INFLUENZA VIRUS INFECTION IN PIGS

Janice Reis Ciacci Zanella*

Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Suínos e Aves, Embrapa Suínos e Aves.

ABSTRACT
Influenza A virus is a zoonotic agent of great importance to human and animal health. Swine is an important host to the dynamics and epidemiology of the infection due to its susceptibility to viruses of both the avian and mammalian virus lineages. The great genetic variability of influenza viruses is caused by two main genetic mechanisms: point mutations and gene reassortment. The latter, by letting the exchange of gene segments between two different influenza viruses infecting the same cell, allows a rapid evolution of influenza viruses and the emergence of reassortant viruses against which there is no immunity in the host (human or animal). Influenza is endemic in pigs in many countries and the emergence of new viruses has been challenging its control and diagnostics. Although infections with influenza A virus (IAV) are endemic in most of the pork producing countries throughout the world, in Brazil this pathogen has not received much attention. Since the emergence of the 2009 pandemic H1N1 influenza virus in pigs (H1N1pdm09), many outbreaks of respiratory disease were observed in Brazilian swine populations. Currently, in Brazil, H1N1, H3N2 and 2009 pandemic H1N1 (A(H1N1)pdm09) influenza A viruses (IAVs) circulate in domestic swine herds.

Keywords: Influenza A, swine influenza, H1N1, H3N2
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INTRODUCTION
Influenza A virus (IAV) is a zoonotic agent of great importance for human and animal health (Olsen, 2002). The pig, particularly, is an important host and plays a role in the dynamics and epidemiology of infection, since this species can become infected with IAV subtypes originated from different animal species, including human, avian and swine (Vincent et al., 2008; 2009b). In cells of the porcine respiratory system, a phenomenon known as genomic reassortment can occur between different influenza viruses (Nelson et al., 2012; Vincent et al., 2009). This reassortment may give rise to more pathogenic influenza viruses for which animals (or humans) have no immunity.

Although influenza is considered an acute respiratory disease that affects human and several animal species worldwide, IAV infection in swine herds in Brazil is considered a recent health problem. For the reason that the influenza pandemic that occurred in humans in 2009 was a major public health concern, prompting the human and veterinary medicine must be in tune (OIE, 2011). The 2009 pandemic H1N1 (H1N1pdm09) influenza virus is a new virus with gene segments originating from human influenza viruses, swine and poultry from different continents (Garten et al., 2009; Smith et al., 2009). Following H1N1pdm09 identification in Brazil (Rajao et al., 2013; Schaefer, 2011) other IAV besides H1N1pdm09 were identified in swine herds in Brazil. Even with high prevalence, it is necessary to perform rapid identification of circulating viruses for the understanding of the epidemiology, pathogenesis and control of new subtypes. Surveillance of IAV in pigs is subject to official veterinary services in Brazil. This situation varies between countries, but generally lack resources, this being the main reason for the absence of regular surveillance. To control the transmission of this virus to humans, as part of a global plan, the World Health Organization (WHO), World Organization for Animal Health (OIE) and the Food and Agriculture Organization of the United Nations (FAO) recommended the monitoring of influenza viral infections in pigs. The OFFLU, joint OIE/FAO worldwide scientific network provides support to veterinary services in the control of swine influenza. Such monitoring, surveillance, research and collaboration is essential in order to allow appropriate control and prevention of the infection in different species (OIE, 2011).

The objective of this paper is to review the current situation of IAV infection in pigs in Brazil and

* Corresponding author.
E-mail address: janice.zanella@embrapa.br
worldwide. In this review will be discussed research results on swine flu held at Embrapa Swine and Poultry in a project funded by CNPq (National Council of Scientific and Technological Development) and MAPA (Ministry of Agriculture, Livestock and Food Supply) for the global surveillance and information held by a working group "OFFLU" composed by OIE, WHO and FAO.

