Swine Influenza Virus and Association with the Porcine Respiratory Disease Complex in Pig Farms in Southern Brazil

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Impacts

• Swine have been proposed to serve as intermediate ‘mixing-vessel’ hosts for the evolution of novel reassortant viruses because of their capacity to be infected with both avian and human influenza viruses, which can exchange genome segments, generating new viruses with pandemic potential.
• The circulation of novel variants of swine influenza A viruses in pigs and the possibility of introduction of such viruses into humans highlight the importance of keeping vigorous epidemiological surveillance on influenza in pigs.
• Monitoring of the diversity of swine influenza A viruses circulating in pig populations is important (for both human and animal health) in consonance with the ‘one health’ concept.

Keywords:
Influenza A virus; A(H1N1)pdm09; H1N2; swine; respiratory disease outbreaks

Summary

Despite the putative endemic status of swine influenza A virus (swIAV) infections, data on the occurrence of swine influenza outbreaks are scarce in Brazil. The aim of this study was to detect and subtype swIAVs from six outbreaks of porcine respiratory disease complex (PRDC) in southern Brazil. Nasal swabs were collected from 66 piglets with signs of respiratory disease in six herds. Lung tissue samples were collected from six necropsied animals. Virus detection was performed by PCR screening and confirmed by virus isolation and hemagglutination (HA). Influenza A subtyping was performed by a real-time reverse transcriptase PCR (rRT-PCR) to detect the A(H1N1)pdm09; other swIAV subtypes were determined by multiplex RT-PCR. In lung tissues, the major bacterial and viral pathogens associated with PRDC (Pasteurella multocida, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Haemophilus parasuis and PCV2) were investigated. In some affected pigs, clinico-pathological evaluations were conducted. Influenza A was detected by screening PCR in 46 of 66 swab samples and from five of six lungs. Virus was recovered from pigs of all six herds. Subtype A(H1N1)pdm09 was detected in four of six herds and H1N2 in the other two herds. In lung tissues, further agents involved in PRDC were detected in all cases; Pasteurella multocida was identified in five of six samples and Mycoplasma hyopneumoniae in three of six. Actinobacillus pleuropneumoniae (1/6), Haemophilus parasuis (1/6) and PCV2 (1/6) were also detected. These findings indicate that subtypes A(H1N1)pdm09 and H1N2 were present in pigs in southern Brazil and were associated with PRDC outbreaks.
Introduction

Respiratory diseases in pigs are a major health concern in swine production (Opiessnig et al., 2011). One of the agents of porcine respiratory disease that are particularly relevant is influenza virus, not only due to its capacity to cause disease in pigs but also due to its zoonotic potential (Vincent et al., 2008). In pigs, the first clinical description of swine influenza A virus (swIAV) dates to 1918, coinciding with the human influenza known as Spanish flu (Koen, 1919). Since then, swIAVs have often been associated with the ‘porcine respiratory disease complex’ (PRDC), where swIAV may act synergistically with other pathogens such as Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) (Vincent et al., 2008).

Swine influenza viruses are members of the family Orthomyxoviridae, genus Influenzavirus A. Virions contain eight segments of single-stranded RNA of negative sense (Palese and Shaw, 2007). Currently, three main subtypes of swIAVs (H1N1, H1N2 and H3N2) with different lineages are circulating in the swine population throughout the world (Moreno et al., 2012). However, the distribution of swIAV subtypes is in constant evolution; the A(H1N1)pdm09 has been isolated from pigs in several countries (Moreno et al., 2010; Forgie et al., 2011; Schaefer et al., 2011). Furthermore, identification of reassortant viruses involving the pandemic A(H1N1)pdm09 and endemic viruses lineages has been frequently reported (Ducatez et al., 2011; Moreno et al., 2012; Schmidt et al., 2014). Therefore, the evolutionary dynamics of IAVs are complex and determined by their multihost ecology, viral structure and segmented genome which allows genetic reassortment between different viruses (Nelson and Vincent, 2015).

In Brazil, coinciding with the 2009 influenza pandemics in humans, outbreaks of acute respiratory infections in pigs were reported in which the subtype A(H1N1)pdm09 was identified (Schaefer et al., 2011; Rajao et al., 2013). The genome of this virus contains six gene segments (PB2, PB1, PA, HA, NP and NS) descending from the triple-reassortant swIAV of the North American swine lineage, plus two gene segments (M and NA) derived from a Eurasian lineage of swine influenza (Dawood et al., 2009). Recently, a human-like H1N2 was identified in Brazil in which the HA and NA genes clustered with influenza viruses of human lineages, whereas the internal genes (PB1, PB2, PA, NP, M, and NS) clustered with the subtype A(H1N1)pdm09 (Schmidt et al., 2014; Schaefer et al., 2015). Apart from this information, data on swIAV infections and swIAV variability in Brazil are still scarce.

