palate tonsil, mediastinal and tracheobronchial lymph nodes). Bronchoalveolar lavage fluid (BALF) was obtained from each pig using 80 mL of phosphate-buffered saline. The BALF samples were then centrifuged (600 × *g*/10 min/4 °C), and the cell pellets were homogenised with 1 mL of culture medium. All samples were preserved in liquid nitrogen until use. Sections of the cranial lung lobe and mediastinal and tracheobronchial lymph nodes were fixed in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

## 2.5. Histopathological and immunohistochemical evaluation

The formalin-fixed lung samples were embedded in paraffin wax, sectioned, and stained with haematoxylin-eosin. These sections were evaluated by light microscopy for histopathological changes. For analysis, four specific changes were assessed: bronchiolar-associated lymphoid tissue hyperplasia, development of perivascular lymphoplasmacytic infiltration near to respiratory bronchioles, interstitial pneumonia, and increase of alveolar macrophages. Lesion severity was scored as follows: 0, null; 1 mild; 2, moderate; 3, marked; and 4, very marked. The pathological scores were averaged for each group analysed. For the immunohistochemical evaluation, two monoclonal antibodies were used, the first directed to the HN protein of PorPV (kindly donated by Dr. Sandra Cuevas-Romero) and the second to the HA1 protein of the influenza virus (MAB8253; Merck Millipore, Billerica, MA). The procedure was performed according to standardized protocols. Immunoreactivity was evidenced using the ImmunoDetector kit (BioSB, Santa Babara, CA).

## 2.6. Haemagglutination inhibition (HI)

The sera were inactivated and adsorbed to remove nonspecific inhibitors of the haemagglutination. HI was conducted following previously described protocols (Oie, 2008; Ramirez et al., 1996). The serum samples were placed in a 96-well U-shaped plate, double serial dilutions were prepared in PBS, and eight haemagglutinating units of virus (PorPV or SIV) were added to each well. For PorPV, the dilutions ranged from 1:2 to 1:4096, while for swH1N1, the range was 1:40 to 1:5120. HI titres $\geq 8$ (3 $\log_2$) of PorPV and $\geq 80$ (6.32 $\log_2$) for swH1N1 were considered as positive. The titre of the haemagglutination-inhibiting antibodies was expressed as the maximum dilution in which the serum was capable of inhibiting the haemagglutinating activity of the virus analysed. The titres were transformed to $\log_2$ values.

## 2.7. Virus isolation

The fluids collected from the nasal and oral cavities were thawed and centrifuged ($3000 \times g/10$ min/$4\,^\circ$C). The supernatants were inoculated in duplicates in MDCK cells at 80% of confluence. A culture medium supplemented with 2 $\mu$g/mL of trypsin-TPCK (Sigma–Aldrich, St. Louis, MO) was added, the inoculum were left to adsorb for 60 min at 37 $^\circ$C. Afterwards, the inoculum was discarded, and culture medium with 2% foetal bovine serum was added. The cell cultures were incubated at 37 $^\circ$C for 72 h in a 5% $CO_2$

atmosphere. Tissue samples (RT and SLO) were analysed. Approximately 1 g of each tissue was homogenised in 5 mL of culture medium, centrifuged and inoculated as described for the swab samples. After 72 h, the cell culture plates were fixed with 4% paraformaldehyde to perform indirect immunofluorescence assays according to a previously described protocol (Rivera-Benitez et al., 2013c).

