Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil[1]

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ABSTRACT.- **Schaefer R., Zanella J.R.C., Brentano L., Vincent A.L., Ritterbusch G.A., Silveira S., Caron L. & Mores N.** 2011. **Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil.** *Pesquisa Veterinária Brasileira 31(9):761-767.* Embrapa Swine and Poultry Research Center, BR153, Km110, Vila Tamanduá, Concórdia, SC 89700-000, Brazil. E-mail: rejane@cnpsa.embrapa.br

Influenza A virus (IAV) infections are endemic in pork producing countries around the world. The emergence of the pandemic 2009 human H1N1 influenza A virus (pH1N1) raised questions about the occurrence of this virus in Brazilian swine population. During a 2009-2010 swine influenza virus research project at Embrapa Swine and Poultry (CNPSA), an outbreak of a highly transmissible H1N1 influenza A virus disease was detected in a pig herd in Santa Catarina State, Brazil. The virus caused a mild disease in growing pigs and sows without mortality. Three clinically affected piglets were euthanized. Gross lesions included mild to moderate consolidation of cranioventral areas of the lung. Microscopically, the lesions were characterized by necrotizing obliterative bronchiolitis and bronchointerstitial pneumonia. Immunohistochemistry using a monoclonal antibody against type A influenza virus nucleoprotein revealed positive staining in the nuclei of the bronchiolar epithelial cells. Lung tissue from three piglets and nasal swabs from five sows and four piglets were positive for influenza A by RT-PCR. Influenza virus was isolated from one lung, later confirmed by the hemagglutination test (HA titer 1:128) and RT-PCR. Sequence analyses of Hemmaglutinin (HA) and Matrix (M) genes revealed that the virus was consistent with the pandemic (A/H1N1) 2009 influenza virus strain that circulated in humans. This is the first report of an outbreak of pandemic A/H1N1 influenza virus in pigs in Brazil.

INDEX TERMS: Swine, influenza virus, pandemic influenza H1N1 virus, hemagglutinin gene, Brazil.

RESUMO.- [**Isolamento e caracterização do vírus da influenza pandêmico H1N1 em suínos no Brasil.**] A infecção causada pelo vírus Influenza A (IAV) é endêmica em suínos no mundo inteiro. O surgimento da pandemia de influenza humana pelo vírus A/H1N1 (pH1N1) em 2009 levantou dúvidas sobre a ocorrência deste vírus em suínos no Brasil. Durante o desenvolvimento de um projeto de pesquisa do vírus de influenza suína em 2009-2010, na Embrapa Suínos e Aves (CNPSA), foi detectado em um rebanho de suínos em Santa Catarina, Brasil, um surto de influenza altamente transmissível causado pelo subtipo viral H1N1. Este vírus causou uma doença leve em suínos em crescimento e em fêmeas adultas, sem mortalidade. Tres leitões clinicamente afetados foram eutanasiados. As lesões macroscópicas incluiam consolidação leve a moderada das áreas cranioventrais do pulmão. Microscopicamente, as lesões foram caracterizadas por bronquiolite necrosante obliterativa e pneumonia broncointersticial. A imunohistoquímica, utilizando um anticorpo monoclonal contra a nucleoproteína do vírus influenza A, revelou marcação positiva no núcleo das células epiteliais bronquiolares. O tecido pulmonar de três leitões e os suabes nasais de cinco fêmeas e quatro leitões foram positivos para influenza A pela RT-PCR. O vírus influenza foi isolado de um pulmão, mais tarde sendo confirmado pelo teste de hemaglutinação (título HA 1:128) e por RT-PCR. A análise das seqüências de nucleotídeos dos genes da hemaglutinina (HA) e proteína da matriz (M) revelou que o vírus isolado foi consistente com o vírus pandêmico A/H1N1/2009 que circulou em humanos no mesmo período. Este é o primeiro relato de um surto de influenza causado pelo vírus pandêmico A/H1N1 em suínos no Brasil.

TERMOS DE INDEXAÇÃO: Suínos, vírus influenza, vírus pandêmico H1N1, gene da hemaglutinina, Brasil.