Pigs may act as ‘mixing vessels’ for the generation of reassortant viruses, eventually capable of infecting humans (Scholtissek, 1995). Thus, influenza surveillance on that species is essential to monitor viruses circulating at the human–animal interface. With this aim, this study was conducted in search for influenza viruses in six outbreaks of porcine respiratory disease occurring in a region with intensive pig production in southern Brazil. In addition, the major bacterial and viral pathogens, usually associated with PRDC, were investigated for to provide an insight on the occurrence of possible co-infections in the examined herds. Clinico-pathological evaluation was also conducted in one of the affected piglets from each of the farms sampled.

Materials and Methods

Animals and sample collection

This study was approved by the Ethics Committee on Animal Use (CEUA-IPVDF) under protocol number 04/2013. All protocols used are in accordance with the requirements of ethics and animal welfare proposed by the Brazilian College of Animal Experimentation (COBEA, Law 6638 of May 8, 1979).

Sample collection was carried out in middle autumn/winter months. Six swine producing herds linked to a cooperative located in the centro-oriental meso-region of the state of Rio Grande do Sul (Vale do Taquari) were selected. Samples were collected from 1- to 5-week-old piglets during outbreaks of respiratory disease in four ‘farrow-to-wean’, as well as two feeder pig production systems. In the ‘farrow-to-wean’ system, the gilts were raised on the same farm, while in the feeder pig production system, piglets were purchased from other farms.

None of the farms had previous swIAV vaccination history. Clinical data were obtained from herd owners and veterinarians. Although sampled farms had no clear epidemiological link between each other, all farm owners reported the occurrence of repeated bouts of PRDC, particularly in cold months. Eleven animals were sampled per farm, based on an estimated 50% prevalence of swine influenza, with a 95% confidence interval (Thrusfield, 2007). Nasal swabs were collected from 66 piglets displaying signs of acute respiratory disease (cough, sneezing, respiratory distress and hyperthermia). Nasal swabbing was performed using sterile synthetic swabs, which were then placed in viral transport medium – minimum essential medium (Gibco), supplemented with 2% antibiotic and anti-mycotic solution (10 000 units/ml penicillin, 10 000 µg/ml streptomycin and 25 µg/ml Fungizone® – Gibco) and 1% bovine serum albumin (Sigma-Aldrich). The swabs were kept frozen (−80°C) until further processing.
In each of the farms, one piglet considered representative of the signs displayed by affected animals was euthanized following recommended ethics and animal welfare procedures (Rivera et al., 2012). Six lung tissue samples (from six different herds) were collected and kept under refrigeration for further processing. Half of each lung samples were fixed in 10% buffered formalin for histopathological examinations.

Cells

The Madin-Darby canine kidney-L cell lineage (MDCK-L) (Jin et al., 1996) was cultivated in MEM (minimum essential medium – Gibco), supplemented with antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml; Gibco), 1% L-glutamine (Life technologies, Carlsbad, CA, USA) and 5% foetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA). Cell multiplication procedures were performed by following standard procedures (Freshney, 2005).

Nucleic acid extraction and reverse transcription

Total RNA was extracted from nasal swabs and lung tissues as follows. Nasal swabs were squeezed in 1 ml of virus transport medium. Lung tissue homogenates were prepared to 10% (w/v) in the same medium. Five hundred microlitres of each of these suspensions was subjected to RNA extraction with TRIZOL® (Life Technologies), according to the manufacturer’s instructions. Ten microlitres of the RNA obtained was submitted to reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturer’s instructions. The primer used in such reactions (Uni12: 5’-AGCAAAAG CAGG-3’) consisted of an oligonucleotide complementary to the conserved 12 nucleotides of the 3’ end of the eight viral RNA segments (Hoffmann et al., 2001).

Lung tissue samples were further submitted to DNA extractions using the PureLink® Genomic DNA Mini Kit (Life Technologies), according to the manufacturer’s specifications. The DNA extracted was used in searches for bacterial or viral nucleic acid.

Influenza A screening by PCR

The cDNA synthesized was submitted to amplification by a screening PCR to detect all influenza A genomes. The primer pair M52C (5’- CTT CTAACC GAG GTC GAA AC G -3’) and M253R(5’- AGG GCA TTT TGG ACA AAG/T CGT CTA -3’), which targets a fragment of 244 bp of the gene encoding the matrix (M) protein, was used (Fouchier et al., 2000). Samples found to contain swIAV genomes were subsequently subjected to additional PCRs for subtyping and virus isolation attempts.