**INFLUENZA A VIRUS IN SWINE**

Influenza viruses belong to the family Orthomyxoviridae that contains five genera: Influenza virus A, B and C, Thogoto virus and Isavirus. Of these, only influenza A viruses are zoonotic, infecting mammals and birds, while the influenza virus B and C are human pathogens. The viral genome consists of seven (C) or eight (A and B) RNA segments linked and protected by a protein called viral nucleoprotein (NP) (Brown, 2000). Influenza viruses are classified into subtypes according to the serological reactivity of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), the major targets for the host immune response (Brown, 2000; Olsen, 2002).

The high genetic variability of influenza viruses is due to two main genetic mechanisms: point mutations and gene reassortment. Point mutations occur due to high frequency rate of errors introduced by the viral RNA polymerase during the copy process for the replication of the viral genome. This phenomenon results on mutations in different genes ("antigenic drift"). When such mutations occur in genes encoding the surface glycoproteins (HA and NA), changes may occur in sites recognized by neutralizing antibodies allowing the virus to escape the host immune response, ensuring its perpetuation and transmission to new hosts. Due to the segmented genome of influenza viruses, exchange of gene segments between different viruses may also occur when a host cell is infected with two (or more) different IAVs. This mechanism is called "antigenic shift", allowing the generation of progeny virus with a reorganization of genes (Brown, 2000; Olsen, 2002; Vincent et al., 2009b).

In mammals, IAV replicates mainly in cells of the respiratory tract, usually followed by clinical signs of acute respiratory illness characterized by fever, lethargy, reduced feed intake, difficulty breathing, coughing, sneezing, nasal discharge and conjunctivitis (Vincent et al., 2009b).

Influenza in swine is a livestock disease, characterized by high morbidity (up 100%) and low mortality (≤ 1%) spread worldwide. Influenza in swine is part of porcine respiratory disease complex (PRDC) along with other agents such as *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, reproductive syndrome virus and swine respiratory (PRRSV), and porcine circovirus type 2 (PCV2) (Vincent et al., 2008).

Several experiments have demonstrated the susceptibility of pigs to H1N1pdm09, which resulted in an highly efficient spread among herds with presentation of clinical signs and lesions consistent with IAV infection (Lange et al., 2009; Pasma and Joseph, 2010; Pereda et al., 2010; Vincent et al., 2010).

**IAV IN SWINE WORLDWIDE**

Influenza is endemic in pigs, which are considered carriers of subtypes A/H1N1, A/H3N2 and A/H1N2 (Van Reeth et al., 2008; Vincent et al., 2009b). Besides these IAV, the H1N1pdm09 is also present in the swine population in countries around the world. In North America, Europe and Asia the IAV circulating in pigs is genetically different, mainly to independent introductions and maintenance of genomic strains of avian origin, human and swine.

In North America, specifically in the USA, the IAV H1N1 classic (cH1N1) was the major cause of swine influenza until 1998. Since the emergence of H3N2 influenza virus recombinants, containing a triple unique combination of internal genes known as the TRIG or triple reassortant internal gene cassette, was observed an increased rate of genetic changes of IAV isolates in North America, consequently, different antigenic and genetic clusters started to evolve (Vincent et al., 2009c). The IAV containing the TRIG are characterized by having the gene segments NP, M, NS from the cH1N1, the PB2 and PA segments from avian influenza virus and the PB1, HA and NA of human influenza viruses. Thus, in the USA and Canada, the H1 and recombinant H3N2 viruses are endemic and most contains the TRIG, regardless of subtype. The TRIG is also present in Korea, Vietnam and China (Lorusso et al., 2012; Ngo et al., 2012). Another important event was the emergence, from 2005 in the U.S. (and 2003 in Canada), of the H1N1 and the H1N2 HA gene derived from human IAV (and different from cH1N1). These viruses have emerged and spread in North American herds and called cluster delta. Between 2008-2010 the IAV cluster delta evolved drastically that were classified as subclusters delta 1 and delta 2 (H1N2 and H1N1, respectively). The HA cluster delta are matched with N1 or N2 of the human lineage. Therefore, the HA virus strain cH1N1 evolved and formed clusters: alpha, beta, gamma and delta, and in the four HA gene clusters can be found neuraminidase subtypes N1 or N2 (Lorusso et al., 2012). Consequently, currently circulating in the U.S. swine population are the H1N1pdm09 viruses (and other gamma clusters), H1N1 clusters (alpha, beta, and delta 2), H1N2 (delta 1) and cluster IV H3N2.