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INTRODUCTION

Swine Influenza (SI) is an acute and infectious respiratory disease of swine caused by type A Influenza viruses within the *Orthomyxoviridae* family. Swine influenza virus (SIV) is responsible for outbreaks of respiratory disease in non-immune herds. Orthomyxoviruses have negative stranded, segmented RNA genomes, which allow the occurrence of genetic exchange or reassortment, among different influenza viruses during mixed infections. The genetic reassortment of influenza viruses has been implicated in the origin of novel strains of influenza with pandemic potential (Easterday & van Reeth 1999). Influenza virus infection has worldwide distribution in all pig producing countries, caused by various SIV subtypes with genetic material from multiple origins, including avian and human viruses (Brown 2000, Karasin et al. 2000, Zhou et al. 2000, Olsen 2002).

In recent years, isolation of influenza viruses with high genetic variability has been carried out in populations of U.S. pigs. It was shown that pig populations, previously infected by a single viral subtype and genotype, currently are reservoirs for at least six different genotypes of influenza virus, containing a genetically similar constellation of internal genes with different surface glycoproteins (Webby et al. 2004, Vincent et al. 2008, Brockwell-Staats et al. 2009). Also, the evidence that pigs can be infected naturally with other influenza virus subtypes (H4, H5 and H9), raises concern about the risk of interaction of these viruses with those of human origin, thus increasing the risk of the emergence of new pathogenic viral strains for humans (Ninomiya et al. 2002).

Since the beginning of the influenza pandemic in humans, in April 2009, there were concerns about the possibility of the spillover of this novel influenza virus A/H1N1 to swine populations worldwide. Several experimental studies demonstrated the susceptibility of pigs to this novel virus, ensuing a fast and efficient viral spread among swine (Lange et al. 2009). Moreover, Vincent et al. (2010) demonstrated an insufficient protection of North American pigs against H1N1 2009 by the immune response from previous exposure or vaccination (Vincent et al. 2010).

Consequently, two years after the emergence of A/H1N1 in humans, 19 countries have notified to the OIE the presence of Pandemic influenza A/H1N1 in pigs herds (OIE, 2011). This novel H1N1 contains genes from both North American and Eurasian swine influenza lineages and there is evidence that its gene segments have been circulating undetected among swine herds for more than 10 years (Garten et al. 2009, Smith et al. 2009). The disease in pigs has been described as mild, both in natural and in experimental conditions. Clinical signs in naturally infected pigs include fever, coughing, sneezing, nasal discharge and inappetence with mortality rates remaining unaffected, before and after the outbreak (Pasma & Joseph 2010, Pereda et al. 2010). In some cases, experimentally infected pigs developed clinical respiratory symptoms similar to naturally infected pigs (Lange et al. 2009).

In Brazil, although the swine production is economically very important (4th leading global pork producer and exporter), few studies investigated the presence of SIV antibodies or virus isolates in pig herds. Since the late 30s, the presence of SIV in pigs was suspected in Brazil, but the first SIV isolation from sick pigs was described only in 1978 (Cunha et al. 1978). Hemagglutination inhibition (HI) tests showed that this virus was closely related to A/swine/Illinois/1/63. More recently, Mancini et al. (2006) reported the isolation of influenza virus from oro-nasal samples from pigs in São Paulo state, Brazil. However, the isolates have not been characterized. Initial serologic studies analyzing swine sera collected from 1996-1999 indicated the presence of antibodies cross-reactive to subtype H1N1/Texas/1/77 (2.2%) and H3N2/New Jersey/76 (16.7%) in pig herds from 10 Brazilian states (Brentano et al. 2002). In Brazil, pig herds are not vaccinated against SIV and notification of the presence of the virus is not mandatory. Respiratory diseases in Brazilian swine are usually diagnosed clinically, and secondary infections are controlled with antibiotics or bacterins. Studies showing a high prevalence of macroscopic pneumonia (lesions consisting of 42.6 to 63.6% of the lung tissue) were reported in pigs from commercial herds during federal inspection on slaughterhouses and were suggestive of infection with *M. hyopneumoniae* and other agents, potentially including SIV (Sobestiansky et al. 1990, Silva, 2006).