Influenza A subtyping

From each of the sampled herds, one sample containing IAV genomes at screening (five samples from lungs and one from a nasal swab) was selected and submitted to additional PCR assays for influenza A subtyping. Subtyping aiming detection of A(H1N1)pdm09 was performed in a set of three independent real-time reverse transcription PCRs (rRT-PCRs) with the following targets: (i) influenza A (InfA) matrix (M) gene, (ii) swine influenza (SwInfA) NP gene and (iii) pandemic A(H1N1)pdm09 virus (SwH1) targeting the HA gene (Shu et al., 2011). Samples which resulted negative at A(H1N1)pdm09 amplification attempts were tested in two additional multiplex RT-PCRs designed to differentially amplify segments of the HA (H1 or H3) and NA (N1 or N2) genes of swIAV (Choi et al., 2002). The PCR products were visualized by electrophoresis in 1% agarose gels stained with ethydium bromide.

Virus isolation

Swab eluates or lung tissue homogenates were inoculated onto confluent MDCK-L cells in 12-well cell culture plates and let to adsorb at 37°C in a 5% CO₂ atmosphere for 60 min. The inoculum was then removed and replaced by 1 ml of serum-free MEM (SFM) supplemented with 2 μg/ml TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (Sigma-Aldrich) and antibiotic/anti-mycotic solution (penicillin 100 units/ml, streptomycin 100 μg/ml, anfotericin B 0,25 μg/ml). The plates were incubated at 37°C for 72 h and checked daily for cytopathic effect (CPE). After 3 days incubation, the supernatants were collected and tested for hemagglutination (HA) activity with 0.5% turkey red blood cell (RBC) suspensions as previously described (World Health Organization, 2002). Samples negative at HA were again inoculated onto fresh MDCK-L cells and the procedures repeated up to three passages. After the third passage, samples without CPE and HA activity were considered negative for IAV.

Detection of pathogens associated with PRDC by PCR

The six lung tissue samples were submitted to PCRs in search for genomes of the following pathogens: Mycoplasma hyopneumoniae, Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis and PCV2.

Detection of Mycoplasma hyopneumoniae was performed as reported previously (Stakenborg et al., 2006), targeting a 1000 bp fragment of the 16S rDNA gene. For Pasteurella multocida, a PCR targeting a fragment of 460 bp of the gene KMT1 was used (Townsend et al., 2001). Detection of Actinobacillus pleuropneumoniae was performed by multiplex PCR using a primer set targeting all the genes.
encoding the APX toxins in different serotypes of *Actinobacillus pleuropneumoniae* (Rayamajhi et al., 2005). Detection of *Haemophilus parasuis* was based on the amplification of a 821 bp fragment of the 16S gene (Oliveira et al., 2001). For PCV2 detection, a PCR was carried out using primers previously described (Dezen et al., 2010).

**Results**

**Influenza A detection by PCR**

A summary of the findings on swIAV detection in the piglets sampled in the present study is presented on Table 1. Positive results at the screening PCR on 46 of the 66 swab eluates confirmed the presence of the swIAV genomes in all six farms. In lung tissue samples, five of the six lungs were found to contain swIAV genomes. Five lung samples and one nasal swab sample were selected for swIAV subtyping.

**Influenza A subtyping**

The subtyping of the viruses recovered from the six herds sampled is presented on Table 2. Four of the six samples were subtyped as A(H1N1)pdm09, whereas the two other samples were classified as subtype H1N2. One sample of the H1N2 subtype was fully sequenced and published elsewhere (Schmidt et al., 2014). Analyses of the eight segments revealed that the HA and NA genes clustered with human influenza viruses (H1-8 cluster and N2), whereas the internal genes (*PB1, PB2, PA, NP, M*, and NS) clustered along with subtype A(H1N1)pdm09 (Schmidt et al., 2014).

**Virus isolation**

All lungs and nasal swab samples in which swIAV genomes were detected were inoculated in MDCK-L cells. Infectious virus was recovered in 37 of 46 swabs. In lungs, infectious virus was recovered from five of five tissue samples (Table 1). In all isolation attempts, virus was detected at the first passage. Infected cells displayed a CPE characterized by the formation of plaques and quick detachment of cells from the surface of the flask at around 24–48 h post-infection. The supernatants from infected cultures were assayed by HA (Table 1).