In Europe and Asia circulate different IAV TRIG in swine (Brown, 2000). Although the cH1N1 virus have circuated in Europe and Asia (as in other parts of the world), in Europe they were replaced by other subtypes. At the same time of the emergence of the cH1N1 virus in pigs in 1976, also emerged the human H3N2 virus (human-like). In 1979, there was avian influenza virus...
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H1N1 (avian-like), which quickly replaced cH1N1 virus, becoming the dominant strain and subsequently undergoing a reassortment with the human H3N2 virus, resulting in virus HA gene containing the and NA of human origin and the internal genes from avian origin (Castrucci et al., 1993). From the 90s, H1N2 viruses emerged containing different combinations of genes (Balint et al., 2009; Kyriakis et al., 2009). Most swine IAV circulating in Europe are also found in Asia, but there are several viral strains that are found only in Asia. China is a key point in the epidemiology of IAV in Asia, due to the high density and extensive pig production, but also because management practices (Zhu et al., 2011). IAV isolated from pigs in China include cH1N1, H1N1pdm09, Eurasian H3N2, H3N2 of human origin or North American H3N2, H1N1 Eurasian and H1N2 containing the TRIG, among others. Thus, China isolates include IAV from North America and Europe, reassortants IAV from North America and Europe and IAV of avian origin only (Zhu et al., 2011). Thus, China isolates include IAV from North America and Europe, reassortants IAV from North America and Europe and IAV of avian origin only. In South Korea, besides the cH1N1, TRIG H1N2 and H3N2, the H3N2 of human origin was also introduced (Lee et al., 2008). Likewise, the H3N2 human origin containing the TRIG also circulates in Vietnam (Ngo et al., 2012). In Thailand IAV similar to Eurasian IAV, such as cH1N1, H1N1, H1N1pdm09 and H3N2 (human) are also present.

In South America, a surveillance for influenza was conducted in Argentina at INTA, SENASA and Universidad Nacional de La Plata between the years 2008 and 2011. In late 2008 one of human origin H3N2 was isolated from pigs with typical signs of influenza (Cappuccio et al., 2011). The experimental reproduction of the disease showed that the virus was transmitted efficiently between the pigs and the inoculated animals presented typical influenza lesions. This suggested suggesting that this virus is completely adapted to swine and has the potential to be retained in the swine population. Also in 2009, it was reported infection of pigs with the virus H1N1pdm09 (Pereda et al., 2011) and in 2010 and 2011, was also reported the isolation of reassortant viruses with internal genes of H1N1pdm09 and the surface genes (HA and NA) of human H1. Therefore, there are some indirect evidences of circulating human IAV as H1 in Argentina (Cappuccio et al., 2011). In 2011, another recombinant virus was isolated containing the surface genes of the human H3N2 virus, first isolated in 2008, and all the internal genes of H1N1pdm09. The clinical signs observed in all cases correspond to the typical signs of influenza (fever, dyspnea, coughing, sneezing, etc.). The lack of influenza vaccines for pigs and the characteristics of pig production in Argentina may contribute to the emergence of new reassortments (Ariel Pereda, personal communication). In Colombia IAV surveillance and diagnostic performed at Universidad Nacional de Colombia verified the presence of cH1N1 and H1N1pdm09 in several swine farms (Jairo Jaime, personal communication). Peru, Bolivia, Ecuador have also started surveillance and positive IAV diagnostic have been (Ariel Pereda, personal communication).