Herein we describe an outbreak of respiratory disease in pigs caused by the pandemic influenza virus (A/H1N1/2009) through molecular characterization of the HA gene, in an experimental herd maintained by Embrapa Swine and Poultry Research Center (CNPSA-SC), during sampling of pigs for an ongoing research project.

MATERIALS AND METHODS

Susceptible pigs and clinical signs. On 30 January, 2010, a pig farm consisting of a 175-sow farrowing- nursery operation with 754 animals that ranged from newborn piglets to nursery pigs presented animals with signs of respiratory disease consistent with SIV infection. Nearly 29% of the pigs were affected (5 sows and 213 nursery pigs), showing fever, cough and loss of appetite, which lasted about 10 days. No clinical signs were observed in newborn piglets and no mortality was reported.

Field samples. Nasal swabs were collected from five sows and four piglets showing respiratory signs. Nasal swabbing was performed using a rayon swab with a plastic handle that was placed in a viral transport medium (Vincent et al. 2010). The swabs were refrigerated and submitted to the virology laboratory of Embrapa Swine and Poultry (CNPSA, Concordia, Santa Catarina, Brazil). Three clinically affected piglets were euthanized and submitted for post-mortem examination. Lung samples were fixed in 10% buffered formalin and processed for histopathologic examination. Immunohistochemical (IHC) analysis for influenza virus antigens in lung tissues was conducted using a monoclonal antibody against type A nucleoprotein, as previously described (Vincent et al. 1997). Viral isolation was carried out in SPF embryonated chicken eggs and viral detection was performed by RT-PCR and sequencing.

Pathologic examination of lungs. At necropsy, lungs were removed and examined macroscopically. Tissue samples from the affected lobes were fixed in 10% buffered formalin for histopathological examination. Tissues were routinely processed and stained with hematoxylin and eosin (Luna 1968).

Immunohistochemical (IHC) analysis for influenza A antigens. The formalin-fixed, paraffin-embedded lung tissues were sectioned and processed for IHC, according to described protocol (Kitikoon et al. 2006). The influenza antigen was detected using a biotin-streptavidin-peroxidase kit (LSAB kit + System - HRP, Dako), employing anti-influenza A nucleoprotein monoclonal antibody

(Mab) HB-65 (Vincent et al. 1997) as primary antibody and 3-amino-9-ethyl-carbasole (AEC) as a chromogen. Paraffin blocks containing influenza positive lung tissues and the Mab HB-65 were imported from United States Department of Agriculture/ National Animal Disease Center (USDA /NADC) following authorization by the Brazilian Agriculture Ministry.

Virus detection. Total RNA was extracted from nasal swabs or lung tissue collected from pigs, using the RNeasy mini kit (Qiagen). The resulting RNA was reverse-transcribed into cDNA using SuperScript II RT Kit (Invitrogen) together with a primer complementary to the conserved 12 nucleotides of the 3' end of viral RNA (Uni12: 5'-AGCAAAAGCAGG-3') (Hoffmann et al. 2001). The cDNA served as a template for amplification of the gene encoding the matrix (M) protein using primers with the sequences: 5'- CTT CTA ACC GAG GTC GAA ACG -3' and 5'- AGG GCA TTT TGG ACA AAG/T CGT CTA -3' (Fouchier et al. 2000). Virus isolation was carried out in 9 to 10-day-old Specific Pathogen Free (SPF) embryonated chicken eggs. After four days of inoculation, allantoic fluids were harvested and tested for the ability to agglutinate 0.5% suspensions of Turkey red blood cells. Allantoic fluids were also tested for the presence of influenza A nucleic acid by RT-PCR.

Nucleotide sequencing and phylogenetic analysis. The coding region of hemagglutinin (HA) gene of the isolated virus, named influenza 12A/2010, was amplified in a one-step RT-PCR (Qiagen, Valencia, CA) using a primer set for the pandemic H1N1/ HA gene (WHO), 2009). The primers used for sequencing the M gene were those described by Chan et al. (2006). The sequencing reactions employed BigDye Terminator chemistry and the products were run on an Applied Biosystems 3130xl Genetic analyzer. The sequences were assembled with SeqScape v2.5 software (Applied Biosystems, Foster City, CA) and were submitted to NCBI database, GenBank Accession No. JF421756. NCBI-BLAST analysis was conducted to identify related references available in GenBank.