## 2.8. Real-time RT-PCR quantification for PorPV and swH1N1

RNA was extracted from the samples that contained cells (tissues and BALF) and from the supernatants of the nasal and oral swabs using the RNeasy™ Mini Kit and QIAamp Viral RNA™ Mini Kit (Qiagen, Dusseldorf, Germany), respectively. All procedures were conducted in accordance with the manufacturer's protocol. Initial extraction was performed in 140 $\mu$L of supernatant from nasal and oral swabs and 350 $\mu$L lysates of BALF and unfiltered homogenised tissue (approximately 110 mg); elution of total RNA was performed in a volume of 60 $\mu$L and 80 $\mu$L, respectively. The total RNA was quantified using spectrophotometry at a wavelength of 260 nm (NANODROP, ND-1000, Wilmington, DE). RNA integrity was determined by the ratio of the $OD_{260}/OD_{280}$ reading, and the inclusion criterion was a ratio greater than 1.8. The RNA extracted from the samples was used in the quantification of the N gene of the PorPV. Real-time RT-PCR quantification was carried out following a previously described procedure (Rivera-Benitez et al., 2013b). For swH1N1, a previously described protocol was used that quantifies a fragment of the HA gene of swine influenza (Richt et al., 2004). This protocol was conducted in accordance with the established procedure, but with the following modifications: 5 $\mu$L of total RNA was used, and the amplification program was run for 40 cycles. Both TaqMan® hydrolysis probes were marked on their 5' ends with 6-FAM (6-carboxyfluorescein) and on their 3' ends with BHQ1 (Black Hole Quencher™-1). Briefly, a one-step, single-tube qRT-PCR assay was performed using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, Life Technologies, Carlsbad, CA). The 25-$\mu$L reaction mixture contained 5 $\mu$L of 5 × buffer, 1 $\mu$L of enzyme mixture (reverse transcriptase and Taq polymerase), 11.25 $\mu$L of nuclease-free water, 400 nM of each primer, 300 nM of probe, and 5 $\mu$L (mean of 500 ng) of total RNA. Thermal cycling was conducted in a SmartCycler real-time PCR thermal cycler (Cepheid, Inc. USA). The cycling protocol involved an initial incubation at 50 $^\circ$C for 15 min, followed by a denaturation step at 95 $^\circ$C for 2 min, then 40 cycles of 95 $^\circ$C for 15 s and 60 $^\circ$C (for PorPV) or 54 $^\circ$C (for swH1N1) for 30 s (annealing step). The accumulated fluorescent signal was collected during the
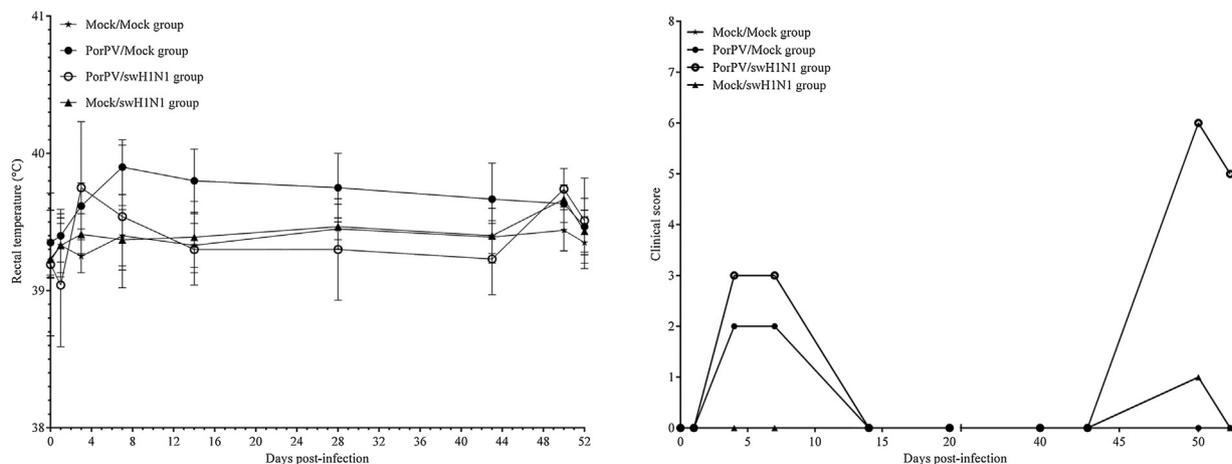


**Fig.1.** Mean of the rectal temperatures ($\pm$SD) and clinical score in Mock/Mock ($\star$), PorPV/Mock ($\bullet$), PorPV/swH1N1 ($\circ$) and Mock/swH1N1 ($\blacktriangle$) groups after simple inoculation with PorPV, swH1N1 and co-infection with both viruses. No significant differences between the observed groups ($P > 0.05$).

annealing step at each cycle. All assays were also performed with a no-template control (NTC), RNA extracted from nasal and oral swabs and tissues taken from uninfected pigs. In both cases, tenfold serial dilutions ($10^{-1}$ to $10^{-10}$) of the in vitro-transcribed RNA were prepared in our laboratory and used to construct a standard curve. The detection limit of quantitative real-time RT-PCR assay was $1 \times 10^2$ viral copies/$\mu$L (the slope of the line was $-3.13$ and $-3.10$. $R^2$ was 0.99 and 0.99 for PorPV and swH1N1, respectively). Viral concentration was expressed as viral copy number per millilitre of total RNA extracted from samples.

### 2.9. Statistical analysis

SPSS v. 15 was used for all statistical analyses. The microscopic lesion score was evaluated using a non-parametric test (Kruskal–Wallis). A Student's t-test assuming unequal variance and a significance level of $P \leq 0.05$ was used to compare rectal temperatures and the viral load of PorPV and swH1N1 in different samples (nasal and oral swabs, respiratory tissues and SLO) between the single-infected groups to the co-infected group.

## 3. Results

### 3.1. Clinical signs

In the PorPV/Mock and PorPV/swH1N1 groups, 3 of the 6 pigs in each group presented nasal secretions and conjunctivitis at 4 DPI. In these same groups, pyrexia (>39.7 °C) was registred at 3 and 7 DPI in 3 pigs (Fig. 1a). The clinical feature in the PorPV/Mock and PorPV/swH1N1 groups consisted of apathy and respiratory distress at 7 DPI. No additional alterations in clinical signs were recorded on the remaining days in the PorPV/Mock group (except in the rectal temperature). In the co-infection group (PorPV/swH1N1), an increase of rectal temperatures above 39.7 °C was observed at 6 DPI of swH1N1 in 3 pigs, but later only one pig had a temperature of 40.1 °C on 8 DPI. This same pig was observed to have dyspnoea and nasal discharge. In the Mock/swH1N1 group, the rectal temperature was increased at 6 DPI of SIV inoculation. No significant differences in rectal temperatures were observed between the four groups at different times, and no clinical alterations or increases in rectal temperature manifested in the pigs of the Mock/Mock group. The clinical score for respiratory signs are shown Fig. 1b.