In Colombia there is evidence for circulating classical H1N1 and A(H1N1)pdm09 in pig populations. Previous studies indicate that the IAV was first detected in 1971 in the Department of Antioquia, with a prevalence of 14%, but not defined strain acting. In 1977, through the HI test virus was detected a reactivity of 21%. In a study of A. Cucaita (2005), demonstrated in 1991 a reactivity of 6.5% for the H3N2 strain. Among 1997 - 1999 a serological monitoring was conducted and it was found reactivity of 43.5% for the H3N2 virus and 0.8% for H1N1 (Mogollon D. et al, 2003.). The same group reported reactivity of 10% for H3N2 on years 2000 - 2001. Moscoso C. Neira and G. (2001), showed a reactivity of 10% by HI test on 30 out of 300 sera tested for A/SW/Texas/4199H3N2 virus. In addition, a seroreactivity of 19.85% for multiparous sows and of 3.13% for gilts was noticed. In 2008, the Ministry of Agriculture and Rural Development (MADER) sponsored a program to study viral diseases of pigs, with emphasis on Circovirus and Influenza by the research group of Microbiology and Epidemiology, National University of Colombia. Preliminary results showed serologic reactivity of 12.82% for the H3N2 virus and 0.82% for H1N1 in three regions (Antioquia, Oeste and Central) in 2008 and 2009. Furthermore, it was found seropositivity for H3N2 in all age groups (sows, growing pigs and piglets) in all regions studied, whereas for H1N1 serological reactivity was demonstrated only for sows and nursing piglets. Regarding serologic reactivity by swine farms, there is evidence of H3N2 infection in 45.07% of the 71 farms surveyed in the study. As regards the H1N1 virus 4.23% of the farms were positive, represented in 3 of the 71 farms in the study (Mancipe, 2012). This study does not exclude the occurrence of other subtypes that may be present in the field and it is not possible to determine if there are components of influenza viruses of other species such as avian or human.

It was observed in Chile in 2009 an abrupt increase of cough in farrowing and growing phases in addition to increased respiratory signs in finishing animals, being indicative of IAV infection. These cases were characterized by an increase in respiratory treatments, increased mortality associated with bacterial respiratory signs, increasing culling and changing of production parameters (Ruiz, 2011). A serological study was conducted and sampled 13 production units (11 companies) and 382 pigs (17.3% reproduction, 59.4% finishing and the remainder from other phases). ELISA tests showed that 48% of pigs react positively to H1N1 and 22% to H3N2. Based on this sampling, 100% of
the farms were positive for H1N1 and 83% for H3N2, predominantly in breeding animals (68% for H1N1 and 21% for H3N2) (Ruiz, 2011). Chile was one of the first South American country to identify the A(H1N1)pdm09 in turkeys, the first confirmed case of transmission of this virus from birds to humans and the first to detect in another species besides humans or pigs. The observed signs were egg drop production in hens and the turkeys farms where the A(H1N1)pdm09 was detected belonged to the same private company near the town of Valparaízo (Moreno, 2010).

In Peru there is serological evidence by immunodiffusion test (AGID), but has no virus isolation or PCR positive diagnostic. Members of OFFLU are performing training for diagnostic testing of IAV in research laboratories in Bolivia, Ecuador, Colombia and Peru.

**Infection caused by Influenza Virus in Brazilian Swine Herds**

In Brazil, before 2010, there were few studies in infection of pigs with IAV. Despite the IAV to be a major player responsible for acute respiratory infections in pigs, acting as primary agent or part of the porcine respiratory disease complex (PRDC) in Brazil, until recently, was not considered as important. After the emergence of the H1N1pdm09 virus, research, diagnosis and control of influenza became extremely important to the health of swine herds in Brazil. Even so, few data and results are available to be described here.