The complete HA gene segment of 12A/2010 isolate was aligned with other representative influenza viruses from swine, human and avian origins using Clustal W (BioEdit Sequence alignment Editor) (for Accession Numbers see Fig.3). Phylogenetic analysis of the HA gene segment was performed using the Neighbor-Joining method in the MEGA 5.01 software (Tamura et al. 2007) based on nucleotide sequences. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site.

RESULTS

Virus detection and viral isolation

Lung tissue (from three piglets) and nasal swabs (from five sows and four piglets) were tested using the RT-PCR for diagnosis of influenza type A. These samples were also analyzed for *Mycoplasma hyopneumoniae* and porcine circovirus type 2 (PCV2). All samples were positive for influenza A by RT-PCR and negative for mycoplasma. Three swabs and two lung samples from piglets *were also positive for PCV2.* Lung samples were submitted to virus isolation in SPF embryonated eggs. One of the lung samples was positive for influenza A virus, whose identification was confirmed by HA (HA titer 1:128) and for the amplification of the Matrix gene by RT-PCR (Fouchier et al. 2000).

Pathologic findings

All necropsied piglets (3/3) had gross lesions in the lungs with an average lung involvement of 30%. The apical and cardiac lobes of the lungs had multifocal to coalescing firm, well-demarcated reddish areas.

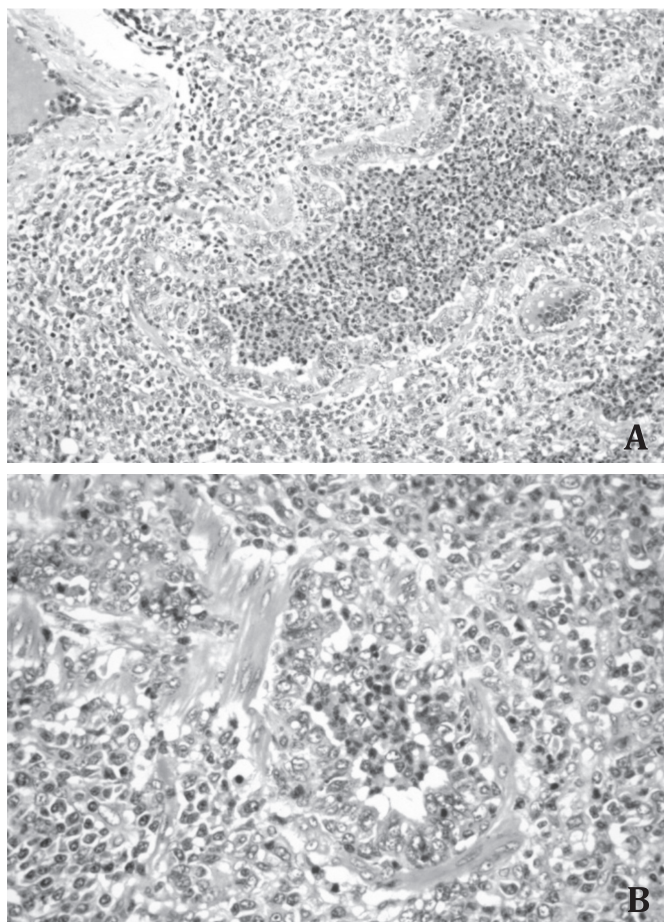

Fig.1. (**A**) Intense inflammatory exudate obliterates the lumen of the bronchiole. The surrounding parenchyma is infiltrated by moderate numbers of lymphocytes. Hematoxylin and eosin. (**B**) Higher magnification of an affected bronchiole. The lumen is infiltrated by numerous neutrophils. Hematoxylin and eosin.

Microscopic lesions in the lungs were typical of influenza. These lesions were observed primarily in bronchi, bronchioles, and alveoli. They were characterized by necrotizing bronchiolitis or bronchointerstitial pneumonia. Inflammatory exudate with predominance of neutrophils and desquamated necrotic epithelial cells were present in airway lumen, causing obliterative bronchiolitis (Fig.1). Infiltration of lymphocytes was present in the alveolar walls, primarily around the blood vessels, characteristic of interstitial pneumonia.