### 3.2. Macroscopic, microscopic lesions, and immunohistochemical staining

Necropsies were performed at 46 and 52 DPI. Macroscopic lesions were observed only in one pig in the Mock/swH1N1 group, who was found to have mild pneumonia localised in the cranial and diaphragmatic lobes (8 DPI-swH1N1). None of the other pigs from the analysed groups manifested macroscopic lesions. Microscopic lesions were observed and scored (Table 2). In all infected groups, the most recurrent lesion was hyperplasia of the bronchiolar-associated lymphoid tissue (Fig. 2). Very marked interstitial pneumonia was observed only in one pig at 52 DPI in the co-infected group (Fig. 2b). In the other groups, the severity of the interstitial pneumonia was found to be moderate (Fig. 2c,d). The presence of multinucleated cells in the lumen of the alveoli was a feature that was recurrently observed in 2/6 pigs of the PorPV/swH1N1 group and in 2/6 pigs of the PorPV/Mock group (Fig. 2e). It was also possible to observe the formation of syncytia in the alveolar lumen in infected pigs with PorPV (Fig. 2f). Pigs of the Mock/Mock group presented no related lesions in the lungs (Fig. 2a). Regarding immunohistochemical staining, positive reactivity to PorPV was observed in 2/6 lung sections of pigs of the PorPV/Mock group (Fig. 2i,j). Immunoreactivity to influenza was observed in 3/6 pigs of the Mock/swH1N1 group (Fig. 2k, 2l). As for the co-infection group, positive immunoreactivity to PorPV was observed in 1/6 pigs and for influenza in 4/6 pigs. Only one pig presented immunoreactivity to both viruses in the the bronchiolar epithelium (Fig. 2g, h). Immunoreactivity to both viruses in mediastinal and tracheobronchial lymph nodes was seen only in two cases.

### 3.3. Serology

The presence of haemagglutination-inhibiting antibodies against PorPV was identified from 7 DPI (PorPV/Mock group), and there was increased until maximum production was reached at 28 DPI (4.66 $\log_2$). In the PorPV/swH1N1 co-infection group, the titre of antibodies remained unchanged at an average of 6 $\log_2$ until the conclusion of the experiment. No antibodies against PorPV were observed in the Mock/swH1N1 and Mock/Mock groups (Table 3). With respect to the haemagglutination inhibition assay for swH1N1, antibodies were detected from the moment the pigs arrived in the PorPV/swH1N1 group. Those antibodies corresponded to passive immunity because the sows are routinely vaccinated against swine influenza virus (subtype H1N1). The titres of antibodies for both viruses are shown in the Table 3.

### 3.4. Virus isolation

Viral isolation for PorPV and swH1N1 was performed using the nasal and oral fluids and tissue samples. In the nasal swab samples, PorPV was isolated at 3 DPI (4/6 pigs) and up to 14 DPI (3/6 pigs) in the PorPV/swH1N1 group. In the PorPV/Mock group, PorPV was isolated at 3, 7 and 14 DPI in 4 of 6 pigs analysed. In the oral swabs, it was isolated from day 1 (4/6 pigs) up to 14 DPI (2/6 pigs) in the PorPV/swH1N1 group. In the PorPV/Mock group, PorPV was isolated at 3, 7 and 14 DPI (3/6 pigs). In the Mock/swH1N1 group, swH1N1-positive samples were detected only in the nasal fluids at 2 (4/6 pigs) and 6 (2/3 pigs) DPI. In the co-infection group, no positive samples for swH1N1 were recorded.

**Table 2**
Mean histopathology lung scores in experimentally infected groups.

| Lesion type[a] | Group | | | |
| --- | --- | --- | --- | --- |
| | PorPV/Mock | PorPV/swH1N1 | Mock/swH1N1 | Mock/Mock |
| Bronchiolar-associated lymphoid tissue hyperplasia (score/4)[b] | 1.67 ± 0.52 | 1.67 ± 1.21 | 2.17 ± 0.75 | 0.78 ± 1.07 |
| Development of perivascular lymphoplasmacytic infiltration near to respiratory bronchioles (score/4) | 1.50 ± 0.55 | 1.0 ± 1.10 | 1.17 ± 0.41 | 0.55 ± 0.96 |
| Interstitial pneumonia (score/4) | 1.33 ± 0.52 | 1.17 ± 1.17 | 1.17 ± 0.98 | 0.67 ± 1.15 |
| Increase of the number of alveolar macrophages (score/4) | 0.17 ± 0.41 | 0.50 ± 0.55 | 0.67 ± 0.52 | 0.67 ± 0.58 |
| Total mean | 1.17 ± 0.76 | 1.08 ± 1.06 | 1.29 ± 0.86 | 0.67 ± 0.94 |
| Total score/16 | 4.67 | 4.33 | 5.17 | 2.66 |