**Detection of co-infecting agents**

Lung tissues were also tested in search for identification of co-infecting pathogens (Table 2). All of those contained at least one of the pathogens frequently associated with PRDC. *Pasteurella multocida* was identified in five of six samples, *Mycoplasma hyopneumoniae* in 3 of 6, *Actinobacillus pleuropneumoniae* in one of six, *Haemophilus parasuis* in one of six and PCV2 in one of six.

**Clinical findings, macroscopic and microscopic lesions**

Clinical signs more frequently detected were hyperthermia (which often reached 41°C), respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge. An additional sign reported by farm owners was decreased food intake and consequent reduction in expected growth rates. Furthermore, piglets co-infected with swIAV and *Mycoplasma hyopneumoniae* displayed slightly more severe clinical signs such as coughing, sneezing and difficult breathing.

Necropsy of the six animals revealed macroscopic lesions characterized by purple-red cranioventral consolidation of lung tissues, the absence of pulmonary collapse due to interstitial pneumonia and foamy exudates in the air passages in five of six piglets. Fibrious pleuritis and pleural adhesions to the chest wall were observed in one piglet.

**Table 1.** Detection of swine influenza virus in nasal swabs (n = 66) and lung tissues (n = 6) from piglets from six farms sampled.

<table>
<thead>
<tr>
<th>Influenza A PCR</th>
<th>Virus Isolation</th>
<th>HA* titre (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Herd n°</strong></td>
<td>Nasal Swabs</td>
<td>Nasal Swabs</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>Lung</td>
</tr>
<tr>
<td>1</td>
<td>8/11</td>
<td>8/8</td>
</tr>
<tr>
<td>2</td>
<td>7/11</td>
<td>6/7</td>
</tr>
<tr>
<td>3</td>
<td>6/11</td>
<td>6/6</td>
</tr>
<tr>
<td>4</td>
<td>7/11</td>
<td>6/7</td>
</tr>
<tr>
<td>5</td>
<td>10/11</td>
<td>6/10</td>
</tr>
<tr>
<td>6</td>
<td>8/11</td>
<td>5/8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>46/66</td>
<td>37/46</td>
</tr>
</tbody>
</table>

*Hemagglutination assay.

**Table 2.** Subtyping of swine influenza virus in lung or nasal swab samples and detection of co-infecting agents in lung samples from piglets displaying signs of respiratory disease.

<table>
<thead>
<tr>
<th>Sample</th>
<th>swIAV subtype</th>
<th>M. hyo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P. multocida&lt;sup&gt;b&lt;/sup&gt;</th>
<th>App&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Hps&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PCV2&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piglet 1</td>
<td>H1N2</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piglet 2</td>
<td>H1N2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piglet 3</td>
<td>A(H1N1) pdm09</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piglet 4</td>
<td>A(H1N1) pdm09</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piglet 5</td>
<td>A(H1N1) pdm09</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Piglet 6</td>
<td>A(H1N1) pdm09</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mycoplasma hyopneumoniae, <sup>b</sup>Pasteurella multocida, <sup>c</sup>Actinobacillus pleuropneumoniae, <sup>d</sup>Haemophilus parasuis, <sup>e</sup>Porcine circovirus type 2.
Microscopic lesions were observed predominantly in bronchi, bronchioles and alveoli, characterized by necrotizing bronchiolitis or bronchointerstitial pneumonia. Inflammatory exudate with predominance of neutrophils and desquamated necrotic epithelial cells were present in the airway lumen, causingobliterative bronchiolitis in five of six piglets. Infiltration of lymphocytes was present in the alveolar walls, primarily around the blood vessels (four of six piglets), characteristic of interstitial pneumonia. One of the animals presented only moderate interstitial pneumonia, without characteristic influenza lesions such as bronchiolitis (Piglet 6). One of the necropsied animals also showed moderate amount of eosinophilic material associated with histiocytes and lymphocyte infiltrates in the lung pleura.

Discussion

This study reports the detection and subtyping of swIAVs identified in six swine farms with ongoing outbreaks of respiratory disease in southern Brazil. Aspects of the clinical–pathological features of influenza infection and concurrent pathogens associated with PRDC were also examined.