Immunohistochemical findings

Strong positive staining for influenza A-H1N1 antigen was detected in the nucleus of the bronchial and bronchiolar epithelial cells (Fig.2). Strongly positive cells, most likely desquamated epithelial cells, and in lesser degree inflammatory cells, were present in the airway lumen. Unaffected areas did not show any IHC reaction (not shown).

Sequence analysis

The complete CDS (<31-1733) of HA (1769bp) and partial M gene (897bp) were constructed with SeqScape v2.5 software

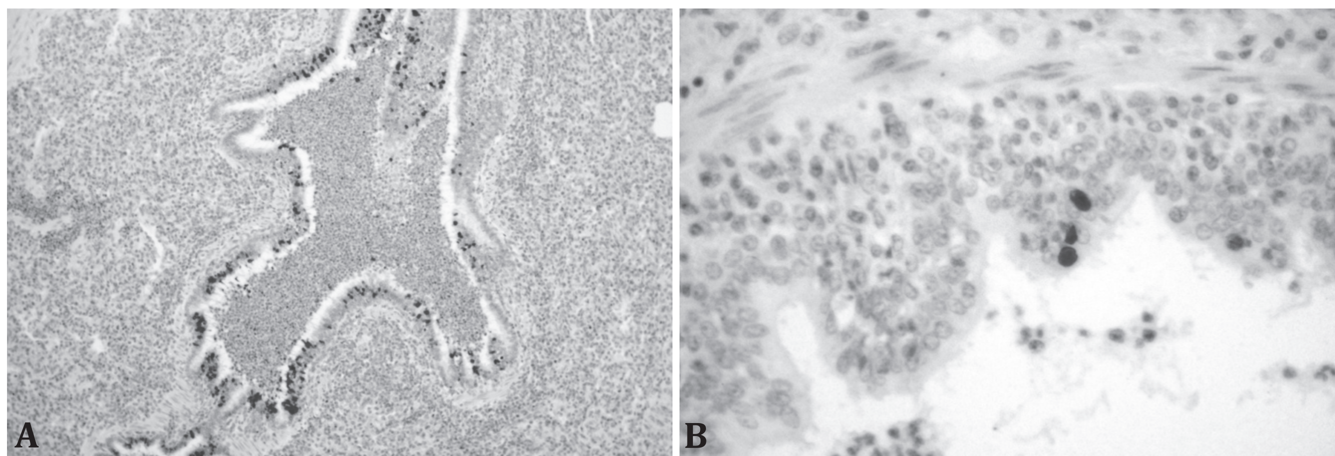

Fig.2. Immunohistochemistry (IHC) for influenza A antigens. (**A**) Many epithelial cells of the bronchioles show strong positive reaction. (**B**) Positive staining in the nuclei of bronchiolar epithelial cells.

Table 1. Influenza A viruses with the highest nucleotide sequence identity to H1N1 swine influenza virus isolated in this study (A/swine/Brazil/12A/2010) as determined by Blast search in NCBI

Gene	Identity (%)	E value	Virus designation	Subtype	Genbank accession no.
HA	99	0.0	(A/Guangdong/55/ 2009 [H1N1])	H1N1	HQ011423.1
M	99	0.0	(A/Kenya/0026/ 2009 [H1N1])	H1N1	HQ214452.1

and subjected to NCBI-Blast analyses (Table 1). Blast analysis showed that the two genes from the Brazilian SIV isolate (A/ swine/Brazil/12A/2010) were closely related with pandemic A/ H1N1 influenza viruses that have been circulating in humans. Phylogenetic analysis (Mega 5.01 software, Neighbor-Joining, Jukes-Cantor model with 500 bootstraps) of the HA gene of the Brazilian influenza virus isolate with GenBank sequences (Fig.3), indicated that the Brazilian SIV isolate is clustered with other pandemic A/H1N1 viruses isolated from human, turkey and swine during 2009-2010.