[a] No significant differences between the observed groups ($P > 0.05$).
[b] Microscopic lesion severity or condition was scored as follows: 0, null; 1, mild; 2, moderate; 3, marked; 4, very marked.
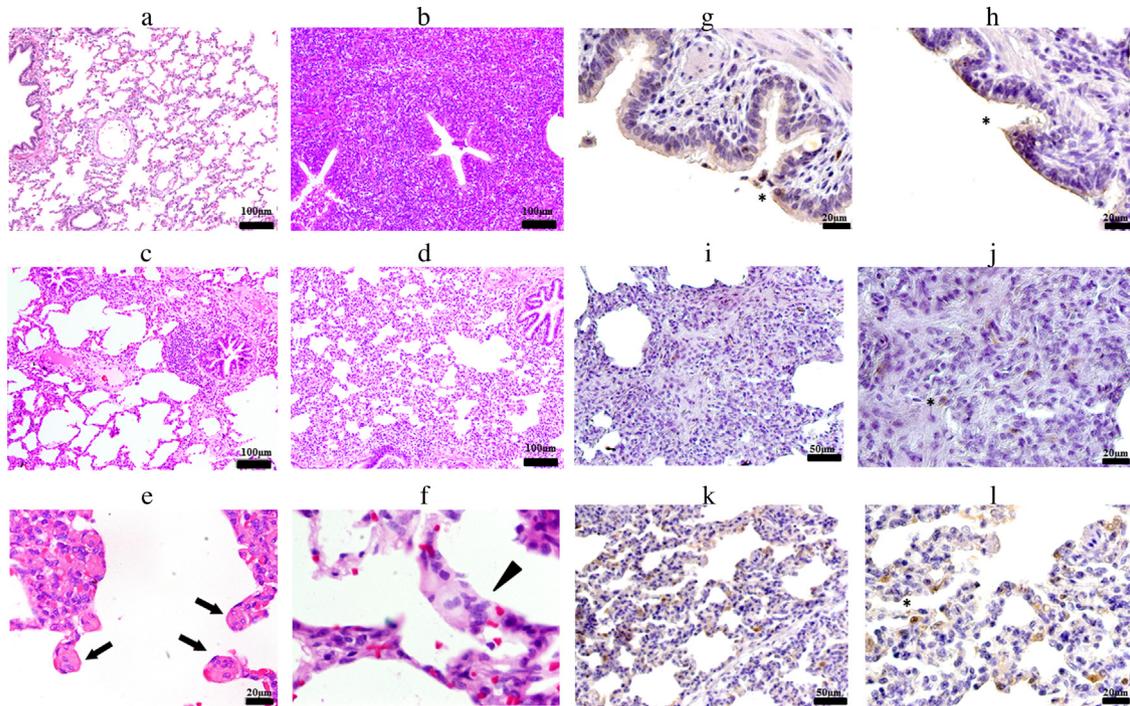
**Fig. 2.** Haematoxylin-eosin stained sections of lungs. (a) A Mock/Mock pig; (b) a pig co-infected with PorPV and swH1N1 at 52 DPI (PoRV/swH1N1 group); (c) a pig infected with PorPV at 52 DPI (PorPV/Mock group); (d) a pig infected with swH1N1 at 4 DPI (Mock/swH1N1 group); (e) a pig co-infected with PorPV and swH1N1 at 52 DPI, note the presence of multinucleated cells in the lumen of the alveoli (arrows); (f) a pig infected with PorPV at 52 DPI (PorPV/Mock group), the arrowhead indicates the presence of syncytia in the alveolar lumen. Immunohistochemistry of sections from the apical lung lobe. (g) Immunoreaction against HN of a lung section from a pig of group PorPV/swH1N1. (h) Immunoreaction against HA1 of a lung section from a pig of group PorPV/swH1N1. (i, j) Immunoreaction against HN of a lung section from a pig of group PorPV/Mock. (k, l) Immunoreaction against HA1 of a lung section from a pig of group Mock/swH1N1. The asterisk indicates the presence of positive immunoreaction. Magnification: $\times 100$ (a-d), $\times 200$ (i, k), $\times 400$ (e, g, h, j and l) and $\times 640$ (f).

Isolation of PorPV and swH1N1 was negative in all tissue samples of the evaluated groups. In the Mock/Mock group, no positive samples for PorPV or swH1N1 were recorded.

### 3.5. Quantification of PorPV by real-time RT-PCR

In the nasal swabs, samples that tested positive for PorPV were detected from 24 h post-infection up to 28 DPI (PorPV/Mock and PorPV/swH1N1 groups) (Fig. 3a), and there were no differences ($P > 0.05$) in the mean of viral loads at any time analysed for these two groups. In oral swabs, the excretion of PorPV was more prolonged, and positive samples were detected from day 1 up to 43 DPI in the PorPV/Mock group. In the PorPV/swH1N1 group after co-infection with swH1N1, negative samples were recorded at 46 DPI, and viral load was detected again at 50 and 52 DPI (Fig. 4a). With respect to BALF, positive samples occurred in two pigs euthanised at 46 DPI in PorPV/Mock group. After co-infection with swH1N1 (in the PorPV/swH1N1 group), no positive samples were

observed (Table 4). In SLO samples, the assay detected 94% of positive samples. In samples from the tonsils and the mediastinal and tracheobronchial lymph nodes, the highest viral load was noted at 52 DPI in the PorPV/swH1N1 group. In all cases, PorPV persisted at 100% at 52 DPI in the PorPV/Mock and PorPV/swH1N1 groups (Table 4). In SLO only at 46 DPI were significant differences ($P = 0.015$) observed between the PorPV/Mock and PorPV/swH1N1 groups. With regard to tissues from the respiratory tract, positive samples were identified in all of the tissues analysed (*i.e.*, nasal mucosa, anterior trachea, bronchial trachea, and lung), except in the trachea and bronchial trachea of the PorPV/swH1N1 pigs at 46 DPI. In the nasal mucosa, positive samples were detected from 46 to 52 DPI, with the highest viral load recorded on 46 DPI (4.59 $\log_{10}$) (the PorPV/swH1N1 group). In the anterior trachea, the highest viral load was recorded at 46 DPI, whereas positive samples were detected up to 52 DPI (the PorPV/Mock group). A similar distribution was observed in the bronchial trachea, with the highest viral load found at 46 DPI. In both sections

**Table 3**
Mean of haemagglutination inhibition titres from sera of pigs of experimental groups.

| Group | Mean HI PorPV titres | | | | | | | Mean HI swH1N1 titres | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Days post-infection (weeks of age) | | | | | | | Days post-infection (weeks of age) | | | | | | |
| | 0 (6) | 7 (7) | 14 (8) | 28 (10) | 46 (12) | 50 (13) | 52 (13) | 0 (6) | 7 (7) | 14 (8) | 28 (10) | 46 (12) | 50 (13) | 52 (13) |
| PorPV/Mock | 0 | 3.16 | 4.33 | 4.66 | 4.50 | 4.33 | 4.33 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 |
| PorPV/swH1N1 | 0 | 3.33 | 5.81 | 6.67 | 6 | 6 | 6 | 6.75 | <5.32 | <5.32 | <5.32 | 6.33 | 7.56 | 10.03 |
| Mock/swH1N1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | <5.32 | <5.32 | <5.32 | <5.32 | 6.61 | 8.3 | 9.3 |
| Mock/Mock | 0 | 0 | 0 | 0 | 0 | 0 | 0 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 | <5.32 |

