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

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ABSTRACT

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.



## 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

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(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 ($600 \times g$/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.