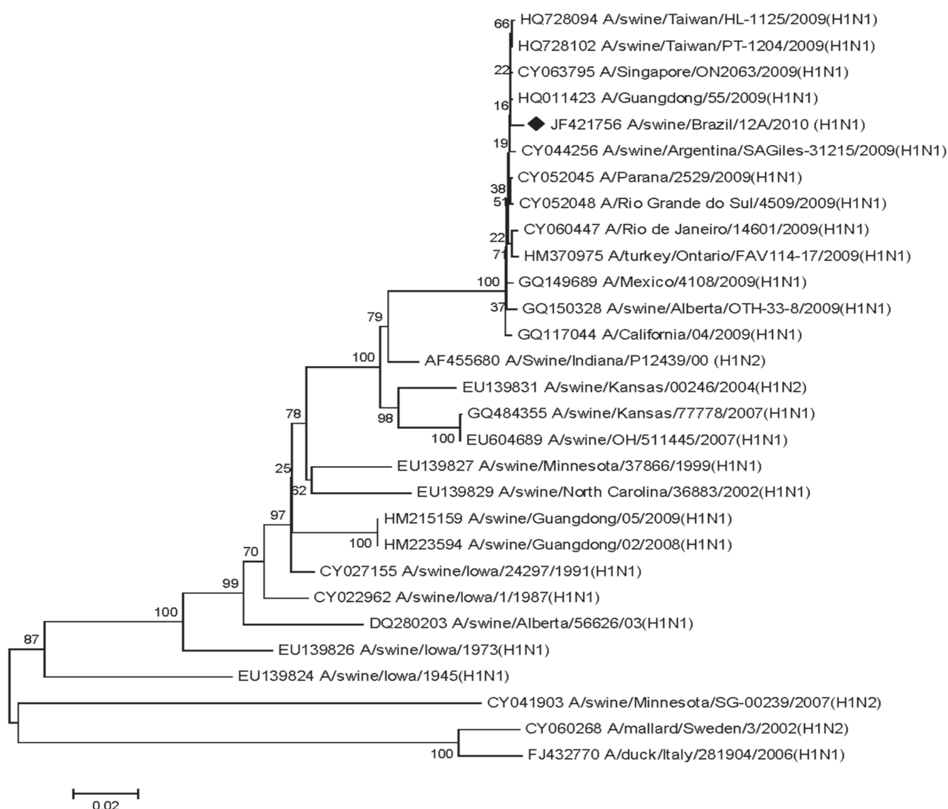

Fig.3. Nucleotide phylogenetic tree of HA gene segment from H1N1 and H1N2 influenza A virus isolates from swine, human and avian origin. The swine influenza viruses were representative from previous and current isolates from North America and Eurasia. Diamond shaped symbol indicate the H1N1 influenza virus isolate sequenced in this study. The tree was inferred using the Neighbor-Joining method.

DISCUSSION

The first notification to the World Organization for Animal Health of the occurrence of swine infection by the Pandemic influenza virus (pH1N1) was on May 2, 2009 in Canada (Promed-mail), 2009). Since then, several countries have identified this novel influenza virus in pigs (Howden et al. 2009, Hofshagen et al. 2009, Weingartl et al. 2010, Pasma & Joseph 2010, Pereda et al. 2010). Similar to what have been previously described, clinical signs in infected pigs were typical of an acute respiratory SIV disease, with pyrexia, cough, dyspnea and anorexia (Howden et al. 2009, Pasma & Joseph 2010, Pereda et al. 2010). Around 29% of growing pigs were affected and no death has been reported, with full recovery of sick pigs within 10 days. Histopathologic analysis of lungs showed a necrotizing bronchiolitis with mild to moderate interstitial pneumonia, later confirmed by IHC as being caused by influenza virus A. Genetic analyses of HA and M genes confirmed that the influenza virus found in sick pigs in Brazil was similar to the Pandemic influenza viruses (pH1N1) that have been detected in humans and pigs herds around the world in the last two years.