$\geq 3 \log_2$ cut-off for PorPV serology.
$\geq 6.32 \log_2$ cut-off for swH1N1 serology.
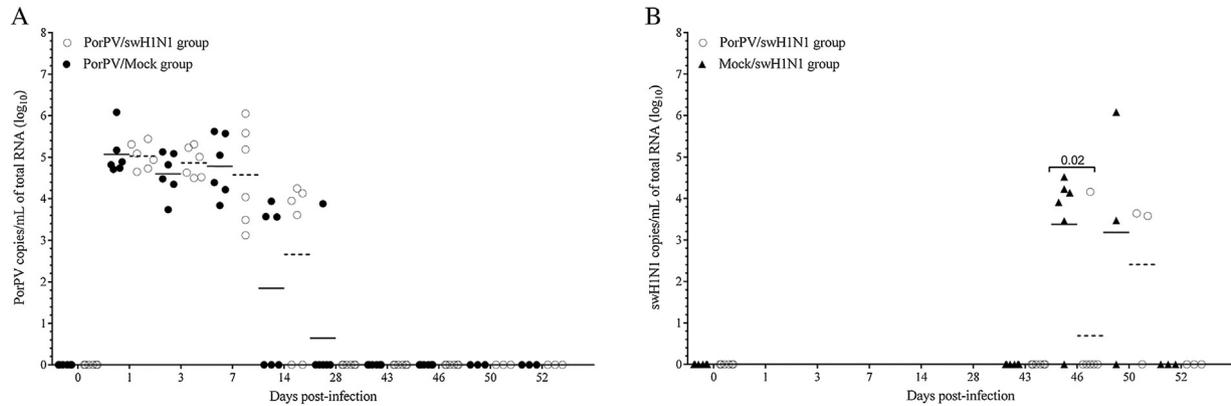
**Fig. 3.** The individual and mean PorPV and swH1N1 viral load in nasal swabs in PorPV/Mock (●) and PorPV/swH1N1 (○) groups (A), and Mock/swH1N1 (▲) and PorPV/swH1N1 (○) groups (B), respectively. The dotted and solid lines indicate the mean of the viral quantification in each group.

of the trachea, negative samples were observed at 46 and 52 DPI in the PorPV/swH1N1 group; however, after co-infection with swH1N1, positive samples were observed once again at 52 DPI (Table 4). In lung tissue, 83% of pigs tested positive, with the highest viral loads quantified at 4.28 $\log_{10}$ at 52 DPI (the PorPV/swH1N1 group). None of the samples from the Mock/swH1N1 and Mock/Mock groups tested positive.

### 3.6. Quantification of swH1N1 by real-time RT-PCR

In the PorPV/swH1N1 group, we detected positive samples for swH1N1 in nasal swabs on 2–6 DPI after co-infection with SIV. In the Mock/swH1N1 group, positive tests were detected at 2 and 6 DPI after inoculation with swH1N1 in significantly ($P = 0.02$) more pigs than in the co-infected group (Fig. 3b). For oral swabs, pigs were positive at 6 DPI only in the Mock/swH1N1 group. In the BALF samples, the highest viral load was recorded at 2 DPI for the PorPV/swH1N1 group and 8 DPI for the Mock/swH1N1 group (Table 5). In SLO, the presence of swH1N1 was negative in the tonsils of all the groups evaluated. The quantification of swH1N1 was more frequent in the mediastinal and tracheobronchial lymph nodes in the PorPV/swH1N1 group. In the Mock/swH1N1 group, only one positive sample was recorded in the tracheobronchial lymph node, at 2 DPI. Tissues from the respiratory tract were also analysed for the presence of swH1N1, but no positive samples were recorded from the nasal mucosa, except in one pig of Mock/swH1N1 group at 8 DPI. In the anterior trachea, two samples with an average viral load of 3.40 $\log_{10}$ were detected at 2 DPI (PorPV/swH1N1 group). In the Mock/

swH1N1 group, positive samples were detected at 2 and 8 DPI. In the bronchial trachea, positive samples were observed in the two groups analysed (PorPV/swH1N1 and Mock/swH1N1). In both tissue samples (anterior and bronchial trachea), the positivity observed at 2 DPI in the co-infection group decreased at 8 DPI, and negative samples were registered. In lung tissue, positive samples were detected more frequently in the co-infection group at 2 and 8 DPI, with viral loads of 6.27 and 5.13 $\log_{10}$, respectively (Table 5). No positive tests were recorded in any tissues from pigs from the PorPV/Mock and Mock/Mock groups. No significant differences in the viral load of SLO and RT were observed between the four groups at two different times of necropsy.