Since 2009, the genetic diversity of IAVs in swine populations has expanded globally, generating additional pandemic threats such as the novel variant H3N2v swIAV that infected humans in the United States during 2011–2013 (Epperson et al., 2013). In Brazil, swIAV infections have been reported; however, only few isolates were subtyped (Rajao et al., 2013; Schmidt et al., 2014; Schaefer et al., 2015). Among these, the pandemic A(H1N1)pdm09 has been identified and appears to be circulating in Brazilian swine population since 2009 (Schaefer et al., 2011; Rajao et al., 2013). In addition, a novel human-like H1N2 influenza virus, resulting from the reassortment of the A(H1N1) pdm09 virus with human-like swIAVs, was detected in 2011 in an outbreak of acute respiratory disease in nursery piglets in Paraná State, Brazil (Schaefer et al., 2015).

The results obtained here confirmed the concurrent circulation of subtypes A(H1N1)pdm09 and H1N2 in swine population in southern Brazil during 2013 and 2014. The subtype A(H1N1)pdm09 was identified in four of the six herds sampled. In the other two herds, swIAV subtype H1N2 was detected. Interestingly, at the time of collection of the samples in this study (2013–2014), the incidence of A(H1N1)pdm09 in humans was in decline, as it has been since the 2009 pandemics, though still circulating (Secretaria de Vigilância em Saúde – Ministério da Saúde, 2013). Nevertheless, it remained circulating in the swine population, although it was not possible to determine whether such circulation was associated with separate introductions of the virus from humans into the holdings, or due to enzootic circulation of the virus in pigs.

As regards subtype H1N2, there is no evidence indicating the circulation of this subtype in humans in Brazil. The circulation of these subtypes in swine and the possibility of introduction of such viruses into humans stresses the importance of keeping a vigorous epidemiological surveillance on influenza in pigs. It is worth highlighting the possible risk that circulation of IAV in swine represents to humans, especially for workers who are employed at commercial swine farms. In this sense, it is very important that particularly workers who handle swine receive the seasonal influenza vaccine annually (Gray and Kayali, 2009). Protection of workers can not only safeguard their own health and their contacts, but may also contribute to health in swine herds.

Recovery of swIAVs in cell cultures may be relatively difficult and it is directly related on the time of sampling. Sample collection needs to be based on the differentiation of acutely infected pigs producing large amounts of viruses from subacute pigs producing little or no virus. Influenza virus titre peaks at 48 h post-infection, and there is little virus shed 6–8 days after infection (Kothalawala et al., 2006). In this study, all samples were collected during the acute phase of infection and swIAV can be recovered in MDCK-L cells in all six tested herds.

Regarding co-infections with other agents, Pasteurella multocida was the main pathogen associated with swIAV infection, as it was identified in five of the six lungs tested, followed in frequency by Mycoplasma hyopneumoniae in which was detected in three of six lung samples. These findings were expected as Pasteurella multocida and Mycoplasma hyopneumoniae are recognized as major pathogens associated with PRDC (Choi et al., 2003). Previous reports demonstrated that pigs infected with swIAV and Mycoplasma hyopneumoniae exhibited more severe clinical disease (Thacker et al., 2001). In this study, piglets co-infected with swIAV and Mycoplasma hyopneumoniae displayed more intense clinical signs such as coughing, sneezing and difficult breathing when compared to animals where no Mycoplasma hyopneumoniae co-infection was detected.

The disease caused by influenza viruses in pigs resembles flu episodes in humans, in that, usually, high morbidity, low mortality and fast recovery are the rules. In this study, swIAV infections were manifested as acute respiratory disease characterized by high morbidity, fever, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge. The high morbidity observed in affected herds may lead to considerable production losses in function of the reduction in food intake and weight gain (Fouchier et al., 2003).

Macroscopic lesions detected in necropsied piglets were similar to those reported previously (Vincent et al., 2008; Sreta et al., 2009). In the piglets necropsied here, lungs had...
purple-red, multifocal to coalescing areas of consolidation, predominantly in the cranioventral portions of the lung. Microscopically, the necrosis of bronchiolar epithelial cells and sloughing of these cells into airway lumen were the most relevant and most commonly observed microscopic changes. In addition to the typical influenza lesions, one of the animals presented pleural adhesions to the chest wall and a moderate amount of eosinophilic material associated with histiocytes and lymphocytes infiltrate on the pleura. It is likely that these lesions were due to secondary bacterial infections, as this animal was also infected with *Pasteurella multocida*.

In summary, this study evidenced that subtypes A (H1N1)pdm09 and H1N2 were, at the time of sampling, circulating in pigs in southern Brazil and were involved in the PRDC outbreaks reported here. These findings emphasize the need for continued surveillance and swIAV subtyping to better understand the evolutionary mechanisms that increase swIAV diversity in order to safeguard both human and animal health, thus improving the overall health status, consistent with the ‘one health’ concept.

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