Brazilian initial reports are cases of bronchopneumonia in pigs from 1938 to 1943, described by Bueno as an illness compatible with IAV [reviewed by (Cunha et al., 1978)]. However, a filterable agent was not isolated at the time. Conversely, in 1978, IAV was first isolated from a pig three months old from Minas Gerais that was slaughtered in Rio de Janeiro (Cunha et al., 1978). The agent was isolated in embryonated chicken eggs and it was characterized from chicken hyperimmune serum produced against A/Swine/Illinois/1/63 virus, which the Brazilian isolated produced HI neutralizing titer of > 1:320, but showed no cross-reaction against the A / Hong Kong/1/68 virus. Still, no further studies was performed to determine the subtype of this virus. More recently, (Mancini D.A.P, 2006) reported the IAV isolation from oro-nasal swine samples from São Paulo state. Nevertheless, no subtype characterization or genomic sequencing were performed. Studies at Embrapa Swine and Poultry have analyzed nasal secretion samples collected from 281 pigs from 29 commercial farms in the state of Santa Catarina in 2005-2006 revealing the presence of IAV RNA by RT-PCR (Schaefer, 2008). However, no isolates or further characterization of those samples was successful.

Serological studies from samples collected from pigs of 10 Brazilian states in the period from 1996 to 1999 indicated the presence of antibodies against viral subtypes H1N1/Texas/1/77 (2.2%) and H3N2/New Jersey/76 (16.7%) (Brentano, 2002). Other studies found higher prevalence of inhibitory antibodies against H1N1 subtypes (85.29%), H3N2 (85.29%) against influenza type B (91.17%), which was restricted to São Paulo state samples (Mancini D.A.P, 2006). The study by Caron et al. (2010) analyzed serum from pigs from Paraná state. The authors reported that 46% of the farms analyzed were positive for influenza virus, with a prevalence of 20% of antibodies against swine H3N2 subtype (Caron, 2010).

Recently, coinciding with the pandemic influenza A (H1N1) in humans in Brazil numerous outbreaks of acute respiratory infection in pigs of various age groups have been reported. The analysis of one of these isolates by sequencing of the viral genome revealed that the viruses found in pigs is the same that circulates in humans (A/H1N1/2009) (Schaefer, 2011). In addition, a new H1N2 IAV was identified in a recent study. The new H1N2 virus contains H1 and N2 genes of human seasonal origin (delta cluster) and the internal gene (M) from the H1N1pdm09 (unpublished data). Furthermore, the analysis of sera collected from pigs in Brazil revealed that HI antibodies against the H1N1pdm09 were not detected in pigs in Brazil prior to 2009 (Ciacci-Zanella, 2011).

The research project: "Diagnostic, molecular characterization and pathogenesis studies of infectious agents economically important for the Brazilian Swine Production” was sponsored by National Council for Scientific and Technological Development (CNPq) and the Ministry of Agriculture, Livestock and Food Supply (MAPA) and the Brazilian Agriculture Research Corporation (Embrapa). The objective of this work was to perform and implement diagnostic methods for economical important infectious agents for swine production, such as influenza A virus (IAV) in swine. Sampling was divided in groups such as 1) samples collected from 16 farrow to finish swine farms from 2009-2012 , named FF farms. 2) Samples from 49 commercial nursery farms 2011-2012 from 7 Brazilian states named commercial farms and 3) Samples from Diagnostic Lab (CEDISA) totaling 86 lungs samples called diagnostic samples. Sampling included: nasal swabs (NS), serum, oral fluid (OF) and lung tissue. The analysis performed included: qRT-PCR (quantitative reverse transcriptase polymerase chain reaction), Elisa IAV NP (nucleoprotein), subtyping RT-PCR, genome sequencing, viral isolation, HI, histopathology (IHC).

Serology results from 15 FF farms indicated a variation on titers and frequency of anti-IAV antibodies. However, an average of frequency on suckling (63%), nursing (45.2%), growing (58%) or finishing (69.4%) phases indicated the dynamics of infection and circulation of IAV in all phases. HI results indicated a major frequency of H3N2 and H1N1pdm09 subtypes
on those FF farms. IHC from lung tissues collected at slaughter indicated high incidence of Pasteurella multocida as secondary agent.