## 4. Discussion and conclusions

The objective of the present study was to evaluate the possible effect of swine influenza virus on growing pigs persistently infected with porcine rubulavirus. In swine farms in the west-central region of Mexico, blue-eye disease has become established as endemic, having reached a seroprevalence of 36% (Escobar-Lopez et al., 2012). Co-infection of PorPV with other viral or bacterial agents increases the negative impact on production in this important swine-producing zone. The seroprevalence of SIV in the west-central region has been identified at 81% for the H1N1 swine subtype (Avalos et al., 2011). Under field conditions, infection and co-infection with these two viral agents has been shown to be related to an increase in the number of pigs that experience respiratory disease. No experimental studies have been conducted that would allow us to assess the effects of a secondary
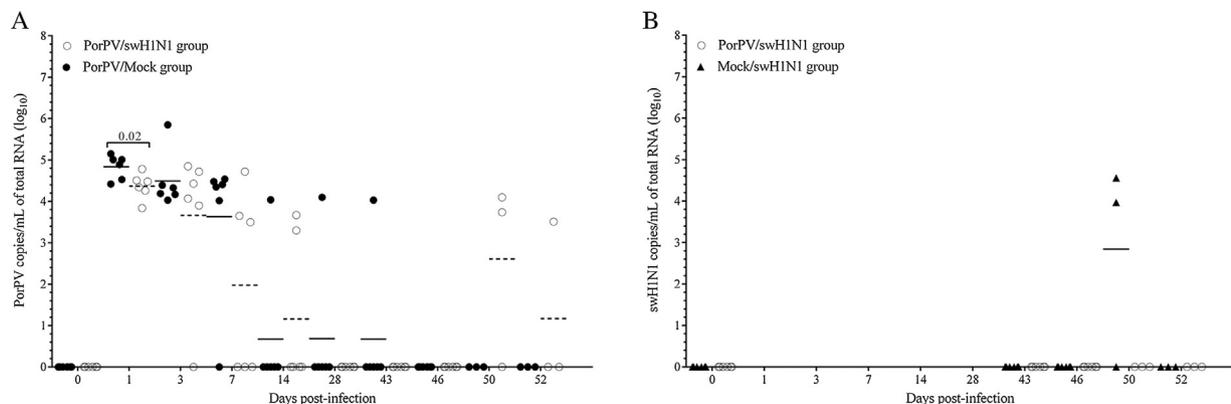


**Fig. 4.** The individual and mean PorPV and swH1N1 viral load in oral swabs in PorPV/Mock (●) and PorPV/swH1N1 (○) groups (A), and Mock/swH1N1 (▲) and PorPV/swH1N1 (○) groups (B), respectively. The dotted and solid lines indicate the mean of the viral quantification in each group.

**Table 4**

Detection of PorPV and viral load RNA in BALF, secondary lymphoid organs (SLO), and respiratory tissues (RT) in PorPV/Mock and PorPV/swH1N1 groups.

| Sample | Number of positive and viral load of PorPV | | | |
| --- | --- | --- | --- | --- |
| | PorPV/Mock group | PorPV/swH1N1 group | PorPV/Mock group | PorPV/swH1N1 group |
| | 46 DPI | | 52DPI | |
| BALF | 2/2 (4.32 ± 1.11) | 0/3 | 0/3 | 0/3 |
| SLO | | | | |
| Tonsil | 3/3 (5.02 ± 0.26) | 3/3 (6.64 ± 0.58) | 3/3 (5.60 ± 0.16) | 3/3 (6.54 ± 1.13) |
| ML | 2/3 (4.34 ± 0.33) | 3/3 (6.88 ± 1.44) | 3/3 (5.88 ± 0.72) | 3/3 (5.86 ± 0.44) |
| TBL | 2/3 (5.42 ± 0.46) | 3/3 (5.09 ± 0.13) | 3/3 (5.36 ± 0.60) | 3/3 (6.56 ± 1.01) |
| Mean and SD[a] | 3.84 ± 2.22 | 6.20 ± 1.14 | 5.61 ± 0.53 | 6.32 ± 0.86 |
| SEM | 0.74 | 0.38 | 0.17 | 0.28 |
| P value | 0.015[*] | | 0.055 | |
| | | | | |
| RT | | | | |
| NM | 3/3 (3.88 ± 0.44) | 3/3 (4.59 ± 0.89) | 1/3 (3.74) | 3/3 (4.22 ± 0.24) |
| Trachea | 3/3 (4.67 ± 0.87) | 0/2 | 1/2 (5.13) | 2/3 (3.64 ± 0.48) |
| BT | 2/3 (3.89 ± 0.20) | 0/3 | 1/3 (3.87) | 1/2 (4.85) |
| Lung | 3/3 (3.39 ± 0.13) | 2/3 (3.91 ± 0.12) | 2/3 (3.86 ± 0.88) | 3/3 (4.28 ± 0.61) |
| Mean and SD | 3.63 ± 1.31 | 1.96 ± 2.30 | 1.85 ± 2.18 | 3.42 ± 1.75 |
| SEM | 0.37 | 0.69 | 0.65 | 0.52 |
| P value | 0.052 | | 0.08 | |

BALF: bronchoalveolar lavage fluid. ML: mediastinal lymph node. TBL: tracheobronchial lymph node. NM: nasal mucosa. BT: bronchial trachea.

[*] Indicates that the PorPV/swH1N1group is significantly different from the PorPV/Mock group ($P < 0.05$).