During the described outbreak (later confirmed as being caused by SIV), some control measures were taken on the pig farm, as recommended by OIE and FAO (OFFLU 2010), including restriction of animal movements until the end of the clinical signs. The movement of animals for slaughter or for sale of breeding stock was restored after 25 days of the onset of clinical illness. At this time, there were no more respiratory signs detected in pigs. According to Lange et al. (2009) and Pasma & Joseph (2010), in pigs naturally or experimentally infected with pandemic influenza H1N1, nasal virus shedding was detected by RT-PCR for up to 20 days after the clinical signs appeared. Other control measures included aerosol disinfection with Vircon®S and TH4+® of the pig housing (during the outbreak period and every 2 days after the end of the clinical signs throughout the farm). Antibiotic therapy to control secondary infections was conducted during the occurrence of clinical signs in susceptible pigs.

In Brazil, the first SIV isolation from pig lungs was described by Cunha et al. (1978). The HI test showed that this virus was antigenically related with A/swine/Illinois/1/63. Afterwards, many attempts to isolate influenza viruses from pig herds were performed, including research projects at Embrapa Swine and Poultry Research Center in the 5 years before the influenza A/H1N1/2009 emerged in humans around the world (Schaefer 2008). Even though no virus was isolated, previous serologic studies have indicated the circulation of SIV in Brazilian pigs (Brentano et al. 2002, Mancini et al. 2006, Caron et al. 2010). However, recent HI analyses performed in our laboratory with sera collected before and after 2009, did not detect the presence of antibodies against the pandemic influenza virus (pH1N1) in pigs in Brazil before 2009 (Zanella 2011). Phylogenetic analyses of the 8 gene segments of the 2009 pH1N1 have shown that neither the 2009 pH1N1 nor closely related progenitor viral genes were present in U.S swine influenza viruses prior to 2009 (Smith et al. 2009). In the outbreak described here, similar to what occurred in other countries (Howden et al. 2009, Weingartl et al. 2010), the detection of the pH1N1 in pigs occurred after the onset of pandemic influenza in humans. Moreover, the farm at Embrapa Swine and Poultry Research Center attends the biosecurity rules in relation to visits, shower and quarantine. Although the farm employees have not been tested for pH1N1 infection, some influenza cases among the employees were reported. It suggests the humans as possible sources of infection of pH1N1 for pigs. Recently, Forgie et al. (2011) have shown data based on epidemiologic and clinical findings that give support to human-to-swine transmission of the pH1N1 in a swine research farm in Alberta, Canada.

In the vast majority of cases of respiratory infections of pigs in Brazil, SIV infection is not considered in the differential diagnosis. Respiratory diseases in swine are usually diagnosed clinically and secondary infections are controlled with antibiotics and bacterins. Even though Brazilian herds are vaccinated against bacterial and viral agents such as *M. hyopneumoniae* and PCV-2, some studies have shown a high prevalence of pulmonary disease characterized by marked consolidation found in pigs from commercial herds during federal inspection in slaughterhouses (Sobestiansky et al. 1990, Silva 2006), suggesting that SIV may have been a component of respiratory diseases and, apparently remains misdiagnosed. We may speculate that upon the occurrence of pH1N1 in humans and the world reports of pandemic virus in swine, or that a perception for even more severe and difficult to control respiratory diseases has finally increased the awareness of the importance of influenza virus infection in pigs. As a result, over the last year sampling of pigs for the diagnosis of respiratory infections has also contemplated a concern with virological analysis, including a better understanding of the more critical timing for harvesting material and sample quality required for influenza virus diagnosis.

In the beginning of the human pandemic of influenza in April, 2009, the association made between the pH1N1 to a swine virus had a negative impact on pork production, resulting in the ban of exported pork from the U.S, even though the contamination of fresh pork meat with the novel virus was experimentally excluded (Vincent et al. 2009). Any event causing the temporary or permanent interruption of pork production has local consequences, as well as the potential to unchain a cascade of other events and may disrupt the balance of the supply of animal protein for the human population. Bernard Vallat, director general of the World Animal Health Organization (OIE), warns that animal diseases have worldwide socio-economic impact through an increase in poverty, since a billion farmers survive on animal production (Vallat 2008).

The role influenza viruses play in respiratory illness in pigs in Brazil is yet to be defined. The findings in this study support the need for implementing a more profound active and passive surveillance of influenza viruses in the national swine population in Brazil, in order to design more appropriate means to improve swine health and the implications of swine influenza for human health as well.

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