Sampling of 49 farms from 7 Brazilian pork producer states showed a high percentage of positive farms of 63% and also a high percentage of positive pigs or 75% of those 8 – 12 week old pigs sampled. HI results indicated higher titers for H3N2, followed by H1N2 and H1N1pdm09 antibodies. detection of IAV in NS and OF by qRT-PCR on 62 swine farms (FF and comercial nursery farms) indicated a global concordance of NS pools and OF of 82.26% for the two tests and the Kappa index was of 0.613. The sensitivity of real-time PCR for IAV and H1N1pdm09 were respectively 66.67% and 57.69% for the OF relative to NS, whereas specificity was 92.11 and 100%, respectively. So the oral fluid test has a high specificity specific and less sensitive. Viral isolation was performed on lung and NS samples and resulted on 50 IAV isolates (3 from NS and 57 from lungs). Subtyping of HA and NA on 28 samples detected H1 (11 samples), H3 (01) and N2 (14), most of the samples were untyped.

Diagnostic samples (2009-2012) analyzed 86 lungs screening for respiratory agents involved in the porcine respiratory disease complex (PRDC). IAV was the most frequent agent (65% of the lungs). Genome sequencing data for HA, NA and M was performed on 26 IAV isolates. HA sequencing grouped most of the Brazilian sequences on the pandemic cluster with 98-99% identity, with the exception of one H1 (delta cluster) sequence. NA sequencing grouped most of the Brazilian sequences on the pandemic N1 cluster, with the exception of one N2 (human-like) sequence. All M gene Brazilian sequences grouped on the pandemic cluster with 98-100% identity.

Populations of wild Suidae, both captive wild boars reared under intensive management or free-range feral pigs of the Pantanal Region in Brazil (Monteiro Pig) were also sampled. In captive wild boars IAV were detected by qRT-PCR in 11 out of 60 lungs samples with typical macroscopic lesions of pneumonia. The M gene sequence showed 98-99% identity with the H1N1pdm09 (Biondo, 2012). In the population of feral pigs analyzed, samples of blood serum indicated the presence of antibodies to IAV in 105 out of 141 samples tested (74.5%).

Serologic detection in 17 pig farms in Minas Gerais state by HI concluded that the IAV H1N1pdm09, H3N2 and a “human” H1N1 were circulating in at least 64.7% of the 11 studied farms (Rajao et al., 2013). A report by the same group has shown genomic sequencing data from hemagglutinin (HA) and neuraminidase (NA) genes of twenty IAV isolated from five Brazilian states farms demonstrating high nucleotide similarity to H1N1pdm09 influenza virus (Rajao et al., 2012).

CONCLUSIONS

Even though influenza in pigs is known for a long time and is considered endemic in most pig producing countries, in Brazil influenza virus was not considered an important infectious agent for swine health. This situation changed with the influenza pandemic in humans in 2009 and the consequent transmission of the virus H1N1pdm09 to pigs, highlighting the importance of zoonosis to human and animal health. As a result, increased the availability of samples for virological diagnosis, which has enabled more detailed studies on the occurrence of influenza in pigs in Brazil could be detained. Data from research conducted by Embrapa Swine and Poultry in commercial herds indicate the prevalence of antibodies against IAV in swine of 60 %. No H1N1pdm09 antibodies was detected by HI analysis of the sera of pigs collected in Brazil before 2009. Results of virus isolation and molecular analysis of samples positive to influenza Brazilian swine herds revealed the circulation of H1N1 pdm09, H1N2 (human-like) reassortant virus, and H3N2 viruses. The occurrence of both H1N1pdm09, which has shown a great ability to spread in pigs worldwide, as well as the new H1N2 influenza virus (human-like) detected in pigs recently, leads us to ask whether these new established swine influenza viruses have replaced the classics, contributing to the emergence of new influenza viruses.

Thus, the research work carried out on swine flu so far emphasize the importance of performing monitoring in order to detect swine influenza virus to increase the data available on the prevalence and genetic evolution of influenza viruses in swine populations Brazil.

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