[a] Mean and standard deviation (SD) of the total viral load in analysed samples for SLO or RT tissues. () Mean ± SD of PorPV copies/mL of total RNA ($\log_{10}$) in positive samples.

infection of SIV in pigs previously infected with PorPV. In a previous study, we showed that PorPV was able to induce a respiratory disease after experimental infection (Rivera-Benitez et al., 2013a). In this study, after infection with PorPV, clinical observations included nasal secretion, conjunctivitis and decreased activity in the first week. After co-infection with swH1N1, only one pig presented with dyspnoea and nasal discharge. These results differ from those reported in other models of co-infection with influenza and *M. hyopneumoniae* (Deblanc et al., 2012; Thacker et al., 2001) and PRRS with influenza (Van Reeth et al., 1996, 2001). The findings from these previous studies included acute respiratory disease; these effects can be influenced by both the duration of the co-infection and the virulence of the strains used. The increase of the rectal temperature observed after PorPV

infection resulted in apathy and a reduction in activity. This increase in temperature lasted for a long period, and it could have been due to an unrelated infection however, this phenomenon was not analysed. In the co-infection group, an increase in rectal temperature was observed in 3/6 pigs. In the pigs infected only with swH1N1, an increase in rectal temperature was noted in 2/6 pigs at 6 DPI. Other studies of co-infection have reported fever after 1-7 DPI (*M. hyo*-influenza) (Deblanc et al., 2012; Thacker et al., 2001) or 4-10 DPI (PRRS-influenza) (Van Reeth et al., 1996) and 2-4 DPI (PRRS-influenza) (Van Reeth et al., 2001). These findings indicated that the signs related to co-infection may occur at a subclinical level compared to other similar models. The scores for respiratory signs were low during the single infection phase with PorPV. The highest score was recorded after co-infection with

**Table 5**

Detection of swH1N1 and viral load RNA in BALF, secondary lymphoid organs (SLO), and respiratory tissues (RT) in Mock/swH1N1 and PorPV/swH1N1 groups.

| Sample | Number of positive and viral load of swH1N1 | | | |
| --- | --- | --- | --- | --- |
| | Mock/swH1N1 group | PorPV/swH1N1 group | Mock/swH1N1 group | PorPV/swH1N1 group |
| | 46 DPI | | 52DPI | |
| BALF | 0/3 | 2/3(7.80 ± 0.88) | 2/3(9.48 ± 1.39) | 0/3 |
| SLO | | | | |
| Tonsil | 0/3 | 0/3 | 0/2 | 0/3 |
| ML | 0/3 | 1/3 (3.5) | 0/2 | 1/3 (4.76) |
| TBL | 1/3 (3.5) | 2/3 (5.11 ± 2.71) | 0/2 | 0/3 |
| Mean and SD[a] | 0.43 ± 1.23 | 1.52 ± 2.52 | 0 | 0.52 ± 1.58 |
| SEM | 0.43 | 0.84 | 0 | 0.52 |
| P value | 0.27 | | 0.34 | |
| | | | | |
| RT | | | | |
| NM | 0/3 | 0/3 | 1/3 (7.41) | 0/3 |
| Trachea | 1/3 (5.75) | 2/2 (3.40 ± 0.13) | 1/2 (3.80) | 0/3 |
| BT | 2/3 (5.92 ± 3.65) | 1/3 (6.84) | 2/3 (5.47 ± 2.94) | 0/3 |
| Lung | 0/3 | 1/3 (6.27) | 1/3 (8.62) | 1/3 (5.13) |
| Mean and SD | 1.46 ± 2.87 | 1.81 ± 2.70 | 2.79 ± 3.55 | 0.47 ± 1.54 |
| SEM | 0.82 | 0.81 | 1.07 | 0.46 |
| P value | 0.77 | | 0.06 | |

BALF: bronchoalveolar lavage fluid. ML: mediastinal lymph node. TBL: tracheobronchial lymph node. NM: nasal mucosa. BT: bronchial trachea.

[a] Mean and SD of the total viral load in analysed samples for SLO or RT tissues. () Mean ± SD of swH1N1 copies/mL of total RNA ($\log_{10}$) in positive samples.

swH1N1. Loeffen et al. (2003) reported similar values in simple influenza infections during an earlier phase (2-3 DPI). The pigs in the Mock/swH1N1 group presented the lowest respiratory signs and rectal temperatures, with no pigs showing a difference in respiration or temperature after experimental infection, a finding that is in accordance with studies that used low-virulence swine influenza virus strains (Busquets et al., 2010). Inspection of the lungs at necropsy revealed mild pneumonia in only one pig of the Mock/swH1N1 group. In other experimental infections with SIV, marked pneumonia has been observed in the cranial lobes. This depends greatly on the virulence of the strain used (Olsen et al., 2006). Histological evaluation of the lung samples indicated the presence of interstitial pneumonia and hyperplasia of the bronchiolar-associated lymphoid tissue in three infected groups. These results confirm that there is an increase in histological lung lesions after single-infection or co-infection. The increase in the presentation of histological lesions in the lungs has been reported in several experiments examining SIV co-infection with other viral and bacterial pathogens (Deblanc et al., 2012; Loving et al., 2010; Pol et al., 1997; Thacker et al., 2001 Yazawa et al., 2004). Persistent infection of PorPV (52 DPI) induces the formation of multinucleated cells and syncytia in the alveolar lumen. This has not been previously described in PorPV infection, and this shows that persistent PorPV infection generates a chronic disease that is indicated by the presence of microscopic lung lesions. The presence of immunopositivity to both viruses indicates a co-infection, at least in the lung and associated lymph nodes. The antibody response for PorPV and swH1N1 was not affected by single-infection or co-infection in all analysed groups. The serological response observed is equal to the normal dynamics previously reported in experimental infection (Cuevas et al., 2009; Rivera-Benitez et al., 2013a Van Reeth et al., 2006). The presence of PorPV in nasal swabs was detected using real-time RT-PCR from 1 to 28 DPI. A similar situation emerged in the first phase sampling with oral swabs, and later, positive samples were detected up to 50–52 DPI in the co-infected group. Based on these results, it can be assumed that a reactivation occurred in the viral excretion of PorPV, possibly influenced by immunostimulation generated by co-infection with swH1N1; however, this is an event that will be studied later by means of immunohistochemical studies. Viral isolation for PorPV was more frequent in nasal and oral fluids in the first 2 weeks post-infection. Viral quantification for PorPV was more frequent in oral swab samples. In cases of infection with the mumps virus in humans (a virus that is closely related to PorPV), these are the samples chosen for viral quantification studies (Boddicker et al., 2007; Krause et al., 2006; Uchida et al., 2005). For SIV, only samples that tested positive by real-time RT-PCR were recorded in nasal swabs, at 2 and 6 DPI in the two swH1N1-infected groups (and viral isolation in nasal fluid was only recorded for single-infected swH1N1 group); however, differences in viral load between the Mock/swH1N1 group and the co-infection group were observed. In oral swab samples, no positive samples were detected in the PorPV/swH1N1 group. Only positive samples were detected in oral fluid of pigs in the Mock/swH1N1 group. These results indicate that the primary infection with PorPV does not cause greater excretion of swH1N1 after co-infection. Other models of infection with influenza and *M. hyo* did not produce increased excretion of SIV in pigs previously infected with *M. hyo* (Deblanc et al., 2012). The BALF samples were collected during the necropsies. In the PorPV group, positive samples were detected at 46 DPI. These observations had not been recorded previously at extended time points, and it is interesting to note that this type of sample may be useful for diagnosis of PorPV. During the evaluation of swH1N1, two samples that were positive by real-time RT-PCR were recorded at 2 and 8 DPI in the single-infected and co-infected pigs, respectively. These results are in agreement with those

obtained in other experimental infections that used the same influenza sub-type. Busquets et al. (2010) reported the presence of influenza H1N1 in bronchoalveolar lavage samples at 2 DPI, and its continuation up to day 7. In that work, quantification was performed using samples of SLO and RT. In the SLO, samples that tested positive for PorPV were detected on two sampling days. The viral load of PorPV in lymphoid organs after co-infection was found to be greater than in the PorPV/Mock group. The tissue that presented the highest viral load was the soft palate tonsils. Previous studies (Cuevas et al., 2009; Wiman et al., 1998) confirmed the persistence of PorPV RNA in lymphoid tissues from day 53 to day 277 post-infection in experimentally infected pigs. In the samples evaluated for swH1N1 in this study, positive results were noted only in the lymph nodes. There were no positive samples from the tonsils. De Vleeschauwer et al. (2009) reported the presence of swH1N1 in tonsils from days 1 to 5 post-infection, and that study emphasised that the route of inoculation (intranasal vs. intratracheal) is an important factor for the distribution of the virus into diverse tissues. The positivity and viral load recorded for swH1N1 in lymphoid organs was more frequent in the co-infected group, which presented with an increase in viral load even in the mediastinal lymph node. In RT, all samples were positive for PorPV. In the analysed samples, no significant difference was observed in the viral load for PorPV or swH1N1. In the nasal mucosa, positive samples for PorPV were found from days 46–52 post-infection, and a higher viral load was observed in the co-infected group. In the case of swH1N1, only one sample was found to be positive in the Mock/swH1N1 group. However, the anterior and bronchial trachea was negative for PorPV in the early stage of swH1N1 co-infection. It is probable that after co-infection with swH1N1, the immune system was reactivated at this site and was able to clear the PorPV in the trachea and bronchial trachea, at least in the early stage of infection with SIV. With respect to swH1N1, viral load was found in samples from the trachea and the bronchial trachea on 2 and 8 DPI, predominantly in pigs of the Mock/swH1N1 group. Previous studies obtained similar results by isolating swH1N1 on days 2–6 post-infection (De Vleeschauwer et al., 2009), revealing a high tropism for this type of tissue. However, in the co-infection group, there was an increase in the presentation of swH1N1 at the early stage of co-infection (2 DPI). The increased viral load of swH1N1 in the trachea and bronchial trachea coincides with the decrease in the viral load of PorPV. In lung tissue, the distribution of PoRV was constant and persisted up to 52 DPI. Wiman et al. (1998) reported the presence of PoRV RNA in lung tissue 53 days after experimental infection. For swH1N1, viral RNA was detected during the first two days in the co-infected group, and in the single-infected group, at 8 DPI, which is similar to data from previous studies (De Vleeschauwer et al., 2009; Weingartl et al., 2009). An interesting effect was observed in BALF and lung tissue: it appears that the pigs of the co-infected group were more susceptible to secondary infection because there was a greater number of a positive sample in this group compared with the single-infected group, thus demonstrating the association between primary PorPV infection and subsequent swH1N1 infection. In conclusion, the results obtained confirm infection, seroconversion, excretion, and distribution of PorPV and swH1N1 in growing pigs. The observations included an increase in the clinical signs in the co-infected group compared to simple infections. There were no significant differences in other measurements such as rectal temperature, macro- and microscopic lesions, and viral loads of both viruses between the analysed groups. However, primary infection with PorPV seems to have a positive impact on the spread and viral load of swH1N1 in respiratory and lymphoid tissues in early stages of co-infection, although viral shedding in nasal and oral secretions was not enhanced. In the present study, the interaction of swH1N1 with PorPV is demonstrated in persistently infected PorPV pigs. Under

field conditions, preventing PorPV infection could also reduce the clinical effect of co-infections with viral or bacterial agents including, as in this case, SIV.

## Conflict of interest

The authors affirm that no financial or personal relationships exist that could have inappropriately influenced the content of this manuscript or the opinions expressed.

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